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• REVIEW •

Hepatocellular Carcinoma-Cause, Treatment and Metastasis

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Abbreviation **HCC-hepatocellular carcinoma, HBV-hepatitis B virus, HCV-hepatitis C virus, HGV-hepatitis G virus, TTV-transfusion transmitted virus, AFB1-aflatoxin B1, IFN-interferon, OLT-orthotopic liver transplantation, PH-partial hepatectomy, RCT-randomized controlled trial, TACE-transcatheter arterial chemoembolization, PEI-percutaneous ethanol injection, PMCT-percutaneous microwave coagulation therapy, RF-radiofrequency, AFP-alpha fetoprotein, VEGF-vascular endothelial growth factor.**

Abstract

In the recent decades, the incidence of hepatocellular carcinoma (HCC) has been found to be increasing in males in some countries. In China, HCC ranked second of cancer mortality since 1990s. Hepatitis B and C viruses (HBV and HCV) and dietary aflatoxin intake remain the major causative factors of HCC. Surgery plays a major role in the treatment of HCC, particularly for small HCC. Down-staging unresectable huge HCC to smaller HCC and followed by resection will probably be a new approach for further study. Liver transplantation is indicated for small HCC, however, some issues remain to be solved. Different modes of "regional cancer therapy for HCC" have been tried. Systemic chemotherapy has been disappointing in the past but the future can be promising. Biotherapy, such as cytokines, differentiation inducers, anti-angiogenic agents, gene therapy and tumor vaccine will probably play a role, particularly in the prevention of tumor recurrence. HCC invasiveness is currently the major target of study. Tremendous works have been done at the molecular level, which will provide clues for biomarker of HCC progression as well as targets for intervention.

Subject headings carcinoma, hepatocellular/etiology; carcinoma, hepatocellular/surgery; carcinoma, hepatocellular/drug therapy; liver neoplasms/etiology; liver neoplasms/surgery; liver neoplasms/drug therapy; human; review

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INTRODUCTION

Liver cancer is the 4th most common cause of death from cancer, the highest age-standardised mortality rate is in China ($34.7/10^5$, the 2nd cancer killer since 1990s), which alone accounts for 53% of all liver cancer deaths worldwide[1]. Recently, the incidence of HCC has been found to be increasing particularly in males in countries such as Japan, Italy, France, Switzerland, United Kingdom and the United States[2-4]. Clinical advances have mainly been made in the fields of medical imaging, surgery, regional cancer therapy and biotherapy. Rapidly growing knowledge in basic science appears at the molecular level, particularly in the study of HCC invasiveness. Although a lot of news from bench to bedside on the advances made in HCC has appeared, the overall dismal outcome of patients with HCC changed very little. In the United States, the relative 5 -year survival for liver cancer only increased from 4% (1974-76) to 6% (1986 -93) in white, and from 1% to 4% in black[5]. In Shanghai, the relative 5-year survival of liver cancer in 1988-91 was 4. 4%[6]. These indicate that there is still a long way to go in conquering HCC.

CAUSE AND PREVENTION

Viral hepatitis B (HBV) and/or C (HCV), aflatoxin and alcohol are major risk factors of HCC

However, the importance of these different factors varies in different geographic areas. HBV is more predominant in Chinese, Southeast Asian and African patients with HCC, whereas HCV is common in HCC patients in developed countries (Japan, France, Italy and others). The prevalence of hepatitis B surface antigen (HBsAg) and antibody to HCV (anti-HCV) in HCC patients were reported to be 63.2% and 11.2% respectively in China[7], which was similar to that reported in the past.Prospective studies showed that there is an additive effect of HCV and HBV infection on HCC development[8]. Cirrhotic patients infected with HCV type 1b carry a significantly higher risk of developing HCC than patients infected by other HCV types[9-11]. An association was found between high serum alanine aminotransferase levels and more rapid development and high incidence rate of HCC in patients with HCV-associated cirrhosis[12]. In a transgenic mice, it was found that the core protein of HCV induces HCC[13].

Hepatitis G virus (HGV) and transfusion-transmitted virus (TTV) infection might not play an important role

Based on the data from China, Japan, Africa, United Kingdom and others, HGV might not play an important role in the development of HCC[14-18]. A case-control study also failed to support the hypothesis of an association between transfusion-transmitted virus (TTV) infection and HCC[19]. However, some authors claimed that HGV and TTV could not be completely excluded as causative agents[20-22].

In China, HBV and HCV (mainly HBV), aflatoxin and contamination of drinking water (such as microcystin, a promoter of hepatocarcinogenesis) remain as major risk factors of HCC, and alcohol should be added in northern China

A study showed that exposure to aflatoxin metabolite M1 (AFM1) can account for a substantial part of the risk of HCC[23].

Other risk factors have also been reported

In Japan, alcohol consumption and cigarette smoking were also risk factors of HCC, and synergism between them was observed[24,25]. In Italy, for attributable risk (AR) of HCC, heavy alcohol intake ranked first (45%), HCV second (36%) and HBV third (22%)[26]. The risk of dietary iron overload was 4.1 for HCC in black Africans[27], which is similar to that of haemochromatosis in Caucasians. A role of family history independent from and interacting with known risk factors for HCC was also reported, the odds ratio was reported to be 2.4[28].

HCC risk is high in individuals with both aflatoxin B1 (AFB1)-DNA adducts and HBsAg, suggesting a viral-chemical interaction

Furthermore, AFB1 exposure correlates with a specific mutation at codon 249 of the p53 tumor suppressor gene in HCC, indicating a molecular pathogenesis[29]. How the four major risk factors (HBV, AFB1, p53 mutation and male gender) for HCC interact to produce malignant liver tumors were also demonstrated in transgenic mouse models[30].

X protein of HBV is one of the target of how HBV induces HCC

The incidence of HCC was as high as 86% in a HBV-X transgenic mice[31]. It was also found that the structure of the X gene is modified in the majority of tumorous livers, suggesting a potential role of mutated X proteins in HBV-related liver oncogenesis[32]. Moreover, HBV-X might play a role in hepatic inflammation by up-regulating interleukin-6 production, which can eventually lead to HCC[33]. Transactivation of transforming growth factor alpha (TGF-alpha) gene by HBV preS1 was observed which provides a clue for understanding viral hepatocarcinogenesis[34]. Synergy between TGF-alpha and HBsAg in hepatocellular proliferation and carcinogenesis was also reported[35].

All of these indicate a multifactorial and multistep development of HCC. Interaction among HBV/HCV, aflatoxin, alcohol, and genetic susceptibility might be important.

The dawn of HCC prevention has been shown

Few approaches of HCC prevention have been emerged, namely: prevention of HBV infection using vaccine, avoid exposure to carcinogens and promoters by changing drinking water, and to prevent viral hepatitis B or C progressing to cirrhosis and HCC by interferon (IFN) therapy. Result from a universal hepatitis B vaccination program indicated that the incidence of HCC in children has declined[36]. A significant declining trend of HCC mortality rate ratios was observed in the vaccination group, but not in the reference group[37]. In Qidong County of China, after people changed their source of drinking water from pond-ditch water (microcystin was found) to deep-well water, the mortality rate of HCC stabilized and even decreased slowly[38]. An analysis of patients with chronic hepatitis, liver cirrhosis, chronic hepatitis bearing HCC and liver cirrhosis bearing HCC, found that the incidence of HCC in the control group was 10.4/100 person-year, while that in the IFN treated group was 1.2/100 person-year[39]. IFN decreased HCC incidence in patients with HBV related cirrhosis. The cumulative occurrence rates of HCC in the treated group and the untreated group were 17.0% and 30.8%, respectively, at the end of 10 years[40]. IFN therapy also decreased the development of HCV related HCC. HCC rates in the IFN treated and untreated groups were 7.6% and 12.4% at the 10th year respectively[41]. Patients with HCV-related cirrhosis also benefit from IFN treatment[42]. IFN therapy significantly reduces the risk for HCC, especially among virologic or biochemical responders of patients with chronic hepatitis C[43]. For those nonresponder, retreatment with IFN-alpha appeared to have the additional effect of suppressing the development of HCC in patients who had incomplete responses to the initial treatment, even when the HCV was not cleared with retreatment[44]. Currently, lamivudine or ribavirin, antiviral agent, is added to the treatment of HBV or HCV, however, long-term follow-up study is needed to evaluated whether this additional treatment will increase the efficacy of HCC prevention.

SURGERY OF HCC

Small HCC resection plays an important role to improve HCC prognosis

Small HCC resection has resulted in marked increase in 5-year survival rate from 20%-30% to 40%-60%. At the author's institution, the 5-year survival rate of 963 patients with small HCC ($\leqslant$5cm) resection was 65.1%, whereas it was only 36.1% for large HCC resection (n=1308); of the 368 HCC patients with 5-year survival, 198 (53.8%) patients received small HCC resection[45,46]. Early HCC with well differentiated cancer containing Glisson's triad has been recognized as an entity with a high rate of surgical cure, the 5-year survival was as high as 93%[47]. A comparison between subclinical HCC and symptomatic HCC revealed that operability was higher (26.8% versus 7.9%), and cumulative survival rate was also higher[48].

Makuuchi *et al* (1998) have performed 367 hepatectomies on 352 patients since 1990, the 5-year survival rate was 47.4%[49]. At the author's institution, HCC resection has been performed on 2119 patients between 1979-1998, the 5-year survival rate was 51.5%[50]. Recently, perioperative blood transfusion and diabetes mellitus were found to be prognostic factors after HCC resection[51,52]. An experimental study indicated that partial hepatectomy was associated with increased levels of TGF-alpha, TGF-beta, and basic fibroblast growth factor (bFGF) in the liver and accelerates local tumor growth[53].

Down-staging of unresectable huge HCC to smaller HCC followed by resection will probably be a new approach for further study

At the author's institution, the 5-year survival of 108 patients with this approach (down-staging by hepatic artery ligation, cannulation, cryosurgery, etc.) was 64.7%[45]. Another 65 patients with unresectable HCC down-staged by transcatheter arterial chemoembolization (TACE) followed by resection, the 5-year survival was 56.0%[54]. The 5-year survival rates

were similar to that of small HCC resection, which coincided with a reduction of median tumor size from 10.0cm to 5.0cm during the resection of this approach[55]. However, a well designed randomized trial is needed for a final evaluation.

Orthotopic liver transplantation (OLT) is a reasonable treatment for small HCC if partial hepatectomy (PH) is impossible

For decades, the role of OLT in the treatment of HCC has been unclear. In the early 1990s, it was accepted that small HCC was indicated for OLT. However, only retrospective data were available for the comparison between OLT and PH in the treatment of HCC. The 5-year survival rate of 422 HCC patients with OLT was 44.4%, and tumor histologic grade and tumor size (>5cm) were linked to recurrence-free patient survival[56]. A comparison between PH (n = 294) and OLT (n = 270) showed that survival was comparable, but operative mortality was lower in PH group, and concluded that HCC developing in a well compensated cirrhotic liver initially may be treated with PH, and OLT should be applied selectively to those patients with tumor recurrence and/or progressive hepatic failure[57]. A proper selection of candidates for PH gives better results than OLT, because of the increasing waiting time for OLT[58]. OLT is a reasonable treatment for patients with early stage tumors if PH is impossible. The oncological advantage of OLT compared with PH, however, is questionable[59]. As survival after PH and OLT for early stage HCC does not reveal a significant difference, resection of these tumors is still justifiable[60]. When compared with PH, OLT for resectable HCC offers substantial survival benefit among well-targeted subgroups of patients as long as an organ donor is available within 6 to 10 months time delay. However, the marginal cost-effectiveness ratios incurred by this strategy are higher than that of many other current medical interventions[61]. This might be of particular impact for developing countries where HCC is endemic.

NONSURGICAL THERAPIES FOR HCC

There is still a long way to go of nonsurgical therapies for HCC

Nonsurgical therapies for HCC generally include regional cancer therapies, radiotherapy, chemotherapy and biotherapy. Unfortunately, a systemic review of 37 RCTs to examine the effect of different treatments for non resectable patients indicated that only 3 modalities were minimally and uncertainly effective (embolization, tamoxifen and IFN)[62]. Another overview of 30 RCTs for unresectable HCC found that no treatment has clearly proven efficacy in survival. 5-Fluorouracil, adriamycin and transarterial chemotherapy were not associated with survival benefit at 1 year. The number of RCTs was insufficient to enable a conclusion to be reached for IFN and PEI (percutaneous ethanol injection). Controversy persists concerning tamoxifen efficacy[63].

Regional cancer therapy for HCC is one of the nonsurgical therapies that develops recently

Based on the advances of early detection and medical imaging, more HCCs can be diagnosed with small and localized lesions. As a result, regional cancer therapies have developed in the recent decades. Unfortunately, the number of RCTs was insufficient to make any conclusion as yet.

Transcatheter chemoembolization (TACE) is one choice of the treatment for unresectable but not far advanced HCC, particularly for patients with multifocal HCCs and with acceptable liver functions. However, some RCTs failed to demonstrate that TACE improve the survival with unresectable or advanced HCC[64,65]. A RCT found that the 4-year survival of intrahepatic-arterial ^{131}I-labeled lipiodol (2.2 GBq) was 10% when compared with 0% in TACE group (70mg cisplatin)[66]. Another RCT indicated that styrene maleic acid neocarzinostatin in Lipiodol was better than epirubicin in Lipiodol[67]. A comparison of planned periodic TACE and TACE based on tumor response found that the 3-year survival rates were 0% and 15% respectively in Okuda 2 stage, the mean time between the first and the third courses of TACE was 4 months and 14 months respectively, indicating the efficacy of TACE increased when it was used selectively and was repeated only when necessary[68]. The overall 5-year survival rate after TACE treatment is around 6%-8%. TACE resulted in prolongation of survival in patients with tumor volumes of less than 200mL, tumor-to-liver volume ratios of less than 5%, and iodized oil retention greater than or equal to 75%[69]. Complications of TACE were encountered in 4.4% of cases, of which, hepatic failure and down-staging of cirrhosis remain a problem[70].

Percutaneous ethanol injection (PEI) is a treatment choice of unresectable small HCC. The 4-year survival rate was 39% in 47 small HCC patients with cirrhosis, however, the 4-year recurrence rate was as high as 79%[71]. Local recurrence depends predominantly on the biologic characteristics of the tumor (histologic grade and intrahepatic recurrence), regardless of the efficacy of PEI[72]. For large (>5cm) HCC, PEI performed in a single session under general anesthesia was an alternative. In a series of 108 patients, the 4-year survival rates were 44% for single encapsulated HCC (5 cm - 8.5 cm), 18% for single infiltrating HCC (5cm-10cm) or multiple HCC and 0% for advanced disease, the mortality was 0.7% and major complications 4.6%[73]. A RCT study comparing 50% acetic acid and PEI indicated that local recurrence rate was lower and 2-year survival rates higher with acetic acid[74].

Percutaneous microwave coagulation therapy (PMCT) is an extension of PEI, the 5-year survival for patients with well-differentiated HCC treated with PMCT and PEI were comparable, however, among the patients with moderately or poorly differentiated HCC, 5-year survival with PMCT (78%) was better than with PEI (35%)[75]. PEI is difficult for small HCC on the surface of liver, however, PMCT can be performed safely in such patients[76]. After PMCT, a second biopsy on 19 patients showed complete destruction of tumor in 18 patients[77].

Radiofrequency (RF) hyperthermia is another mode of regional cancer therapy. Of the 73 HCC patients treated with RF and evaluated by CT, complete response rate was 10%, partial response rate 21%, and 5-year survival 17.5%[78]. A comparison was made between RF and PEI in the treatment of small HCCs. It was found that RF ablation resulted in a higher rate of complete necrosis (90% versus 80%) and requires fewer treatment sessions than PEI. However, the complication rate was higher with RF ablation than with PEI[79].

The inadequacy for complete control of cancer nodule is one of the major problems of regional cancer therapies. Therefore, surgery remains the choice of treatment for curatively resectable HCC with Child A cirrhosis until a RCT clarifies the situation. In general, TACE is a treatment choice

for multinodular and large unresectable HCC (a part of TNM Stage II, IIIA, IIIB and IVA; with Child A or Child B cirrhosis). The other regional therapies may be used on unresectable small HCC which is not multinodular.

Three-dimensional conformal radiotherapy will probably play a role for HCC treatment in the future

A pilot study indicated that three-dimensional conformal radiotherapy helped to avoid excessive exposure of the liver and adjacent organs and made it a safer treatment modality for unresectable HCC[80]. Selective internal radiation therapy using 90Y microspheres (median 3.0 GBq) was effective for selected cases of nonresectable HCC. There was a 50% reduction in tumor volume in 26.7% of patients after the first treatment, the nontumorous liver appeared more tolerant to internal radiation than external beam radiation. This treatment may help to convert nonresectable tumors to resectable ones[81]. At the author's institution, long-term follow-up study indicated that a combination of surgery and intrahepatic arterial infusion of ^{131}I-anti-HCC mAb improved survival of unresectable HCC[82].

Systemic chemotherapy for HCC has been disappointing in the past, but in the future can be promising

Neither complete response nor partial response was observed using paclitaxel (175mg/m^2 q3w) for unresectable HCC[83]. However, a phase II study with cisplatin, doxorubicin, 5-fluorouracil, and IFN-alpha in advanced unresectable HCC demonstrated that complete pathological remission was possible, partial response rate was 26%, no viable tumor cells were found in four out of nine resected specimens[84]. Based on the study of the expression of drug resistance-related genes in three human hepatoma cell lines, it was demonstrated that IFN-alpha modulated the mechanism of resistance to cisplatin in liver cancer[85]. Individual patient with complete remission of multiple HCC associated with HCV-related decompensated liver cirrhosis by oral administration of enteric-coated tegafur/uracil has been reported[86].

Biotherapy will play a role in the treatment of HCC in the future

However, the results were still controversial. Many RCTs of tamoxifen for advanced HCC were negative[87-90]. A lack of efficacy of antiandrogen treatment was found for unresectable HCC in a RCT[91]. Oral beta all transretinoic acid (50mg/m^2 t.i.d.) was also ineffective against HCC[92]. Interestingly, Octreotide, a somatostatin analogue, improves survival of inoperable HCC in another RCT[93]. Randomized controlled trial of interferon treatment for advanced HCC indicated that its administration prompts no benefit in terms of tumor progression rate and survival[94].

Gene therapy-"progress but many stone yet unturned[95]"

Gene therapy for HCC remains an attractive field. Experimental studies using cytokine genes (tumor necrosis factor, interleukin-2, interferon), suicide and *p*53 genes; using retrovirus, adenovirus and Epstein-barr virus as vectors; using AFP enhancer; using intraarterial administration, etc. have been reported[96-108]. Data from both the literature[109-112] and from the author's institution[113] demonstrated that human malenoma antigen (MAGE) gene expression is frequent in HCC, suggesting that HCC patients may be good candidates for specific immunotherapy (tumor vaccine) using MAGE encoded antigen. Dendrite cells are good candidates for this particular purpose[114].

METASTASIS OF HCC

Invasiveness of HCC has become a major target of recent research

The high recurrent rate in the liver with mainly intrahepatic metastatic spread remains a major obstacle to further improvement on the long-term survival after curative HCC resection. Therefore, research on the invasiveness of HCC has become a major target. Clinically, targets include prediction, treatment and prevention; in the laboratory, investigations include metastatic model, molecular events, angiogenesis, intervention, etc.

A routine biomarker for prediction of metastasis and recurrence is not yet available

Although many biomarkers have been tried, such as AFPmRNA, circulating VEGF and PD-ECGF[115,116], human macrophage metalloelastase gene[117], p27[118], p53 mutation[119], expression of p73[120], telomerase activity[121], etc.

Both pre- and postoperative chemotherapy or chemoembolization have not adequately proved to be effective for prevention of metastatic recurrence

Convincing evidence is lacking to support systemic preoperative chemoembolization in patients with initially resectable HCC[122]. Although many authors supported the strategy of postoperative chemoembolization, its effectiveness might be due to suppression of intrahepatic micrometastases rather than multicentric carcinogenesis[123]. Postoperative intraarterial chemotherapy has also been claimed to improve survival[124,125]. Recently, a RCT showed that postoperative adjuvant systemic chemotherapy using epirubicin and mitomycin C has a tendency to reduce recurrence rate[126]. However, two RCTs failed to demonstrate the effectiveness of postoperative adjuvant therapy. The adjuvant chemotherapy with epirubicin and carmofur after radical resection of HCC was not effective[127]. Postoperative chemotherapy using intravenous epirubicin and intraarterial iodized oil and cisplatin was associated with more frequent extrahepatic recurrences and a worse outcome[128]. Interestingly, a RCT revealed that oral polyprenoic acid prevents second primary HCC after surgical resection, and reconfirmed after longer follow-up study[129,130]. Recently, a RCT study indicated that a single 1850 MBq dose of intra-arterial ^{131}I-lipiodol increased the 3-year overall survival from 46.3% in the control to 86.4% in the treatment group[131].

The molecular basis of "HCC invasiveness" is similar to that of other solid cancers, its complexity represents as multi-genes involvement and multi-step process

Numerous papers have been published concerning the molecular basis of "HCC invasiveness" in the literature[132-150]. At the author's institution, studies concerning HCC invasiveness could be summarized into the followings: a Factors that positively related to invasiveness included: p16 and p53 mutation, H-ras, c-erbB-2, mdm2, TGFα, epidermal growth factor receptor (EGF-R), matrix metalloproteinase-2 (MMP-2), urokinase-type plasminogen activator (uPA), its receptor (uPA-R) and inhibitor (PAI-1),

intercellular adhesion molecule-1 (ICAM-1), vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF), basic fibroblast growth factor (bFGF), etc. On the other hand, factors that negatively related to HCC invasiveness included: nm23-H1, Kai-1, tissue inhibitor of metalloproteinase-2 (TIMP-2), integrin α5, E-cadherin, etc. b The biological characteristics of small HCC was slightly better than that of large HCC c The following blood test have been tried with potential clinical implication: thrombomodulin, ICAM-1, PAI-1, VEGF, bFGF, etc. Serum ICAM-1 content was higher in patients with metastasis than those without metastasis. Loss of heterozygosity (LOH) at D14S62 and D14S51 (on chromosome 14q) in plasma DNA were also related to metastatic recurrence. The combination of several items that mentioned above increased sensitivity[151-164].

Comparison between primary HCC tumors and their metastatic lesions using comparative genomic hybridization (CGH) indicated that chromosome 8p deletion might contribute to HCC metastasis[165]. The presence of at least three novel tumor suppressor loci on 8p in HCC was reported[166], and DLC-1 might be one of the related tumor suppressor gene[167].

Metastatic human HCC model in nude mice (LCI-D20) and HCC cell line with metastatic potential (MHCC97) have been established[168,169]

Using corneal micropocket model in nude mice, the difference of angiogenesis induced by LCI-D20 and LCI-D35 (a low metastatic model) was also demonstrated[170]. Highly metastatic HCCs induced in male F344 rats and a transplantable lymph node metastatic mouse model of HCC were also reported[171,172]. These will provide a tool for the study of the mechanism and the intervention of metastasis.

Angiogenesis is closely related to HCC invasiveness

Vascular endothelial growth factor (VEGF) gene and protein expression are involved in the progression of HCC[173-175], and that VEGF 121 and 165 isoforms play a critical role in angiogenesis of HCC[176]. However, some author reported that VEGF might be associated with the angiogenic process of the cirrhotic liver, but not with the angiogenesis of HCC[177]. VEGF level increased after TACE, indicating that VEGF may be a marker for tumor ischemia[178]. Angiogenesis in HCC depends on the net balance between human macrophage metalloelastase (a potent angiogenesis inhibitor) and VEGF gene expressions[179]. Platelet-derived endothelial cell growth factor (PD-ECGF), another angiogenic factor, is also involved in HCC progression[180]. The enhanced gene expression of angiopoietin-2 may also contribute to the hypervascular phenotype[181]. Angiogenesis in HCC can be evaluated by CD34 immunohistochemistry[182,183]. At author's institution, using CD34 staining to measure microvessel density (MVD), we found that MVD was only useful for small HCC resection, the 5-year survival after resection of hypovascular type small HCC was double to that of hypervascular type, being 74.6% versus 34.7%[184]. As small HCCs increase in size and become increasingly dedifferentiated, the number of portal tracts apparently decreases and intratumoral arterioles develop. These findings may reflect changes in the hemodynamics as the HCC develops[185].

Experimental intervention of HCC metastases is progressing

Many approaches have been tried in preventing metastases, and anti-angiogenesis is one of the major target. For example, anti-angiogenic agent TNP-470, a derivative of fumagillin, was found to inhibited tumor growth and metastasis in nude mice bearing human HCC and suppressed the progression of experimentally-induced HCC in rats[186,187]. High-dose and long-term therapy with IFN-alpha inhibited tumor growth and recurrence in nude mice bearing human HCC xenografts with high metastatic potential in a dose-dependent manner, and the preventive effect was mediated by anti-angiogenesis[188]. However, its clinical significance has to be assessed by a RCT. Other experimental interventions for metastasis were also reported, such as matrix metalloproteinase inhibitor BB-94[189], 4-[3,5- Bis (trimethylsilyl) benzamido] benzoic acid (TAC-101)[190], antisense H-ras oligodeoxynucleotides[191], synthetic β peptide[192], etc.

In short, much has been done and much remains to be done. Well designed RCTs are needed for a more clear conclusion in many treatment modalities that are in debate. Some agents that have not been effective for advanced HCC may still be tried in the prevention of metastases and recurrence with a much smaller tumor burden. In the 21st century, prevention is doubtlessly of prime importance, however, detection of small HCC and studies on HCC invasiveness remain critical issues for further improvement of prognosis of HCC.

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• REVIEW •

Fertility and pregnancy in inflammatory bowel disease

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disorder affecting young adults in the reproductive years. It is common for both female and male patients with IBD to ask questions about IBD's effect on their relationships, sexual and reproductive function, in particular fertility, the outcome of pregnancy and its possible effects on the disease. An open discussion of the social situation and education targeted at these issues therefore forms an essential part of the management of any young person with IBD. The questions that are most commonly asked are summarised in Table 1. In order to answer these questions we need evidence. There are few large prospective case controlled studies to provide the information which is required but the available data, some of it from small observational studies, will be summarised in this chapter.

Table 1 Questions commonly asked by IBD patients

Sexual health	Will I be able to have normal relationships and a family?
Inheritance	Will my children inherit IBD?
Fertility	Will my fertility be impaired by IBD or its treatment?
Outcome of pregnancy	Will I have a normal, healthy baby?
Disease activity in pregnancy	Will my IBD flare up in pregnancy?
Drug and other treatments	I don't want to take any drugs during pregnancy?
Breast feeding	Is breast feeding advisable and safe

SEXUAL HEALTH

It is well established that general measures of quality of life are impaired in patients with IBD[1]. Sexual health is an important aspect of quality of life which is often overlooked in a routine gastroenterological consultation. Sexual problems in IBD often seem to be focused around three major factors: body image problems, difficulties with social relationships and impaired sexual function[2]. Crohn's disease (CD), in particular has been shown to have an impact on self-image, social relationships and sexual function[1]. Body image concerns are frequently found in IBD patients relating either to the direct physical effects of their disease such as weight loss, growth retardation as a result of chronically active disease in childhood, fistulae or perianal disease. The effect of surgery especially when a stoma is involved is associated with low self-esteem and poor body image. The side effects of steroids and other medications may lead to weight gain, hirsutism, skin changes and other features which promote feelings of unattractiveness. There is some evidence that psycho-social effects of stoma surgery performed in childhood, before puberty may be less severe than if such surgery is performed during the teenage and early adult years[3]. Partners of IBD patients with stomas have been found to be more likely to be able to accept the stoma than the patient themselves[4]. Psychological fears of loss of control of bodily functions and the fear of rejection by new or established partners in an intimate relationship all contribute to difficulties in social and sexual relationships. Counselling and practical advice and support is frequently helpful, but the need for it is not always identified in general gastroenterological practice.

Some studies have reported an increase in sexual difficulties including dyspareunia in women who have had surgery for IBD and there is some evidence that patients with IBD may delay or even defer pregnancy because of their disease[4,5]. Most of the reports of sexual dysfunction are in women with Crohn's disease. There are no specific reports of sexual dysfunction in women with ulcerative colitis (UC) who have not had previous surgery.

In men with IBD the risk of impotence after proctocolectomy is the main concern[4]. Advances in surgical technique have decreased although not eliminated post-operative sexual dysfunction, and this seems to apply to both conventional proctocolectomy and pouch surgery with a reported incidence of impotence of around 4%-8%[6]. It is important to remember that patients are often quite reluctant to discuss such delicate matters and tactful prompting and adequate time during the consultation is the key to their detection.

CONTRACEPTION

As there is clear evidence that the outcome of pregnancy is better in women with IBD who have quiescent disease at the time of conception, advice about contraception is keenly sought and opinions have differed over the years. In women who do not smoke and who have quiescent or mildly active Crohn's disease (CD), the use of a low dose combined oral contraceptive is not associated with increased disease activity compared with non-users[9,10]. There are no data about the thrombo-embolic complications of the oral contraceptive pill in IBD but this should be considered especially in patients with active UC.

INHERITANCE OF IBD

Individuals with IBD are often concerned that their children may inherit the illness. There is a familial increased risk in IBD which is stronger if the parent has CD. This risk also appears to be greater in Jewish families. The life-time risk factor for a child of a parent with CD is around 7%-9% of developing CD and about 10% for the development of IBD[11]. If both parents are affected the risk for any children may be up to 35%.

FERTILITY

Women with UC generally appear to have normal fertility, although one retrospective study from Scotland reported that women who had had surgery for IBD had increased infertility compared to the general population (25% *vs* 7%)[12]. Voluntary childlessness in people with IBD is still probably greater than in the general population however, possibly relating to fear of pregnancy or even inappropriate professional advice suggesting that pregnancy might be dangerous. In CD, fertility is probably normal in individuals with inactive disease[13,14]; however, fertility is impaired in women with active Crohn's disease. This relates directly to disease activity and fertility appears to revert to normal with the induction of remission. Women whose IBD develops before their first pregnancy have been shown to have fewer pregnancies than population controls. In women who had had a pregnancy prior to the onset of IBD, however, they seem to have a similar reproductive history to a control population[13].

Increasingly, men are concerned about fertility and other issues of reproductive health. Sulphasalazine has been known for many years to cause reversible semen abnormalities with impairment of fertility in up to 60% of men on the drug. This effect is reversed two months after withdrawing sulphasalazine. Men express concerns about the safety of immunosuppressive agents in terms of fertility and the risk of congenital abnormality, but there are no reliable data at all for guidance in this area[15].

PREGNANCY OUTCOME IN IBD

In recent years there has been an increased interest in the outcome of pregnancy in IBD and a number of epidemiological surveys and case controlled studies have been published. In UC, there is a large body of evidence suggesting that the frequency of spontaneous abortion, still birth and congenital abnormality are no different to that in the general population[16-19]. In quiescent Crohn's disease, pregnancy outcome, in terms of spontaneous abortion, still birth and congenital abnormality, is also no different from the general population[14,17]. Active Crohn's disease at the time of conception or during pregnancy significantly increases foetal loss and pre-term delivery and it appears that disease activity rather than medical treatment accounts for the adverse outcomes[20,21].

A large prospective population-based study looked at adverse pregnancy outcomes in 239773 single pregnancies in Sweden over a two year period. This included 756 pregnancies in women with IBD. This is the expected number of IBD pregnancies for this population[22]. No significant increase in the most serious adverse outcomes of still birth or infant death in the first year of life was found in the IBD mothers. There was also no significant increase in babies which were small for gestational age. There was however, a significantly increased risk of pre-term birth (odds ratio 1.81, 95% confidence intervals 1.06-3.07) at less than 33 weeks and for 33-36 weeks (odds ratio 1.48, confidence intervals 1.0-1.19), and of low birth weight (less than 1500g, odds ratio 2.15 confidence intervals 1.11-4.15). IBD patients also had an increased caesarean section rate (15% *vs* 10%). These estimates were not affected by adjustments for maternal age, parity and smoking. There was, however, not any information about whether the mothers experiencing pre-term delivery and low-birth weight babies had UC or CD, or whether these were women with active disease during their pregnancies. More recent studies from France and Denmark have confirmed this small increase in pre-term birth and low birth weight, particularly in CD[23,24].

INFLUENCE OF PREGNANCY ON IBD ACTIVITY

In any woman with quiescent IBD at the time of conception, the likelihood of a flare-up of IBD during pregnancy or the puerperium is no greater than in any other year of her life. Active UC at the time of conception is associated with continuing disease activity in about two thirds of pregnant women. Chronic activity will continue throughout pregnancy in about a quarter of these patients and in about 45% the activity may actually worsen[14]. This therefore constitutes a strong indication for aggressive medical treatment, since if remission can be induced by medical therapy, the course of pregnancy is similar to that in patients with quiescent disease at conception. About two thirds of women with active Crohn's disease at the time of conception will continue to have disease activity throughout the pregnancy and in about half of these there will be a deterioration during the pregnancy (Table 2). Therefore it is inadvisable to conceive when CD is active, but if conception occurs, an aggressive therapeutic strategy is indicated as there is clear evidence in Crohn's disease that disease activity is associated with pre-term birth and low-birth weight and some suggestion that early miscarriage may be increased.

MANAGEMENT OF IBD IN PREGNANCY

All the evidence suggests that maintenance treatment, certainly with aminosalicylates, should be continued throughout pregnancy and flare-ups of disease activity should be investigated and treated appropriately as in a non-pregnant patient. All pregnant women are very concerned about taking medication during gestation and it is essential if at all possible that these issues are broached and discussed well in advance of a planned pregnancy enabling informed discussion with the patient and her partner once she becomes pregnant.

Nutrition is extremely important in pregnancy, the average weight gain during a normal pregnancy being between 11 and 16kg. Folic acid supplementation is recommended for all pregnant women but in IBD, patients who may have folic acid deficiency or be taking drugs which interfere with folic acid metabolism, a dose of 5mg daily should be recommended rather than the usually advised dose of 400μg daily. It is extremely important to remember that early nutritional intervention is indicated in a woman with active disease who may not be gaining weight. Women with active CD in pregnancy have received an elemental diet as primary therapy with rapid resolution of symptoms[25] and supplemental feeding may be required in sick IBD patients who are failing to achieve the expected weight gain during pregnancy.

Proper investigation of gastrointestinal symptoms is not contraindicated during pregnancy and indeed it is important in order to ensure that appropriate treatment is advised. Blood

investigations are often difficult to interpret in pregnancy due to haemodilution, and therefore sigmoidoscopy and indeed colonoscopy may be indicated in some circumstances. Both these investigations have been shown to be safe in a small study[26]. Monitoring foetal heart rate during endoscopy has not shown any adverse effects and there has been no evidence of increased premature labour or foetal abnormalities following endoscopy in pregnancy. Radiographic imaging should obviously be avoided unless obstruction, perforation or toxic megacolon are suspected and if possible in this situation, plain abdominal films should be used rather than CT or barium studies which involve much higher radiation exposure. Ultrasound may be useful, for example to identify an intra-abdominal collection in patients with Crohn's disease.

Table 2 Influence of pregnancy on IBD activity
Meta-analysis data from Reference 17

INACTIVE disease at conception. Likelihood of relapse during pregnancy

	Ulcerative colitis	Crohn's disease
Number of pregnancies	528	186
Relapse	34%	27%

ACTIVE disease at conception. Pattern of disease activity in pregnancy

	Ulcerative colitis	Crohn's disease
Number of pregnancies	227	93
Better	27%	34%
No change	24%	32%
Worse	45%	33%

TREATMENT OF IBD IN PREGNANCY

Drug treatment

The safety (or risk) of drug therapy during pregnancy is of prime concern to any pregnant woman. In women with IBD, the most important factors in relation to treatment are to emphasise the importance of planned pregnancy when the disease is quiescent and the fact that, if conception occurs with active IBD, inducing remission with medical therapy carries less risk than continuing a pregnancy without treatment[26].

First line agents

Aminosalicylates and sulphasalazine have been widely used in pregnancy in IBD. They are safe in conventional doses and should be used for maintenance or induction of remission in the same way as in a non-pregnant individual. Both aminosalicylates and sulphasalazine are poorly systemically absorbed and there is little placental transfer from mother to foetus[27-29]. No evidence of teratogenicity has been demonstrated and the outcome of pregnancy has been shown to be similar to that in healthy women. There have however, been reports of nephrotoxicity in the foetus of a woman taking a high dose of mesalazine[30]. High dose aminosalicylates are not therefore advisable during pregnancy.

Corticosteroids are well tolerated in human pregnancy. They cross the placental barrier but there has been no convincing evidence of teratogenesis despite reports of cleft lip and palate in the past. Immune deficiency in the new-born infant is theoretically possible, but is very rarely reported in clinical practice. In IBD patients taking corticosteroids during pregnancy, no increase in foetal complications have been found compared to the general population[29]. It is, therefore, important to use corticosteroids in women with moderate to severe disease activity in pregnancy in the same way as in a non-pregnant patient.

Second line agents

Azathioprine and 6-mercaptupurine have never been demonstrated to be teratogenic in humans and do not have any effects on human interstitial cell function or gametogenesis in the doses used in clinical practice[31,32]. There is extensive experience of the use of these drugs in pregnancy in renal transplant recipients and in patients with systemic lupus erythematosus who are unable to discontinue immunosuppressive treatment, with very little evidence of adverse effect[33,34]. However, because of the theoretical possibility of teratogenesis in animals, gastroenterologists have been very cautious in advising discontinuation of azathioprine prior to pregnancy or even termination of pregnancy in women conceiving on azathioprine. In a small retrospective study on the use of azathioprine in pregnancy and IBD there were no serious adverse outcomes. All the women conceived while taking the drug and half of them continued to take it throughout gestation[35]. In a larger study, looking at pregnant women with IBD on 6-mercaptupurine, there were also no adverse outcomes of pregnancy[36], although in this study only a small number of patients actually continued to take the drug throughout their pregnancy.

In general therefore, if a patient is established and well on azathioprine or 6-mercaptupruine and it is felt to be essential to continue this drug to retain remission, after full discussion with the patient and her partner, it is reasonable to decide to continue treatment during pregnancy. It is essential that this decision is made by the patient who has been presented with the evidence. In view of the complications which may arise at the start of treatment with these agents, it is not advisable to commence treatment for the first time during pregnancy.

Cyclosporine has been used in patients with severe UC which has not responded to steroids in an attempt to avoid surgery which is said to carry a high risk of foetal mortality[37]. Cyclosporine is not teratogenic and has been extensively used in transplant recipients and lupus patients without increased adverse effects[38-41]. Cyclosporine is a highly toxic drug however, carrying the risk to the mother of hypertension, nephrotoxicity and hepatotoxicity and it would therefore appear to be undesirable in almost all circumstances except the avoidance of urgent colectomy in a patient with fulminant UC.

Methotrexate is mutagenic and teratogenic and is therefore contraindicated in pregnancy or immediately prior to conception. There are reports of women with IBD who have conceived while taking methotrexate, who had a high incidence of severe congenital abnormalities in the babies born from these pregnancies, with neural tube defects and other severe deformities[42,43]. In a woman who conceives on methotrexate and will not agree to a therapeutic abortion, however, the methotrexate must obviously be stopped immediately and high dose folic acid replacement is indicated.

Anti-TNF antibodies There are currently no data about pregnancy in patients receiving therapy. By definition this therapy is used for people with severe active CD, and for the present pregnancy should be discouraged during this treatment.There is no evidence that it is safe to continue with the pregnancy if conception occurs during treatment with anti-TNF antibody.

Antibiotics: Metronidazole has not been shown to have

adverse effects and has been used extensively in pregnancy by gynaecologists to treat bacterial vagionosis. There is not any evidence of increased risk of spontaneous abortion or congenital abnormality in humans[44,45]. Ciprofloxacin and other quinolone antibiotics have been suggested to be associated with musculoskeletal problems in foetuses in animal studies but this has not been substantiated in humans. Ciprofloxacin has been used during pregnancy with no increased incidence of spontaneous abortion or congenital abnormality and follow-up of the children born from these pregnancies is ongoing[46].

Surgery

Patients who have undergone previous surgical intervention for UC or CD do not appear to have any increase in problems during pregnancy compared to the general population. Patients who have undergone colectomy and ileostomy or ileal pouch operations can expect a normal outcome of pregnancy. In women with an ileostomy, stomal prolapse has been reported following hyperemesis. This can cause discomfort and require revision of the ileostomy post-partum. Opinions vary about the need for delivery by caesarean section following pouch surgery. Some centres have published data suggesting that vaginal delivery is appropriate. There are no published long-term studies of the effect of vaginal delivery on pouch function although one study demonstrated no short-term deterioration of pouch function[7,8]. Some surgeons advise elective caesarian section to avoid risk of sphincter damage.

Surgery for acute indications during pregnancy has been reported to carry a high risk of foetal loss and is generally felt to be inadvisable. Reports of a 60% risk of foetal loss for urgent colectomy in UC may be an overestimate in the 21st century. A small case report from Manchester recently reported six women who had surgery for intraperitoneal sepsis in Crohn's disease during pregnancy. Five healthy babies resulted from these pregnancies although one miscarriage occurred in a patient with a surgical complication[47].

Breast-feeding

Breast-feeding is the best option for mother and baby in most circumstances. Concerns about breast-feeding are related to worries about the secretion of drugs in breast milk. Sulphasalazine and the aminosalicylates are poorly absorbed from the bowel and very small amounts are excreted in breast milk. It is safe to breast-feed while taking these medications with the small reservation that it is inadvisable to take high doses of amino-salicylates as there is one report of renal impairment in a child of a mother on a high dose of mesalazine[37].

Prednisolone is also concentrated poorly in breast milk and the amount received by the infant is minimal[48]. If breast-feeding is deferred until 4 hours after taking steroids, this further decreases the dose to the infant. It is therefore considered safe to breast-feed while taking these first-line agents.

There are almost no data on the safety of breast-feeding while taking other agents used to treat IBD. Breast-feeding is not recommended by the manufacturers of azathioprine or 6-mercaptopurine. Many transplant recipients and patients who take these drugs for rheumatological disorders and who must remain on azathioprine have breast-fed without reports of ill effect to the baby. This is another situation where full discussion with the mother and her partner may allow them to make an informed decision on the basis of what little evidence is available.

CONCLUSION

The key to the management of the pregnant IBD patient is to have discussed the issues relating to reproductive health prior to conception. Counselling from teenage years in young patients with IBD will help them to understand the importance of planned pregnancy. Fertility can be expected to be normal except in women with active Crohn's disease. The outcome of pregnancy is usually normal but the risks are associated with active disease and more strongly with Crohn's disease. Disease activity is definitely associated with premature delivery and low birth weight. Drug treatment should be discussed in advance of pregnancy and it would seem logical that women should be encouraged to continue maintenance treatment with aminosalicylates or sulphasalazine during pregnancy. If an attack occurs it should be investigated and treated as in a non-pregnant patient, except that use of x-rays should be minimised. Patients maintained on azathioprine may wish to continue with the drug if it is important to retain remission after full discussion. A patient with quiescent IBD can expect normal fertility, normal outcome of pregnancy and there is no contraindication to breast-feeding. The management of IBD in pregnancy is a good example of the therapeutic partnership between patient and doctor. Education and communication are key, active disease is the greatest risk to the outcome of pregnancy and drug therapy may be necessary and if so is safe.

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• REVIEW •

Non-invasive investigation of inflammatory bowel disease

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Abstract

The assessment of inflammatory activity in intestinal disease in man can be done using a variety of different techniques. These range from the use of non-invasive acute phase inflammatory markers measured in plasma such as C reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) (both of which give an indirect assessment of disease activity) to the direct assessment of disease activity by intestinal biopsy performed during endoscopy in association with endoscopic scoring systems. Both radiology and endoscopy are conventional for the diagnosis of inflammatory bowel disease (IBD). However these techniques have severe limitations when it comes to assessing functional components of the disease such as activity and prognosis. Here we briefly review the value of two emerging intestinal function tests. Intestinal permeability, although ideally suited for diagnostic screening for small bowel Crohn's disease, appears to give reliable predictive data for imminent relapse of small bowel Crohn's disease and it can be used to assess responses to treatment. More significantly it is now clear that single stool assay of neutrophil specific proteins (calprotectin, lactoferrin) give the same quantitative data on intestinal inflammation as the 4-day faecal excretion of 111Indium labelled white cells. Faecal calprotectin is shown to be increased in over 95% of patients with IBD and correlates with clinical disease activity. It reliably differentiates between patients with IBD and irritable bowel syndrome. More importantly, at a given faecal calprotectin concentration in patients with quiescent IBD, the test has a specificity and sensitivity in excess of 85% in predicting clinical relapse of disease. This suggests that relapse of IBD is closely related to the degree of intestinal inflammation and suggests that targeted treatment at an asymptomatic stage of the disease may be indicated.

Subject headings inflammatory bowel diseases; permeability; NCAM; membrane glycoproteins

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INTRODUCTION

Distinguishing irritable bowel syndrome from inflammatory bowel disease

Gastroenterologists are often faced with the diagnostic difficulty of differentiating patients with the irritable bowel syndrome (IBS) from those with organic intestinal pathology, in particular inflammatory bowel disease (IBD). Many symptoms are common to both conditions including abdominal pain, bloating, excessive flatus and altered bowel habit while other clinical features such as a predominance of diarrhoea and rectal bleeding will increase the likelihood of organic disease. Although symptoms are a surprisingly good guide to a diagnosis, most clinicians proceed to and rely on laboratory tests to aid in the differential diagnosis. Certainly, fulfilling the ROME criteria[1,2] and having a normal full blood count, routine biochemical screening, ESR and CRP are reassuring indicators pointing to IBS. As a result a number of investigators[1,3,4] have recommended a straightforward approach to evaluation and treatment of patients with IBS based on the use of the Rome criteria as a means of cost effective management. Despite this the use of the Rome criteria has not been universal and is largely confined to use as entry criteria into research studies of patients with IBS. The concern for gastroenterologists is that some patients with organic intestinal disease will be incorrectly diagnosed if excess reliance is placed upon these criteria. They may therefore feel compelled to exclude all organic disease using invasive diagnostic investigations as objective evidence for there being no other significant pathology. This has significant implications for health care costs as well as exposing patients to the inherent risks associated with invasive procedures.

Managing inflammatory bowel disease

Once IBD is diagnosed the treatment involves induction and subsequently maintenance of remission based largely on clinical disease activity indices[5,6] and the physicians global assessment of well-being. The problem with the use of clinical disease activity scores is that they are a composite of quantitative subjective symptoms that are affected by non inflammatory processes such as fibrous strictures, fistulas and previous surgical intervention. As a guide to clinical decision making, many clinicians therefore use nonspecific laboratory tests to document relapse of disease and radiology and radio isotopic techniques to distinguish between actively inflamed disease and fibrotic strictures. In addition a number of blood tests (erythrocyte sedimentation rate (ESR), orosomucoid, C -reactive protein (CRP), platelet, and white cell counts, IL-6, TNF-α, IL-1β)[7-11] which reflect the systemic consequences of inflammation, have been proposed as predictors and/or markers of clinical relapse of IBD with varying degrees of success. However, the overall predictive values of these different variables in identifying patients at risk of relapse have in general been disappointing. This is possibly due to the fact that these measures are non-specific, affected by a variety of non-intestinal diseases[12] and most importantly do not measure the intestinal inflammation directly. Patients with clinically active IBD can have normal serological inflammatory indices while clinically quiescent disease may be associated with abnormal blood tests. In particular, there is a major discrepancy between severity of

symptoms and macroscopic evaluation of disease activity in patients with Crohn's disease limited to the colon.

Intestinal function tests

Although imperfect the above approach to diagnosis and management of patients with IBD remains the norm and in general it works well for the vast majority of patients. However, few would argue with the notion that there is scope for improvement. Where is such improvement to come from? Recently, investigators have turned to direct tests of intestinal function. Such tests provide new, direct and different information. They have the potential to be used as a diagnostic screen for intestinal disorders as well as providing prognostic information for the behaviour of the disease. At present there are three kinds of intestinal function tests that could fulfill the above promise, two of which (intestinal permeability and white cell scans) have a 20 year history. The third, namely direct assay of faeces for inflammatory markers, we suspect has the greatest potential. There follows a brief outline of how these tests can provide information that is not obtainable by other methods and their possible use in the day-to-day management of patients with IBD.

INTESTINAL PERMEABILITY

Permeability refers to that property of a membrane that enables passage of a solute by unmediated diffusion. The diffusion of a solute across a simple membrane is determined by the structure of the membrane (in terms of its composition, charge, thickness, etc.), the physicochemical properties of the solute (like molecular size, shape, charge and solubility) and its interaction with the media or solvent. Intestinal permeability is assessed non-invasively *in vivo* by measuring urinary excretion of orally administered substances. The ideal permeability probe is water-soluble, non-toxic, non-degradable and not metabolised before, during or after permeating the intestine[13]. The probes should preferably not be naturally present in urine, be completely excreted in the urine following intravenous administration and be easily and accurately measurable. Fordtran *et al*[14] were instrumental in the development of ideas for assessing intestinal permeability in man but it was Menzies who introduced oligosaccharides as test substances for the non-invasive assessment of intestinal permeability[15] in 1974, and later formulated the principle of differential urinary excretion of orally administered test substances[16]. The importance of the differential urinary excretion principle is that it overcomes most if not all the problems associated with the use of a single test substance, where urinary excretion is dependent on a number of pre- and post-mucosal factors as well as intestinal permeability. The differential principle advocates that a nonhydrolyzed disaccharide (i.e. lactulose) and a monosaccharide (L-rhamnose or mannitol) are ingested together. As the pre- and post-mucosal determinants of their excretion affects the two test substances equally and the differential 5 hour urinary excretion ratio (ratio of lactulose/L-rhamnose) is not affected by these variables the urinary excretion ratio becomes a specific measure of intestinal permeability.

Tests of intestinal permeability were initially designed to allow reliable non-invasive detection of patients with untreated coeliac disease[16]. The tests have since come to be viewed as synonymous with assessing intestinal barrier function. In clinically active small bowel Crohn's disease the vast majority of patients (>95%) have an increase in the differential urinary excretion of ingested di-/mono-saccharides (lactulose/L rhamnose or mannitol) and half of those with Crohn's colitis are abnormal[13]. These figures are marginally improved with the use of 51CrEDTA, which requires a 24-hour, as opposed to a 5-hour urinary collection. The vast majority of patients with ulcerative colitis have normal small intestinal permeability when assessed by these methods. However, tests of intestinal permeability have not found widespread application as screening tests to discriminate between patients with Crohn's disease and IBS. The reason for this is probably that the urinary sugar analysis is time consuming and demanding, and there may be some concern that the tests lack specificity being abnormal in a variety of small intestinal diseases (Table 1). At first sight the test appears to identify a number of "clinically irrelevant" diseases, which usually translates into disease for which no treatment is available, but in practice the tests seem often to identify small intestinal pathology where none was previously expected, thus expanding the number of identifiable small bowel pathologies.

There have been attempts to use intestinal permeability as an index of disease activity in Crohn's disease. In general these have been disappointing because the degree of increase in the differential urinary excretion of lactulose/L-rhamnose or the excretion of ^{51}CrEDTA is dependent on localisation and extent of disease within the small bowel as well as activity of the inflammation[13]. Abnormalities in intestinal permeability may, however, be used as a predictor of imminent relapse of quiescent Crohn's disease. Three studies have now shown that, in patients with Crohn's disease in clinical remission, an increased intestinal permeability can predict those at significant risk of relapse of disease in the next few months[17-19]. The strength of this association is difficult to assess from the published studies. Nevertheless, less than 20% of those with normal intestinal permeability appear to relapse over the ensuing 6 months. Interestingly, elevated levels of IL-6 in serum, which can be viewed as a surrogate marker of intestinal inflammation, also has a predictive value for relapse of Crohn's disease[8], but receiver operating curve (ROC) analysis shows relatively low sensitivity and specificity (70 and 50%, respectively). The permeability ratio differs from such indices in that it is not based on concentrations of plasma proteins but rather represents functional changes in the intestinal mucosa, a direct consequence of intestinal inflammation.

The clinical implications of these findings are discussed later.

WHITE CELL SCANS AND FAECAL EXCRETION

Intense neutrophil recruitment to the intestinal mucosa is a feature common to inflammatory bowel diseases[20]. When a patient's own radiolabelled neutrophils are re-injected they migrate to sites of acute inflammation as well as to the liver, spleen and bone marrow[21]. Segal, Saverymuttu and Chadwick were instrumental in the introduction, validation and application of the 111Indium white cell technique for use in gastroenterology[20,22]. The technique visualises inflamed segments of bowel and quantitates the degree of inflammatory activity[20,23-26].

A number of studies have established that abdominal scans are abnormal in virtually all patients with active IBD; their accuracy in localisation of disease and distinguishing between actively inflamed and fibrous stricturing disease has implications for treatment. It was suggested that the technique could be used to discriminate, with an accuracy approaching 100%, between patients with IBD and IBS at the first outpatient visit. In practice this suggestion was not followed up with relevant research.

When combined with measurement of the 4 day faecal

excretion of labelled white cells for quantitation of the inflammatory activity the technique becomes a formidable tool for research and investigation. The faecal excretion of the labelled white cells quantitate inflammation accurately and can be used to document therapeutic efficacy of various treatments in IBD[25,27]. It has also been used to define a number of enteropathies (NSAIDs, alcohol, chronic renal failure, hypogammaglobulinaemia, HIV-AIDS, etc.) where none were suspected or impossible to demonstrate by techniques other than perhaps the intestinal permeability tests (Table 1)[28]. The method is not disease specific, resembling that of the permeability tests, but it is specific for intestinal inflammation. This is not a drawback as it is a simple matter to distinguish between the inflammatory activity in patients with IBD and the above enteropathies, colonic cancer, diverticulitis, etc., since patients with active IBD have excretion values often an order of magnitude higher than the others.

Table 1 Some conditions reported to be associated with increased intestinal permeability

Nonsteroidal anti-inflammatory drugs	Inflammatory bowel disease
Alcohol	Ankylosing spondylitis
Renal failure	Coeliac disease
Abdominal radiation	Intestinal ischaemia
Cytotoxic drug treatment	Hypogammaglobulinaemia
Abdominal surgery	HIV infection
Fasting	Endotoxinaemia
Total parenteral nutrition	Multiorgan failure
Food allergy	Diabetic diarrhoea
Multiple sclerosis	Scleroderma
Cystic fibrosis	Reactive arthritis
Recurrent abdominal pain of childhood	Intestinal infections / bacterial overgrowth
Neomycin	Whipples disease
Acute and chronic liver disease	Sarcoidosis

Why has the white cell technique not been universally adapted for use as a diagnostic screen in IBD, and to assess disease activity? It requires expensive labeling facilities including labelling cabinets. The labeling procedure is time consuming, taking over 2 hours. The cost of isotope and material is in excess of £200 (US $300) and the radiation dose is not trivial if abdominal scans are carried out, being equivalent to that of a barium enema[29,30]. A complete 4-day faecal collection is also demanding and unpleasant for patients, occasionally requiring hospital admission.

Other methods have attempted to build on this success. One such is 99mTc labeling of white cells[31]. This is purported to give superior quality abdominal scintigraphy (which is not clinically important), but does not allow late (>4 hours) scanning, because the label comes off and is excreted into the bowel independent of white cell excretion. Furthermore a faecal collection provides no quantitative information on intestinal inflammation (as the Technetium comes off the white cells and is excreted in faeces) and the labeling requires the same facilities as the white cells.

Newer techniques include E-selectin scanning[32]. This method is derived from the more conventional labelled white cell scintigraphy, but uses a labelled antibody to E-selectin, which is over-expressed in endothelial cells at sites of inflammation. It has the advantage of studying a more fixed entity that (unlike white cells) will not be shed at a variable rate into the bowel lumen and is applicable to the occasional patient with intestinal inflammation who is neutropenic.

In our opinion, the greatest impact that the white cell technique has had is that it ① emphasised that if a sensitive method is to be established for assessing intestinal function there are no shortcuts. Neurologists assess spinal fluid, respiratory physicians assess sputum, urologists urine and the gastroenterologist needs to come terms with the fact that faecal analysis is essential to obtain maximal information about the state of the intestine. ② emphasised that there is life beyond morphological assessment of the gut (x-ray and colonoscopic studies). ③ raised the possibility of dramatically changing our views on the treatment of IBD. Many patients with IBD in full clinical remission are shown to have significant intestinal inflammation[27,33]. At present treatment is non-specifically directed at maintaining remission (5-ASA, azathioprine, etc.). It seems highly probable that those patients with substantial inflammatory activity should be targeted for more aggressive therapy, in particular if they can be shown to be at significant risk of clinical relapse of disease. The analogy with the treatment of rheumatoid arthritis springs to mind. Here, first line treatment is directed to wards reducing the acute inflammatory component of the disease followed by a number of second line agents that can alter the natural history of the disease, reduce the frequency, duration and severity of relapses as well as reducing the joint damage.

FAECAL MARKERS

Faecal analysis is unpleasant but has been with us for a long time. Measure of electrolytes and osmolality helped in the differential diagnosis of diarrhoea in children. Faecal fats were a widespread screening test for steatorrhoea for a while and faecal occult bloods have become the yardstick for colorectal screening with which other methods need to be compared. An improvement on these techniques was the introduction of radioisotopically labelled compounds (labelled red blood cells, proteins, white cells) which provided quantitative and functional data and which was event specific (blood loss, inflammation, protein losing enteropathy, etc.) but non-specific for disease.

The inflamed hyperpermeable mucosa of patients with inflammatory bowel disease is associated with increased protein loss into the bowel lumen[34]. Studies using radiolabelled proteins have demonstrated that there is faecal protein loss in patients with active Crohn's disease and it may therefore be a useful marker of disease activity. Other studies have shown faecal α1 antitrypsin clearance to be a useful indicator of protein losing enteropathy[35] and that in patients with inflammatory bowel disease, 72 hour faecal clearance of α1 antitrypsin is a useful method for quantitating intestinal protein loss[36,37]. Faecal clearance of α1 antitrypsin correlates with that of ^{51}Cr-albumin, and moderate rectal bleeding does not affect the α1 antitrypsin determination[36]. Random faecal α1 antitrypsin levels have been shown to be as useful as more prolonged collection in measuring Crohn's disease activity[38] and correlated with several other laboratory measures that have been proposed as indicators of Crohn's disease activity[39].

Concerns about costs, radiation, and the need for prolonged faecal collections all worked against these techniques for routine use, although many remain very important for research studies. The idea then emerged that it might be possible to assay for cell proteins or substances that are specifically associated with a certain cell type and which would then provide information on a specific component of the inflammatory cascade. Ferguson's Edinburgh group was instrumental in expanding this idea[40]. Concerned about bacterial degradation of markers they used a whole gut lavage method involving ingestion of polyethylene-based purgatives

(Kleenprep or GoLitely) for obtaining clear liquid faecal samples for analysis. The analysis took to various markers, such as immunoglobulins, neutrophils-specific elastase, andhaemoglobin. Separate studies showed that Crohn's disease could be identified with ease, and that the method had a greater sensitivity for colorectal cancer than the conventional faecal occult blood technique. Ideally suited for research, the method has as yet not found wide application for routine screening purposes, possibly because of the drawback of patients needing to ingest large volumes of liquid.

Direct analysis of markers in faeces would be a major advance on this method. Here the problem is initially the bacterial degradation of the marker necessitating swift sample handling. One such marker, TNF, has been successfully used in children and in HIV infection in adults[41,42]. However, it is now clear that it is not necessary for the marker to be completely non-degraded, provided that the antibody (most of these assays are ELISA's or radio immunoassay) is directed at an epitope of the molecule which resists degradation. One such assay is that for lactoferrin[43]. Lactoferrin is a relatively specific marker for neutrophils, in which it is present in cytoplasmic granules.

Faecal calprotectin

The greatest experience with analysis of faecal proteins is with calprotectin[44-48]. It accounts for up to 50% of the neutrophilic cytosolic protein while being resistant to colonic bacterial degradation. It is easily measured in faeces by a commercially available ELISA.

Calprotectin was first isolated from granulocytes by Fagerhol *et al*[49] and named L1 protein, but was later named calprotectin upon identification of its calcium binding and antimicrobial properties[50]. The protein is a heterocomplex protein consisting of two heavy (L1H) chains and one light (L1L) chain[51] which are non-covalently linked[52]. Calprotectin appears to play a regulatory role in the inflammatory process[53] and functions in both an antimicrobial[50,54] and antiproliferative capacity[55-57]. It has both bactericidal and fungicidal properties with minimal inhibitory concentrations comparable to those of many antibiotics[50]. It is released from the cells during cell activation or cell death. The C-terminal sequence of the L1H chain has been shown to be identical to the N-terminus of peptides known as neutrophil immobilising factors (NIF)[58]. It has been suggested that NIF activity of the L1H chain depends upon its phosphorylation[59] and that such an activity of calprotectin could be important for the accumulation of granulocytes, while calprotectin released from dead neutrophils, macrophages and epithelial cells might exert antimicrobial activity, possibly by depriving microorganisms of zinc[60,61]. Calprotectin may inhibit metalloproteinases[62] which may also involve the deprivation of zinc suggesting that it may limit their participation as enzymatic cofactors for invading organisms. Interest in calprotectin as a marker for inflammation in the gut followed the realisation that 111 Indium labelled granulocyte scans could be used to both visualise and quantitate the acute inflammation in the gut of patients with inflammatory bowel disease[20,23]. These findings led to the idea that an increased influx of granulocytes into the intestinal mucosa in conditions of inflammation might give increased levels of proteins from such cells in faeces.

Others[63] have demonstrated that eosinophilic granulocytes are the main cellular source of calprotectin in the normal gut mucosa. However, relatively high levels of calprotectin are found in the stools of normal individuals- about six times the plasma levels (which are about 0. 5mg/L) . This is compatible with data suggesting that in normal individuals most circulating neutrophils migrate through the mucosal membrane of the gut wall and thereby terminate their circulating life[64]. Subsequent lysis within the gut lumen and release of cytosolic calprotectin thereby accounts for the median faecal levels of 2.0mg/L seen in healthy controls[46,65]. The diagnostic use of faecal calprotectin in a broad spectrum of intestinal diseases has been studied by a number of groups with remarkable agreement between the results to date.

Inflammatory bowel disease

It is almost possible to extrapolate all the findings obtained with the white cell faecal excretion technique to the calprotectin method. Both techniques correlate with histopathological assessment of disease activity in ulcerative colitis and there is a very good correlation between the 4-day faecal excretion of white cells and faecal calprotectin concentrations[33,45], a correlation which is maintained when single stool calprotectin concentrations are used as opposed to 1 or 4 day collections. The faecal calprotectin concentration has a narrow normal range with an upper limit of 10mg/L. As with the white cells, faecal calprotectin has potential as a screening procedure to differentiate between patients with IBD and IBS and it may be useful for documenting a fall in intestinal inflammation in response to successful treatment of disease. Calprotectin concentration is rarely within the normal range in patients with IBD despite full clinical remission and is therefore a highly sensitive method for detecting such patients irrespective of disease activity. In over 100 patients with Crohn's disease of varying severity and activity only 4 had normal calprotectin concentrations[33].

Since the method is so much simpler than the white cell technique, requiring only a single stool sample, extraction and an ELISA, it has potential as a screening test to distinguish between patients with IBD and IBS in an outpatient setting. One study in over 225 patients showed that a cut off of 30mg/L had a 100% sensitivity and 94% specificity for this purpose[33]. Another showed that this was also the case when over 600 unselected consecutive patients were studied. Indeed a patient presenting with positive ROME criteria and a normal faecal calprotectin has virtually no chance of having IBD[66]. As a result of these studies it is now our practice not to investigate such patients by radiology or colonoscopy with considerable cost saving implications. The white radiolabelled cell technique demonstrated reduced intestinal inflammation in response to 5-ASA treatment and elemental diets. We have shown (unpublished) that improvement in calprotectin parallels the improvement in the excretion of labelled white cells in response to treatment with elemental diets. These techniques prove to be much more reliable and reproducible than the changes in clinical disease indices. It seems likely that the assay of faecal calprotectin will become an integral part of the assessment of therapeutic efficacy of the acute inflammation in future treatment trials in patients with IBD.

Apart from screening and assessing response to treatment, the faecal calprotectin has a further major advantage over the white cell labeling technique in predicting relapse of IBD. It has been shown that, in patients with clinically quiescent IBD (ulcerative colitis and Crohn's disease), faecal calprotectin values above 50mg/L may be used to predict clinical relapse of disease within a few months with over 80% sensitivity[67]. Symptoms of inflammatory bowel disease often appear to be the direct consequence of the inflammatory process itself and often vary dependent upon

the location of the inflammation. Most patients with quiescent IBD have low-grade inflammation[27] and it is possible that symptomatic relapse occurs only when the inflammatory process reaches a critical intensity. Furthermore, as inflammation is a continuous process it may be that direct assessment of the level of inflammatory activity may provide a quantitative pre-symptomatic measure of imminent clinical relapse of the disease.

The clinical implications of this, if substantiated, are considerable as it might offer targeted treatment at an earlier stage, with less side effects, to avert the relapse, as well as assessment of new therapeutic strategies to maintain symptomatic remission[68]. At present this is done with some degree of success with the rather indiscriminate use of sulphasalazine, 5-ASA and azathioprine, all of which are associated with side effects. However the calprotectin method offers guidance as to whom to treat at this stage and with what kind of vigour. Theoretically such treatment should lead to a dramatic reduction in the frequency and severity of clinical relapses with an improvement in the patient's quality of life.

In addition, the identification of patients at high risk of relapse will improve the design of clinical trials to assess the efficacy of therapeutic regimes designed to maintain patients in remission. In most such trials, patients studied tend to be a heterogeneous mix of those with high and low risk of relapse. This introduces possible bias when assessing the response to a particular treatment regime due to the imbalance of high risk patients in each treatment arm. Stratification by risk group using faecal calprotectin would reduce the possibility of such a bias. It is also possible that a lack of power in detecting a response to treatment may be due to the study of a large number of patients at low/intermediate risk of relapse, in whom all treatments may show the same efficacy, and therefore clinical trials studying a homogenous high risk group may be more powerful in detecting a difference in treatment efficacy.

Much work remains to be done, some is already on its way, but what is clear is that gastroenterologists need to move with the times and start thinking along the lines that rheumatologists do, that is, to implement treatments that alter the natural history of the disease. We are now in possession of tests that have the potential to revolutionise our approach to treatment of patients with IBD. There are some hurdles to overcome. The most frequent criticism of the "faecal" tests is that they are unacceptable to patients and unpleasant to work with.

The faecal calprotectin and lactoferrin methods are the first wave of techniques that allow non-invasive assessment of specific and selective cellular components of the intestinal inflammatory cascade. At present these are useful for a variety of purposes, outlined above, but it is likely that it will be possible to estimate the participation of other cells. Many other cells of the inflammatory cascade are numerically increased in biopsy specimens from patients with a variety of gastroenterological conditions. Some, such as mast cells and eosinophils, are thought to play a central role in mediating intestinal allergic reactions[69]. However, both types of cell are found to be activated in a number of other gastrointestinal inflammatory diseases such as inflammatory bowel disease, coeliac disease, eosinophilic gastroenteritis[69] and collagenous colitis[70], suggesting that both cell types may be involved in the pathogenesis of chronic intestinal inflammation. It may therefore be possible, as for neutrophils and calprotectin, to identify mast cell granule proteins, such as tryptase and chymase, in faecal samples and use them as markers of a specific component of the intestinal inflammatory response. The long-term objective might be to fully automate a faecal sample assay method that provides specific information on the activity of acute inflammation (neutrophils), chronic inflammation (T-cells) and allergy (mast cells).

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• REVIEW •

Acute variceal bleeding: general management

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TREATMENT STRATEGIES FOR ACUTE VARICEAL BLEEDING

Background

Acute variceal bleeding has a significant mortality which ranges from 5% to 50% in patients with cirrhosis[1]. Overall survival is probably improving, because of new therapeutic approaches, and improved medical care. However, mortality is still closely related to failure to control haemorrhage or early rebleeding, which is a distinct characteristic of portal hypertensive bleeding and occurs in as many as 50% of patients in the first days to 6 weeks after admission *et al*[2]. Severity of liver disease is recognised as a risk factor for both early rebleeding and short-term mortality after an episode of variceal bleeding[3]. Active bleeding during emergency endoscopy (e.g. oozing or spurting from the ruptured varix) has been found to be a significant indicator of the risk of early rebleeding[4,5]. Increased portal pressure (HVPG>16mmHg) has been proposed as a prognostic factor of early rebleeding in a study of continuous portal pressure measurement immediately after the bleeding episode[6], and recently Moitinho *et al*[7] have shown a failure to reduce portal pressure more than 20% from baseline is associated with a worse prognosis as well as early rebleeding.

Effective resuscitation, accurate diagnosis and early treatment are key to reducing mortality in variceal bleeding. The aims are not only to stop bleeding as soon as possible but also to prevent early re-bleeding. Early rebleeding, as with the peptic ulcer disease, is significantly associated with worsening mortality[5]. Thus treatments regime should be evaluated not only in terms of immediate cessation of haemorrhage, but also in terms of providing a bleed free interval of at least 5 days. This allows some recovery of the patient, and provides an opportunity for secondary preventative therapy to be instituted.

Diagnosis

Upper gastrointestinal endoscopy is essential to establish an accurate diagnosis, as 26%-56% of patients with portal hypertension and GI bleeding will have a non-variceal source[8], particularly from peptic ulcers and portal hypertensive gastropathy. Endoscopy should be performed as soon as resuscitation is adequate, and preferably with 6 hours of admission. This may need to be done with prior endotracheal intubation if there is exsanguinating haemorrhage or if the patient is too encephalopatic because of the substantial risk of aspiration.

Despite many authors and junior doctors proclaiming endoscopic prowess, it is the authors opinion that a definitive endoscopic diagnosis during or shortly after upper GI bleeding can be difficult, because the view can be obscured by blood. A diagnosis of bleeding varices is accepted either when a venous (non pulsatile) spurt is seen, or when there is fresh bleeding from the O-G junction in the presence of varices, or fresh blood in the fundus when gastric varices are present. In the absence of active bleeding (approximately 50%-70% of cases) either the presence of varices in the absence of other lesions, or a "white nipple sign" - a platelet plug on the surface of a varix[9,10], suggests varices as the source of haemorrhage.

When the diagnosis is in doubt, repeat endoscopy during re-bleeding is mandatory as it will show a variceal source in over 75% of patients[8]. Gastric varices are particularly difficult to diagnose, because of pooling of blood in the fundus. Endoscoping the patient on the right side, with the head up may help. If the diagnosis is still not made, splanchnic angiography will establish the presence of varices, and may display the bleeding site if the patient is actively haemorrhaging.

In the true emergency situation in which the patient is exsanguinating and varices are suspected on the basis of history and examination, a Sengstaken Blakemore tube (SBT) should be passed[11]. If control of bleeding is obtained, varices are likely to be the source of haemorrhage. If blood is still coming up the gastric aspiration port, then varices are less likely to be the cause of blood loss (although fundal varices are not always controlled by tamponade). In practice the position of the SBT has to be re checked and adequate traction applied. If there is still continued bleeding the diagnosis of variceal bleeding should be questioned or fundal bleeding suspected, and emergency angiography performed.

Therapeutic aims in acute variceal bleeding

The important point is to treat the patient and not just the source of bleeding (Figure 1). The specific aims are: ① Correct hypovolaemia; ② Stop bleeding as soon as possible; ③ Prevent early rebleeding; ④ Prevent complications associated with bleeding; ⑤ Prevent deterioration in liver function.

It is important to identify those at high risk of dying during the initial assessment. Individuals in this category should have early definitive therapy, the precise treatment regimen depending on availability Predictive factors for early deaths are: Severity of bleeding[12], Severity of liver disease[13], Presence of infection[14], Presence of renal dysfunction[15], Active bleeding[4,5], Early

rebleeding[5,6,10,16,17], Presence of cardiorespiratory disease, and Portal pressure[7].

Early rebleeding is also associated with many of the same factors associated with mortality including infections[18] and is a strong indication of increased mortality.

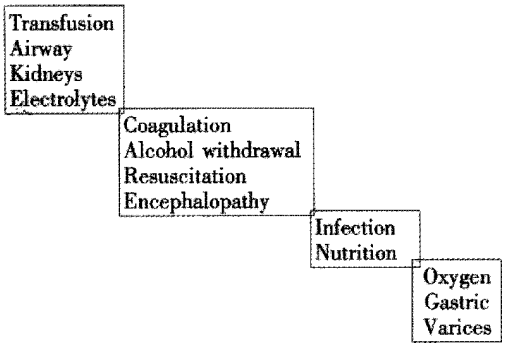


Figure 1 Take care in OGV.

Transfusion

Optimal volume replacement remains controversial. Following variceal bleeding in portal hypertensive animal models, return of arterial pressure to normal with immediate transfusion results in overshoot in portal venous pressure, with an associated risk of further bleeding[19]. This effect may not be relevant in clinical practice as volume replacement is always delayed with respect to the start of bleeding. Over-transfusion should certainly be avoided, and it is usual to aim for an Hb between 9-10g/dL, and right atrial pressure at 4 to 8mmHg, but fluid replacement may need to be greater in the presence of oliguria to be sure that the circulation is filled.

Large volume transfusion may worsen the haemorrhagic state, as well as lead to thrombocytopenia so that fresh frozen plasma and platelets need to be replaced. Optimal regimens for this are not known. It is reasonable to give 2 units of FFP after every 4 units of blood, and when the PT is >20 seconds. Cryoprecipitate is indicated when the fibrinogen level is less than 0.2g/L.

Platelet transfusions are necessary to improve primary haemostasis and should be used if the baseline count is 50×10^9/L or less. Platelet count may show little change following platelet transfusion in patients with splenomegaly. It is also routine to give intravenous vitamin K to cirrhotics, but no more than three doses of 10mg are required. Many cirrhotics have a background tendency of fibrinolysis. Transfusion of more than 15 units of blood results in prolongation of the prothrombin and partial thromboplastin time[20] in normal individuals, and occurs with smaller volumes of blood transfusion in cirrhosis.

Massive transfusion may cause pulmonary microembolism, and the use of filters is recommended for transfusions of 5L or more in normal humans[21]. Therefore, the routine use of filters in variceal haemorrhage could be considered, but rapid transfusions cannot be administered with these, limiting their application.

Further measures in patients who continue to bleed despite balloon tamponade include the use of desmopressin (DDAVP)[22] and antifibrinolytic factors. In stable cirrhotics the former produces a 2-4 fold increase in factors VIII and VWF, presumably by release from storage sites and may shorten or normalize the bleeding time[23]. However, in one study of variceal bleeding DDAVP in association with terlipressin was shown to be detrimental compared to terlipressin alone[24].

The use of antifibrinolytics has not been studied in variceal bleeding, although their role has been established in liver transplantation in our unit as well as others[25]. Their clinical utility should be established in clinical trials, and preferably when increased fibrinolysis has been documented. Recombinant factor VIII may be useful in variceal bleeding as it has been shown to normalize prothrombin time and bleeding times in cirrhotics[26].

PREVENTION OF COMPLICATIONS AND DETERIORATION IN LIVER FUNCTION

Infection control and treatment

Bacterial infections have been documented in 35%-66% of patients with cirrhosis who have variceal bleeding, with a incidence of SBP ranging from 7%-15%. However if only patients with ascites and gastrointestinal bleeding are considered, the incidence of SBP is very high. A recent meta-analysis has demonstrated that antibiotic prophylaxis significantly increased the mean survival rate (9.1% mean improvement rate, 95% CI: 2.9-15.3, P = 0.004) and also increased the mean percentage of patients free of infection (32% mean improvement rate, 95% confidence interval: 22 -42, $P<0.001$)[27](Figures 2,3). Finally our group has recently shown that bacterial infection, diagnosed on admission, is an independent prognostic factor of failure to control bleeding or early rebleeding[4]. These data may support a role of bacterial infection in the initiation of variceal bleeding[28]. All cirrhotics with upper gastrointestinal bleeding should receive prophylactic antibiotics whether sepsis is suspected or not. The optimal regimen is yet to be decided but oral or intravenous quinolones have been used.

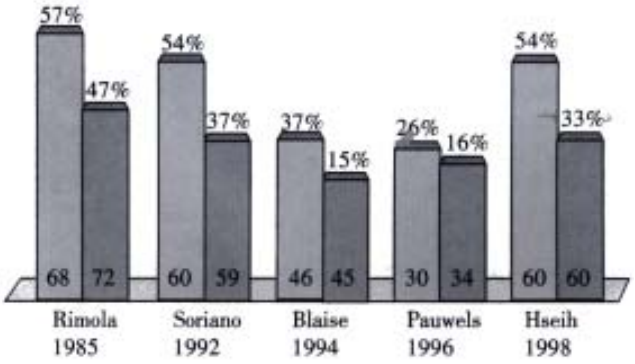


Figure 2 Antibiotics in GI bleeding in cirrhotics (Bernard 1999). Free of infection-mean improvement 32% ($P<0.001$).

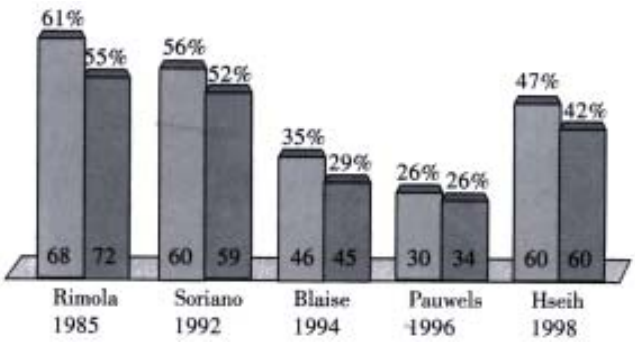


Figure 3 Antibiotics in GI bleeding in cirrhotics (Bernard 1999). Survival-mean improvement 9% (P = 0.0042).

Ascites and renal function

Renal failure may be precipitated by a variceal bleed, usually due to a combination of acute tubular necrosis, and hepatorenal syndrome (HRS) associated with deterioration in liver function and sepsis. HRS is associated with an over 95% mortality. Thus any iatrogenic precipitants must be avoided. The intravascular volume should be maintained preferably with Human Albumin Solution or blood initially. Normal saline should be avoided as it may cause further ascites formation. Catheterisation of the bladder and hourly urine output measurement is mandatory and nephrotoxic drugs should be avoided, especially aminoglycosides and non-steroidal drugs.

Dopamine was the first drug used due its vasodilator effect when given in subpressor doses. Dopamine is frequently prescribed to patients with renal impairment, and yet no studies have ever shown any convincing benefit[29,30]. It is our impression that occasionally a patient responds with an increase in urine output. It is therefore our practice to give a 12-hour trial of dopamine, and stop treatment if there has been no improvement of urine output.

Increasing ascites may occur shortly after bleeding, but should not be the main focus of fluid and electrolyte management until bleeding has stopped and the intravascular volume is stable. If there is a rising urea and creatinine, all diuretics should be stopped, and paracentesis performed if the abdomen becomes uncomfortable, re-infusing 8 gr. of albumin for every litre removed.

When the patient has stopped bleeding for 24 hours, nasogastric feeding can be commenced with low sodium feed. This avoids the need for maintenance fluid, and removes the risk of line sepsis. An unexplained rise in creatinine and urea may indicate sepsis. Evidence of sepsis should be sought by blood, ascitic, cannulae, and urine culture, and non-nephrotoxic broad-spectrum antibiotics commenced, regardless of evidence of sepsis. An undiagnosed delay in effective treatment of infection may increase mortality. In advanced cirrhosis, endotoxins and cytokines play an increasingly important role in advancing the hyperdynamic circulation and worsening renal function[31].

There is now increasing evidence for the use of vasopressin analogues in patients who develop renal impairment during the variceal bleeding episode, probably through maintenance of renal perfusion. Indeed, the beneficial effect of terlipressin with respect to bleeding and survival in trials to date may be through the prevention of this catastrophic complication[32-34].

Porto systemic encephalopathy

The precipitant factors include: haemorrhage, sepsis, sedative drugs, constipation, dehydration, and electrolyte imbalance. These should be evaluated and corrected. Hypokalaemia, hypomagnesaemia and hypoglycaemia may precipitate encephalopathy and should be aggressively corrected (e.g. a patient with ascites and a serum potassium of 3.0mmol/L is likely to require in excess of 100mmol over 24 hours).

As soon as the patient is taking oral fluid, lactulose 5mL -10mL QDS can be started. Phosphate enemas are also useful.

Alcohol withdrawal

It is important to be alert to the possibility of withdrawal from the patient's history. Encephalopathy and withdrawal may co-exist, and careful use of benzodiazepines may be required. Short acting benzodiazepines or oral chlormethiazole can be used. Intravenous preparations should be avoided, particularly of chlormethiazole, because of the risk of oversedation and aspiration.

Nutrition

Only a few cirrhotics are not malnourished[35], particularly with severe liver disease. This may be exacerbated in hospital, as often they do not want to eat, are "nil by mouth" because of investigations, and the food itself may be "unappealing".

A fine bore nasogastric tube should be passed 24 hours after cessation of bleeding to commence feeding. There is no evidence that this may precipitate a variceal bleed, and it allows treatment of encephalopathy in comatose patients and makes fluid management easier. It is extremely rare that parental nutrition is required.

Vitamin replacement: All patients with a significant alcoholic history should be assumed to be folate and thiamine deficient, and be given at least three doses of the latter intravenously. It is easier and more practical to assume all such patients are vitamin deficient rather than delay treatment whilst awaiting red cell transketolase activity levels.

Transfer of the patient with bleeding varices and use of balloon tamponade

Inter-hospital transfer should not be attempted unless the bleeding has been controlled, either with vasopressor agents/ endoscopic therapy or tamponade. If there is any suggestion of continued blood loss, and the source is known to be variceal, then a modified Sengstaken tube must be inserted prior to transfer.(i.e. with an oesophageal aspiration channel such as the Minnesota Tube).

DRUG THERAPY IN ACUTE VARICEAL BLEEDING

Pharmacological agents

The number of placebo controlled trials is relatively small, whilst in the case of octreotide there have been a large number of trials comparing with another form of therapy another drug, or sclerotherapy.

Vasopressin

Only 4 trials compared the efficacy of vasopressin with a placebo[36-39], and two of these studies used an intrarterial route of administration[38,39]. Using meta-analysis, there was a significant reduction in failure to control bleeding (Pooled Odds Ratio 0.22, 95%CI 0.12-0.43), but no benefit in terms of mortality.

Glypressin

The 3 trials are shown in Table 1. There has been criticism of these studies. The trial by Walker *et al* included other therapies, the timing of which is unclear, and the other two trials are hampered by insufficient patient numbers to avoid a type 2 error. These issues will be addressed in forthcoming trials.

A recent study in which terlipressin together with a nitrate patch or placebo was administered before arriving at hospital, based on reasonable evidence of bleeding varices, also showed a reduced mortality of glypressin in grade C patients. How this may have occurred deserves further examination, as there was no difference in blood pressure or early blood product requirements between the drug and placebo arms of the trial, and only three doses of the drug were given. Nonetheless, Terlipressin is a powerful splanchnic

vasoconstrictor, and may be preserving renal blood flow and hence preventing the development of hepatorenal syndrome, in a situation similar to the use of vasopressin anolgues in established hepatorenal failure.

It remains to be seen whether these data can be reproduced. Terlipressin is not licensed in the USA.

Table 1 Randomized studies of terlipressin

Study (ref)	Number of patients C/T	Child C %	Failure to control bleeding C/T	Death C/T
Terlipressin *vs* placebo				
Walker, 1986[40]	25/25	50	12/5	8/3
Freeman, 1989[41]	16/15	29	10/6	4/3
Soderlund, 1990[42]	29/31	33	13/5	11/3
Levacher, 1995[43]	43/41	81	23/12	20/12
POR (95% CI)			0.33 (0.19-0.57)	0.38 (0.22-0.69)
P value			0.0001	0.001

Somatostatin/Octreotide

The first 2 placebo controlled trials came to opposite conclusions. The trial by Valenzuela *et al* suggested that somatostatin is no more effective than placebo, when the end point was a bleed-free period of 4 hours[44]. Furthermore, the 83% placebo rate is the highest reported in the literature. In contrast, the trial by Burroughs *et al* reported a statistically significant benefit for somatostatin in controlling variceal bleeding over a 5 day treatment period, with failure to control bleeding seen in 36% of patients receiving somatostatin, compared with 59% of patients receiving placebo[45]. This emphasizes the problem of differences in end point selection, making meaningful comparisons difficult. The third study[46] also showed no effect of somatostatin, but it took 5 years to recruit 86 patients.

There has been only one randomised placebo controlled trial examining the efficacy of octreotide, and this has only been published in abstract. 383 patient admissions were randomised to 5 days octreotide or placebo. 58% of bleeding episodes in the drug arm were controlled compared with 60% in the placebo arm[45].

Trials comparing drug with drug have shown little statistical difference, though the side effect profile has generally favoured somatostatin/octreotide[47]. Trials in which drugs are examined in association with sclerotherapy are reviewed below.

Randomised controlled trials of emergency sclerotherapy in the management of acute variceal bleeding

Injection sclerotherapy, first introduced in 1939 and "rediscovered" in the late 1970's, rapidly became the endoscopic treatment of choice for the control of acute variceal bleeding. Paradoxically the best evidence for the value of sclerotherapy in the management of acute variceal bleeding has come from a comparatively recent published study by the Veterans Affairs Cooperative Variceal Sclerotherapy Group[48]. In this study sclerotherapy, compared to sham sclerotherapy, stopped haemorrhage from actively bleeding esophageal varices (91% in sclerotherapy arm compared to 60% in sham sclerotherappy, $P<0.001$) and significantly increased hospital survival (75% *vs* 51%, $P = 0.04$).

Today it is generally accepted that sclerotherapy should be performed at the diagnostic endoscopy, which should take place as soon as possible, because there is evidence that this is beneficial compared with delayed injection. Volumes of sclerosant should be small, 1-2 mls in each varix and should be applied in the distal 5cm of the oesophagus. More than two injection sessions are unlikely to arrest variceal bleeding within a 5-day period and are associated with significant complications rate including ulceration and aspiration[2]. Several sclerosing agents have been used for injection ie. polidocanol 1%-3%, ethanolamine oleate 5%, sodium tetradecyl sulfate 1%-2% and sodium morrhuate 5%. There is no evidence that any one sclerosant can be considered the optimal sclerosant for acute injection. As it has been shown that a substantial proportion of intravariceal sclerosant ends up in the paravariceal tissue and vice-versa there is no evidence that one technique is better than the other. One of the main shortcomings of sclerotherapy is the risk of local and systemic complications-although this varies greatly between trials and may be related to the experience of the operator.

Sclerotherapy vs drugs

There are 10 studies, including 921 patients: vasopressin was used in 1[49] terlipressin in 1[50], somatostatin in 3[51-53], and octreotide in 5[54-58], involving 921 patients. The evaluation of the treatment effect was performed at the end of the infusion of the drug (from 48hrs to 120hrs). The overall efficacy of sclerotherapy was 85% (range 73%-94%) in studies of 12 to 48h drug infusion[49,52-56,58] and 74% (68% -84%) in studies of 120hrs drug infusion[49,51,57]. There was significant heterogeneity ($P<0.05$) in the evaluation of failure to control bleeding in these studies was, which was mainly due to the different extent of benefit from sclerotherapy rather than different outcomes in individual studies: only two of the ten studies[55,56], reported that drugs were better than sclerotherapy but in neither did this reach statistical significance. Failure to control bleeding was statistically significantly less frequent in patients randomized to sclerotherapy (Der Simonian and Laird method: POR, 1.68 [95%CI, 1.07-2.63]). The NNT to avoid one rebleeding episode is 11 (95%CI, 6-113). Publication bias assessment showed that 9 null or negative studies would be needed to render the results of this meta analysis non-significant.

There was no significant heterogeneity in the evaluation of mortality in these studies: only two studies[54,56] reported a lower mortality in the drug arm but in neither was this statistically significant. Overall there were statistically significantly fewer deaths in patients randomized to sclerotherapy (POR, 1.43 [95% CI, 1.05-1.95]). The NNT to avoid one death is 15 (NNT, 15 [95% CI, 8-69]).

Finally the type of complications recorded in 8 studies[50-57] differed considerably, resulting in a significant heterogeneity ($P=0.04$). Four studies reported more complications in the sclerotherapy arm[51-54] while 3 reported more complications[50,55,57] in the drug arm and one found equal numbers in both arms[56]. The meta analysis showed a trend in favour of drug treatment but the result was not statistically significant (Der Simonian and Laird method:

POR, 0.71 [95% CI, 0.41-1.2]).

Sclerotherapy plus drugs vs sclerotherapy alone

This group comprised 5 studies[59-64] including 610 patients which compared sclerotherapy plus somatostatin, octreotide, or terlipress in with scleotherapy alone. Only three studies were placebo controlled[59,60,62]. Combination therapy was more effective (POR 0.42; 95%CI 0.29-0.6; failure to control bleeding sclerotherapy + drugs 22%, sclerotherapy alone 38% ARR 16, NNT=6, 95%CI 4-10). No effect on mortality was demonstrated. Only two studies provided data on complication[59,60]. There were no significant differences between arms.

Sclerotherapy vs variceal ligation

There are only two studies specifically designed to compare sclerotherapy with variceal ligation for the management of the acute bleeding episode[65,66]. Other data come from 10 studies of long term sclerotherapy versus variceal ligation[67-76]. There was no statistical heterogeneity (P=0.21) in the analysis of failure to control bleeding from the twelve studies[66-77], including a total of 419 patients. There was no difference between the two treatment modalities, although there was a trend in favour of variceal ligation (POR, 0.66 [95% CI, 0.36-1.18]). Short-term mortality was reported only in two studies[66,77] in both there was a trend in favour of variceal ligation but the result was not statistically significant. Finally only the two studies specifically designed to compare emergency sclerotherapy with variceal ligation[66,77] reported incidence of complications. Complications were less frequent in the variceal ligation arm in both studies and the result reached statistical significance in one[66].

Randomised controlled trials of emergency surgery in the management of acute variceal bleeding

Four randomised trials, performed during the previous decade, compared sclerotherapy to emergency staple transection[78-81]. Failure to control bleeding was reported only in two of these studies, with divergent results. Teres *et al*[80] reported that efficacy of transection in their study was only 71%, the lowest in the literature, compared to 83% in the sclerotherapy arm. In contrast in the largest study performed by Burroughs *et al*[81], a 5 day bleeding free interval was achieved in 90% of the transected patients (none rebled from varices) compared to 80% in those who had 2 emergency injection sessions. There was no difference in mortality between the two treatment modalities. Cello *et al* showed that emergency porta-caval shunt was more effective than emergency sclerotherapy (followed by elective sclerotherapy) in preventing early rebleeding (19% *vs* 50%)[78]. Hospital and 30 day mortality was not significantly different. Finally Orloff *et al* reported, in a small study[82], that portacaval shunt, performed in less than 8 hours from admission, was significantly better than medical treatment (vasopressin/balloon tamponade) in the control of acute variceal bleeding. Survival was also better in the shunted patients but the difference was not statistically significant.

Randomized controlled trials of tissue adhesives in the management of acute variceal bleeding

Two types of tissue adhesives (n-butyl-2-cyanoacrylate [Histoacryl] and isobutyl-2-cyanoacrylate [Bucrylate]) have been used for the control of variceal bleeding[83]. The adhesives could offer better immediate control of bleeding because they harden within seconds upon contact with blood[84]. However extra care must be taken to ensure that the adhesive does not come into contact with the endoscope and blocks the channels of the instrument. This can be prevented if the adhesive is mixed with lipiodol to delay hardening. Ideally, the sclerotherapy needle should be carefully placed within the varix prior to injection, to avoid leak of the adhesive[84]. Two randomised controlled trials compared sclerotherapy alone with the combination of sclerotherapy and Histoacryl for the control of active variceal bleeding[85,86]. The combined treatment was more effective than sclerotherapy alone in both studies. Two further studies compared Histoacryl to variceal ligation for the control of bleeding from oesophageal[87] or oesophagogastric varices[88]. The overall success rate for initial haemostasis of both treatment modalities was similar in these studies. However Histoacryl was superior to variceal ligation for the control of fundic variceal bleeding, but it was less effective for the prevention of rebleeding (67% *vs* 28%). Finally, in a recent small study[89], a biological fibrin glue (Tissucol) was more effective than sclerotherapy with polidocanol in the prevention of early rebleeding and had significantly lower incidence of complications. More studies are necessary to confirm these data and examine the potential risks of activation of coagulation, systemic embolism and transmission of infections with the human plasma derived fibrin glue.

Emergency transhepatic porto-systemic stent shunt

The first reports of TIPS used clinically were in patients with uncontrolled variceal bleeding. In this very ill group of patients TIPS was found to have a life saving role, stopping bleeding in over 90% of patients; over half of these were leaving hospital alive, figures that were unachievable before. Prolonged expensive postoperative ventilation on intensive care was avoided, a situation with a well-defined high mortality[90]. Perhaps as a consequence of this apparent efficacy, there have been no controlled trials of emergency TIPS against other forms of salvage therapy.

One of the biggest problems in discussing uncontrolled variceal bleeding is that there is no accepted definition for this clinical situation. At Baveno 2[91], the panel was unable to reach a consensus as to what this term meant and what its defining parameters were. A re-evaluation of this was recommended at Baveno 3[92] and justified by a prospective evaluation in France[93]. However the reduced efficacy of repeated sclerotherapy is well established, with bleeding control achieved in 70% after the first session and 90% after two sessions. Risk of aspiration, complications of sclerotherapy itself and the general deterioration of the patient render further endoscopy potentially hazardous, as established by Bornman *et al*[94] and at our unit[95] with no improvement in the control of bleeding. Thus with respect to esophageal varices, many use the definition of continued bleeding despite two sessions of therapeutic endoscopy within 5-day period of the index bleed. To this group of patients one

can add those who continue to bleed despite a correctly positioned Sengstaken Blakemore tube (approximately 10% of patients with Sengstaken tube[96], and those patients who continue to bleed from gastric or ectopic varices despite vasoconstrictor therapy.

The results of emergency TIPS are shown in Table 2. There is a predictable proportion of patients with Pugh's C cirrhosis and patients with bleeding gastric varices. In the largest series of salvage TIPS, gastric varices were shown to be no different in terms of bleeding characteristics and portal haemodynamics when compared with oesophageal varices[97].

Patients with bleeding varices that are inaccessible to an endoscope or respond poorly to sclerotherapy are well suited to TIPS. Typical cases include fundal varices, small bowel varices (classically around anastomotic or surgical resection sites[98,99] intraabdominal varices (punctured during large volume paracentesis[100], stomal varices[101,102] (usually in patients with inflammatory bowel disease and sclerosing cholangitis), and bleeding rectal varices[103]. These sites are also amenable to embolization via shunt. TIPS have been successfully placed in infants[104,105], and children[106-108] with similar efficacy.

The results of emergency TIPS are good, especially when compared historically with surgery[109], but the mortality in these series of patients with uncontrolled bleeding is high. There is a need to try to improve patient selection. A number of markers of outcome have been identified. Including the APACHE score[110], presence of hyponatremia and child C liver disease[111], hepatic encephalopathy before TIPS, presence of ascites and serum albumin[112]. Artificial neural network have been developed and validated[113], though many of these series have mixed patients having elective TIPS and those having the procedure as an emergency. The authors feel that this latter group of patients is likely to be different from patients having an elective procedure, with characteristics of haemodynamic instability, worse liver function, lower platelets counts, and higher serum urea concentrations.

At Royal Free Hospital a score system was developed on 54 patients undergoing emergency TIPS as salvage therapy[115]. A prognostic index was developed, based on six factors independently predicted death on multivariate analysis: presence of moderate/severe ascites, requirement for ventilation, white blood cell count, platelet count, serum creatinine and partial tromboplastine time. The score was validated in a further 31 patients and shown to be accurate in predicting mortality. The use of TIPS as a salvage therapy in patients who have uncontrolled variceal bleeding is likely to remain the most established indication for TIPS, and clinical experience to date suggest that this procedure will be required in 10%-20% of patients presenting portal hypertension related bleeding.

CONCLUSION

Today the therapeutic approach in cirrhotic patients with bleeding varices must include the prophylactic use of antibiotics, early endoscopic diagnosis and endoscopic therapy, probably combined use of vasoactive agents. The best evidence for an improvement in mortality is for terlipressin, but only data for 48-hour treatment exists; for somatostatin, which is an alternative, there is data for 5-days use. However mortality is unchanged in trials of endoscopic therapy versus endoscopic therapy combined with vasoactive agents. Further trials are indicated in this area. Although no randomized trials on emergency TIPS exist, this procedure is very effective in stopping bleeding and has virtually eliminated the need for emergency surgery, and reduced ITU stays. If new trials are to be done, comparison with glues or thrombin may be justified.

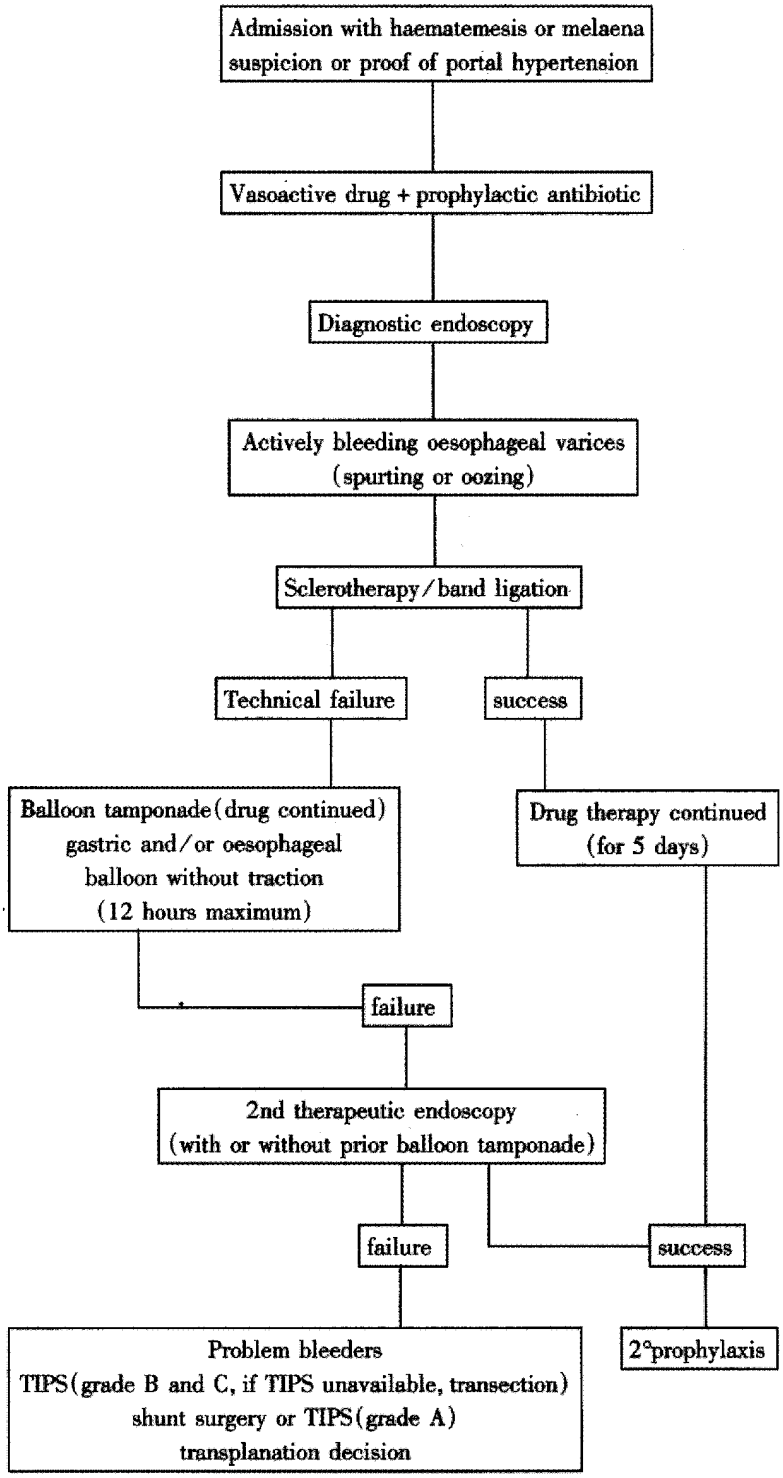
All studies should now adhere to consensus definition[92] so that the field can accumulate evidence for optimal treatment strategies.

Table 2 Reports of TIPS for acute variceal bleeding

Authors	Patients	Child score A / B	Source of bleeding, Gastric / Oesophageal / 42 days (%)	Immediate control of bleeding (in completed TIPS)	Early rebleeding (within 1mo)	Mortality
Other						
McCormick (1994)[116]	20	1 / 7 / 12	3 / 17 / -	20 / 20	6	11(55)
Jalan (1995)[109]	19	3 / 3 / 13	- / 19	17 / 17	3	8(42)
Sanyal (1996)[117]	30	1 / 7 / 22	4 / 26 / -	29 / 29	2	12(40)
Chau (1998)[97]	112	5 / 27 / 80	28 / 84 / -	110	15	41(37)
Gerbes (1998)[114]	11	1 / 3 / 7	8 / 11	10	3	3(27)
Banares (1998)[112]	56	11 / 22 / 23	19 / 37 / -	53 / 55	8	15(28)

Key Concepts:

- Aim is not only stop bleeding but prevent early rebleeding as this significantly impairs survival
- Prophylactic use of antibiotics is manadatory
- Vasoactive drugs administered before diagnostic endoscopy for at least 48 hours, and perhaps 5 days
- No more than 2 sessions of endoscopic therapy during the first 5 days of admission
- TIPS as salvage therapy for continued bleeding



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• REVIEW •

Management of necrotizing pancreatitis

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Abstract

Infection complicating pancreatic necrosis leads to persisting sepsis, multiple organ dysfunction syndrome and accounts for about half the deaths that occur following acute pancreatitis. Severe cases due to gallstones require urgent endoscopic sphincterotomy. Patients with pancreatic necrosis should be followed with serial contrast enhanced computed tomography (CE-CT) and if infection is suspected fine needle aspiration of the necrotic area for bacteriology (FNAB) should be undertaken. Treatment of sterile necrosis should initially be non-operative. In the presence of infection necrosectomy is indicated. Although traditionally this has been by open surgery, minimally invasive procedures are a promising new alternative. There are many unresolved issues in the management of pancreatic necrosis. These include, the use of antibiotic prophylaxis, the precise indications for and frequency of repeat CE-CT and FNAB, and the role of enteral feeding.

Subject headings pancreatitis, acute necrotizing/drug therapy; pancreatitis, acute necrotizing/surgery; biopsy, needle; tomography, x-ray computered; enteral nutrition; human

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INTRODUCTION

Acute pancreatitis is common, the incidence in recent European studies varying between 20 and 70 cases per 100 000 population with an overall mortality of between 3% and 8%[1-6]. Most cases are secondary to gallstones or excess alcohol consumption. Activation of trypsinogen within pancreatic acinar cells is the critical initiating event[7]. This leads to autodigestion of the pancreas, with a localised and then systemic inflammatory response, which if marked leads to the development of multiple organ dysfunction syndrome (MODS) and death[8,9]. Approximately half of deaths from acute pancreatitis occur in the first week following an attack. In patients who survive the initial attack a proportion develop areas of pancreatic and peripancreatic necrosis. Secondary infection then leads to persisting sepsis, MODS, and accounts for the majority of the remaining late deaths[1,10].

IDENTIFYING PATIENTS WITH NECROSIS

Nearly all patients who suffer a mild attack of acute pancreatitis make a complete recovery[11]. About one third of patients with a severe attack, who develop organ failure during the first week, will however, subsequently develop pancreatic necrosis involving more than 30% of the gland. There are several methods that are routinely used to identify early those patients who are likely to develop organ failure and those who will be at risk of pancreatic necrosis. Specific clinico pathological scoring systems include those described by Imrie[12] and Ranson[13]. These, however, are only accurate 48 hours after hospital admission, when they correctly categorise around 80% of patients into mild and severe. An APACHE II score $\geqslant$9 on hospital admission correctly identifies around 85% patients who will suffer a severe attack[14]. Unfortunately the relative complexity of the APACHE II system limits its clinical use.

Plasma C reactive protein levels (CRP), greater than 150mg/L 48 hours after admission, are widely used to predict a severe attack of pancreatitis[15,16]. CRP levels do not however peak until seventy-two hours after onset of symptoms thus CRP levels, like the Imrie and Ranson scores are limited in predicting a severe attack during the first few hours following admission (Table 1). Plasma levels of other direct inflammatory mediators, such as interleukin-8 and interleukin-6 are elevated earlier in the course of an attack of acute pancreatitis and relate to the severity of the systemic inflammatory response[17]. Although the levels of these mediators are as accurate at the time of admission as the APACHE II score, the assay systems are not suitable for widespread clinical use. Urinary levels of trypsin activation peptide (TAP), the cleavage peptide released following the activation of trypsinogen, become significantly elevated with the onset of an attack and measuring TAP has been shown to be a valuable predictor of severe disease[18] and urinary TAP levels may ultimately form the basis of a simple bedside urine test (Table 1).

Intravenous contrast-enhanced computerised tomography (CE-CT) has also been used to predict the severity of an attack of acute pancreatitis[19]. Balthazar described a CT severity index, based on a combination of peripancreatic inflammation and degree of pancreatic necrosis as seen at initial CT study. Patients with a high CT severity index had 92% morbidity and 17% mortality; patients with a low CT severity index had 2% morbidity, and none died[20]. This type of scoring system using CT offers no advantages as compared to clinico-biochemical scoring systems for the prediction of severe disease[21]. Rather the value of CE-CT is in the detection of pancreatic necrosis and definition of its extent and distribution (Figure 1A)[22-25] as well as in helping to delineate any associated collections[26]. Serial CT scans also allow the progression of the disease to be followed and are an essential adjunct when surgical intervention is required.

Table 1 Prognostic accuracy of the APACHE II, the Imrie and Ranson scores, plasma CRP and urinary TAP levels[18]

Scoring System	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
Post-symptom 24 hrs					
Urinary TAP >35nmol/L	58	73	39	86	70
Plasma CRP >150mg/L	0	90	0	75	69
Plasma CRP >150mg/L or urinary TAP >35nmol/L	58	72	37	86	69
Plasma CRP >150mg/L and urinary TAP >35nmol/L	0	92	0	74	70
Post-symptom 48 hrs					
Urinary TAP >35nmol/L	81	71	42	94	73
Plasma CRP >150mg/L	65	73	37	90	72
Plasma CRP >150mg/L or urinary TAP >35nmol/L	86	60	35	94	65
Plasma CRP >150mg/L and urinary TAP >35nmol/L	60	85	50	90	80
Post-hospitalisation 24 hrs					
Urinary TAP >35nmol/L	68	74	44	89	73
Plasma CRP >150mg/L	47	82	42	84	74
Plasma CRP >150mg/L or urinary TAP >35nmol/L	74	66	38	90	68
Plasma CRP >150mg/L and urinary TAP >35nmol/L	40	91	57	83	79
APACHE II ⩾8	63	73	38	88	71
Post-hospitalisation 48 hrs					
Urinary TAP >35nmol/L	83	72	44	94	74
Plasma CRP >150mg/L	86	61	37	94	66
Plasma CRP >150mg/L or urinary TAP >35nmol/L	94	49	32	97	58
Plasma CRP >150mg/L and urinary TAP >35nmol/L	74	85	58	92	83
APACHE II ⩾8	56	64	30	85	63
Imrie Score ⩾3	77	75	44	93	76
Ranson Score ⩾3	89	64	38	96	69

PPV=positive predictive value; NPV=negative predictive value.

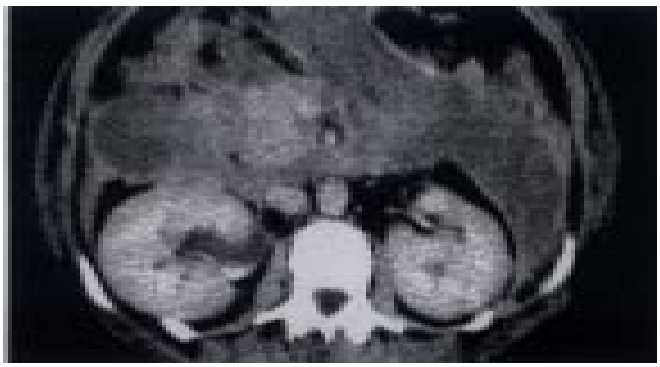

Figure 1A Extensive retroperitoneal pancreatic necrosis.

DETECTION OF INFECTION

In addition to the amount of pancreatic necrosis the outcome in severe pancreatitis is also determined by the presence or absence of infection within the necrotic tissue[27]. Clinical indicators that suggest the presence of infection include pyrexia, hypotension, continuing tachycardia, and a leukocytosis, but these features of sepsis syndrome are identical to those in patients with severe pancreatitis irrespective of the presence of pancreatic infection[28]. Beger *et al* studied 144 patients who underwent open necrosectomy. The proportion of patients who had demonstrable bacterial contamination at the time of necrosectomy increased from 24% during the first week to 36% in the second and peaked at 72% during the third week suggesting that infection is not immediate but that its frequency increases with time[29]. Table 2 shows organisms found within infected necrotic pancreas in their study, which was conducted prior to the routine use of prophylactic antibiotics. The profile of infecting organisms suggests origin from the gastrointestinal tract.

Table 2 Bacteria isolated from operative specimens taken at necrosectomy prior to the introduction of routine antibiotic prophylaxis, Beger *et al*, 1986[29]

Bacteria isolated	No. of patients
Gram - ve aerobic	
Escherichia coli	24
Enterobacter aerogenes	16
Pseudomonas aeruginosa	5
Proteus species	5
Klebsiella pneumonia	3
Citrobacter freundi	1
Gram - ve anaerobic	
Bacteroides species	5
Gram + ve aerobic	
Streptococcus faecalis	6
Staphlococcus aureus	4
Streptococcus viridans	1
Staplococcis epidermidis	1
Others	
Mycobacterium tuberculosis	1
Candida species	3

Several studies have shown that persistently elevated CRP is associated with infected pancreatic necrosis[30]. The presence of gas within an area of necrosis shown by CE-CT is highly suggestive of infection (Figure 1B), although it is desirable to detect the presence of infection before this becomes apparent. CE-CT guided fine needle aspiration, however, allows direct sampling of the necrotic tissue and subsequent microscopy and bacteriology (FNAB) will confirm the presence of infecting organisms (Figure 1C)[31,32].

Figure 1B Infection of pancreatic necrosis with gas forming organisms.

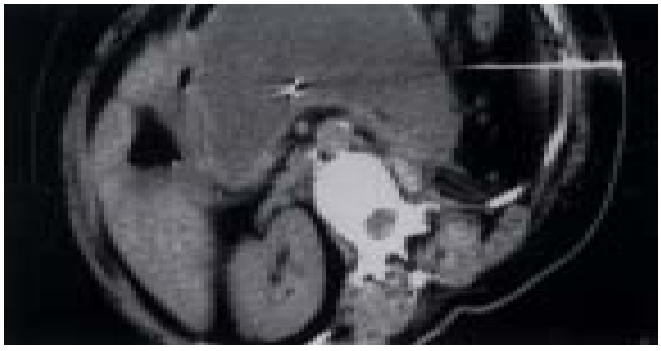

Figure 1C CE-CT guided fine needle aspiration for bacteriology.

The nature of the inflammatory response may also be modified by the presence of infection and recent studies have attempted to identify circulating factors that might confirm this. Serum procalcitonin is a potential marker for non-invasive prediction of infected necrosis[33]. Rau *et al* studied 50 patients with acute pancreatitis, 18 patients with oedematous pancreatitis, 14 patients with sterile necrosis, and 18 patients with infected necrosis. Levels of procalcitonin were measured in plasma during the first two weeks of admission. If levels reached 1.8ng/mL on at least two days during this time, sensitivity, specificity, and accuracy for the prediction of infected necrosis were 94%, 91%, and 92% respectively. This was not confirmed however in a more recent study[34].

PREVENTION OF PANCREATIC NECROSIS

Reducing the severity of the initial attack of acute pancreatitis might reduce the incidence and magnitude of pancreatic necrosis. Unfortunately at the present time, in the absence of effective intervention, management of the acute attack is predominantly supportive. One exception is the use of endoscopic retrograde cholangio-pancreatography and sphincterotomy in patients with predicted severe gallstone pancreatitis, which reduces the severity of an attack. Patients with severe acute pancreatitis due to gallstones need to undergo endoscopic sphincterotomy during ERCP, irrespective of the presence of acute cholangitis and ERCP should be undertaken within forty-eight hours of diagnosis[35-37].

ANTIBIOTIC PROPHYLAXIS

Prophylactic antibiotic use may reduce the incidence of septic complications particularly infection involving areas of pancreatic necrosis. In the 1970s three randomised placebo controlled studies assessed the role of prophylactic antibiotics in acute pancreatitis and found no effect on mortality or morbidity[38-40]. These studies, which were small, consisted almost entirely of patients with mild disease and without necrosis and thus no conclusions can be drawn.

In 1993 Pederzoli *et al* reported a multi-centre randomised study in which 74 patients with pancreatitis from all causes and with confirmed necrosis on CT at the time of admission were randomly assigned to imipenem or to no antibiotic[41]. The incidence of pancreatic sepsis, which was determined by fine needle aspiration or culture of intra-operative specimens, decreased from 30% in those untreated with antibiotics to 12% in the antibiotic treated group. There was, however, no significant difference in the rate of surgical intervention or mortality.

In a subsequent study from Finland, 60 patients with severe alcohol-induced necrotising pancreatitis as determined by CT and CRP estimation were randomly assigned to treatment with cefuroxime or to no antibiotic. One (3%) patient in the antibiotic treated group died compared to seven (23%) patients in the untreated group, ($P<0.05$)[42]. Surprisingly given the large difference in mortality there was no significant difference in the overall incidence of sepsis or the number of patients requiring surgery. Further, given the relatively small size of the study it is probable that there was heterogeneity in the randomisation as shown by the greater number of patients with fulminant pancreatitis on admission in the control group.

More recently, 60 patients with severe acute pancreatitis and necrosis affecting at least 50% of the pancreas, were randomly allocated to receive intravenous treatment for 2 weeks with pefloxacin, (30 patients), or imipenem, (30 patients), within 120 hours of onset of symptoms. The incidence of infected necrosis and extra-pancreatic infections was 34% and 44% respectively in the pefloxacin group and 10% and 20% in the imipenem group. Although imipenem proved significantly more effective in preventing pancreatic infections ($P<0.05$), there was no significant difference in mortality nor in the number of patients requiring surgery between the two treatments[43]. A feature of this last study and of other recent series[44,45] in which prophylactic antibiotics have been used is the increasing incidence of drug resistant or unusual organisms, including fungi, cultured from pancreatic tissue removed at necrosectomy. When such organisms are present the mortality following necrosectomy may be increased[46,47]. Thus the data imply that the use of prophylactic antibiotics promotes drug-resistant organisms and the growth of fungi. In the absence of further studies routine antibiotic prophylaxis in patients with acute pancreatitis cannot be recommended at present.

TRANSLOCATION OF GUT ORGANISMS

The gastrointestinal tract is thought to be the major source of organisms infecting necrotic pancreatic tissue. Increased translocation of bacteria and toxins is known to occur in acute pancreatitis[48,49]. Anaerobic bacteria are less likely to translocate from the gut lumen. Thus selective digestive decontamination (SDD) with appropriate antibiotics may change the intestinal flora to one that is less invasive. Between 1990 and 1993, 102 patients with severe pancreatitis from 16 centres in the Netherlands were randomized to selective digestive decontamination plus standard treatment or standard treatment alone[50]. There was a significant reduction in the incidence of gram-negative pancreatic infection in treated patients. Although deaths were reduced from 35% in the control group to 22% in the treatment group this difference was not significant. A short course of systemic antibiotics (cefotaxime) was used in the SDD group so that interpretation of the data with regard to the specific effects of gut decontamination as opposed to antibiotic prophylaxis is difficult[50].

Early re-introduction of nutrition via the gastrointestinal tract may also help to restore mucosal integrity and reduce translocation. A number of studies in patients with major trauma, surgery and burns showed that enteral nutrition significantly decreased the acute phase response and incidence of septic complications when compared with total parenteral nutrition[51,52]. In acute pancreatitis therefore early reintroduction of feeding via the gastro intestinal tract might also reduce the incidence of pancreatic infection.

Two randomized studies have compared enteral and parenteral nutrition in patients with severe acute pancreatitis. In the first study, 38 patients received enteral nutrition through a nasoenteric tube with a semi-elemental diet or parenteral nutrition through a central venous catheter. Patients who received enteral feeding experienced fewer total complications ($P<0.05$) and were at lower risk of developing septic complications ($P<0.01$) than those receiving parenteral nutrition. The cost of nutritional support was three times higher in patients who received parenteral nutrition[53].

In a second study from Leeds, 34 patients with acute pancreatitis received either parenteral or enteral nutrition for seven days and were then re-evaluated. The frequency of SIRS, sepsis, organ failure and the need for ITU admission was reduced in the enterally fed patients[54].

In a third study from Edinburgh, 27 patients with predicted severe acute pancreatitis were randomised to early introduction of enteral nutrition via a nasojejunal tube or conventional therapy, i.e. nil by mouth with re-introduction of oral intake with return of gut function. There were no significant complications as a consequence of enteral nutrition. The introduction of enteral nutrition did not affect the serum concentrations of IL-6 (P=0.28), soluble tumour necrosis factor-α receptor (P=0.53) or CRP (P=0.62) over the first 4 days of the study. Although there were no significant differences in intestinal permeability between the two patient groups at admission, by day four abnormal intestinal permeability occurred more frequently in patients receiving enteral nutrition (P=0.03).

Thus it can be concluded that enteral nutrition is safe in patients with severe acute pancreatitis and there is some evidence that it may be preferable to parenteral nutrition. The power of these three studies was too low to show any differences with respect to surgical intervention, incidence of pancreatic infection or mortality and the effect of nutrition route and timing on these outcomes requires further study.

NON-OPERATIVE TREATMENT OF PANCREATIC NECROSIS

Although there are isolated case reports of patients with pancreatic infection surviving with medical treatment alone[55] and limited success using percutaneous drainage[56,57], the presence of infection in necrotic pancreatic tissue is accepted to be an absolute indication for surgical intervention (Table 3). The situation in patients with extensive areas of sterile necrosis is less clear. Bradley *et al* reported on 38 patients with necrosis on CT who were initially treated medically and underwent FNAB if they remained persistently febrile. Infected pancreatic necrosis was demonstrated in 27 (71%) of the 38 patients with pancreatic necrosis who were treated by open drainage, with a mortality rate of 15%. All 11 patients with sterile pancreatic necrosis, including six with pulmonary and renal insufficiency, were successfully treated without surgery[58]. On the basis of this and subsequent studies sterile necrosis, should initially be managed non-operatively[59].

Table 3 Indications for surgical intervention

Absolute	• Presence of infected pancreatic necrosis shown by CE-CT or FNAB.
Relative	• In a patient with >50% pancreatic necrosis, failure to improve appreciably after 2 - 3 weeks, unexplained deterioration, or a suspicion of infected pancreatic necrosis even in the absence of firm evidence on CE-CT and FNAB. In a patient with >50% pancreatic necrosis, • prolonged illness with an unacceptably slow recovery

The optimal frequency of CE-CT imaging and FNAB has not been clearly established. In the recent study from Bern, all patients underwent contrast-enhanced CT within 24 to 48 hours of admission and this was repeated weekly in those patients whose clinical condition did not improve[45]. Fine needle aspiration under CT guidance with subsequent microscopy and bacteriological culture was undertaken to rule out infection in patients who developed signs of metabolic disorders, those with deteriorating function of lung, kidney or the cardio circulatory systems and those with persistent leukocytosis or fever (>38.5℃).

A second issue is the treatment of patients with sterile necrosis who remain unwell. In this group surgical intervention has been suggested for patients with persisting or advancing organ complications despite intensive care therapy[59]. In contrast in a recently published single-centre study, pancreatic infection, if confirmed by fine-needle aspiration, was considered an indication for surgery, whereas patients without signs of pancreatic infection were treated medically[45]. Eighty-six (42%) of the patients in this study had necrotizing disease, of which two thirds had sterile necrosis. The death rate was 1.8% (1/56) in patients with sterile necrosis managed without surgery versus 24% (7/29) in patients with infected necrosis (P<0.01). Two patients whose infected necrosis was not diagnosed in time died whilst receiving medical treatment. Thus, an intent to treat analysis (non-surgical *vs.* surgical treatment) produced a death rate of 5% (3/58) with conservative management versus 21% (6/28) with surgery. The authors concluded that non-surgical management, including early antibiotic treatment, should be used in all patients with sterile pancreatic necrosis[45]. In contrast other authors have observed a similar mortality in patients undergoing necrosectomy between those with sterile and those with infected necrosis[60].

TIMING OF SURGERY

Timing of surgery is critical. Necrosectomy is technically difficult during the first week but becomes progressively easier with time. One controlled trial has addressed the role of early surgery. Forty-one patients with pancreatic necrosis on CT were randomized to early necrosectomy (within 48 to 72 hours of onset) or late necrosectomy (at least 12 days after onset). Both groups continued with open packing and staged necrosectomies. Although the mortality rate (58% versus 27%) did not reach statistical significance, the odds ratio for mortality was 3.4 times higher in the early group and for this reason the study was terminated early[61]. Thus the contemporary management of patients with extensive necrosis involves repeated imaging using contrast-enhanced CT in association with fine needle aspiration for microscopy and bacteriology with immediate surgery if infection is detected.

OPEN NECROSECTOMY

Necrosectomy has traditionally been undertaken by an open route. Following laparotomy the lesser sac is opened if possible, the colon is mobilised downwards and the pancreas identified. Necrotic pancreas is debrided by blunt finger dissection and wide bore suction drainage. If opening of the lesser sac is not possible, direct access from the infracolic compartment via the left transverse mesocolon (space of Riolan) is an alternative. Adequate debridement is usually achieved with a single visit to theatre. Any associated fluid collections are drained by the most direct route. Large drains and irrigating catheters are left within the retroperitoneal area and continuous irrigation is continued post surgery[62]. The use of open packing with multiple visits to theatre prior to secondary closure over drains has been described but hospitalisation can be significantly reduced by using prolonged lavage rather than pre-planned multiple laparotomies. Mortality rates in recent series are generally between 20%-40%[45,58,60-67], but may be higher even in specialised centres[33].

Several developments have led to a reassessment of the role and the extent of surgery in acute pancreatitis. Percutaneous drainage has been advocated as a means of

treating pancreatic necrosis[56,57,68]. Unfortunately it is impossible to achieve adequate debridement of solid pancreatic debris by this route except in a minority of cases and it may lead to secondary infection in pancreatic necrosis that is initially sterile. Aggressive percutaneous drainage has been proposed as a means of treating infected pancreatic necrosis. A major reason for failure however is the variable amounts of infected solid material that cannot be removed. Indeed Paye *et al* [69] found percutaneous drainage to be largely inadequate requiring surgical intervention in the majority of cases.

In an attempt to reduce the high mortality from surgical necrosectomy less traumatic approaches than open laparotomy have been advocated. Fagniez *et al*[70] described a retroperitoneal approach for pancreatic necrosectomy through the left flank just anterior to the 12th rib. There was an overall mortality of 33% in 40 patients with severe pancreatic necrosis and 18% in the 22 patients in whom this was the only abdominal procedure performed. Similarly good results have been reported in three other small series[71-73]. Morbidity rates, including colonic fistulae and haemorrhage were, however, high.

Another factor that has led to the re-evaluation of the extent of surgery has been the concept of the two-hit response. This hypothesis states that many patients with a severe attack of acute pancreatitis are primed to mount an inappropriate and exaggerated inflammatory response to a second traumatic challenge[8,9]. Thus a subsequent hit, for example from an open procedure to debride the infected necrotic pancreas, may lead to an overwhelming systemic inflammatory response and death. This would account in part for the continuing high mortality that follows open surgical necrosectomy. Unfortunately patients liable to have such an abnormal response cannot be identified at present although markers of genetic susceptibility are being sought.

A new technique of minimally invasive pancreatic necrosectomy via a left loin approach, analogous to the open technique of Fagniez *et al*[70] was recently pioneered in Glasgow[74]. The advantages of this technique are two-fold. First the peritoneal cavity is not transgressed, and second, tissue damage is limited-thus reducing the magnitude of the systemic inflammatory response of the second hit. Mortality in the 10 patients treated by this technique was only 20%. We have used this technique in a further 14 patients with 3 (21%) deaths[75]. We believe that these results are encouraging and that in the future a significant proportion of patients with infected pancreatic necrosis may be managed by this technique.

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• REVIEW •

Adjuvant therapy in pancreatic cancer

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Abstract

The outlook for patients with pancreatic cancer has been grim. There have been major advances in the surgical treatment of pancreatic cancer, leading to a dramatic reduction in post-operative mortality from the development of high volume specialized centres. This stimulated the study of adjuvant and neoadjuvant treatments in pancreatic cancer including chemoradiotherapy and chemotherapy. Initial protocols have been based on the original but rather small GITSG study first reported in 1985**. There have been two large European trials totalling over 600 patients (EORTC and ESPAC-**1**) that do not support the use of chemoradiation as adjuvant therapy. A second major finding from the ESPAC-**1 **trial (**541 **patients randomized) was some but not conclusive evidence for a survival benefit associated with chemotherapy. A third major finding from the ESPAC-**1 **trial was that the quality of life was not affected by the use of adjuvant treatments compared to surgery alone. The ESPAC-**3 **trial aims to assess the definitive use of adjuvant chemotherapy in a randomized controlled trial of** 990 **patients.**

Subject headings pancreatic neoplasms/drug therapy; pancreatic neoplasms/radiotherapy; human; review

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INTRODUCTION

Pancreatic cancer is the 5th most common site of deaths due to cancer among all cancer sites in the Western world. Low cure rates ensure that the mortality is nearly as high as the incidence. It is responsible for 7 000 deaths per year in the UK[1], 40 000 per year in Europe and 28 000 in the USA[2]. The peak mortality ages are estimated to be between 65 and 72 years in Europe and 55 and 75 years in the USA[2]. The incidence of this deadly disease has been rising during the last century. In the past 20 years however, there have been vast improvements in the surgical management of patients with pancreatic cancer. The surgical procedures have been improved and become more standardized between centres and countries. The level of pre- and post-operative support for these patients has been optimized, particularly in established centres with a high throughput[3]. These measures have ensured that the outlook for patients with resectable disease has certainly improved, particularly in the short term. Extending patient survival still remains a problem. The overall five-year survival for all patents with pancreatic cancer is only 0.4%[4]. Patients who are suitable for resection have five-year survival rates of between 10% and 24%[5-8] and are virtually never cured. Therefore, even for the 10% to 15% of patients who undergo surgery, there appears to be no guarantee of cure or indeed long-term survival. These outcomes would suggest a role for the use of additional or adjuvant therapy to attempt to improve patient survival and quality of life.

RADICAL SURGERY

Japanese groups amongst others have been enthusiastic in pursuing radical resection as means of increasing disease free margins and thus hopefully improving patient survival. Radical surgery includes extensive lymph node dissection and retroperitoneal connective tissue clearance as well as pancreatic resection. The Japanese groups have suggested that these approaches are superior to conventional Kausch-Whipple resection but several studies have not shown significant survival advantages when compared with conventional resection[9-13]. Kayahara *et al* found that radical resection in patients with Stage I and II disease (Japanese classification) and clear margins (R0) resulted in a reduction of local recurrence but did not translate into improved survival because of hepatic metastases[14]. Interestingly highly detailed serial section analysis of presumed R0 specimens has revealed microscopic margin disease (R1) in up to 38% of specimens[14].

Difficulties are encountered when comparing survival figures of Western and Japanese studies because of the different staging systems used (UICC *vs* JPS respectively). This is because of the phenomenon of 'staging system migration' that may give apparently better survival for each stage in one system compared to the other even though there is no overall difference in survival. Satake *et al*[15] compared the Japanese and UICC staging systems in a large cohort of patients. Stage for stage the Japanese system revealed a better survival from Stage I to IV compared with UICC system. The overall five-year survival however, was the same (11%) because the systems had been analysed in identical patients.

The majority of radical resection studies have been non-randomized and performed in single institutions. The radical lymph node dissection allows more accurate staging of disease and these tumours will tend to be upstaged because of this. Thus it is necessary to examine overall group survival within the context of randomized studies by an intention to treat analysis. There has been one multicentre prospective randomized trial comparing traditional partial pancreatoduodenectomy with and without a more extensive lymph node dissection[16]. Eighty-one patients were randomized to receive a standard (n=40) or extended (n= 41) lymphadenectomy and retroperitoneal soft tissue clearance. The standard lymphadenectomy included removal of lymph nodes situated at the anterior and posterior

pancreatoduodenal, pyloric, main bile duct, superior and inferior pancreatic head and pancreatic body stations. The extended lymphadenectomy also included the removal of lymph nodes from the hepatic hilum, along the aorta from the diaphragmatic hiatus to the inferior mesenteric artery, laterally to both renal hila and clearance of the coeliac trunk and superior mesenteric artery. There was no significant difference of overall survival between the two groups. Patients who had lymph node positive disease demonstrated better survival following an extended resection compared to those who did not have an extended lymphadenectomy but must be regarded as a statistically invalid manoeuvre as this was a post-hoc subgroup analysis. In light of these findings the ultimate benefit of extended lymphadenectomy surgery still needs to be proven with further critical evaluation.

There appears to be no additional survival benefit associated with total pancreatectomy compared to Kausch-Whipple pancreatoduodenectomy and at the present time the pylorus preserving pancreatoduodenectomy has been shown to produce similar results to the more traditional Kausch Whipple procedure[17,18]. The latter approach is now the procedure of choice in most centres.

The lack of survival benefit associated with radical resection may be due in part to the pattern of disease recurrence in resected pancreatic cancer. Most tumour recurrences are local, peritoneal and hepatic[19-24]. The early appearance of hepatic metastases following resection almost certainly indicates the presence of hepatic micrometastases at the time of surgery. Microscopic peritoneal disease also tends to occur early in contrast to the relatively later presentation of local recurrence. Pancreatic cancer cells tend to spread within a range of peripancreatic tissues. Lymphatic infiltration and perineural invasion may be found in 90%-100% of resected specimens[25]. Reasons for recurrence following an apparently curative resection include residual retroperitoneal disease, perineural invasion, hepatic micrometastases and lymph node involvement. The pattern of relapse after surgery reflects the natural course of the disease without resection. The most commonly affected organs include abdominal lymph nodes (72%-83%), liver (64%-80%), peritoneum (40%-53%) and lung (27%-50%)[14]. An R0 resection in patients with no lymph node metastases cannot be achieved in more than about half of the patients undergoing resection. Kayahara *et al*[14] at post mortem examined 15 patients who had undergone radical resection. The local recurrence rate in this group of patients was 80%. The local recurrences were associated with perineural invasion, lymphatic invasion and soft tissue infiltration. High rates of local recurrence have been confirmed in numerous studies of patients who have undergone pancreatoduodenectomy and the majority occur within 1 to 2 years of surgery[23,24].

Identification of extrapancreatic disease at the pre-operative stage has improved due to accurate imaging techniques[26] and laparoscopy[27]. Peritoneal cytology has been shown to be positive in 58% of patients who may have unresectable tumours or have a limited postoperative survival[27]. The best predictors of outcome following surgery also reflect the causes of disease relapse. These include tumour stage (which also includes the lymph node status), grade of primary tumour and resection margin status[28-32]. Not surprisingly patients with stage I or II disease and negative resection margins tend to demonstrate the best survival.

The poor overall survival of patients with pancreatic cancer, even following optimal surgical intervention, and the pattern of disease progression and recurrence are clear indications for the use of additional treatment modalities.

CHEMOTHERAPY

Advanced pancreatic cancer

There have been many studies of chemotherapy in patients with advanced pancreatic cancer. Single agents and combination regimens have been used. At the present time there is no accepted standard chemotherapeutic agent for the treatment of pancreatic cancer. 5-fluorouracil (5-FU) remains the most effective and most frequently used single chemotherapy agent. 5-FU works partly by interference with enzymes such as thymidylate synthase and partly by incorporation of 5-FU metabolites into DNA and RNA. The response rates of -15% with a median survival of 3-5 months[33]. The addition of the modulator folinic acid has produced marginal survival benefit over 5-FU alone but this has not been a significant increase[33]. The addition of other modulators such as phosphonacetyl-L-aspartate (PALA), and interferon has also not produced significant improvements in survival[34,35]. Comparisons of 5-FU alone and 5-FU with a combination of other agents have not shown any advantage for the combination groups in randomized trials[33].

A new agent, gemcitabine has been compared to 5-FU in a randomized multi-centre phase III clinical trial[36]. Gemcitabine is a deoxycytidine analogue that is phosphorylated to an active form and competes with dCTP for incorporation into DNA. The study, in which over 70% of patients had stage IV disease, randomized 63 patients to receive gemcitabine and 63 patients to receive 5-FU. Median survival in the gemcitabine group was 5.7 months compared to 4.4 months in the 5-FU group but no patient survived beyond 19 months. The clinical benefit response was also significantly higher in the gemcitabine group[36]. Despite the fact that this is the only trial with a straight comparison between the two agents gemcitabine has been recommended as the drug of choice in the USA. Gemcitabine has also been combined with 5-FU in several phase II studies. It is generally well tolerated but can have unpredictable side effects such as neutropaenia, abnormal liver function tests and nausea and vomiting. In patients who have had previous radiotherapy to the mediastinum there have been unpredictable reactions[37].

There is good evidence from several randomized controlled trials comparing chemotherapy with a no treatment group that chemotherapy is of benefit in patients with advanced pancreatic cancer. Mallinson *et al*[38] demonstrated a median survival of 11 months for patients treated with 5-FU, cyclophosphamide, methotrexate, vincristine and mitomycin C compared to 2.2 months for the untreated control group. This regimen did not produce a significantly greater survival when compared to 5-FU alone in a much larger randomized control trial[39]. Another rather poorly controlled study compared 5-FU and carmustine to untreated controls[40]. There was no significant survival benefit associated with this regimen but the majority of patients in this study received only a single treatment and did not finish the course. A further trial of the combination of 5-FU, doxorubicin and mitomycin C (FAM) resulted in median survival of 33 weeks compared with median survival of 15 weeks in untreated control patients[41]. A recent study compared the use of 5-FU + folinic acid (+/- etoposide) with best supportive care and showed that the median survival in the treated group was 6 months compared to 2.5 months in the control group[42]. Moreover there was better overall

quality of life score for the treated patients.

Adjuvant chemotherapy

There have been only a few studies of adjuvant chemotherapy in pancreatic cancer and (up until the ESPAC-1 trial) there was only one randomized controlled trial comparing surgery and chemotherapy with surgery alone (Table 1)[43-46]. Splinter *et al* [43] reported no evidence of improvement or survival using a FAM regimen in 16 patients who had undergone pancreatoduodenectomy with a three year survival of 24% compared to a three year survival of 28% in 36 patients who had undergone surgery only. Patients from different time periods were included in the two groups and there were only nine patients with pancreatic ductal adenocarcinoma in the adjuvant group and 18 in the surgery only group. Baumel *et al*[46] reported adjuvant chemotherapy in 43 selected patients with a median survival of 12 months but there was no difference in median survival from those patients who underwent surgery only (12 months). Bakkevold *et al*[45] randomized 61 patients who had undergone pancreatoduodenectomy for pancreatic cancer or ampullary cancer to receive either six courses of FAM or no chemotherapy. There was a significant difference in the median survival rates between the two groups: 23 months. Unfortunately this did not translate into a significantly improved long-term survival however: the 5-year survival rates were 4% for the treatment arm versus 8% for the surgery only arm. There was also considerable toxicity encountered with the FAM regimen. Only 24 out of 30 patients randomized to treatment actually started therapy. Sixteen patients needed hospitalization after the first chemotherapy course and a total of 13 patients managed to complete all six cycles of FAM.

The European Study Group for Pancreatic Cancer (ESPAC) has commenced the ESPAC-3 trial with the objective of definitively defining the role of adjuvant chemotherapy following curative resection for pancreatic ductal adenocarcinoma. Two adjuvant regimens are being studied against a no chemotherapy control: ① 5-FU + folinic acid for 24 weeks versus ② gemcitabine for 24 weeks versus ③ observation. All patients will have undergone potentially curative resection for pancreatic ductal adenocarcinoma. A total of 990 patients (330 in each arm) will be recruited over the next few years and survival analysis will be completed after two years of follow-up. At the present time recruitment is underway from centres across Europe with further centres in Canada, Australia and New Zealand due to join.

RADIOTHERAPY (CHEMORADIOTHERAPY)

Advanced pancreatic cancer

External beam radiotherapy (EBRT) although used in the treatment of advanced pancreatic cancer, has never been compared with an untreated control arm in any randomized controlled trial. The most commonly used and probably the best radiosensitizer used with EBRT for advanced pancreatic cancer is 5-FU. Many retrospective studies of EBRT, usually in relatively small groups of selected patients report median survival times of 10 - 15 months with good palliation of symptoms[47,48].

The improved local control of disease achieved with EBRT has not translated into significantly longer survival times, so there have been various refinements in an attempt to enhance the effectiveness of radiotherapy. Wide field irradiation has been used to address the problem of hepatic micrometastases. A Radiation Therapy Oncology Group (RTOG) study of 79 patients who received pancreatic and hepatic irradiation resulted in a median survival of 8.4 months but at the expense of considerable toxicity[49]. Intraoperative radiation therapy (IORT) aims to deliver higher doses of radiation with greater precision and thus reducing the exposure of neighbouring organs. Experimentally, its effectiveness may be as high as five times the equivalent dose given by EBRT. In advanced pancreatic cancer the survival times achieved using IORT have not been encouraging (median survival -6 months) and it has been mainly used to boost EBRT[50,51]. A study comparing EBRT + IORT + 5-FU with EBRT +5-FU demonstrated no significant survival difference (12 and 13 months respectively)[50,51]. Complications encountered during IORT include, duodenal and gastric ulceration, vascular sclerosis and pancreatic abscess[52]. IORT offers good local control and pain relief but cannot be recommended as a standard treatment as it has not been possible to demonstrate any advantages over conventional therapy.

Adjuvant radiotherapy (chemoradiotherapy)

Adjuvant EBRT and IORT have been used alone and in combination in the adjuvant setting (Table 2)[21,32,53-60]. The majority of studies indicate that EBRT alone or in combination with IORT has a significant survival advantage over the use of IORT alone. In a randomized trial IORT was observed to reduce the local recurrence rate by 50% following surgery, but this did not translate into a significant survival advantage (3-year survival with IORT=7% *vs* no IORT 3%)[21]. In selected patients IORT and resection produced 3 and 5-year survival rates of 53% and 29%[61,62]. The use of IORT however requires specialised facilities and can be associated with severe complications.

EBRT (with concomitant chemotherapy) following resection is generally well tolerated but the degree of survival advantage, if any, is uncertain. To address this issue a multicentre Phase III trial organized by the European Organisation for Research and Treatment of Cancer (EORTC) compared chemoradiotherapy in patients following potentially curative surgery for pancreatic cancer with surgery alone[58]. Between 1987 and 1995, 218 patients were randomized to receive either chemoradiotherapy or no chemoradiotherapy following curative surgery for pancreatic or ampullary cancer. Ninety-three out of 110 patients randomized to treatment received a total of 40 Gy with concomitant continuous infusion of 5-FU. There were a total of 54 patients in the observation group and 60 patients in the treatment group with pancreatic ductal adenocarcinoma. There was no significant difference in median survival (with treatment 17.1 months *vs* 12.6 months with observation) and in five year survival [with treatment 20 (95% CI, 5-35)% *vs* 10 (0-20)% with observation]. Similarly there was no significant difference in survival between the treatment and observation groups in patients with ampullary cancer. This study showed that there was no survival advantage for adjuvant chemoradiotherapy for pancreatic and ampullary cancer but has been criticized because it was almost certainly underpowered.

REGIONAL THERAPY

Advanced pancreatic cancer

Regional therapy has been developed with the objective of

delivering high doses of cytotoxic drug to the tumour. The systemic side effects should be reduced with this approach. The coeliac and hepatic arteries and portal vein have all been used to deliver chemotherapeutic drugs to the tumour bed. Good control of hepatic metastases has been reported, with disease progression mainly due to local progression or peritoneal deposits. The combination of 5-FU, folinic acid and cisplatin produced median survival times of 9-14 months in selected patients[63,64]. It has also been reported that some apparently irresectable tumours have become resectable following regional therapy[64].

Adjuvant regional therapy

There have been several studies which have demonstrated improved survival in patients receiving regional chemotherapy following pancreatic resection largely in comparison with historical controls (Table 3)[65-68]. Link *et al*[69] found a median survival of 21 months in 18 patients who had undergone pancreatoduodenectomy for pancreatic ductal adenocarcinoma and then coeliac artery infusion of 5-FU, folinic acid, mitoxantrone and cisplatin compared to 9.3 months for historical controls. Disease progression occurred principally locally or in the peritoneum and was rarely detected in the liver. The rate of hepatic recurrence was greatly reduced using a combination of hepatic artery and portal vein infusion in patients with resected pancreatic, which in one study cancer produced a 54% three-year survival compared to 34% in historical controls[65]. Ozaki *et al*[66] found a 5-year survival rate of 32% patients treated with extended resection, IORT and hepatic artery and portal vein. The encouraging results of these small studies have prompted the ESPAC-2 trial, which is a multicentre, prospective randomized controlled Phase III trial. This study will compare adjuvant intra-arterial chemotherapy (cisplatin, 5-FU, folinic acid and mitoxantrone) and radiotherapy (Arm A) with surgery alone (Arm B) in patients who have undergone potentially curative resection for pancreatic ductal adenocarcinoma or ampullary carcinoma. The trial will recruit 110 patients into each arm and will be completed by 2007 aiming to provide a definitive answer to the role of adjuvant regional therapy for pancreatic cancer.

Table 1 Survival following surgery and adjuvant chemotherapy for pancreatic cancer

Series	Period	Number		Regimen	Median survival (months)	Actuarial survival (%)		
		Total	PDAC			1 year	3 year	5 year
Splinter *et al*[43]	1977-1984	36	18				28	
	1980-1984	16	9	5-FU / DOX / MMC			24	
Livingstone *et al*[44]	N / A	285	285	N / A				9
Bakkevold *et al**[45]	1984-1987	30	23	5-FU / DOX / MMC	23	70	70	4
		31	24		11	45	30	8
Baumel *et al*[46]	1982-1988	43	43	Not specified	12			
	1982-1988	527	527		12			

5-FU = 5-fluorouracil; DOX = doxorubicin; MMC = mitomycin C; * randomised cont rolled trial, PDAC = pancreatic ductal adenocarcinoma.

Table 2 Survival following surgery and radiotherapy for pancreatic cancer

Series	Year	Number	EBRT(Gy)	IORT(Gy)	Median survival (months)	Actuarial survival (%)		
						1 year	3 year	5 year
Willett *et al*[32]	1993	16 (nm)	40-50		21			29
		23 (pm)	40-50		11			0
Johnstone *et al*[53]	1993	26	45-55	20	18			
Zerbi *et al*[21]	1994	43		12.5-20	19	71	7	
		47			12	49	10	
Di Carlo *et al*[54]	1997	27			14			
		27		12.5-2017				
Dobelbower *et al*[55]	1997	14			6.5	15	0	0
		6		10-20	9	50	35	33
		14	50-67		14.5	64	28	0
		10	27-54	10-25	18	70	10	0
Farrell *et al*[56]	1997	14	60	12-25	16	62	22	15
Hishinuma *et al*[57]	1998	34	24 EBRT	13 EBRT + IOR	13	59		19
Klinkenbijl *et al**[58]	1999	54pdc			12.6			10
		60pdc	40		17.1			20
Mehta *et al*[59]	2000	52	45-54	8 IORT	32	75	38	
Lee *et al*[60]	2000	22					47	
		13	49				81	

EBRT = external beam radiotherapy; IORT = intraoperative radiotherapy; nm = nega tive resection margin; pm = positive resection margin; pdc = pancreatic ductal adenocarcinoma.

Table 3 Adjuvant regional therapy for pancreatic cancer

Series	Year	Number	Regimen	Median survival (months)	Actuarial survival (%)		
					1 year	3 year	5 year
Ishikawa *et al*[65]	1994	20	HAI + PVI			54	
Ozaki *et al*[66]	1994	24	IORT + HAI +/- PVI				32
Link *et al*[67]	1997	20	CAI	21			
		29		9.3			
Beger *et al*[68]	1999	24	CAI	23			54 (4 year)
		nd		10.5			9.5 (4 year)

HAI = hepatic arterial infusion; PVI = portal vein infusion; CAI = coeliac artery infusion

NEOADJUVANT THERAPY

The rationale for pre-operative therapy includes (a) the avoidance of long delays following surgery before starting adjuvant therapy and (b) an attempt to downstage the tumour and thereby increase the prospect of resection (Table 4)[70-77]. Pre-operative radiotherapy produced only modest increases in resectability and so chemotherapy was added in an attempt to improve the efficacy of this approach. Recent studies have reported resection rates as high as 60%[70-77] but not surprisingly those tumours >4cm-cm, encase the superior mesenteric artery or obstruct the superior mesenteric/ hepatic portal vein are less likely to be resected[71,73]. Pre-operative chemoradiotherapy may also increase the incidence of clear resection margins to as high as 90%, compared to the accepted norm of 60%-80%.

The effect of any long-term survival benefit from neoadjuvant treatment, if any, is not known due to the lack of randomized controlled studies. Twenty- four out of 53 patients with pancreatic ductal adenocarcinoma initially treated with 5-FU, mitomycin C and 50.4 Gy were able to undergo resection with a medi an survival of 15.7 months[74]. There was a significant level of toxicity associated with this regimen with two treatment related deaths. Another recent non-randomized study found a median survival of 19.2 months for patients with pancreatic cancer treated by pre-operative chemoradiation compared to 22 months for those treated by post-operative chemoradiation. Neither survival nor the pattern disease of recurrence was significantly different between the two groups[73].

COMBINATION THERAPY

Advanced pancreatic cancer

The combination of chemoradiation and follow-on chemotherapy may enable good local control with systemic destruction of the disease. The Gastrointestinal-Tumour Study Group (GITSG) randomized patients with advanced pancreatic cancer to receive either 60Gy EBRT (with radiosensitizing 5-FU) with or without follow-on 5-FU versus 40Gy EBRT with radiosensitizing 5-FU and follow-on 5-FU. The median survival times were 40, 23 and 42 weeks respectively, indicating a- likely valuable role for radiosensitizing (± follow-on)chemotherapy but not for increased radiotherapy[78]. Other combinations have been evaluated in the palliative setting[79]. A randomized trial of IORT versus IORT and methotrexate/5-FU produced a median survival of 4.8 and 8.5 months respectively[80].

Adjuvant combination therapy

The regimen originally adopted by the GITSG for patients with advanced pancreatic cancer was used in the adjuvant setting for a randomized trial in the 1970's . Forty-three patients were randomized to receive either 40Gy (with radiosensitizing 5-FU) then weekly 5-FU or surgery alone. The median survival in the treated group was 20 months compared to 11 months in the surgery only group and the two year survival rates were 42% and 15% respectively[81]. To increase numbers in the treatment group a further 30 patients received adjuvant therapy. The median survival was 18 months with a two year survival of 46%[82]. The number of patients that received treatment as part of the randomized study however was far too small for convincing conclusions to be drawn. The results were encouraging enough for other studies to adopt this protocol and investigate its role in the treatment of pancreatic cancer (Table 5)[81-88]. Yeo *et al*[84]compared three different regimens in selected patients who had undergone pancrea toduodenectomy: ① 40-45Gy EBRT plus follow-on 5-FU for 4 months (standard); ② 50-57Gy EBRT plus hepatic radiation plus 5-FU + folinic acid for 4 months (intensive); ③ no adjuvant treatment. The median survival was 21 months and the two-year survival was 44% for the group given standard adjuvant treatment, significantly better compared to 13.5 months and 30%, respectively in the no treatment group. There was no significant survival difference however between patients that had received the intensive treatment and those that had received no treatment. The main drawbacks to this study are the retrospective data that suffer from patient selection bias and no specification of patient performance status, which is an extremely important independent prognostic factor.

A phase III randomized controlled study organised by the Radiation Therapy Oncology Group (RTOG) in the USA is currently recruiting patients who have undergone resection for pancreatic adenocarcinoma. This study aims to compare 5-FU versus gemcitabine pre- and post- chemoradiotherapy all following surgery. The trial has already accrued the original 330 patient target but is still recruiting, presumably because of a lack of a significant therapeutic effect so far.

The UK Pancreatic Cancer Trials Group (UKPACA)[85] utilized the GITSG protocol for an open phase II study of 40 patients (34 with pancreatic ductal adenocarcinoma and 6 with ampullary tumour) who had undergone-pancreatoduodenectomy between 1987 and 1993 were recruited. Patients received 40Gy (with 5-FU as a radiosensitizer) plus 5-FU weekly for a maximum of 24 weeks. After a median of eight treatments there were no treatment related deaths and no hospitalizations even with a prolonged course of post-operative chemotherapy. The median survival for patients with pancreatic ductal adenocarcinoma was 13.2 months and the five-year survival rate was 15%.

The findings of these studies were instrumental in the design of the ESPAC-1 trial, which commenced in 1994. This trial was established to compare the effects of three adjuvant treatments with a control group. The four groups were: ① chemoradiotherapy (40Gy with radiosensitizing 5-FU); ② chemotherapy (5-FU plus folinic acid for six months); ③ combination chemoradiotherapy followed by chemotherapy; and ④ best supportive care. Patients were eligible following potentially curative resection for pancreatic ductal adenocarcinoma. Between February 1994 and April 2000 a total of 591 patients were randomized of which 541 had pancreatic ductal adenocarcinoma. A total of 61 centres recruited patients from UK, Ireland, France, Sweden, Spain, Italy, Germany, Switzerland, Greece, Hungary, Belgium and Austria. Randomization was stratified by resection margin status. Clinicians could randomize patients into a 2×2 factorial design (obse rvation, chemoradiation, chemotherapy or combination) or into one of the main treatment comparisons (i.e. chemoradiation *vs* control or chemotherapy *vs* control). Two-hundred and eighty five patients were randomized to the 2×2 factorial design, a further 68 patients were randomized to chemoradiation *vs* no chemoradiation and 188 patients were randomized to chemotherapy *vs* no chemotherapy. Tumour grade, size, nodal status and resection margin status were all significantly associated with survival. The overall results showed no benefit for chemoradiation *vs* no chemoradiation (median survival 15.5 months *vs* 16.2 months respectively). There was evidence of a survival benef it for

chemotherapy (median survival 19.7 months) compared to those patients who did not receive chemotherapy (median survival 14 months). The effect was reduced when taking into account whether patients had also received chemoradiation suggesting that chemoradiation may decrease the benefit of chemotherapy[89]. Moreover quality of life analysis showed no significant difference between any of the groups indicating that adjuvant therapy in pancreatic cancer is worthwhile, provided there is a significant prolongation of life[90].

The results of ESPAC-1 have provided for the first time a clear indication of a potential benefit for the use of chemotherapy in the treatment of pancreatic cancer. Even more importantly both ESPAC-1 and the EORTC trials have rejected the use of chemoradiation as adjuvant therapy in pancreatic cancer. Thus the focus of future studies such as ESPAC-3 will be on new and efficacious chemothe rapy regimens. This study is randomizing patients to three arms: 5-FU+folinic acid, gemcitabine and observation. The power of the study is such to detect a 10% difference in 2-year survival between any of the groups. Thus ESPAC-3 will establish (a) the benefit of adjuvant chemotherapy for pancreatic cancer and (b) if such, then the best form of chemotherapy.

Table 4 Neoadjuvant therapy for pancreatic cancer

Series	Year	Number	Regimen	Resection rate		Positive resection margin (*n*)	Median survival (months)	Actuarial survival (%)	
				n	%			3 year	5 year
Ishikawa *et al*[70]	1994	23	EBRT	17/23	74				22
Coia *et al*[71]	1994	27	EBRT + 5-FU + MMC	13/27	48	0/13	16	43	
Staley*et al*[72]	1996	39	EBRT + 5-FU + IORT	39/39	100	7/39	19		19 (4 year)
Spitz *et al*[73]	1997	41	EBRT + 5-FU	41/91	51	5/41	19.2		
Hoffman *et al*[74]	1998	53	EBRT + 5-FU + MMC	24/53	45		15.7		
White *et al*[75]	1999	25	5-FU + EBRT + MMC +CPP	5/25	20				
Wanebo *et al*[76]	2000	14	5-FU + EBRT +CPP	9/14	64				
Snady *et al*[77]	2000	68	EBRT+5-FU+STREP+CPP	20/68	29		32	32	
				48 NR	71		21	13	

EBRT = external beam radiotherapy; 5-FU = 5-fluorouracil; MMC= mitomycin C; IORT = intraoperative radiotherapy; STP = streptozocin; CDDP = cisplatinum; FA = folinic acid; DPD = dipyridamole; n = number; CPP=cisplatin; NR = not resectable.

Table 5 Results of combination therapy in patients who have undergone resection for pancreatic cancer

Series	Year	Number	Radiotherapy (Gy)	Chemotherapy	Median survival (months)	Actuarial survival (%)			
						1 year	2 year	3 year	5 year
Kalser *et al**[81]	1985	21	EBRT 40	5-FU	20	67	42	24	18
		22			11	50	15	7	8
GITSG[82]	1987	30	EBRT 40	5-FU	18		46		
Conlon *et al*[83]	1996	56	EBRT 45	5-FU	20		35		
Yeo *et al*[84]	1997	53			13.5		30		
		99	EBRT 40-45	5-FU	21		44		
		21	EBRT 50-57	5-FU + FA	17.5		22		
UKPACA[85]	1998	35	EBRT 40	5-FU	13	56	38	29	15
Abrams *et al*[86]	1999	23	EBRT	5-FU + FA	15.9				
Paulino *et al*[87]	1999	30	EBRT	5-FU	26				
		8	EBRT		5.5				
André *et al*[88]	2000	10	EBRT	5-FU + FA + CPP	17				

EBRT = external bean radiotherapy; 5-FU = 5-fluorouracil; FA = folinic acid; CPP = cisplatin

CONCLUSION

This is a very encouraging time for the treatment of pancreatic cancer. The results of these large European trials have at last given clear indications for future therapies of pancreatic cancer. By using this information, reasoned approaches are being developed to improve the treatment of patients with pancreatic cancer without sacrificing quality of life.

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• ORIGINAL RESEARCH •

Experimental and clinicopathologic study on the relationship between transcription factor Egr-1 and esophageal carcinoma

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Abstract

AIM **To observe the growth suppression effect of exogenous introduction of early growth response gene-1 (Egr-1 gene) on esophageal carcinoma tissue as well as on esophageal carcinoma cell line Eca109 and to explore the potential application of Egr-1 gene in gene therapy of tumor.**

METHODS **Eukaryotic expression vector of PCMV-Egr-1 plasmid was introduced into Eca109 cell line which expressed no Egr-1 protein originally with lipofectamine transfection method. The introduction and expression of PCMV-Egr-1 plasmid into Eca109 cell line was confirmed by G418 selection culture, PCR amplification of neogene contained in the vector, Western blot analysis and immunocytochemical analysis. The cell growth curve, soft agar colony formation rate and tumorigenicity in SCID mice were examined to demonstrate the growth suppression effect of exogenous Egr-1 gene on Eca109 cell line. The Egr-1 mRNA and Egr-1 protein were also detected in 50 surgical specimens of esophageal carcinoma by *in situ* hybridization and immunohistochemistry.**

RESULTS **Exogenous Egr-1 gene was introduced successfully into Eca109 cell line and expressed Egr-1 protein stably. The transfected Eca109 cell line grew more slowly than control Eca109 as shown by cell growth curves, the soft agar colony formation rate (4.0% *vs* 6.9%, *P*<0.01) and the average growth rate of tumor in SCID mice (35.5±7.6 *vs* 65.8±7.6, *P*<0.05). The expression level of Egr-1 mRNA and protein significantly increased in dysplastic epithelia adjacent to cancer rather than in cancer tissues (65.8% *vs* 20.0% by ISH and 57.9% *vs* 14.0% by IHC, *P*<0.01).**

CONCLUSION **Exogenous Egr-1 gene shows the strong effect of growth inhibition in Eca109 cell line. Egr-1 in the cancer tissue shows down-regulated expression that supports the inhibited function of Egr-1 in cancer growth and suggests Egr-1 may have an important role in gene therapy of esophageal carcinoma.**

Subject headings esophageal neoplasms/pathology; tumor cells, cultured; genes, immediate early; gene expression; transfection

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INTRODUCTION

Esophageal carcinoma is one of the most common malignant tumors in China[1-5]. Its pathogenesis and development are closely related with some of proto-oncogenes and their products[6-17]. Early growth response gene-1 (Egr-1 gene) is known as a member of immediate early gene (IEG) family characterized by rapid and transient expression in response to stimulation, modulating gene transcription positively or negatively depending on the cell types and playing an important role in the early cell growth. But its relationship with esophageal carcinoma is not well understood so far. In order to evaluate the growth inhibition in the cell line of esophageal carcinoma (Eca109) and analyze the expression of Egr-1 in the cancer tissue of esophageal carcinoma, the relationship between Egr-1 and esophageal carcinoma is explored by the methods of gene transfection, tumorigenicity in severe combined immunodeficient (SCID) mice and *in situ* hybridization.

MATERIALS AND METHODS

Human esophageal carcinoma cell line Eca109

Eca109 cell line from Chinese Academy of Medical Sciences was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 100mL·L^{-1} fetal bovine at 37℃ and 50mL·L^{-1} CO_2 incubator. The cancer tissue with adjacent mucosa and the esophageal mucosa in the upper surgical margin was from the same surgical specimen of 50 patients with esophageal carcinomas who had received neither chemotherapy nor radiotherapy before surgery. These were fixed in 40mL·L^{-1} neutral formalin with 1/1000 diethyl pyrocarbonate (DEPC, Sigma Chemical Co, USA) and embedded in paraffin, the sections were cut in 5μm.

Eukaryotic expression vector of PCMV-Egr-1 plasmid

The plasmid was donated by Dr RP Huang (Molecular Medicine, Northwest Hospital, WA, USA). The final construct contains the neogene (5.5kb fragment) driven by the respiratory syncytial virus (RSV) promoter and the Egr-1 gene (2.1kb fragment) driven by the human cytomegalovirus (CMV) promoter. The plasmid was confirmed by amplification, purification and tested by cutting endonuclease.

Gene transfection

The eukaryotic expression vector of PCMV-Egr-1 plasmid was transfected to human esophageal carcinoma cell line Eca109 by the lipofectamine (Gibcobrl Company, USA) according to the manufacturer's instructions. After transfection, the cells were trypsinized and reseeded at 1:2 ratio for selection culture with G418 at 600mg·L^{-1}. Four weeks later, the resistant colonies were formed.

Detection of transfected cell neogene with polymerase chain reaction

Extracting the cell DNA followed by performing polymerase chain reaction (PCR) to amplify 327bp of neogene with primer 1 (5'ACAAGATGGATTGCACGC AGG3') and primer 2 (5'TTCTCGGCAGGAGCAAGGTGAG3'). The cycling procedure was: denatured at 95℃ for 1 min, annealed at 55℃ for 1.5 min, extended at 72℃ for 1 min, and after 30 cycles, lengthened at 72℃ for 5 min. The untransfected Eca 109 cell line was used as a negative control.

Western blot and immunocytocheimstry

Cells (3×10^5) were harvested and dissolved by the addition of sodium dodecyl sulfate (SDS)-containing lysis buffer. The lysate was used for SDS-polyacrylamide gel electrophoresis on a 75g·L^{-1} gel. The proteins were electrophoretically transferred from the gel to NC membrane. The transferred membrane was treated with polycolonal antiserum against Egr-1 protein (1:200, Santa Cruz Biot Co, USA) followed by detection with peroxidase-labeled goat anti-rabbit (1:1000, Dako, USA). The resulting complexes were detected with the ECL reagent (Amersham Company, UK) according to the manufacturer's instructions. The Egr-1 protein was detected by immunocytochemistry.The untransfected Eca 109 cell line was used as a negative control.

Detection of biological feactures in transfected cells

Growth curve assays Cells (1×10^4) were seeded in each well of a 24-well plate and were allowed to grow for varying periods. The trypsinized cells were counted by cell counter plate. The growth assays were made by counting in triplicate on every other day of culture for up to 7d followed by constructing the cell growth curves.

Soft agar culture The anchorage independent growth was examined by seeding 1000 cells in 3g·L^{-1} agar medium into 60mm plates previously lined with 5g·L^{-1} agar medium. The plates (in triplicate and repeated twice) were cultured at 37℃, 50mL·L^{-1} CO_2 incubator for 14d. The average number of colony formation (any colony containing >50 cells was counted as a colony) and the colony formation rate (the number of colonies/the number of seeded cells) were calculated.

Tumorigenicity in SCID mice Two groups were divided randomly. The experimental group that consisted of 6 mice was used for Eca 109 cells with transfected Egr-1 injection. The control group of 4 mice was used for Eca 109 cells without transfected Egr-1 injection. Trypsinized cells (5×10^6) were injected into the subcutaneous sites on the shoulders of SCID mice. Animals were inspected at regular intervals for the appearance of visible tumors to measure the time of first appearance. Thirty days later, the mice were sacrificed and the tumors were carefully removed by blunt dissection. The tumors were weighed and their average growth rates were measured as mg·d^{-1}.

Egr-1 in situ hybridization

The expression of Egr-1 was detected by digoxigenin-labeled gene probe from a commercial kit (Boster Company, China) according to the manufacturer's instructions. The human breast tissue and the mouse brain tissue were used as the positive control. Either the sections detected with incubation solution instead of the probe or the sections digested with ribonucleases (RNase) (10mg·L^{-1}) before Egr-1 detection was designed for the negative control. The positive expression showed the brown staining signal in the cytoplasm.

Immunohistochemistry

Egr-1 was analyzed by using Egr-1 rabbit polyclonal antiserum with the SABC method according to the manufacturer's instructions (Boster Company, China) following treatment with 3.3'-diaminobenzidine (DAB) staining. The human breast tissue and the mouse brain tissue were used as the positive control. Negative control was designed using phosphate-buffered saline (PBS) instead of Egr-1 rabbit polyclonal antiserum in detection. The positive expression showed the brown staining signal in nuclei.

Statistical analysis

The data were statistically analyzed using Student's *t* test and the difference of results was analyzed by *U* test and χ^2 test.

RESULTS

Identification of plasmid

The eukaryotic expression vector of PCMV-Egr-1 plasmid was tested by PCR amplification, purification and restriction endonuclease and confirmed to be consistent with the plasmid map. With single digestion of *Sac* II, a 7.6kb band was obtained which represents the whole lengh of the plasmid DNA. With double digestion of *Sac* II combining with *Sma* I, both 5.5kb and 2.1kb bands were obtained which represented the fragment of the vector and Egr-1 gene fragment respectively (Figure 1).

Gene transfection

Eca109 was transfected with PCMV-Egr-1 plasmid by lipofectamine transfection method. Four weeks after G418 selection culture, the resistant colonies were formed. PCR

indicated that 327bp of neogene was shown in transfected Eca109 compared to the negative band in control Eca109 (Figure 2), demonstrating that PCMV-Egr-1 plasmid had been introduced into Eca109 cell and integrated into the genomic DNA.

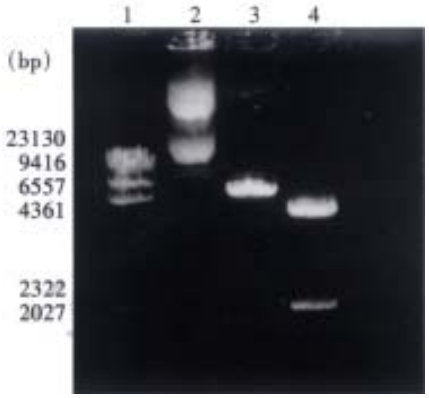

Figure 1 Identification of PCMV-Egr-1 plasmid.
1. Marker; 2. Uncut plasmid; 3. Cut with *Sac* II, showing 7.6kp fragment of whole plasmid; 4. Cut with *Sac* II and Sma I, showing 5.5kp fragment of vector and 2.1kp fragment of Egr-1 gene.

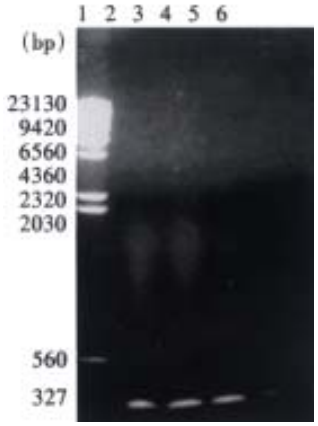

Figure 2 PCR amplification of neogene.
1. Marker; 2-5. For transfected Eca109, showing 327bp of neogene; 6. Negative control Eca109, no band was shown.

Detection of Egr-1 protein in transfected Eca109

Western blot The 80kd of Egr-1 protein was shown for transfected Eca109 compared to the negative band of the control Eca109.

Immunocytochemistry The transfected Eca109 was positively brown stained in the nucleus compared to the negative staining for control Eca109 (Figure 3). The results demonstrated that exogenous Egr-1 gene introduced into Eca109 expressed high level of Egr-1 protein.

Growth feature of transfected Eca109

Growth curves The transfected Eca109 grew much more slowly in DMEM medium than the control Eca109 cell (Figure 4).

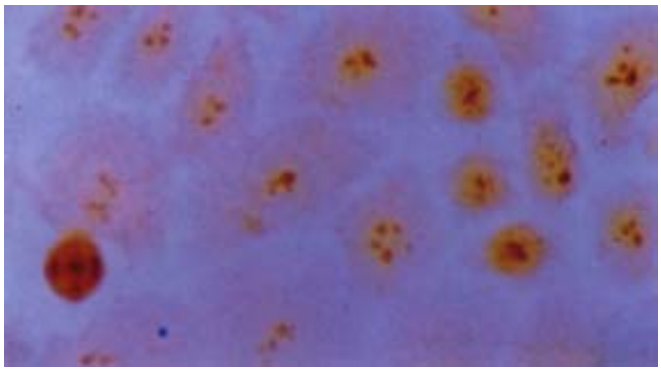

Figure 3 Positive Egr-1 protein in nuclei of transfected Eca109 cells. ICC ×400

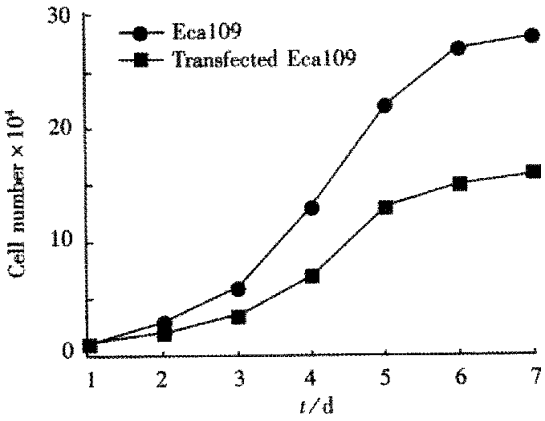

Figure 4 Cell growth curves showing lower growth rate in transfected Eca109 cell than in the control Eca109 cell.

Soft agar growth assay Small and few colonies were formed slowly in transfected Eca109 compared to large and numerous colonies formed quickly in control Eca109. The colony formation rates were 4.0% and 6.9% in transfected Eca109 and in control Eca109 respectively (Table 1) which demonstrated that the anchorage independent growth ability of Egr-1 expressing Eca109 was reduced.

Table 1 Soft agar assays in Eca109 cell line

Cell line	No. of seeded cell	No. of colonies				CFR
		1	2	3	Mean	
Eca109	1000	65	71	73	69±2.4	6.9%
Egr-1-Eca109	1000	40	43	39	40±1.2	4.0%[b]

[b]$P<0.01$, *vs* Eca109, *U* test.

Tumorigenicity in SCID mice The tumorigenicity test showed that the tumors started to appear on the 21st day after injection in the six SCID mice injected with transfected Eca109, and on the 14th day in four SCID mice injected with control Eca109. The tumors of Egr-1 expressing Eca109 grew slowly with an average growth rate of 35.5mg·d^{-1}, and the tumors of control Eca109 grew fast with an average growth rate of 65.8mg·d^{-1} (Figure 5, Table 2). The results demonstrated that the tumorigenicity in SCID mice of Egr-1 transfected Eca109 was inhibited.

Table 2 Tumorigenicity assays in Eca109 cell line

Cell line	Tumorigenicity rate	Growth rate of tumors(mg·d^{-1})						
		1	2	3	4	5	6	Mean
Eca109	4/4	83.3	70.0	46.6	63.3			65.8±7.6
Egr-1-Eca109	6/6	20.0	13.3	33.3	66.6	43.0	36.7	35.5±7.6[a]

[a]P<0.05, *vs* Eca109, Student's t test.

Egr-1 expression on esophageal tissues

In simple hyperplastic epithelia of esophageal mucosa, the expression of Egr-1 was found in the basal layer of the mucosa (Figure 6). In dysplastic epithelia, the expression of Egr-1 increased but significantly decreased in cancer tissues in which only a few cases of well-differentiated squamous cell carcinoma had the Egr-1 expression (Figure 7). The expression of Egr-1mRNA and proteins in the various pathological changes of esophagus are shown in Table 3.

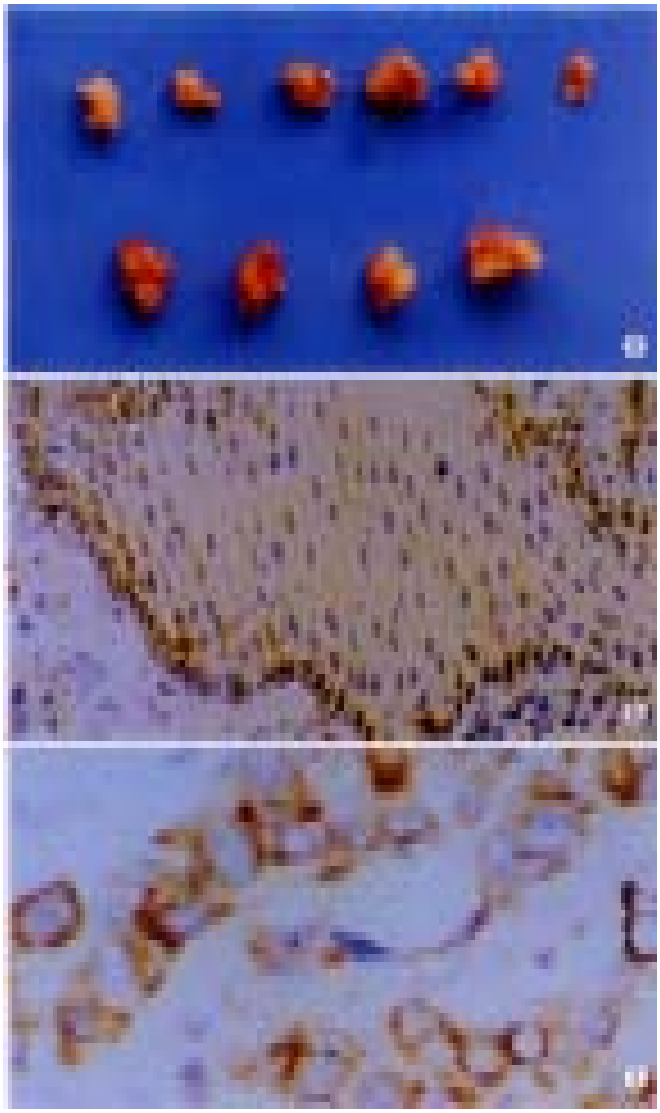

Figure 5 The tumors of transfected Eca109 are smaller than that of control Eca109, *in vitro*, 30 d after injection in tumorigenicity test in SCID mice.

Figure 6 Egr-1 protein expression in basal mucosal layer in simple hyperplastic epithelia of esophagus. IHC ×200

Figure 7 Positive Egr-1 mRNA in cytoplasm of esophageal squamous cell carcinoma. ISH ×400

Table 3 Expression of Egr-1mRNA and proteins in various pathological changes of esophagus, n(%)

Group	n	ISH	IHC
Simple hyperplasia	30	12 (40.0)	9 (30.0)
Dysplasia	38	25 (65.8)	22 (57.9)[a]
Esophageal cancer	50	10 (20.0)	7 (14.0)[b]

[a]P<0.05, *vs* simple hyperplasia, χ^2 test; [b]P<0.01, *vs* dysplasia,χ^2 test.

DISCUSSION

As we know, the oncogenes and tumor suppressor genes are involved in the pathogenesis and development of esophageal carcinoma[18-32]. Recent studies in dicate that Egr-1 gene as a tumor related-gene is correlated with the tumor development. It was reported that Egr-1 proteins were decreased and even disappeared in several kinds of cancer tissues. Furthermore, the Egr-1 mRNA expression was consistent with the expression level of Egr-1 protein which had been verified in the down-regulation of Egr-1 occurred at the transcription level in cancer cells[33]. Southern blot analysis indicated no deletion, no rearrangement or mutation of Egr-1 on DNA level. The exogenous introduction of Egr-1 could inhibit the growth of tumor cells accompanied in a dose-dependent manner, for example, the ability of anchorage independent growth and tumorigenicity in SCID mice of human HT1080 fibrosarcoma without original Egr-1 was significantly inhibited after the exogenous introduction of Egr-1. If the antisense Egr-1 was introduced to the cells, it would inhibit the endogenous Egr-1 expression and promote the malignant transformation of the cells. In the present study, eukaryotic expression vector of PCMV-Egr-1 plasmid was introduced into Eca109 cell line that expressed no Egr-1 protein originally with Lipofectamine transfection method. The introduction and expression of PCMV-Egr-1 plasmid into Eca109 cell line was confirmed by G418 selection culture by which colony formation persisted in next generations and by PCR amplification of neogene contained in the vector. Furthermore, the strong expression of Egr-1 protein in transfected Eca109 was detected by Western blot and immunocytochemistry, which verified the success of gene transfection. Growth inhibition of the transfected cells shown by the growth curves, the colony formation rates in the soft agar and tumorigenicity in SCID mice demonstrated that exogenous Egr-1 gene inhibited the growth of Eca109.

Recent studies suggest that the mechanism of suppression of tumor growth by Egr-1 is the Egr-1 protein with a zinc-finger domain which regulates the transcription of many downstream genes by binding to the GC rich element in the promoter region and modulates genes transcription and various biological effects[34-45]. Egr-1 can compete with transcription activator SP1 in binding to an overlapping consensus binding motif in the promoter region of GCE which commonly exists in oncogene and tumor suppressor gene and abolishes the function of SP1, leading to the transcription inhibition of downstream genes and growth inhibition of tumor cells[46,47]. In addition, the exogenous Egr-1 may inhibit the growth by binding to the GEE of TGF-β1 and activating the transcription of TGF-β1 and subsequently activating p21 gene or by down-regulating Bcl-2 gene to influence the tumor cells[48,49]. Some

studies indicated that the activation of some oncogenes e.g. wild type *p*53 gene, cell apoptosis, the TNF-α and the concentration of calcium may be involved in the mechanism of growth inhibition property of Egr-1[50,51]. On the whole, various and complicated mechanisms may be involved in the suppressive property of Egr-1 for tumor growth.

Egr-1 is one of the immediate early gene family which regulates the cellular growth and differentiation by activating Cyclin D1 to promote the cells from the G_0/G_1 phase into the G2/M phase[52]. The mechanism of down-expression of Egr-1 in cancer tissues is not clear up to now. It was hypothesized that the high concentration of Egr-1 proteins produced by overstimulation of Egr-1 interacted with the promoter region of EBS and subsequently the gene transcription was inhibited, which was concordant with the present results that showed strong expression of Egr-1 in dysplastic cells adjacent to esophageal carcinoma and weekly expression in cancer cells.

Since only downregulation expression without gene mutation occurred in Egr-1 DNA level, it was different from some tumor suppressor genes such as *p*3, *p*16 and Rb with mutation gene on DNA level. It is more convenient to introduce normal exogenous gene into the tumor cells than to repair mutation gene in gene therapy. The present study substantiates that exogenous Egr-1 as a target gene has a potential application in gene therapy of esophageal carcinoma.

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• ORIGINAL RESEARCH •

Sleep deprivation increase the expression of inducible heat shock protein 70 in rat gastric mucosa

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Abstract

AIM **To investigate if sleep deprivation is able to increase the expression of inducible heat shock protein 70 in gastric mucosa and its possible role in mucosal defense.**

METHODS **Rats for sleep disruption were placed inside a computerized rotating drum, gastric mucosa was taken from rats with 1, 3 and 7d sleep deprivation. RT-PCR, immunohistochemistry and Western blotting were used to determine the expression of heat shock protein 70. Ethanol (500mL·L^{-1}, i.g.) was used to induce gastric mucosa damage.**

RESULTS **RT-PCR, Western blotting and immunostaining confirmed that the sleep deprivation as a stress resulted in significantly greater expression of inducible heat shock protein 70 in gastric mucosa of rats. After the 500mL·L^{-1} ethanol challenge, the ulcer area found in the rats with 7d sleep deprivation (19.15±4.2)mm^2 was significantly lower (P<0.01) than the corresponding control (53.7± 8.1) mm$_2$.**

CONCLUSION **Sleep deprivation as a stress, in addition to lowering the gastric mucosal barrier, is able to stimulate the expression of inducible heat shock protein 70 in gastric mucosa of rats, the heat shock protein 70 may play an important role in gastric mucosal protection.**

Subject headings sleep deprivation; heat shock proteins 70/biosynthesis; gastric mucosa; rats

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INTRODUCTION

Stress has been shown to induce gastric mucosal lesions and lower the effectiveness of the mucosa as a barrier[1-6]. In rats, gastric ulcers can be produced by cold-restraint stress[7-9] and it is frequently employed as a model for the study of the mechanisms of stress on ulcer formation. Cold-restraint stress, however, is not normally encountered in human subjects while sleep deprivation is a common experience among city dwellers. swift workers and medical professionals. It imposes stress on the body, and produces a variety of health problems[10-14]. Sleep deprivation is associated with poor congitive ability, and shortening of longevity[15]. Studies have found that the cognitive function of doctors after a night shift was considerably decreased. Sleep deprivation is also a major problem in the intensive care units and it has been suggested to affect the healing process of patients thus contributing to an increase in morbidity and mortality[16]. Sleep deprivation has been shown to induce typical dermatitis in experimental animals. Severe ulcerative and hyperkeratotic skin lesions localized to the paws and tails developed in rats deprived of sleep[17]. This effect of sleep deprivation may affect the epithelial linings of the gastrointestinal tract, because stress has been demonstrated to produce gastric mucosal lesions in rats[18,19]. Our previous works showed that the food and water consumption in sleep disturbed rats were not affected but they had a smaller percentage gain in body weight. The locomotion activities of sleep disturbed rats were similar to the controls, however, their adrenal weights were increased[20]. The mucosal epithelial cell proliferation rate was also suppressed by sleep disturbance. Although various factors have been proposed to account for this process, the precise mechanism of how sleep deprivation affects the gastric mucosa barrier, especially at the molecular level, still remains unclear. Previously, we used cDNA expression arrays to identify genes that abnormal expressed in gastric mucosa of sleep deprivation rats[20]. In this project, inducible heat shock protein 70, one candidate gene emerging from cDNA array for its potential significance was further analyzed.

MATERIALS AND METHODS

Rats and reagents

Male Sprague Dawley rats weighing 180g-200g were used in the experiments. They were housed in a temperature 22℃ ± 1℃ and humidity 65% - 70% controlled room with a day night cycle of 12h. The rats were given standard laboratory diet (Ralston Purina Co., Chicago, IL) and tap water *ad libitum*. Rats were starved for 24h and water withdrawn 1 hour prior to any oral or intragastric administration of agents in order to obtain a uniform distribution of those agents onto the gastric mucosa. All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, USA) unless specified otherwise. The present study has been examined and approved by the Committee on the Use of Live Rats for Teaching and Research of the University of Hong Kong.

Sleep disturbance

Rats for sleep disruption were placed inside a computerized rotating drum while the control animals were left undisturbed in a stationary drum. The drum was rotated 180° in 30s at 5 minutes intervals and was programed to switch off for 1h

every day at 13:00 to allow for an hour of undisturbed sleep. Sleep disturbance was continued for 1wk before the animals were killed.

Collection of gastric mucosa

Rats were killed by ether anesthesia followed by cutting off the abdominal aortic artery. The stomachs were removed rapidly, opened along the greater curvature, and rinsed with cooled normal saline thoroughly. A longitudinal section of gastric tissue was taken from the anterior part of the stomach and then fixed in 100mL·L^{-1} buffered formalin for 24h. It was cut into sections of 5μm and then used in immunostaining. Gastric mucosa was taken from the remaining part of the stomach by scraping with a glass slide on a glass dish on ice. They were wrapped by a piece of aluminum foil, immediately froze in liquid nitrogen and stored at -70℃ until assayed.

Detection of inducible heat shock protein 70 *mRNA expression by RT-PCR*[21-23]

Total RNA was extracted from gastric mucosa of rats by using Trizol reagent (Gibco BRL, Gaitherburg, MD). First-strand complementary DNAs were synthesized from 5μg RNA by using oligo dt primer and Thermoscript RT-PCR system (Gibco BRL, Gaithersburg, MD). The PCR cycle was performed for in ducible heat shock protein 70 and β-actin from the same complementary DNA sample using a PCR Thermal Cycler (Gene Amp PCR System 9700, The Perkin-Elmer Corporation, Norwalk, CT). The sequence of the oligonucleotide primers are as follows: sense inducible heat shock protein 70, 5'-TGCTGACCAAGATGAAG-3'; antisense inducible heat shock protein 70, 5'-AGAGTCGATCTCCAGGC-3'[24] and sense β-actin, 5'-GTGGGGCGCCCCAGGCACCA-3'; antisense β-actin, 5'-CTCCTTAATGTCACGCACGATTTC-3'[25]. After-denaturation for 10 min at 95℃, 30 cycles of amplification were carried out followed by final extension of 10 min at 72℃, each step for 1 min. After amplification, 10μL of PCR products were electrophoresed in a 1% agarose gels containing 0.5μg/mL ethidium bromide.

Immunohistochemical detection of inducible heat shock protein 70 in gastric mucosa[26-30]

Fixed tissue sections (5μm) were mounted on Vectabond Reagent-coated slides, deparaffinized and rehydrated through xyline, graded ethanol to distilled water. After blocking endogenous peroxidase with 3mL·L^{-1} hydrogen peroxide in methanol for 30 min, sections were treated with 0.05mol·L^{-1} phosphate-buffered saline containing 30mL·L^{-1} normal horse serum and 3g·L^{-1} Triton X-100 for 30min, then the sections were rinsed with PBS, incubated with mouse anti-rat monoclonal antibody (StressGen Biotechnologies Corp. Victoria, Canada, Cat# SPA-810) at dilution of 1:200 overnight at 4℃ in a humidity chamber. The secondary, biotinylated antibody (LSAB kit, Dako, Denmark) was then applied for 30 min followed by rinsing with PBS. Staining was performed by the addition of streptavidin (LSAB kit, Dako, Denmark) for 30 min, rinsed in PBS and developed in 3, 3'-diaminobendine tetrahydrochloride for about 3 min. Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted.

Detection of inducible heat shock protein 70 *proteins expression by Western blotting*

The gastric mucosa were homogenized at 4℃ in RIPA buffer (50 mmol·L^{-1} Tris-HCl, pH 7.5, 150 mmol·L^{-1} NaCl, 1g·L^{-1} sodium dodecyl sulfate, 5g·L^{-1} a-cholate, 2 mmol·L^{-1} EDTA, 10 g·L^{-1} Triton X-100, 100 mL·L^{-1} glycerol) containing 1 mmol·L^{-1} PMSF and 10 mg·L^{-1} aprotinin. After centrifuged at 10000×*g* at 4℃ for 20 min, the supernatant (50μg of total protein) were denatured and separated on 75g·L^{-1} sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA)[31,32]. The membranes were blocked with blocking buffer (50mL·L^{-1}) nonfat milk in wash buffer (20 mmol·L^{-1} PBS, pH 7.5 containing 100mmol·L^{-1} NaCl and 1g·L^{-1} Tween 20) for 1h at room temperature, and subsequently incubated at 4℃ with mouse monoclonal antibody against rat inducible heat shock protein 70 (StressGen Biotechnologies Gorp. Victoria, Canada, Cat # SPA-810) diluted in blocking buffer (1:1000). Membranes were washed 6 times and incubated with a rabbit-anti-mouse immunoglobulin G conjugated with the horseradish peroxidase (1:4000) (Bio-Rad Laboratories) for 1h . After six times additional washes, membranes were developed by a commercial chemiluminescence system (Amersham, Arlington Heights, IL) and exposed to X-ray film. Protein determinations were made with Bio-Rad protein assay kit with bovine serum albumin as a standard. Prestained molecular-weight standards (Bio-Rad) were used as markers.

Ethanol-induced gastric mucosal damage

Rats were starved for 24h before 1mL of 500mL·L^{-1} ethanol was administered orally to induce acute gastric mucosal damage[33]. Rats were killed 2h later by a sharp blow on the heads followed by cervical dislocation. The stomach was removed and opened along the greater curvature. The gastric lesion area (mm^2) was traced onto a glass plate and subsequently measured on a graph paper with 1mm^2 division[34]. The lesion index was calculated by dividing the total lesion area with the number of rats in each group.

Statistics

The data were statistically analyzed with the unpaired two-tailed Student's *t* test.

RESULTS

Sleep deprivation increase the expression of inducible heat shock protein 70

Total RNA and protein were isolated from rats' gastric mucosa at various times of sleep deprivation. As shown in Figure 1, RT-PCR results indicated that the expression of inducible heat shock protein 70 mRNA was low in normal gastric mucosa. Following the sleep deprivation, inducible heat shock protein 70 mRNA expression was elevated. The pattern of inducible heat shock protein 70 protein accumulation showed a similar trend to mRNA expression (Figure 2).

Inducible heat shock protein 70 immunohistochemistry in gastric mucosa

To further prove that inducible heat shock protein 70 is increased in gastric mucosa of sleep deprived rats, confirm their expression at protein level, and determine their cellular sources, immunostaining for iHsp 70 was performed on gastric mucosa of sleep deprived rats and normal rats. The results showed that inducible heat shock protein 70 staining was nearly absent in normal mucosa but in mucosa of sleep deprived rats was detected in epithelium (Figure 3).

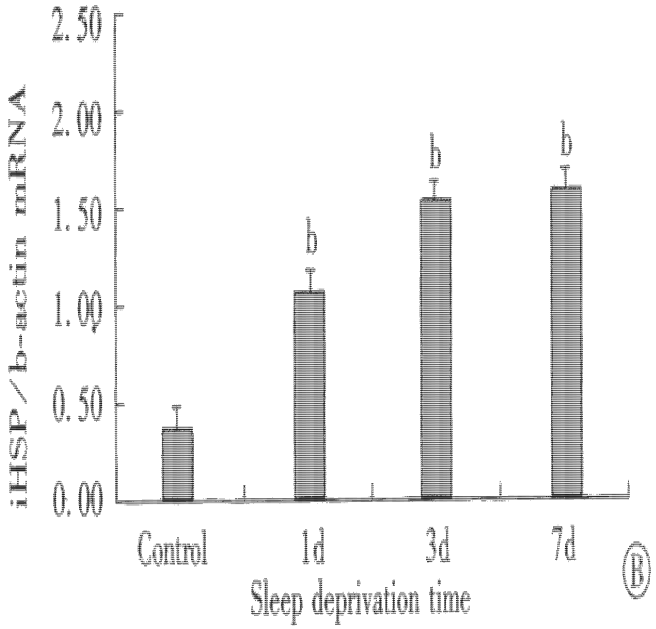

Figure 1 Effect of sleep deprivation in inducible heat shock protein 70 mRNA expression in gastric mucosa of rats. Inducible heat shock protein 70 mRNA was determined by RT-PCR.
(A). Gel photograph of PCR-amplified inducible heat shock protein 70 and β-actin cDNA derived from inducible heat shock protein 70 and β-actin mRNA. Lane 1: normal control; Lane 2: 1 day sleep deprivation; Lane 3: 3 days sleep deprivation; Lane 4: 7 days sleep deprivation.
(B). Bar graph showing the relative amount of inducible heat shock protein 70 mRNA quantified by densitometry and expressed as mean of inducible heat shock protein 70 mRNA: β-actin mRNA ratios. Error bars represent SE, n=8 for each group. $^bP<0.01$ *vs* control group.

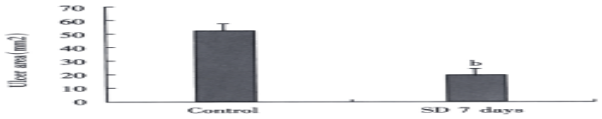

Figure 2 Western blotting analysis of inducible heat shock protein 70 from gastric mucosa of sleep deprivation rats. Lane 1 and 2: control; Lane 3 and 4: 1 day sleep deprivation; Lane 5 and 6: 3 days sleep deprivation; Lane 7 and 8: 7 days sleep deprivation.

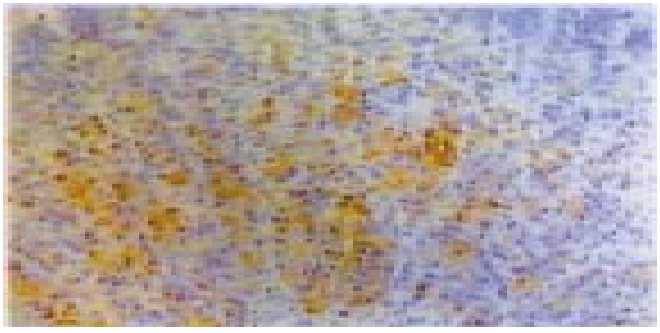

Figure 3 Inducible heat shock protein 70 immunohistochemistry in gastric mucosa of rats with 7 days sleep deprivation.

Sleep deprivation decrease ethanol induced gastric ulceration

After the 500mL·L^{-1} ethanol challenge, the ulcer area found in the rats with 7d sleep deprivation (19.2±4.2) mm^2 was significantly lower (P<0.01) then the corresponding control (53.7±8.1) mm^2, as shown in Figure 4.

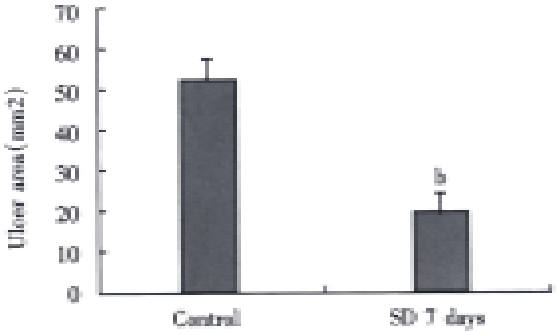

Figure 4 Effect of sleep deprivation in ethanol induced (50% ethanol 1mL p.o. for 2h) gastric ulceration in rats. Error bars represent SE, n=10 for each group. $^bP<0.01$ *vs* control group.

DISCUSSION

In our previously experiment, cDNA arrays were used to search for genes that were differentially expressed in gastric mucosa of sleep deprivation rats compared to gastric mucosa of control rats. More than 10 differentially expressed genes were found in total 588 genes, most of these were digestive enzyme related genes, one of the overexpression gene was inducible heat shock protein 70 gene[20]. A variety of chemicals, viruses, and noxious stimuli such as trauma, hypoxia, or ischemia trigger the heat shock response and the subsequent synthesis of heat shock proteins[35-43]. The results of our experiment indicated that sleep deprivation as a stress resulted in significantly greater expression of inducible heat shock protein 70 in gastric mucosa of rats, which was confirmed by RT-PCR, Western blotting and immunostaining. Substantial evidence showed that heat shock is capable of protecting cells, tissues and organs, and animals from a subsequent, normally lethal heating, as well as from other types of noxious condition[44]. The protective effect of heat shock is likely mediated by overexpressed heat shock protein 70, because there is a lag between heat shock and the development of protection correlated with the production of heat shock protein 70, and protection is affected when heat shock protein 70 production is inhibited by treatment with inhibitors[45-47]. Microinjection of anti-heat shock protein 70 antibody into fibroblasts to neutralize heat shock protein 70 increases the vulnerability of the cells to sublethal temperatures[48]. Furthermore, heat shock protein 70 also provides protection when induced by methods other than heat shock, such as rats that overexpress heat shock protein 70 induced by methods other than heat shock display protection of the lungs from sepsis-induced injury[49] and a reduction in hepatocyte apoptosis induced by tumor necrosis factor-α[40]. Transgenic mice that overexpress heat shock protein 70 demonstrate resistance to adverse effects of lethal heat or ischemia[41]. Similar evidence has been derived from the study of cultured cells after heat shock or heat shock protein 70 gene transfection to promote overexpression of heat shock protein 70[50,51]. Our results showed that sleep deprivation was able to decrease the gastric mucosa damage caused by 50% ethanol. Although we cannot completely preclude the possibility that the vascular, neural, and hormonal factors may be involved in this mucosa protective effect, our previous work showed that sleep deprivation decreased the gastric mucosal blood flow, hampered the ability of the gastric mucosa to repair itself by slowing down its cellular replication rate, and depressed gastric potential difference. In conclusion, our results lead us

to speculate that the heat shock protein 70 may play an important role in gastric mucosal protection.

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• ORIGINAL RESEARCH •

Expression of vascular endothelial growth factor and its role in oncogenesis of human gastric carcinoma

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Abstract

AIM **To establish the role of vascular endothelial growth factor (VEGF) in the oncogenesis of human gastric carcinoma more directly.**

METHODS **The expression of VEGF and its receptor kinase-domain insert containing receptor (KDR) in human gastric cancer tissue were observed by immunohistochemical staining. VEGF levels were manipulated in human gastric cancer cell using eukaryotic expression constructs designed to express the complete $VEGF_{165}$ complimentary DNA in either the sense or antisense orientation. The biological changes of the cells were observed in which VEGF was up-regulated or down-regulated.**

RESULTS **VEGF-positive rate was 50%, and VEGF was mainly localized in the cytoplasm and membrane of the tumor cells, while KDR was mainly located in the membrane of vascular endothelial cells in gastric cancer tissues and peri-cancerous tissue. In 2 cases of 50 specimens, the gastric cancer cells expressed KDR, localized in both the cytoplasm and membrane. Introduction of $VEGF_{165}$ antisense into human gastric cancer cells (SGC-7901, immunofluorescence intensity, 31.6%)) resulted in a significant reduction in VEGF-specific messenger RNA and total and cell surface VEGF protein (immunofluorescence intensity, 8.9%) (P<0.05). Conversely, stable integration of $VEGF_{165}$ in the sense orientation resulted in an increase in cellular and cell surface VEGF (immunofluorescence intensity, 75.4%) (P<0.05). Lowered VEGF levels were associated with a marked decrease in the growth of nude mouse xenografted tumor (at 33 days postimplantation, tomor volume:** 345.40±136.31 **mm^3) (P<0.05 *vs* control SGC-7901 group:** 1534.40±362.88 **mm^3), whereas up regulation of VEGF resulted in increased xenografted tumor size (at 33 days postimplantation, tomor volume:** 2350.50±637.70 **mm^3) (P<0.05 *vs* control SGC-7901 group).**

CONCLUSION **This study provides direct evidence that VEGF plays an important role in the oncogenesis of human gastric cancer.**

Subject headings endothelial growth factor/analysis; angiogenesis; solid tumor; stomach neoplasms/pathology; endothelium; vascular

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INTRODUCTION

It is known that malignant tumors depend on neovascularization for their growth and metastasis[1,2]. Recently, many studies have shown that the secretion and activation of various endothelial growth factors, called angiogenic factors, by tumor cells plays a crucial role in the formation of neovasculature[3-8]. VEGF is a powerful mitogen for vascular endothelial cells, both *in vitro* and *in vivo*. In addition, VEGF has the property of inducing vascular permeability *in vivo*. Three evidences for the central role played by VEGF in tumor angiogenesis were: ① the detection of high levels of VEGF expression in palisading cells around regions of necrosis in a number of solid tumor systems, coupled with the rapid induction of VEGF when tumor cells are grown under hypoxic conditions[9,10]; ② injection of antibodies against VEGF markedly reduced the *in vivo* growth of s.c. injected tumor cells, which are known to produce robust tumor angiogenesis[11]; and ③ introduction into tumor endothelial cells of a dominant-negative version of VEGF-R_2 by means of retroviral transfer markedly reduced tumor size[12]. The current study was designed to establish the role of VEGF in the oncogenesis of human gastric cancer more directly.

SUBJECTS AND METHODS

Immunohistochemistry

Resected specimens from 50 patients with gastric carcinoma who underwent gastrectomy at our institute were studied. The patients ranged in age from 27 to 76 years (average 56.3 years); 40 were men, and 10 were women. No patient had received chemotherapy or radiation therapy before surgery. Specimens were fixed in a 40g·L^{-1} formaldehyde solution and embedded in paraffin. Five μm thick sections were cut and mounted on glass slides. Immunohistochemistry was performed using the avidin-biotin complex method. Sections were dewaxed in xylene, taken through ethanol, and then incubated with 30mL·L^{-1} hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. Sections were then washed in phosphate-buffered saline and incubated in 20mL·L^{-1} normal goat serum for 30 minutes to reduce nonspecific antibody binding. The antibody for VEGF and

KDR (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) were used respectively. Specimens were then incubated with a 1:500 dilution of anti-VEGF antibody overnight at 4℃, followed by three washes with PBS. Sections were then incubated with biotinylated goat antirabbit immunoglobulin G (Sino-American Biotechnology Co.) at a dilution of 1:100 for 2 hours followed by 3 washes. Slides were then treated with the complex of Reagent A and Reagent B (ABC kit, Sino-American Biotechnology Co.) for 2 hours at a dilution of 1:100 and were washed with phosphate-buffered saline 3 times. Finally, slides were incubated in phosphate-buffered saline containing diaminobenzidine and 300 $mL \cdot L^{-1}$ hydrogen peroxide for 10 minutes. Normal rabbit immunoglobulin-G was substituted for primary antibody as the negative control.

Plasmid constructs

pGEM-hVEGF is pGEM-3Zf(+) derivative Promega cloning vector plasmid containing the complete complementary DNA (cDNA) sequence of human $VEGF_{165}$ (this plasmid was generous gifts from Judith Abraham, Scios Nova, Inc.)[13]. The $VEGF_{165}$ cDNA was subcloned into the $pCDNA_3$ eukaryotic expression vector using the Eco-RI and *Xba* I or *Eco*R I and *Hin*d III restriction enzyme sites in sense or antisense orientation respectively. Restriction enzyme analysis (*Eco*R I, *Xba* I and *Hin*d III) and dideoxy sequencing method were used to confirm the orientation and quality of the VEGF cDNA in the $pCDNA_3$ vector, respectively.

Gastric cancer cell line culture conditions

Cell line SGC-7901 was derived from a moderately-differentiated gastric adenocarcinoma and has been characterized extensively[14]. The cells were routinely cultured in RPMI 1640 / 100 $mL \cdot L^{-1}$ NCS (heat-inactivated) and 2 $mmol \cdot L^{-1}$ L-glutamine in a humidified atmosphere of 50 $mL \cdot L^{-1}$ CO_2 at 37℃, supplemented with penicillin (100 $KU \cdot L^{-1}$) and streptomycin (100$mg \cdot L^{-1}$).

Liposome-mediated transfection

Human SGC-7901 cells were grown in RPMI 1640 / 100$mL \cdot L^{-1}$ NCS in 6-well tissue culture dishes to 50% confluence. The lipofect Amine-mediated transfection (Gibco/BRL) was performed as described previously using 2μg of recombinant constructs DNA (or $pCDNA_3$ vector alone)[15]. After 48h, they were trypsinized from the plates and dilute cells into selective medium containing Geneticin G_{418} (350$mg \cdot L^{-1}$). Cell death was observed after 3 days in culture, and discrete colonies were apparent by 10 days post-selection. Individual colonies were then isolated and grown in 24-well culture plates. Genomic DNA and total RNA were then isolated from these colonies, and PCR analysis and RNA dot blotting were performed. Clones demonstrated to express the sense and antisense-VEGF constructs were then recloned by growing single cell in 96-well plates.

Detection of expression constructs in genomic DNA by PCR

PCR was used to determine which human gastric cancer cell clones were successfully transfected with the sense or antisense-VEGF construct. PCR was performed on genomic DNA isolated from human SGC-7901 gastric cancer cells and individual clones of transfected cells using a sense primer(a) that corresponds to the 5' initial sequence of VEGF cDNA insert (5'GCACCCATGGCAGAAGGAGGAG 3') or an antisense primer (b) that corresponds to the 3' terminal sequence (5'TCACCGCCTCGGCTTGTCACATC 3') and a primer that corresponds to the SP_6 transcription start of the $pCDNA_3$ expression vector (5'GATTTAGGTGACACTATAG 3'). The PCR reaction was performed using standard protocols with 30 cycles of 50s at 94℃, 50s at 55℃ and 60s at 72℃. Appropriately sized amplification products were verified by agarose gel electrophoresis. Negative controls lacking target DNA or containing a nonhomologous plasmid routinely did not show amplification.

RNA dot blotting

Total RNA was isolated from parental and derivative cell lines using the Trizol reagent (GIBCO/BRL). The samples were serially diluted in diethyl pyrocarbonate-treated water containing placental RNase inhibitor (Boehringer Mannheim) to produce working stocks with a final concentration of 10$mg \cdot L^{-1}$ as spectrophotometrically. Denatured RNA was immobilized on nitrocellulose membranes (Boehringer Mannheim). "Run-off" digoxigenin (DIG) labeled riboprobes specific for the sense or antisense strands of VEGF was synthesized from appropriately linearized plasmid stocks using the DIG-RNA labeling kit (Boehinger Mannheim) and appropriate RNA polymerase (Promega) plus 10U placental RNase inhibitor. The specificity of each probe stock was verified by hybridization to originating plasmids and negative controls. The blots were prehybridized in DIG easy Hyb buffer (Boehringer Mannheim), then hybridized for 18 hours at 60℃ in buffer containing 100$\mu g \cdot L^{-1}$ of sense or antisense-specific DIG-labeled probe. After hybridization, the blots were washed extensively and the extent of hybridization was visualized colorimetrically using reagents from the DIG nucleic acid detection kit, Boehringer Mannheim).

Immunofluorescence staining

Confluent monolayers of parental and derivative cell lines were harvested in calcium and magnesium free Dulbeco's phosphate-buffered saline (CMF) supplemented with 2$mmol \cdot L^{-1}$ EDTA. These stocks were pelleted by centrifugation, resuspended in ice cold CMF with 10$g \cdot L^{-1}$ bovine serum albumin and 0.2$g \cdot L^{-1}$ sodium azide, quantitated for viability by trypan blue dye exclusion, and then diluted to a final total viable cells concentration of $1 \times 10^9 \cdot L^{-1}$. Each stock was then exposed to anti-VEGF for 1 hour at 4℃. The cells were washed three times with ice-cold CMF with 10$g \cdot L^{-1}$ bovine serum albumin, then exposed to a 1:20 dilution of fluorescein isothiocyanate- conjugated goat anti-rabbit IgG (Boehringer Mannheim) for 30 minutes at 4℃. The cells were washed twice in CMF, and extent of surface fluorescence for equal number subpopulations of cells was analyzed by fluorescence-activated cell-sorting (FACSTAR; Becton Dickinson) analysis.

In vitro growth rate

Human gastric cancer cells SGC-7901 and its transfected ones with sense or antisense VEGF were cultured at 4×10^4 cells and grown under standard culture conditions. Cell counts were performed on a hemocytometer, initially at 12h time points, and then every 24h for a total of at least 140h. The total number of cells from duplicate experiments was determined as a function of time (h), and the rate of division was calculated from the exponential phase of growth.

In vivo tumor analysis

Adult male or female (nu/nu) mice (5 - 8 weeks of age) received s.c. injections of SGC-7901 gastric cancer cells or SGC-7901 gastric cancer cells transfected with the sense-VEGF or antisense-VEGF constructs. Approximately 10^6 cells

resuspended in a volume of 100μL of serum free cell culture medium were s.c. injected into the dorsa of mice. The mice were monitored daily, and tumor sizes were determined by tridimensional calliper measurements. Tumor volume was calculated as 1/2×length×width 2 (mm^3). Mean tumor volumes were calculated from measurements performed on five mice in each of two individual experiments. The animals were killed 33 days later. s.c. tumors were removed from mice, formaldehyde fixed, and paraffin-embedded. Five μm thick sections were cut and mounted on glass slides. The sections were stained with hematoxylin and eosin[16-18].

RESULTS

VEGF and KDR expressions in human gastric cancer tissues

Among 50 formalin-fixed, paraffin-embedded surgically resected tissue specimens of gastric carcinoma, 10 specimens were composed of two different histological type cancer cells, most of which were poorly differentiated adenocarcinoma and mucinous cell carcinoma. Normal gastric mucosa was not immunoreactive with an anti-VEGF antibody. VEGF was mainly localized to the cytoplasm or the membrane of the carcinoma (Figure 1). Tumor cells that stained strongly for VEGF were observed more often in the invasive front than in the tumor center. Weakly positive VEGF staining was seen on some endothelial cells. VEGF expression was detected in 25 (50%) tumors. KDR was mainly localized in the cytoplasm or the membrane of vascular endothelial cells in gastric cancer tissue and peri-cancerous tissue (Figure 2). In 2 cases of 50 specimens, the gastric cancer cells expressed KDR, localized in the cytoplasm and membrane (Figure 3).

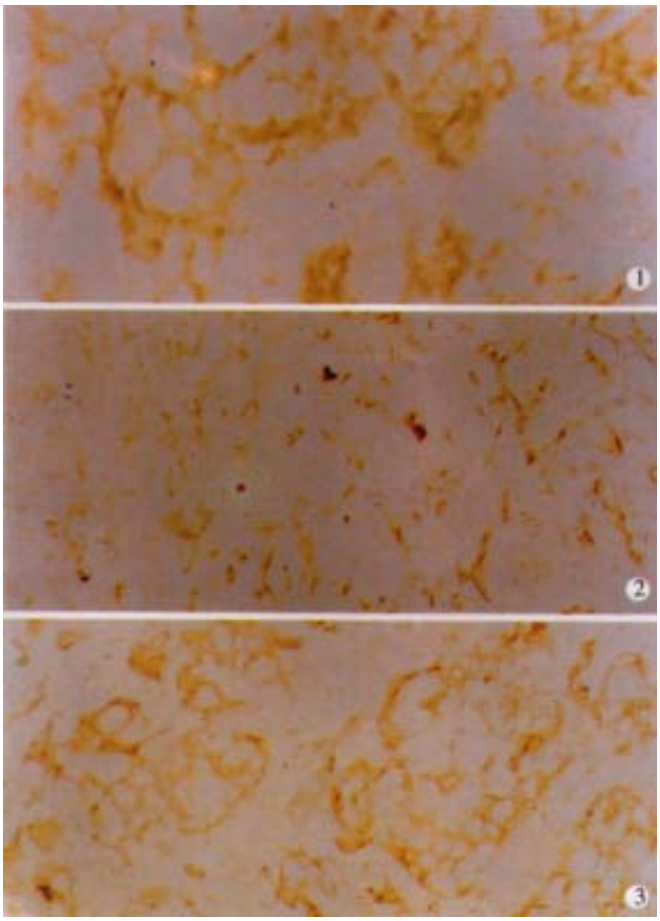

Figure 1 Immunohistochemical staining for VEGF in adenocarcinoma tissues of the stomach. The positive signal was found mainly on the surface and in the cytoplasm of gastric cancer cells. ×400

Figure 2 Immunohistochemical staining for flk-1/ KDR in undifferentiated cancer tissues of the stomach. The positive signal was found on the surface of endothelial cells. ×100

Figure 3 Immunohistochemical staining for flk-1/ KDR in adenocarcinoma tissues of the stomach. It was found mainly on the surface and in the cytoplasm of gastric cancer cells. ×200

Cloning of the sense and antisense VEGF Cdna

The $VEGF_{165}$ insert was cleaved from pGEM-3Zf(+) by restriction enzymes digestion and cloned into the same restriction sites of the eukaryotic expression vector, $pCDNA_3$. Restriction enzyme mapping and dideoxy sequencing assay performed on DNA from transformed clones demonstrated the presence of the $VEGF_{165}$ cDNA insert cloned in the sense and antisense orientation in the $pCDNA_3$ vector (Figure 4).

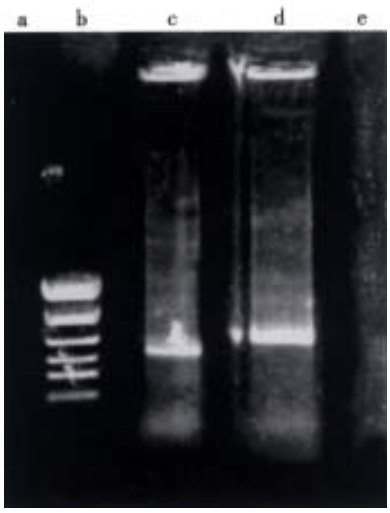

Figure 4 PCR analysis of SGC-s-hVEGF and SGC-as-hVEGF. a. SGC-s-hVEGF/SP_6+b; b. PCR marker; c. SGC-s-hVEGF/SP_6+a; d. SGC-as-hVEGF/SP_6b; e. SGC-as-hVEGF/SP_6+a

SGC-7901 gastric cancer cells expressing sense or antisense VEGF

Following transfection of human SGC-7901 gastric cancer cells with the sense or antisense VEGF construct (or vector alone control) and subsequent antibiotic selection, individual clones were isolated and grown in 24-well culture plates. Polymerase chain reaction (PCR) analysis of DNA isolated from these clones revealed that the positive clones had the sense or antisense VEGF construct. A selection of these clones were then re-cloned at the level of one cell/well, and the PCR analysis was repeated to confirm expression of the cDNA.

VEGF levels in SGC-7901 cells by stable transfection with sense and antisense VEGF constructs

To better determine whether VEGF plays a functional role in the oncogenesis of gastric cancer, gastric cancer cells were transfected with sense or antisense VEGF expression vector, and the effect on the growth of tumor was evaluated. Stable integration of expression constructs into G_{418}-resistant subclones was shown by PCR-based approach that utilized SP6 and VEGF-specific primer pairs and genomic DNA (see Methods). Introduction of the VEGF antisense construct into SGC-7901 cells resulted in a markedly reduction in the expression of VEGF-specific mRNA by dot blot analysis in SGC-7901/as hVEGF compared with the parental cell line.

Conversely, the expression of VEGF in mRNA level was enhanced in SGC-7901 cells transfected with the sense-VEGF construct. Alterations in total cellular VEGF after transfection with sense or antisense constructs were accompanied by similar changes in cell surface VEGF protein as determined by flow cytometer analysis (Figure 5).

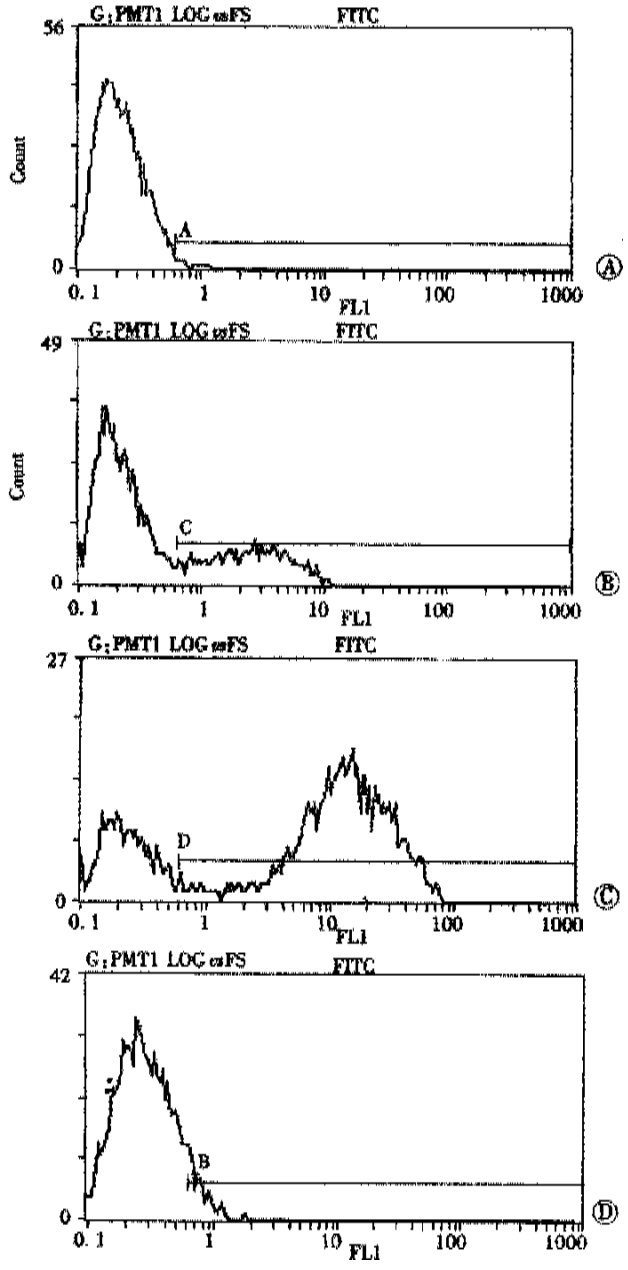

Figure 5 Changes in cell surface VEGF protein as deter mined by flow cytometer immunofluorescence staining analysis.
(A) Flow cytometric detection of VEGF changes of control. (B) Flow cytometric detection of VEGF changes of SGC-7901. (C) Flow cytometric detection of VEGF changes of SGC / s-hVEGF. (D) Flow cytometric detection of VEGF changes of SGC / as-hVEGF.

In vitro growth properties of parental and derivative cell lines

The sense-VEGF cell lines and antisense-VEGF cell lines appeared phenotypically indistinguishable from normal SGC-7901 gastric cancer cells and SGC-7901 transfected vector alone cells, and the growth rates of the derivative cell lines were identical to that of normal SGC-7901 cells (Figure 6).

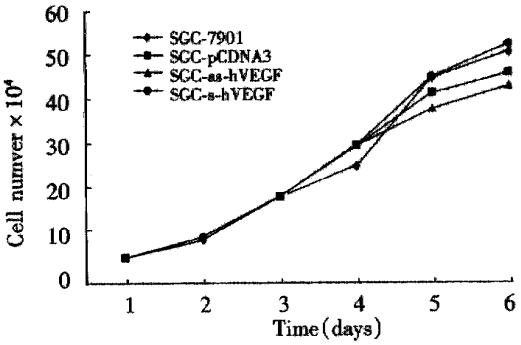

Figure 6 The growth curve of different transfectant.

In vivo growth of parental and derivative cell lines

Control human SGC-7901 gastric cancer cell, sense and antisense-VEGF cell lines were s.c. injected into nude mice, and tumor volumes were measured daily for the duration of the experiments. Tumor growth was detectable and measurable by 8 days postimplantation. At this time point, the parental cells, sense-VEGF cells, and antisense-VEGF cells had produced tumors of 13.25 ± 3.58 mm^3, 13.46±6.04 mm^3 and 12.46 ± 3.01 mm^3. There were no significant differences between their volumes(P>0.05). After 18 days postimplantation, however, the tumors from the sense-VEGF cell lines begun to grow more quickly than that from the parental cells, but the growth rate of tumors from the antisense-VEGF cell lines become slow. At 33 days postimplantation, sense-VEGF, SGC-7901, and antisense-VEGF cell lines produced tumors of 2350.50 ± 637.70 mm^3, 1534.40 ± 362.88 mm^3, and 345.40 ± 136.31 mm^3, respectively (Figure 7). At this time point, the tumor inhibiting rate was 77% through antisense inhibition. Sections of tumors were observed for their degree of tissue necrosis and vascularization. The results demonstrated that there was higher degree of necrosis in the tumors of the antisense-VEGF cell lines in comparison to tumors produced by control SGC-7901 cells, and the number of blood vessels observed in the tumors derived from the sense VEGF cell lines was higher.

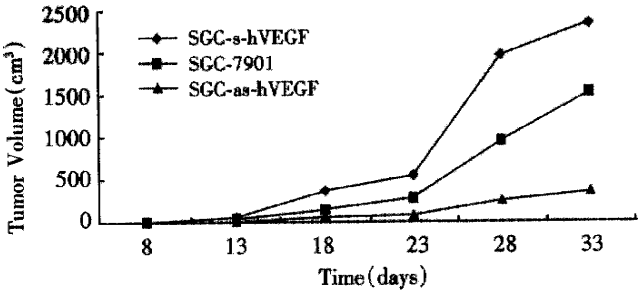

Figure 7 The volume of tumors in nude mice (n=5/ group) s.c. developing from s.c. injection totaling 1.4×10^6 cells / animal of the different transfectants.

DISCUSSION

Chemotherapy is the main treatment for patients with malignant tumor not to be resected, however, acquired resistance to chemotherapy is a major problem during cancer treatment. One mechanism for drug resistance is overexpression of the MDR (multidrug resistance)1 gene encoding the transmembrane efflux pump, P-glycoprotein (P-gp). In recent years, it has attracted much attention and has

been studied as a mechanism of multidrug resistance of tumors to anticancer drugs, however, the application of most agents with the capacity to reverse multidrug resistance (MDR) via modulation of the multidrug transporter P-glycoprotein (Pgp) was shown to be associated with toxic side-effects[19-21]. In addition to it, the heterogenerous of tumor may caused some trouble for choosing sensitive agents. Take altogether, many attempt were made to search for notoxic agents aiming at a common target. Neovascularization is critical for supporting the rapid growth of solid tumors. Tumor angiogenesis appears to be achieved by the expression of angiogenic agents within solid tumors that stimulate host vascular endothelial cell mitogenesis and possibly chemotaxis[22-25]. One such protein, vascular endothelial growth factor, or vascular permeability factor, is a selective endothelial cell mitogen and angiogenic agent induced by several growth factors and cytokines, and elevated expression of either the Ras[26,27], Raf[28], Src[29], or mutant P53[30] oncogenes and hypoxia[31-33] characteristic of rapidly growing solid tumors. VEGF was over expressed in many solid tumors, including breast[34], ovarian[35], lung[36], esophageal[37], and colon[38] cancer. These data suggest a potential role for VEGF in the oncogenesis of solid tumor.

VEGF-positive rate was 50% in human gastric cancer tissues, and it was mainly localized to the cytoplasm and membrane of the tumor cells, while KDR was mainly localized in the endothelial cells. This finding indicated that VEGF might have paracrine effect upon the endothelial cells to promote angiogenesis. In 50 specimens, of 2 cases in which the gastric cancer cells expressed VEGF and its receptor KDR, suggesting that VEGF might have an autocrine effect upon the gastric cancer cells themselves. Analysis of human gastric cancer tissue sections has shown that they are highly heterogeneous[39-41]. The cellular profile of each individual is also heterogeneous in that they contain cells at varying stages of malignancy and have growth factor/receptor expression profile that differ markedly[42-48].

The current study provides a more direct evidence that VEGF plays a role in the growth of gastric cancer. Reduction of VEGF mRNA and protein levels through antisense inhibition significantly lowered the growth rate of the tumors from antisense-VEGF cell lines. Conversely, elevation of VEGF levels after sense transfection resulted in a significant increase of the growth rate of the tumors from sense VEGF cell lines. These results provide strong evidence that VEGF plays an important role in the oncogenesis of the gastric cancer.

A potential therapy based on the interruption of paracrine and/or autocrine growth factor pathways that impinge upon the tumor cells themselves might well prove to be a successful antitumor approach[49-53]. Exploiting the ubiquity of tumor angiogenesis as a suitable target for therapy has been proposed previously to be an important concept for antitumor therapy[54]. This concept has recently been the subject of renewed interest in the development of new therapeutic strategies. Significant evidences are accumulating in favor of the notion that VEGF and its receptor play important roles in the development of solid tumors, such as those derived from gastric cancer origin.

Much indirect evidences indicate that VEGF is an important participant in tumor biology[11,12,55,56]. We now provide direct evidence that VEGF plays an important role in the growth of gastric cancer. VEGF might have mainly paracrine effect upon the endothelial cells to promote angiogenesis[57-62], and it might have an autocrine effect upon the gastric cancer cells themselves. The inhibition of VEGF is sufficient to control tumor growth *in vivo* by the suppression of tumor neovascularization[63-69]. The antisense VEGF strategy offers a new avenue of gene therapy development as an adjuvant treatment for human gastric cancer[70-76].

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• ORIGINAL RESEARCH •

Glutathione S-transferases M1, T1 genotypes and the risk gastric cancer: A case-control study

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Abstract

AIM **Glutathione S-transferases (GSTs) are involved in the detoxification of many potential carcinogens and appear to play a critical role in the protection from the effects of carcinogens. The contribution of glutathione S-transferases M1 and T1 genotypes to susceptibility to the risk of gastric cancer and their interaction with cigarette smoking are still unclear. The aim of this study was to determine whether there was any relationship between genetic polymorphisms of GSTM1 and GSTT1 and gastric cancer.**

METHODS **A population based case-control study was carried out in a high-risk area, Changle County, Fujian Province, China. The epidemiological data were collected by a standard questionnaire and blood samples were obtained from 95 incidence gastric cancer cases and 94 healthy controls. A polymerase chain reaction method was used to detect the presence or absence of the *GSTM1* and *GSTT1* genes in genomic DNA. Logistic regression model was employed in the data analysis.**

RESULTS **An increase in risk for gastric cancer was found among carriers of *GSTM1* null genotype. The adjusted odds ratio (OR) was 2.63 [95% Confidence Interval (95% CI) 1.17-5.88], after controlling for age, gender, cigarette smoking, alcohol drinking, and fish sauce intake. The frequency of *GSTT1* null genotype in cancer cases (43.16%) was not significantly different from that in controls (50.00%). However, the risk for gastric cancer in those with *GSTM1* null and *GSTT1* non-null genotype was significantly higher than in those with both *GSTM1* and *GSTT1* non-null genotype (OR = 2.77, 95% CI 1.15-6.77). Compared with those subjects who never smoked and had normal GSTM1 genotype, ORs were 1.60 (95% CI: 0.62-4.19) for never smokers with *GSTM1* null type, 2.33 (95% CI 0.88-6.28) for smokers with normal *GSTM1*, and 8.06 (95% CI 2.83-23.67) for smokers with *GSTM1* null type.**

CONCLUSIONS ***GSTM1* gene polymorphisms may be associated with genetic susceptibility of stomach cancer and may modulate tobacco-related carcinogenesis of gastric cancer.**

Subject headings glutathione transferase/genetics; genotype; polymorphism (genetics); stomach neoplasm/genetics; case control studies

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INTRODUCTION

Glutathione S-transferases (*GSTs*), a supergene family of detoxification enzymes, appear to form a protection mechanism against chemical carcinogenesis. In human tissues this family consists of four multigene classes, referred to as alpha, mu, pi, and theta. The *GSTM1* gene is classified into the mu class and the *GSTT1* gene belongs to the theta class. They detoxify reactive chemical species, such as polycyclic aromatic hydrocarbon epoxides by catalyzing their conjugation to glutathione. Genes coding for *GSTM1* and *GSTT1* proteins are polymorphic in humans and these genes are absent in 10% -60% of different ethnic populations[1,2]. Accumulating evidence indicates that susceptibility to cancer is mediated by genetically determined differences in the effectiveness of detoxification of potential carcinogens. Genetic differences are likely to be a major source of interindividual variation in susceptibility to cancer[3].

Gastric cancer is the most common cancer in whole China[4-8], especially in Changle County, Fujian Province, China[9,10]. Previous studies have shown that a number of environmental risk factors may play a role in a multistep and multifactorial process[11-13]. Tobacco smoking has been considered a potential risk factor for gastric cancer[14]. Few data have so far been reported on the risk of gastric cancer associated with genetic and environmental exposures. To evaluate the relationships between *GSTM1/GSTT1* and gastric cancer, a molecular epidemiological study was conducted in Changle County.

MATERIALS AND METHODS

Study subjects

Cases and controls were all residents in Changle County, China, which is one of areas with the highest rates of gastric cancer in the world. All primary gastric cancers (n=95) were histologically confirmed or diagnosed by operation between January 1996 to March 1998. Population controls (n = 94) were randomly selected from the same geographical region, and matched to cases by their gender and age. The field staff conducted face-to-face interviews. Cases and controls were interviewed in the same manner using a standard epidemiological questionnaire. Blood samples (5mL) were collected.

GSTM1 and GSTT1 Assay

DNA was isolated from peripheral white blood cells by proteinase K (Huamei Biotechnology, Inc.) digestion and phenol / chloroform extractions. The PCR reactions were performed in 50μL of a solution containing PCR buffer (1.5 mmol·L^{-1} $MgCl_2$, 50 mmol·L^{-1} KCl, 10 mmol·L^{-1} Tris-HCl, pH 8.3), 200 μmol·L^{-1} of each dNTP, 1 μmol·L^{-1} of each primer, 200ng of template DNA, and 2.5 unit of TAQ DNA polymerase (Promega). Primer sequences for *GSTM1* were 5'-GCTTCACGTGTTATGGAGGTTC-3' and 5'-GAGATGAAGTCCTCCAGATTT-3', which produced a 157 base pair band. The *GSTT1* primers were 5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'-TCACCGGATCATGGCCAGCA-3'-3, which produced a 480-base pair band. β-globin was used as an internal positive control, which was amplified with the following primers: 5'-CAACTTCATCCACGTTCACC-3' and 5'-GAAGAGCCAAGGACAGGTAC-3' and produced a 268-base pair band. The primers were synthesized by Sangon and PCR amplifications were carried out in a Thermal Cycler (Perkin Elmer 4800). Main cycling parameters were 94℃ for 8 min, followed by 35 cycles of 94℃ for 30s, 60℃ for 40s and 72℃ for 1 min with a final extension at 72℃ for 10 min. PCR products were detected by electrophoresis in agarose gels (2g·L^{-1} for *GSTM1* and 12g·L^{-1} for *GSTT1*).

Statistical analysis

The Chi-square method was used to test the frequencies of *GSTM1* and *GSTT1* genotypes. ORs and 95% CIs were calculated by logistic regression analysis controlling for possible confounding factors.

RESULTS

GSTM1 and *GSTT1* null genotypes are indicated by the absence of a 157bp band and 480 bp band, respectively. β-globin (268bp) indicating the presence of DNA is co-amplified in all the samples (Figures 1, 2).

Main characteristics of subjects

The main characteristics of cases and controls are presented in Table 1, the distribution of sex and age among cases and controls were not statistically significant (P>0.05).

Table 1 Main characteristics of cases and controls

	Cases (n=95)		Controls (n=94)	
	n	(%)	n	(%)
Age groups / yr				
<50	21	(22.1)	22	(23.4)
50 - 59	23	(24.2)	22	(23.4)
60 - 69	33	(34.7)	34	(36.2)
⩾70	18	(19.0)	16	(17.0)
Mean age	59 ± 11		58 ± 11	
Age range	32 - 78		34 - 79	
Gender				
Male	81	(85.3)	82	(87.2)
Female	14	(14.7)	12	(12.8)
Education				
College	1	(1.1)	1	(1.1)
High school	15	(15.8)	63	(67.0)
Elementary school	61	(64.2)	22	(23.4)
Illiterate	18	(19.0)	8	(8.5)

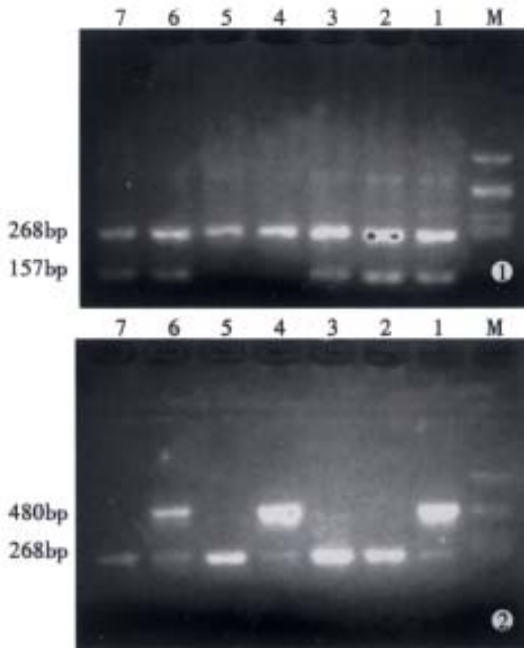

Figure 1 Agarose gel electrophoresis of PCR products. 157bp fragment: *GSTM1*; 268bp fragment: β-globin. Lane M: marker; Lanes 4 and 5:*GSTM1* null; Lanes 1, 2, 3, 6 and 7: *GSTM1* non null.

Figure 2 Agarose gel electrophoresis of PCR products. 480bp fragment: *GSTT1*; 268bp fragment: β-globin. Lane M: marker; Lanes 1, 4, and 6: nun-null; Lanes 2, 3, 5 and 7: *GSTT1* null.

GSTM1 and GSTT1 genotype frequencies in cases and controls

The results showed that GSTM1 null genotype distributed unevenly between gastric cancer cases and controls. The frequency of *GSTM1* null was significantly increased in gastric cancer cases compared with the general controls (χ^2 = 5.75, P=0.0165, Table 2).

Fifty percent (47/94) of individual in the controls exhibited the *GSTT*1 null genotype, and 43.2% (41/95) in gastric cancer cases. The frequencies of *GSTT*1 genotypes in cases and population controls were not significantly different (OR = 0.76, 95% CI 0.1 ~ 1.4). The odds ratio of gastric cancer associated with the combined genotypes of the polymorphisms of *GSTM*1 and *GSTT*1 are shown in Table 3. Persons who carried the *GSTM*1 null genotype and *GSTT*1 non-null had a higher risk of gastric cancer. The odds ratio was 2.77.

Table 2 Association between *GSTM1* and gastric cancer risk

	*GSTM*1 genotype				
	Nonnull	n	(%)	null	n	(%)
Contr		51	54.3		43	45.7
Case		35	36.8		60	63.2
Crude OR (95% CI)		2.03 (1.13-3.65)				
Adjusted OR[a] (95% CI)		2.03 (1.13-3.68)				
Adjusted OR[b] (95% CI)		2.47 (1.21-5.03)				
Adjusted OR[c] (95% CI)		2.63 (1.17-5.88)				

a: Logistic regression adjusted for age and sex; b: Adjusted for age, sex, cigarette smoking and alcohol drinking (yes / no); c: Adjusted for age, sex, cigarette smoking, alcohol drinking (yes / no), and fish sauce intake (continuous).

Table 3 Association between gastric cancer and combinations of *GSTM*1 and *GSTT*1 genotypes

*GSTM*1	*GSTT*1	Case		Contr		OR(95% CI)
		n	%	*n*	%	
Non-null	Non-null	21	22.1	30	31.9	1.00
Non-null	Null	14	14.7	21	22.3	0.95 (0.36~2.50)
Null	Null	27	28.4	26	27.7	1.48 (0.64~3.47)
Null	Non-null	33	34.7	17	18.1	2.77(1.15~6.77)

GSTM1 null genotype and smoking

Because *GSTM*1 may play an important role in the metabolism of tobacco smoke-derived carcinogens, the risk of gastric cancer associated with the polymophisms of metabolic enzymes may depend on the individuals' smoking status. We compared smokers with and without gastric cancer and found that the increased susceptibility to gastric cancer in smokers with *GSTM*1 null phenotype. The subjects which have been exposed to cigarette smoking and *GSTM*1 null genotypes had 8.06 fold risk to develop gastric cancer (Table 4).

Table 4 Risk of gastric cancer in relation to *GSTM1* genotypes by e smoking

Genotype	Smoke	Contr		Case		OR (95% CI)
		n	%	*n*	%	
Nonnull	No	28	29.8	12	12.6	1.00
Null	No	32	34.0	22	23.2	1.60 (0.62-4.19)
Nonnull	Yes	23	24.5	23	24.2	2.33 (0.88-6.28)
Null	Yes	11	11.7	38	40.0	8.06 (2.83-23.9)

DISCUSSION

Changle County is a hyperendemic area of gastric cancer. Familial aggregation of gastric cancer in this area has been reported in previous studies[15,16]. This familial tendency toward gastric cancer may result from a common environment shared by familial members of inherited genetic susceptibility[17]. Gastric cancer is a multistage process[18]. each caused by numbers of factors[19-31]. Environmental and host factors may all contribute to the etiology of gastric cancer[32]. The relationship between polymorphisms of genes involved in carcinogen metabolism and individual susceptibility to the mutagenic and carcinogenic actions of specific chemical exposure is a new field of research[33-35].

Recent studies reported genes that on code enzymes involved in the metabolism of carcioogens or environmental toxins may be related to an increased risk of cancer in some individuals[36,37]. GSTs are multifunctional proteins that catalyze many reactions between glutathione (GSH) and lipophilic compounds with electrophilic centers, including cytotoxic and genotoxic reactions[38]. Polycyclic aromatic hydrocarbons, N-nitrosomines, found in cigarette smoke and food, are potential human carcinogens[39,40]. Deficiency of detoxifying enzymes may affect the metabolic fates of these chemicals and raise cancer risks in exposed individuals[41]. The *GSTM*1 enzyme is involved in detoxifying a number of carcinogenic electrophiles, such as the epoxides of polycyclic aromatic hydrocarbons. Individuals with the homozygous *GSTM*1 null genotypes express no protein and are expected to have reduced abilities of detoxification of hazardous compounds, particularly epoxides.

In this study, *GSTT*1 gene deletion was not associated with gastric cancer. We observed evidence of a relationship between null genotype of *GSTM*1 and risk of gastric cancer. The *GSTM*1 genotype exhibited a higher frequency of gene deletions in cases than in controls. The finding suggests that *GSTM*1 may play a role in gastric cancer susceptibility. Gastric cancer, which is associated with exposure to smoking, may be more striking in individuals who carrying the null genotype *GSTM*1. This result suggests that intervention against smoking may be important for the prevention of gastric cancer in high incidence area because the *GSTM*1 is present in a majority of persons and the potential population impact may be important. However, these results should be considered preliminary. Larger studies will be needed to confirm potential gene-environment interactions.

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• ORIGINAL RESEARCH •

Preparation of single chain variable fragment of MG_7 mAb by phage display technology

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Abstract

AIM **To develop the single chain variable fragment of MG_7 murine anti-human gastric cancer monoclonal antibody using the phage display technology for obtaining a tumor-targeting mediator.**

METHODS **mRNA was isolated from MG_7 producing murine hybridoma cell line and converted into cDNA. The variable fragments of heavy and light chain were amplified separately and assembled into ScFv with a specially constructed DNA linker by PCR. The ScFvs DNA was ligated into the phagmid vector pCANTAB5E and the ligated sample was transformed into competent *E. Coli* TG1. The transformed cells were infected with M13K07 helper phage to form MG_7 recombinant phage antibody library. The volume and recombinant rate of the library were evaluated by means of bacterial colony count and restriction analysis. After two rounds of panning with gastric cancer cell line KATOIII of highly expressing MG_7-binding antigen, the phage clones displaying ScFv of the antibody were selected by ELISA from the enriched phage clones. The antigen binding affinity of the positive clone was detected by competition ELISA. HB2151 *E.coli* was transfected with the positive phage clone demonstrated by competition ELISA for production of a soluble form of the MG_7 ScFv. ELISA assay was used to detect the antigen-binding affinity of the soluble MG_7 ScFv. Finally, the relative molecular mass of soluble MG_7 ScFv was measured by SDS-PAGE.**

RESULTS **The V-H, V-L and ScFv DNAs were about 340bp, 320bp and 750bp, respectively. The volume of the library was up to 2×10^6 and 8 of 11 random clones were recombinants. Two phage clones could strongly compete with the original MG_7 antibody for binding to the antigen expressed on KATOIII cells. Within 2 strong positive phage clones, the soluble MG_7 ScFv from one clone was found to have the binding activity with KATOIII cells. SDS-PAGE showed that the relative molecular weight of soluble MG_7 ScFv was 32.**

CONCLUSION **The MG_7 ScFv was successfully produced by phage antibody technology, which may be useful for broadening the scope of application of the antibody.**

Subject headings antibodies, neoplasms/ biosynthesis; antibodies, monoclonal; stomach neoplasms/ immunology; bacteriophages/genetics

Yu ZC, Ding J, Nie YZ, Fan DM, Zhang XY. Preparation of single chain variable fragment of MG_7 mAb by phage display technology. *World J Gastroenterol*, 2001;7(4):510-514

INTRODUCTION

In our previous studies, MG_7 hybridoma cell line had been successfully prepared by immunization of mouse with KATOIII gastric cancer cells and hybridization of the B cells from the spleen of the immunized mouse with the murine myeloma cell line SP 2/0. This hybridoma cell line generates a kind of monoclonal antibody against gastric cancer which can specifically recognize an ascertained gastric cancer associated antigen[1]. MG_7 antibody was confirmed to be of great value and good potency in the targeting gene therapy of gastric cancer due to the overexpression of its corresponding antigen in a large proportion of patients with gastric cancer. But owing to its murine origin, like many other similar antibodies, MG_7 antibody can elicit human anti-mouse immunoreaction and thus its use in clinical practice is restricted[2,3]. One of the efficient solutions to this problem is to remove the constant region of antibody which makes main contribution to the immunogenicity of the murine antibody to human being. It has been proved that antibody devoid of constant region still maintains its capacity of specific antigen-binding affinity[4-10]. Additionally, antibody without constant region, termed ScFv, is a small molecule and comprises 1/6 of its original antibody in molecular mass. Therefore, ScFv can more readily penetrate into the solid tumor *in vivo* and be easily cleared up from the normal tissue. In the early 90's, the emergence of recombinant phage library represented a great breakthrough in the antibody technology which provides an economical means to prepare the ScFv/Fab of any desired antibody[11-19]. In the present study, the MG_7 recombinant phage antibody derived from MG_7 hybridoma was constructed and screened to prepare the MG_7 ScFv which might help establish an efficient strategy of targeting gene therapy in gastric cancer.

MATERIALS AND METHODS

Detection of antigen-binding affinity of MG_7 antibody

MG_7 hybridoma cells and KATOIII cells were cultured with RPMI 1640 (purchased from Gibco) supplemented with heat-inactivated $100mL\cdot L^{-1}$ fetal bovine serum at 37℃ under $50mL\cdot L^{-1}$ CO_2. MG_7 hybridoma cells were harvested at log phase and stored at -70℃ with aliquot of 10^6 for RNA isolation. Supernatant was collected for detection of antigen-binding affinity of MG_7 antibody by ELISA. KATOIII cells in log phase were transferred into a 96 wellplate and immobilized on the wall by centrifiguation at $1\,000\times g$ for 10 min, finally

fixed by 0.25mL·L^{-1} glutaraldehyde. Supernatant of 0.2mL was applied to each well and incubated at 4℃ overnight, and 0.1mL HRP-labeled goat anti-mouse (HRP-GAM) Ig was added into each well. The absorbance value (*A*) at 492nm of reactant in each well was measured after incubation for 1 hour at 37℃ and staining with OPD.

Construction of MG_7 recombinant phage antibody library

According to the protocol of svtotal RNA isolation system and polyAT tract mRNA isolation system (purchased from Promega), mRNA was isolated from MG_7 hybridoma cells and quantified by gel electrophoresis for following reverse transcription reaction. Subsequently, reverse transcription reaction was performed with 0.3μg mRNA, 2U reverse transcripase (purchased from Promega) mixed together for incubation of 1 hour under 37℃. PCR was conducted with a mixture of 10μg product of reverse transcription, 2U *Taq* DNA polymerase and 2μL V H/V_L primers mix (purchased from Promega) in a total volume of 50μL. The procedure of PCR was arranged in the following order: 95℃×5min; 94℃ ×1min, 55℃×2min, 72℃×2min and 30 cycles; 72℃×10min. After precise quantification of PCR product by gel electrophoresis, 50ng of V_H and V_L product was respectively mixed with 50ng linker primer and 1μL *Taq* DNA polymerase to perform PCR (94℃×1min, 63℃×4min, 7 cycles). Subsequently,50ng RS primers (purchased from Promega) underwent another PCR (94℃×1min, 55℃×2min, 72℃×2min and 30 cycles; 72℃×10min). Two μL *Sfi* I and 0.5μg ScFv product were added into a sterile 0.5mL microtube and incubated at 50℃ for 4 hours. After being purified by PCR purification kit, 0.5μg *Sfi* I digested ScFv product mixed with 2μL *Not* I was incubated at 37℃ for 4 hours and purified again for later use. ScFv (150ng) and pCANTAB5E (250ng) mixed with 2μL T_4 DNA ligase was incubated at 16℃ for 16 hours. Ligated product was transformed into TG1 cell. Transformed product with aliquot of 100μL was placed onto SOBAG plates and incubated overnight at 37℃ to form bacterial clones.

Evaluation of volume and recombinant rate of phage antibody library

Colony count was adopted to exhibit the total number of clones formed on the SOBAG plates. Eleven clones were randomly singled out from the SOBAG plates and passaged into 5mL 2×YT-AG medium for an incubation of 12 hours at 37℃. Plasmid from each clone was respectively isolated and digested by *Eco* RI and *Hin* dIII. Gel electrophoresis was conducted with restriction digested product to examine the recombinant phagmid.

Panning and enrichment of MG_7 recombinant phage antibody

The initial recombinant phage antibody library was incubated for 1 hour at 37℃ with shaking at 250r·min^{-1}, and helper phage M13KO7 was added and incubated for another hour at 37℃ with shaking at 250 r·min^{-1}[20]. The culturing product was spinned at 1000×*g* for 10 min to precipiate the cells. Then the entire sample was gently resuspended in 10 mL 2× YT-AK medium. After an overnight incubation at 37℃ with shaking at 250r·min^{-1}, the culturing product was spinned at 1 000×*g* for 20 min and the supernatant which contained the recombinant phages was collected. Then, 2mL PEG/NaCl was added and placed on ice for 45 min for precipitation of recombinant phage clones. It was spinned at 10000×*g* for 20 min at 4℃ and the pellet was resuspended in 16mL 2×YT medium diluted with 14 mL blocking buffer containing 0.1g·L^{-1} sodium azide and incubated at room temperature for 15 min. Twenty mL of the diluted recombinant phage was then added to the flask which was coated with KATOIII cells and well blocked. The flask was incubated for 2 hours at 37℃, washed 10 times with PBS plus another 10 times with PBS containing 1mL·L^{-1} Tween20. Ten mL log-phase TG1 cells were added to the flask and incubated with shaking at 37℃ for 1 hour for reinfection. After two rounds of panning, reinfected TG1 cells with bound phages directly in the panning flask were plated for colony isolation.

Screening for MG_7 recombinant phages

Recombinant phages were rescued from individual clones and screened for MG_7 binding by ELISA. Microtiter wells were coated with KATOIII cells. Bound phages were detected by incubation with a 1:5000 dilution of conjugate (Pharmacia Biotech). And the detection was achieved by addition of TMB substrate. Clones reacted to KATOIII cells were referred to as positives.

Competitive test of positive selected MG_7 recombinant phages

Microtiter wells were coated with KATOIII cells as mentioned above. The supernatant of the selected positive MG_7 recombinant phages was applied into each well (100mL·well^{-1}) and then incubated for 1 hour at 37℃. After disposing of the supernatant, MG_7 antibody (100mg·L^{-1}, 50μL·well^{-1}) was added and incubated for 1 hour at 37℃. PBST was used to wash 5 times, and HRP-GAM Ig (1:1000 diluted, 50μL·well^{-1}) was added and developed by TMB to measure the absorbance value at 450nm. The inhibiting ratio of selected positive MG_7 recombinant phages with MG_7 antibody for binding of KATOIII cells was calculated by the following formula: Inhibiting ratio = 1- (value of sample/value of control)×100%

Detection of antigen-binding affinity of the soluble MG_7 ScFv

The positive phages were transfected into *E.coli* HB2151 cells for the production of a soluble form of the MG_7 ScFv. Five mL culturing product of transfected *E.coli* HB2151 cells with overnight induction of 1mmol·L^{-1} isopropyl β-D-thiogalactopyanoside (IPTG) was centrifugated at 1000×*g* to collect the sediment and the supernatant (containing extracellular soluble ScFvs). The sediment was given osmotic shock to prepare periplasmic extracts. Microtiter wells were coated with KATOIII cells, and ELISA test was made twice to detect the antigen-binding affinity of soluble MG_7 ScFv.

Measurement of the relative molecular weight of soluble MG_7 ScFv

Periplasmic extracts from transfected *E.coli* HB2151 cells induced by IPTG was adopted to measure the relative molecular weight of soluble MG_7 ScFv by SDS-PAGE.

RESULTS

Antigen-binding affinity of MG_7 antibody

The ELISA showed that the A_{492} absorbance of reactant with presence of MG_7 antibody was up to 0.65 (0.208 in control).

Amplification of V_H, V_L and ScFv gene

On electrophoresis, V_H product formed a band at 350bp and V_L at 320bp, and ScFv was successfully spliced together to form a fragment of 750bp, as shown in Figure 1.

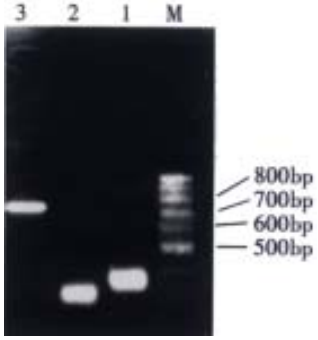

Figure 1 RT-PCR of V_H, V_L and ScFv fragment of MG_7 antibody. 1: V_H; 2: V_L; 3: ScFv; M: 100bp ladder

Volume of MG_7 phage antibody library

Colony counts showed that MG_7 phage antibody library consisted of 2×10^6 clones.

Recombinant rate of MG_7 recombinant phage antibody library

Eight of 11 random clones were found to release a 2.1 kb DNA fragment and confirmed to be recombinant phagmid by restriction analysis and gel electophoresis (Figure 2). The recombinant rate was 72.7%.

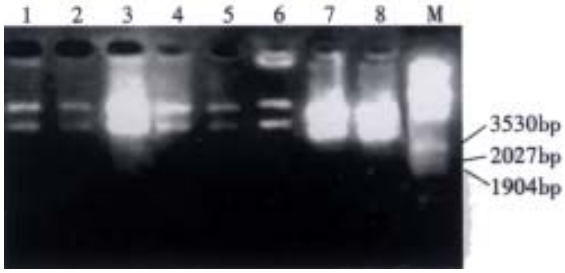

Figure 2 Enzymatic analysis of MG_7 recombinant phage antibody library with *Eco* RI and *Hin* dIII. 1-8: Recombinant clones from library; M: λ / *Eco* RI and *Hin* d III

Screening of MG_7 positive recombinant phages

Using ELISA assay, we yielded six strains of positive clones which had a good reaction with KATOIII cells (Table 1).

Table 1 ELISA results of screening from enriched phage displayed antibody library

Screening	Number of positive clones (A- value)						Neg. ctrl
	1	2	3	4	5	6	
First round	0.495	0.508	0.488	0.805	0.845	0.580	0.157
Second round	0.543	0.606	0.560	0.840	0.796	0.758	0.185

Results of competitive ELISA

Two strong positive clones were found to inhibit the binding of MG_7 antibody and KATOIII cells with the inhibiting ratio of 26.1% and 30%, respectively.

Antigen-binding affinity of soluble MG_7 ScFv

By means of ELISA assay, one of the strong positive clones exhibited the capacity of binding with KATOIII cells (Table 2).

Table 2 ELISA results of the soluble MG_7 ScFv for binding with KATOIII cells

ELISA	Number of strong positive clones (*A* value)		Neg. ctrl
	1	2	
First round	0.776	0.287	0.201
Second round	0.802	0.346	0.223

The relative molecular weight of soluble MG_7 ScFv

From Figure 3, an extra band on the lane of sample was visualized at M_r 32, as compared with the negative control. The relative molecular weight of soluble MG_7 ScFv was 31.

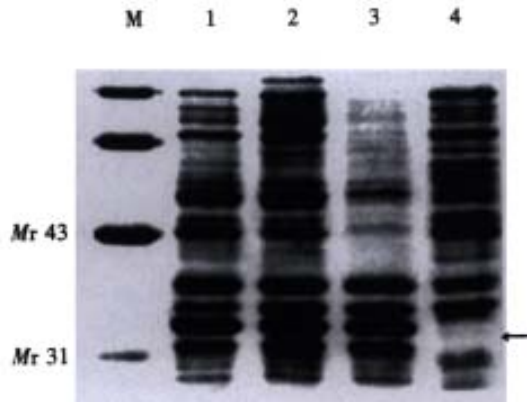

Figure 3 Measurement of the relative molecular weight of soluble MG_7 ScFv. 1-3: Periplasmic extracts; 4: Neg. ctrl; M: Low molecular mass protein marker

DISCUSSION

The phage antibody technique is one of the most remarkable achievements in antibody technology. With this technique, the repertoire of V_H and V_L genes are amplified and joined together by PCR and finally inserted into phagmid[20]. After transformation into *E.coli*, phage with the fusion of exogenous ScFv and pIII protein exposed on the surface was released from *E.coli* with the aid of the helper phage M13KO7. This technique uniforms the phenotype of ScFv to its genotype. By immunosorbance of the immobilized antigen, phage with functional ScFv can be bound and enriched. Subsequently, the desired phage harboring functional ScFv gene can be selected from the enriched phage antibody library by ELISA. The resultant ScFv can be solubly expressed in *E. coli* HB2151. The primary structural information of ScFv of antibody is accessible by DNA sequence of phagmid from the bound phage. Therefore, phage antibody has become an optimal measure to develop the ScFv of desired antibody[21-26].

It is well known that the immune system will be triggered and activated in response to the presence of certain antigens in patients with some kinds of diseases, such as tumor, infective diseases and autoimmune diseases[27-32]. The immune system will produce abundant B lymphocyte clones which can yield and secrete antibody directed against the disease associated antigens in these patients. Therefore, the B lymphocyte

population isolated from PBMC of these patients can be used as an ideal material source for construction of the recombinant phage antibody library[29-31]. Additionally, the B lymphocyte isolated from PBMC of immunized animals with given antigen is an alternative material source[20]. Besides the B lymphocyte population from patients or immunized animals, many kinds of antibody-producing hybridomas are also suitable as a kind of material source for construction of the recombinant phage antibody library[6,19]. Owing to the unraveling of biological functions over many antigen recognized by antibody from hybridomas, hybridomas are more favorable as material source for construction of recombinant phage antibody library.

In order to understand the quality of MG_7 hybridoma as a material source for construction of MG_7 recombinant phage antibody library, we detected the antigen-binding activity of MG_7 antibody in present study. ELISA assay showed that the *A* 492nm value of reactant with presence of MG_7 antibody was 0.65 which was over twice higher than that with absence of MG_7 antibody (0.208 only). It demonstrated that MG_7 hybridoma could secrete functional antibody and could be used as the source of mRNA to amplify the V_H and V_L genes of MG_7 antibody. Colony count and restriction analysis were conducted for evaluating the volume and quality of MG_7 recombinant antibody library. The large volume of MG_7 recombinant antibody library (2×10^6) and high recombinant rate (72.7%) confirmed that MG_7 phage antibody library comprised sufficient repertoire of recombinant clones for further research. ELISA assay and SDS-PAGE showed that the soluble MG_7 ScFv had antigen-binding activity and was M_r 31. Taken together, we have successfully constructed the MG_7 recombinant phage antibody library and prepared the phage-displayed/soluble MG_7 ScFv.

Gastric cancer is a highly prevalent neoplasm and is the first killer among various malignancies. In advanced cases, many current therapeutic approaches, including surgery combined with chemotherapy, appear to be palliative. These therapeutic approaches can not be targeted to and completely annihilate individual tumor cells, which leads to the failure of preventing metastasis and recurrence of many tumors. Besides, some kinds of therapeutic approaches, such as chemotherapy, can cause damage to both the tumor cells and normal tissue cells. Thus, introduction of a new way of targeting therapy for tumor is desperately needed to overcome these obstacles with the conventional approaches, such as surgery and chemotherapy[33-44]. Targeting therapy for tumors in the last decade has become a highlight in the field of tumor therapy. This therapy mediated by antibody still remains as a promising curative modality among the ways of tumor therapy and attracts worldwide attention[45-50].

Developing ScFv of the MG_7 is of great significance in both early diagnosis and treatment of gastric cancer. For instance, MG_7 ScFv fused with avidin can be used as a reagent in immuno-PCR for early diagnosis of gastric cancer. Additionally, a new immunotoxin with curative effect on gastric cancer can be developed by fusing the MG_7 ScFv and A subunit of ricin. MG_7 ScFv can direct the A subunit of ricin to MG_7 positive gastric cancer cells. Thus, the construction of MG_7 phage antibody library and subsequent preparation of MG_7 ScFv may be a step forward in seeking an efficient way for targeting therapy for gastric cancer.

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• ORIGINAL RESEARCH •

Expression, deleton and mutation of *p*16 gene in human gastric cancer

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Abstract

AIM **To investigate the relationship between the expression of *p*16 gene and the gastric carcinogenesis, depth of invasion and lymph node metastases, and to evaluate the deletion and mutation of exon** 2 **in *p*16 gene in gastric carcinoma.**

METHODS **The expression of P**16 **protein was examined by streptavidin-peroxidase conjugated method** (**S-P**)**; the deletion and mutation of p16 gene were respectively examined by polymerase chain reaction** (**PCR**) **and polymerase chain reaction single strand conformation polymorphism analysis** (**PCR-SSCP**) **in gastric carcinoma.**

RESULTS **Expression of P**16 **protein was detected in** 96.25**%** (77/80) **of the normal gastric mucosa, in** 92.00**%** (45/50) **of the dysplastic gastric mucosa and in** 47.54**%** (58/122) **of the gastric carcinoma. The positive rate of P**16 **protein expression in gastric carcinoma was significantly lower than that in normal gastric mucosa and dysplastic gastric mucosa** (***P***<0.05)**. The positive rate of P**16 **protein expression in mucoid carcinoma** 10.00**%** (1/10) **was significantly lower than that in poorly differentiated carcinoma** 51.22**%** (21/41)**, undifferentiated carcinoma** 57.69**%** (15/26) **and signet ring cell carcinoma** 62.50**%** (10/16) (***P***<0.05)**. The positive rate of p16 protein in 30 cases paired primary and lymph node metastatic gastric carcinoma: There was** 46.67**%** (14/30) **in primary gastric carcinoma,** 16.67**%** (5/30) **in lymph node metastatic gastric carcinoma. The positive rate of lymph node metastatic carcinoma was significantly lower than that of primary carcinoma** (***P***<0.05)**. There was of *p*16 gene mutation in exon** 2**, but** 5 **cases displayed deletion of *p*16 gene in exon** 2 **in the** 25 **primary gastric carcinomas.**

CONCLUSIONS **The expression loss of P**16 **protein related to the gastric carcinogenesis, gastric carcinoma histopathological subtypes and lymph merastasis. The mutation of *p*16 gene in exon** 2 **may not be involved in gastric carcinogenesis. But the deletion of *p*16 gene in exon** 2 **may be involved in gastric carcinogenesis.**

Subject headings gastric carcinoma; dysplasis p16/MTS1/CDK4I/CDKN2 gene; mutation deletion; expression; stomach neoplasms; genetics genes

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INTRODUCTION

Carcinogenesis and progression of human gastric cancer are related to the activation of proto-oncogenes and/or the inactivation of anti-oncogenes and they are the results of genetic alteration accumulated. A recently cloned new tumor suppressor *p*16 gene is located in 9p21, with the full-length of 8.5 kb. It consists of 2 introns and 3 exons, encoding P16 protein-whose molecular mass is 15840 M_r, a single strain peptide comprising 148 amino acid, participating in regulating the proliferation of normal cell growth negatively[1,2]. There was a high frequent loss of homozygosis of *p*16 gene in a variety of cancer cell lines such as gliocytoma, melanoma, breast cancer cell lines[2] and in certain primary cancer, for example, leukemia[3], gliomas[4], astrocytomas[5], bladder cancer[6], melanoma[7], oral squamous cell carcinomas[8], squamous cell carcinoma of head and neck neoplasm[9,10]. The frequency of *p*16 gene deletion and mutation is up to 75% in all kinds of human neoplasm, higher than that of the well-known p53 gene. Gastric cancer is common in China[11-30]. In this paper, S-P immunohistochemical staining was used to detect the expression of P16 protein in gastric cancer and precancerous lesions. PCR and PCR-SSCP methods were used to analyse the deletion and mutation of *p*16 gene exon 2. This study aims to evaluate the relationship between P16 protein and the carcinogenesis, progression, histological types as well as biologic behaviors in human gastric cancer, to find a new marker in early diagnosis and to discover the role of deletion and mutation of *p*16 gene in exon 2 in the carcinogenesis and progression of human gastric cancer.

MATERIAL AND METHODS

Specimens and treatment

All specimens were confirmed by pathology. Paraffin-embedded tissue were collected from the department of pathology and fresh resected specimens were from the First Affiliated Hospital of the Nanhua University, among which there were 50 cases of dysplasia of gastric mucosa and 122 cases of gastric cancer (25 cases were resected freshly from September 1995 to December 1996). In the 122 cases of gastric cancer, 29 were well-differentiated adenocarcinoma, 41 were poorly-differentiated adenocarcinoma, 26 were undifferentiated carcinoma, 16 were signet ring cell carcinoma and the other 10 were mucoid carcinoma. There were 81 men

and 41 women, 22 aged below 40 years, 69 aged from 41 to 59 years, and 31 were older than 60 years. The youngest was 15 years and the oldest 79 years (mean 56 years). Superficial muscles, were invated in 50 cases and deep muscles and the full layer in 72. Sixty-nine cases had lymph node metastasis, 53 had no lymph node metastasis. Thirty cases primary and lymph node metastasis cancer selected randomly were paired and compared. According to Borrmann's classification, 15 were type I, 43 were type II, 47 were type III and 17 were type IV. The 25 cases of fresh resected specimens included cancer, cancer-surroundings and normal mucosa selected far from cancer, were cut into 2 - 4 blocks under sterile conditions. Each block was 2-3 mm^3 and stored in -70℃ refrigerator for PCR and PCR-SSCP analysis. The rest tissues were fixed in 100 $mL \cdot L^{-1}$ neutral formalin, resected, dehydrated, cleaned and paraffin-embedded. All paraffin-embedded tissues were cut into sequential slices for 5μm and adhered to the glass which was processed by poly-lys previously.

Reagents and instruments

Rabbit-anti-human P16 protein multiple clonal antibody, streptavidin-peroxidase immunozator kit (S-P kit), and DAB were all bought from Maxim Company, USA. Protase K (Merk, USA), *Sma* I, agar gel, propylene acrylamide, N-N-sulmethyl bipropylene acrylamide, ammonium persulfate, xylene nitrile, bromophenol blue were bought from Shanghai Sangon Company. PCR primer synthesized by Shanghai Sangon, primer sequences of p16 gene exon 2[4].

Sense: 5'-TCT GAC CAT TCT GTT CTC TC-3'

Antisense: 5'-CTC AGC TTT GGA AGC TCT CA-3'

The fragment length of amplification was 384 bp. Primer sequences of β-active served as an internal control.

Sense: 5'-GCG GGG CGC CCC AGG CAC CA-3'

antisense: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'

The fragment length of amplification was 548bp.

Instrument Ultra low refrigerator (Japan) of -70℃, rotary sector (Germany), microscope (Japan), type 480 DNA amplificatory (PE,USA), type 901 ultraviolet spectrophometer (PE,USA), type DY-IIIB vertical eletrophores and all kinds of centrifuges(Beijing Liuyi).

METHODS

S-P immunohistochemical staining

Operated as the specification of sp kit, that was: paraffin-embedded tissue slices deparaffined hydrated→endogenous peroxidase blocked→added first antibody→then bridge antibody→added enzyme labeled S-P reagents→DAB colorized →hematoxylin stained→dehydrated→cleaned and paraffin-embedded→observed by microscope.

Genomic DNA extraction[31]

Frozen tissue of 0.5g was put into liquid nitrogen and powdered immediately, 10× buffer (10$mmol \cdot L^{-1}$ Tris-HCl pH 8.0, 0.1$mol \cdot L^{-1}$ EDTA pH 8.0, 5$g \cdot L^{-1}$ SDS) was added and span in 37℃ water for 1h at the same time, added protase K to the mixture at a final concentration of 100$mg \cdot L^{-1}$ in 50℃ water for 3h and readjusted the protase K as possible reaction. After the mixture lysed completely, 20$mg \cdot L^{-1}$ Rnase reacted in 37℃ water for 1h, saturated phony was put together and bugged slightly for 10 min, centrifuged and extracted up clean liquid transfer to a cleaned plastic tube, saturated phony processed repeatedly 3 times, added 1/10 volume 3$mol \cdot L^{-1}$ NaAc and 2-2.5 times cold ethyl, DNA precipitated by centrifugation, removed ethyl, DNA washed by 700$mL \cdot L^{-1}$ ethyl land centrifuged 3 times, dried, resolved with TE, A260/A280:1.8-1.9, stored at 0℃ for use.

PCR amplification

PCR was performed according to the reference[31] in 50μL reactive volume containing 0.1μg gDNA template, 200$\mu mol \cdot L^{-1}$ each of dCTP, DATP, dGTP, dTTP, 0.25$\mu mol \cdot L^{-1}$ primer, PCR buffer (Tris-HCl 10$mmol \cdot L^{-1}$, pH 8.3, $MgCl_2$ 1.5$\mu mol \cdot L^{-1}$, KCl 50$mmol \cdot L^{-1}$, gelatin 100$mg \cdot L^{-1}$) pre-denatured at 95℃ for 5 min and added 1.5μL of Taq DNA polymerase, 75μL of mineral oil. These samples were subjected to 30 cycles, including: 95℃ 1 min, 60℃ 1 min, 72℃ 1 min, and extended at 72℃ 5 min. Five μL of PCR product and appropriate bromophenol blue was added to the sample point container and electrophoresed at 20$g \cdot L^{-1}$ agarose gel containing 0.5$mg \cdot L^{-1}$ ethidium bromide at tank with 0.5×TBE liquid of electrophoresis, then observed and photographed with ultraviolet radiography.

PCR-SSCP analysis [31]

Five μL digested PCR product mixed with 5μL denatured dissolution (950$mL \cdot L^{-1}$ forman mide, 20$mmol \cdot L^{-1}$ EDTA, 0.05% bromophenol blue, 0.5$g \cdot L^{-1}$ xylene nitrile) denatured at 95℃ 5 min and colded on ice. Solution processed as above was added to the gel containing 80$g \cdot L^{-1}$ polypropylene acrylamide, vertical electrophoresed at 100 V for 4h and gel stained with silver: fixed in 100$mL \cdot L^{-1}$ alcohol for 10 min→oxidized in 100$g \cdot L^{-1}$ nitric acid for 3 min→drip washed for 1 min with double distilled water→stained in 12$mmol \cdot L^{-1}$ silver nitric acid for 20 min→drip washed for 1 min with double distilled water→showed appropriate color in 0.028 mol anhydrous sodium carbonate and 0.19$mL \cdot L^{-1}$ formalin→ended reducing response by 100$mL \cdot L^{-1}$ glacial acetic acid→drip washed with double distilled water→analysis results and photographed. P16 protein expression of confirmed positively cervix carcinoma served as positive control. PBS substituted with first antibody served as negative control.

Immunohistochemical determination

According to Gevadts' standard modefied slightly[32,33], nuclear or plasma stained brown-yellow as positive, (-) indicated no cell stained positive or only plasma stained or the number of nuclear stained positive less than 1 cell, (+) indicated the cells stained weakly or the number of stained cells less than 25%, (++) indicated the cells stained moderately or the stained cells covering about 26%-50%, (+++) indicated cells stained strongly or the number of stained cell more than 50%. The number of nuclear stained positively more than 2 cells per high time sight was considered to be positive. No folding, and no edging-effect fields were chosen to calculate 100 cells per 5 sights and evaluate the average number of positive cells. Positive cells discerned by two

researchers alone and decided on the disagreements together. No products of PCR amplification were loss of homozygosis of *p*16 gene, and abnormal traces found in PCR-SSCP were considered gene mutation.

Statistical analysis

Chiq-square test was used *P* value less than 0.05 was considered to be statistically significant.

RESULTS

P16 protein expression in gastric cancer

The positive rate of P16 protein expression in 80 cases of normal gastric mucosa was 96% (Figure 1), and in 50 cases of dysplasia mucosa was 90% (Figure 2). In these mucosa P16 protein expression could only be seen in partical adenoepithelial cells. We did not find staining in mucosal epithelial cells, matrix fibrocytes, lymphocytes and smooth myocytes. But in gastric cancer, the ratio was 48% (Figure 3). The positive rate of P16 protein expression in gastric cancer was lower than that in normal and dysplasia mucosa ($P<0.05$). There was no significant difference between the normal gastric mucosa and dysplasia mucosa ($P>0.05$, Table 1).

In the 122 gastric cancer, the positive rate of P16 protein expression was 38%, 51%, 58%, 62% and 10% in well-differentiated adenocarcinoma, poorly-differentiated adenocarcinoma, undifferentiated carcinoma, signet ring cell carcinoma and mucoid carcinoma, respectively. The P16 protein expression in mucoid carcinoma was lower than that in signet ring cell carcinoma, undifferentiated carcinoma and poorly-differentiated adenocarcinoma ($P<0.05$). The positive rate of P16 protein expression was 48% (24/50) in gastric cancer invaded superficial muscle layer and 47% (34/72) in gastric cancer invaded deep muscle and full layer. There was no apparent relevance between P16 protein expression and the depth of invasion ($P>0.05$). In 30 cases of paired primary cancer and lymph node metastasis cancer, the rate of P16 protein expression of the lymph node metastasis cancer was 17%(5/30), significantly lower than that of primary cancer, 47% (14/30), ($P<0.05$).

Deletion and mutation of p16 gene exon 2 in gastric cancer

In 25 fresh resected gastric cancer, there were 7 well-differentiated adenocarcinoma, 13 poorly-differentiated adenocarcinoma, 3 undif ferentiated carcinoma, 1 signet ring cell carcinoma and 1 mucoid carcinoma, cancer-arrounding and normal mucosa were taken at the same time. The PCR amplification showed no product in 1 case of well-differentiated adenocarcinoma, 1 case of poorly-differentiated adenocarcinoma and 1 case of mucoid carcinoma; little product found in 1 case of well-differentiated adenocarcinoma and 1 case of poorly-differentiated adenocarcinoma. There were products of PCR amplification in the rest 20 cases of gastric cancer, tumor adjacent tissue and normal mucosa. All experiments were repeated three times. The result was identical. No product of PCR amplification might indicate the loss of homozygosis of *p*16 gene, little product of PCR amplification was possibly loss of heterozygosis of *p*16 gene or loss of homozygosis of *p*16 gene, but contaminated with normal mucosa (Figure 4). Four of these 5 cases were P16 protein negative expression and 1 case expressed weekly by immunohistochemical staining. No gene mutation was observed in PCR-SSCP analysis after the PCR amplification products cut with *Sam* I (Figure 5, Table 1) (the location of restriction site, and the length of fragment are shown in Figure 6).

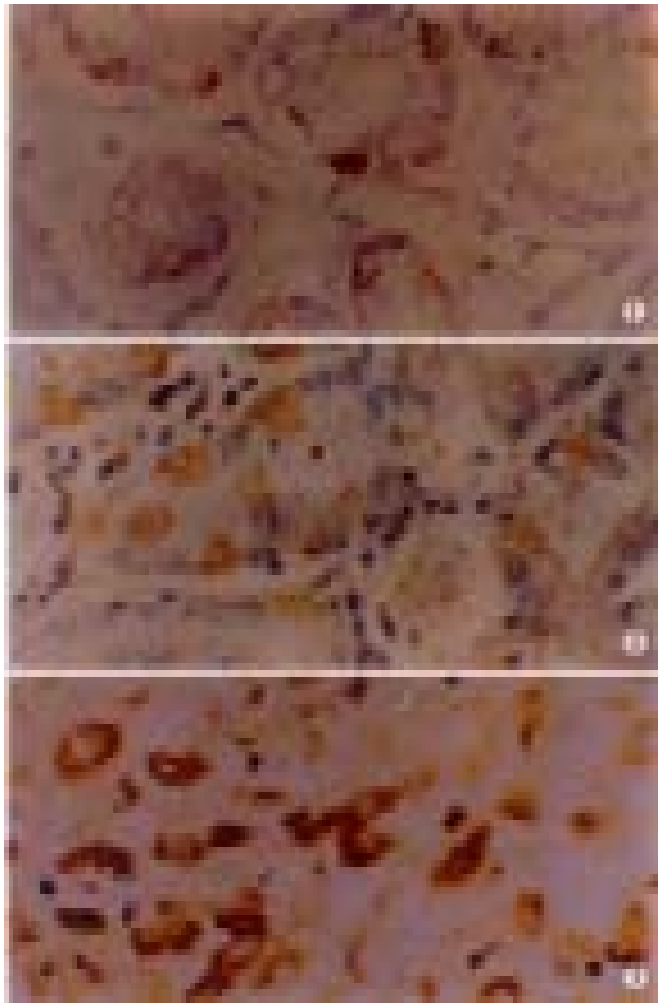

Figure 1 *p*16 protein expression in normal gastric mucosa. ×400
Figure 2 *p*16 protein expression in dysplastic gastric mucosa. ×400
Figure 3 *p*16 protein expression in gastric carcinoma. Immunohistochemic stain. Arrow shows the undifferentiated carcinoma positive cell. ×400

Table 1 P16 protein expression, p16 gene mutation and deletion gastric cancer

Hisological types	*n*	P16 protein				*p*16 gene		
		-	+	++	+++	Positive rate (%)	Mutation	Deletion
A. Normal gastric mucosa	80	3	41	20	16	96	0/25	0/25
B. Dysplasia gastric mucosa	50	5	12	19	14	92	0/25	0/25
C. Gastric cancer	122	64	13	20	25	48	0/25	5/25

A,B *vs* C, $P<0.05$. Mutation and deletion of p16 gene: B is tumor adjacent tissue.

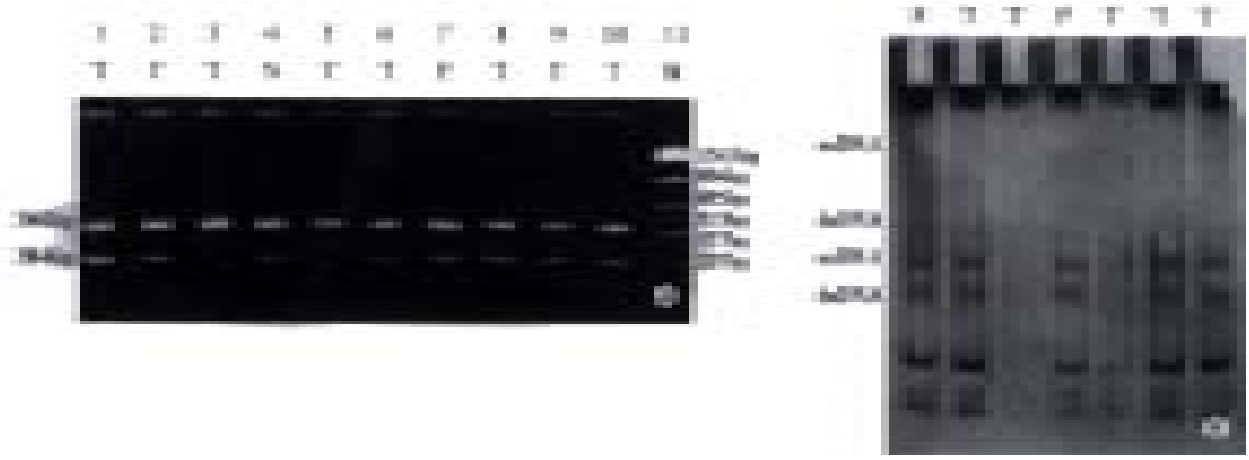

Figure 4 PCR amplification product in exon 2 of p16 gene.
Lines 1-4,5-6,8-10 (T: gastric cancer); Line 7 (P: tumor adjacent tissue); Line 4 (N: normal gastric mucosa); Line 11 (M: marker) Little PCR product in line 3 and no PCR product in line 5.

Figure 5 The exon 2 of p16 gene analyzed by SSCP. segment a 135bp, segment b 249bp.
Lines 2,3,5,6,7 (T: gastric cancer); Line 4 (P: tumor adjacent tissue); Line 1 (N: normal gastric mucosa).
No electrophoresis band on line 3, weak electrophoresis band on line 5, and no abnormal electrophoresis band in all lines.

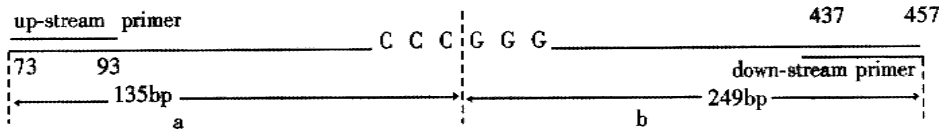

Figure 6 The length of PCR amplification of p16 gene exon 2 and the *Sam* I (CCCGGG) restriction site.

DISCUSSION

The *p*16 gene is a tumor suppressor gene that participates in the negative regulation of the cell growth and proliferation, and a hot spot in the molecular biological research of neoplasm since its discovery in 1993. The product of p16 gene-P16 protein is the inhibitor of CDK4. Its function is to default the activity of CDK4 by binding with CDK4 against Cyclin D_1 competitively, then inhibits the phosphation of Rb protein, transcription factor such as E2F when combined with the dephosphated Rb protein can not be released and activated, thus cells arrest in G_0/G_1 phase, resulting in cell dividing and proliferation suppressed. If p16 gene was abnormal, its function of negative regulation of cell growth would be lost. CDK4 combines to cyclin D_1 and PRb phosphates, lots of transcription factors would be released. The cell from G_1 phase enters into S phase rapidly, cell proliferates excessively and results in carcinogenesis and progression[1,2,34-36]. A lot of investigations show that there were P16 protein deletion and *p*16 gene abnormality in various primary cancers and cancer cell lines. The alteration forms of *p*16 gene were deletion, mutation[2,9,37-43], rearrangement, insertion[44-48], translocation[49-51]and hyper methylation of CpG islands presented in promotor sequence[52-58]. Such alterations consequently change the gene activity, cause abnormality, structure change of the product of *p*16 gene expression and the loss of its physiological functions. Recently, it has been reported that the exoteric *p*16 gene was transfected into the cancer cell which *p*16 gene deletes. The cancer cell restored *p*16 gene expression and cell growth was remarkably inhibited[59-67]. It has also been documented that the CpG island methylated of *p*16 gene cancer cell line was treated with 5-aza-2'-deoxycytidin. Cancer cell restored *p*16 gene expression and showed growth inhibition[68-71]. All these indicated that *p*16 gene and its product played important roles in the carcinogenesis.

This investigation showed that the positive rate of P16 protein expression in gastric cancer was remarkably lower than that in dysplasia and that in normal gastric mucosa ($P<0.05$). The result indicated that gastric carcinogenesis was probably related to the loss of P16 protein expression. But there was no significant difference between the normal mucosa and the dysplasia mucosa of the stomach ($P>0.05$). The positive rate of P16 protein expression in gastric cancer was not identical with other reports[72£¬73]. The cause was not clear. It was possibly related to the different standards of determination, reagents and some uncertain factors. However, the quantity of P16 protein expression increased from normal mucosa to precancerous lesions and gastric cancer ($P<0.05$). Following pathological lesions, P16 protein expression increased. This change may inhibit cell proliferation. The positive rate of P16 protein expression in mucoid carcinoma was significantly lower than in poorly-differentiated adenocarcinoma, undifferentiated carcinoma and signet cell carcinoma ($P<0.05$). The result suggested that the alteration of *p*16 gene was different in various histological types gastric cancer. The discrepancy of P16 protein expression exists in various histological types of lung and esophageal cancer[33,74]. There was difference of P16 protein expression and deletion of *p*16 gene in various differentiation types of gliomas. But the deletion of *p*16 gene concurred with the expression of P16 protein[4]. In 30 cases of primary gastric cancer paired with lymph node metastasis cancer, the positive expression rate of P16 protein in metastasis cancer was lower than in primary gastric cancer

($P<0.05$), which was in agreement with the reported results[75]. The result convincingly suggested that the P16 protein deletion might be related to gastric cancer metastasis and indicate P16 protein expression heterogentity in gastric cancer[76]. What was more intriguing that 2 neighboring lymph nodes metastasis cancer migrated from primary cancer had positive expression P16 protein. Expression of P16 protein is not only related to neoplasms metastasis but also related to prognosis and progression. Expression of P16 protein is low, clinical prognosis is bad[77-81]. We also investigated the relevance between various factors such as age, sex, the depth of invasion and Borrman classification and P16 protein expression in gastric cancer. There was no significant difference ($P>0.05$). The positive expression of P16 protein could merely be observed in partial adeno-epithelial cells of normal and dysplasia gastric mucosa, and weakly positive expression or undetectable in gastric mucosa epithelium cells, interstitial lymphocytes, fibroblasts and smooth muscle cells, which is contrary to some published files[82]. Nevertheless, others confer that the undetectability of P16 protein expression in neoplasm interstice[32], normal lung tissue[83] and normal urinoepithelial cells[82] might attribute to a paucity of P16 molecule in G_0/G_1[84] phase cells or short halftime of P16 protein[85].

Among some human neoplasms, *p*16 gene alterations always resided in exon 2[5]. There was no product of PCR amplification in 3 of 25 cases possibly due to the loss of homozygosis. Little product of PCR in 2 of 25 cases amplification might be the loss of heterozygosis or loss of homozygosis but normal mucosa contaminated. In the 5 cases, the expression of P16 protein was negative in 4 cases and weekly positive in one. The results manifested that 4 cases might be the loss of homozygosis and 1 case might be the loss of heterozygosis among the 5 cases of gastric cancer and the deletion of *p*16 gene is possibly related to the carcinogenesis and progression of gastric carcinoma. The rate of deletion in this study was slightly lower than that reported by others[74]. It was likely that only exon 2 was examined or inadequate for specimens or other unknown factors. Nevertheless, PCR amplification products were found in the rest 20 cases of gastric cancer, normal gastric mucosa and cancer-surrounding mucosa. No abnormal PAGE band and mutation of *p*16 gene was found by SSCP analysis digestion product of PCR amplification. We suggested that *p*16 gene was not involved in the carcinogenesis of gastric cancer, which coincided with other authors[86,87]. We also found that the frequency of *p*16 gene deletion was lower than that of deletion P16 protein expression. P16 protein was undetectable in normal and dysplasia gastric mucosa epithelial cells but in partial adenoepithelium. Some other uncertain mechanisms might exist in the regulation of *p*16 gene and the expression level of P16 protein[88-90], which require further studies.

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• ORIGINAL RESEARCH •

Telomere erosion is independent of microsatellite instability but related to loss of heterozygosity in gastric cancer

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Abstract

AIM **To correlate the length of the telomere to microsatellite instability (MSI) and loss of heterozygosity (LOH) of APC, MCC and DCC genes in gastric carcinomas.**

METHODS **Telomeric restriction fragment (TRF) length of gastric cancer was measured with Southern blot. LOH of APC, MCC and DCC genes, microsatellite instability (MSI) and frameshift mutation of hMSH6, TGF-βRII and BAX genes were analyzed by PCR-based methods.**

RESULTS **Sixty-eight cases of sporadic gastric carcinoma were studied for MSI using five microsatellite markers. MSI in at least one locus was detected in** 17 (25%) **of** 68 **tumors analyzed. Frameshift mutations of hMSH6, TGF-βRII and BAX were detected in** 2,6 **and 3 of gastric carcinomas respectively showing high MSI (**≥2 **loci, *n* =** 8**), but none was found in those showing low MSI (only one locus, *n*=**9**) or MSS (tumor lacking MSI or stable, *n*=**51**). Thirty-five cases, including all high MSI and low MSI, were studied for TRF. The mean TRF length was not correlated with clinicopathological parameters. No association was observed between TRF length and MSI or frameshift mutation. On the contrary, LOH at the DCC locus was related to telomere shortening (*P*<**0.01**). This tendency was also observed in APC and MCC genes, although there was no statistical significance.**

CONCLUSION **The development of gastric cancer can arise through two different genetic pathways. In high MSI gastric cancers, defective mismatch repair allows mutations to accumulate and generate the high MSI phenotype. In gastric cancers showing either low MSI or MSS, multiple deletions may represent the LOH pathway. Telomere erosion is independent of high MSI phenotype but related to the LOH pathway in gastric cancer.**

Subject headings gastric cancer; telomere restriction fragment; microsatellite instability; loss, heterozygosity

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INTRODUCTION

Eukaryotic linear chromosomes are capped by a special structure known as the telomere. In vertebrates, telomeres consist of several kilobases of tandem TTAGGG repeats bound by the related telomere-specific proteins, TRF1 and TRF2[1,2]. TRF1 regulates telomere length[3] and TRF2 maintains telomere integrity[4]. In most human cells, telomeres shorten with each cell division due to the incomplete replication of linear DNA molecules and the absence of telomere-elongating mechanisms[5]. It has been reported that telomeric repeats are often reduced in various human tumors as a consequence of many cell divisions of tumor cells, and the loss of telomeric repeats would cause additional senetic changes as a consequence of chromosomal instability.

The presence of telomeric DNA at the chromosomal termini is essential for genetic stability. Genetic instability may be classified into two different forms in which hypermutability occurs either by means of chromosomal instability or microsatellite instability (MSI)[6-10]. There is now increasing evidence that telomere shortening is involved in chromosomal instability. Indeed telomeres are thought to maintain the chromosome integrity during the cell cycle by allowing a proper segregation during cell division. Broken chromosomes that are uncapped become hotspots for different types of recombination. They may therefore undergo aberrant recombination, end-to-end fusions and bridge-fusion-breakage mechanisms. Being unprotected, the chromosomes are also susceptible to exonucleolytic degradation[11]. Telomeres would also prevent the activation of DNA-damage check-points[12]. Thus, without these essential structures, the chromosome is unstable.

MSI represents an important new form of genetic alteration characterized by widespread instability in repetitive nucleotide sequences. MSI has been found in the majority of tumors associated with hereditary non-polyposis colorectal cancer (HNPCC)[13,14] in which germ-line mutation occurs within the mismatch repair genes hMSH2, hMLH1, hPMS2 or hMSH6[15-17]. Mutations of the transforming growth factor type II receptor gene (TGF-βR II), and BAX gene are strongly correlated with MSI[18-20]. MSI is also a distinctive feature in about 10%-15% of sporadic colorectal tumors and to a varying degree in tumors of other organs, including the stomach[7,21-24]. Although alterations of telomerase activity have been reported in MSI and microsatellite stable (MSS) tumors[25,26], less clear, however, the relevance of progressive telomere shortening as a potential factor in MSI is less clear. The aim of the present study is to correlate telomere status with MSI and loss of heterozygosity (LOH) of APC, MCC and DCC genes in gastric carcinomas.

MATERIAL AND METHOD

Sixty-eight cancer and corresponding normal tissues were obtained from surgically resected gastric carcinoma patients in our hospital. Each specimen was frozen immediately and

stored at -80℃ until analyzed. A 5μm section was cut from each tissue and stained with hematoxylin /eosin in order to ascertain whether the cancer cells in tissues were predominant or not. Genomic DNA was isolated by standard proteinase-K digestion and phenol-chloroform extraction protocols. Of the 68 patients with gastric cancer, 45 were men and 23 were women with an age range of 30-76 years (mean age of 56.2 years at diagnosis). None of the patients included in the present series had a family history suggestive of HNPCC and had received chemotherapy or radiation therapy.

MSI analyses included five microsatellite markers: BAT25, BAT26, BAT40, D2S123, and D5S346. PCR was performed in 15μL of reaction mixture containing 10mmol·L^{-1} Tris-HCl (pH 8.3), 50mmol·L^{-1} KCl, 1.5mmol·L^{-1} $MgCl_2$, 200μmol·L^{-1} each deoxynucleotide triphoshate, 0.5μmol·L^{-1} of each primer, 0.75unit Ampli Taq polymerase (Perkin-Elmer, Norwalk, CT, USA), and 100ng genomic DNA. The reaction was carried out in a thermal cycler at 94℃ for 1min, 55℃-62℃ for 1min, and 72℃ for 1min, for 35 cycles with an initial denaturation step of 94℃ for 5min and a final extension step of 72℃ for 10min. The PCR products were then separated on 5% polyacrylamide, 7M urea denaturing gel, and visualized by autoradiography. MSI was defined as the presence of band shift in the tumor DNA that was not present in the corresponding normal DNA. Based on the number of mutated MSI markers in each tumor, carcinomas were characterized as high MSI if they manifested instability at two or more markers, low MSI if unstable at only one marker, and MSS if they showed no instability at any markers (microsatellite stable)[27-29].

To detect frameshift mutations in coding regions, repetitive mononucleotide sequences, the (A)10 tract of TGF-βRII, the (G)8 tract of BAX and the (C)8 tract of mismatch repair gene MSH6 were amplified using published primers, respectively[30-32]. The reaction condition consisted of 35 cycles at 94℃ for 1min, 55℃ or 56℃ for 1min and 72℃ for 1min using 0.5μCi[33p] dATP into 15μL reaction mixture. DNA denaturation, electrophoresis and autoradiographic procedure were done likewise with MSI analysis, except for TGF-βRII where 8% denaturing gel was used.

Southern blot analysis to estimate telomeric restriction fragment (TRF) length was based on previously reported methods[33]. High molecular weight DNA was prepared from each sample, followed by extraction with phenol chloroform isoamyl alcohol and precipitation with 3M sodium acetate and ethanol. Genomic DNA was digested with *Hin* fI (Promega, USA)at 37℃ for 6 hours and then 10μg of digested DNA was subject to electrophoresis on 1% agarose gels. After electrophoresis, the gel was denatured in 0.5M NaOH and 1.5M NaCl for 30 minutes and neutralized in 0.5M Tris (pH 8.0) and 1.5M NaCl for 30 minutes. The DNA was then transferred onto a nitrocellulose membrane (Promega, USA) overnight. The membrane was dried at 80℃ for 1 hour and subsequently hybridized to α-^{32} P-ATP end- labeled $(TTAGGG)_4$ probe. The membranes were autoradiographed on X-ray films for 24 hours. Each lane was scanned with a densitometer and the data was used to determine the mean TRF length as previously described[34].

The APC, MCC and DCC genes were investigated for LOH and PCR was carried out as described[35,36]. The priming regions were located within specific tumor suppressor genes at sequence, either a restriction fragment length polymorphism (RFLP) or a variable mumber of tandem repeats-type polymorphism (VNTR)[37]. Annealing temperature, extension time and the number of amplification cycles were optimized for each primer set. After amplification, PCR products were digested with appropriate restriction enzymes (for RFLPs) or not digested (for VNTRs) and electrophoresed on 1.5% agarose gels or 8% polyacrylamide gels, which were stained with ethidium bromide and photographed under UV light. When the intensity of one allele in cancerous tissue was less than 50% of the other allele in comparison with the ratio of intensity of the allele in the corresponding normal tissue, it was judged as LOH.

One factor analysis of variance, and Chi-square test with Yates' correction were used. A P value <0.05 was considered significant.

RESUTS

Alterations of electrophoretic patterns of PCR products of five microsatellite markers, TGF-βRII, BAX, and MSH3 genes were compared between tumor and normal DNA in each patient (Figures 1,2). MSI affecting at least one locus was observed in 17 (25%) of 68 tumors, among which eight (11.8%) had high MSI and nine (13.2%) had low MIS. A comparison of MSI status with frameshift mutation is shown in Table 1. The (A)10 frameshift mutation in TGF-βRII gene was detected in 6 of 8 gastric cancers with high MSI, whereas none of the low MSI or MSS tumors showed such a mutation. The mutation in the BAX (G)8 repeat site and hMSH6 (C)8 tracts was detected in 3 of 8 and 2 of 8 gastric cancers with high MSI, respectively, and no mutation was found in tumors with low MSI and MSS.

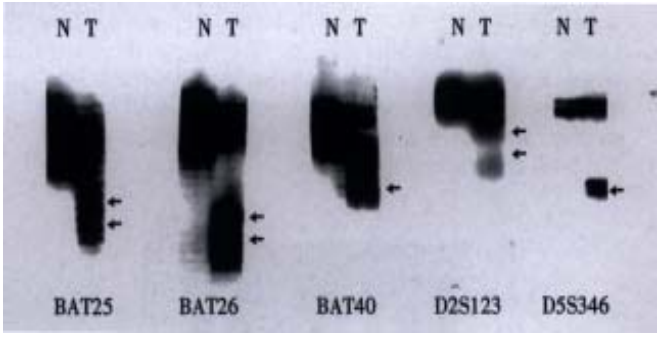

Figure 1 MSI in gastric cancer using 5 microsatellite loci (BAT-25, BAT-26, BAT40, D2S123, and D5S346). Arrows indicate variant conformers. (N: normal DNA pattern; T: tumor specimens containing variant conformers representing MIS)

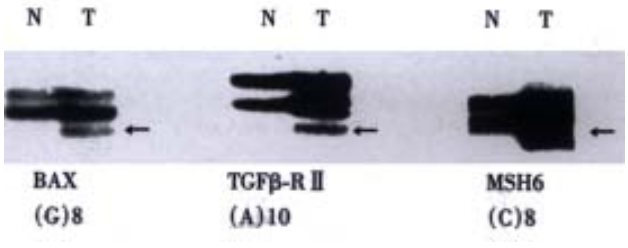

Figure 2 Frameshift mutations of hMSH6 TGF-beta RII, and BAX genes in gastric cancers. Arrows indicate conformational variants associated with frameshift mutations. (N: normal DNA; T: tumor DNA)

Table 1 Relationship between MSI status and frameshift mutation

MSI status	No. of cases	Frameshift mutations		
		MSH6	TGF β RII	BAX
MSI-H	8	2	6	3
MSI-L	9	0	0	0
MSS	51	0	0	0

Thirty-five gastric cancers, including all 8 with high MSI and 9 with low MSI, were examined for alteration in TRF length using the Southern blot technique. Since the signal of telomeric repeats is detected as a smear (Figure 3), we visually determined the peak of signal, which was confirmed using a densitometer. When the TRF length in the tumor was more than 20% shorter or longer than that in corresponding normal tissues, we classified the TRFs as shortened or elongated, as previously described[38]. Compared with the corresponding normal mucosa, 20 (57.1%) had considerable shortening (<80% of corresponding normal tissues), 12 (34.3%) had approximately the same length (between 80%-120%), and 3(8.6%) showed elongation (>120%). No correlation was found between mean TRF length and clinicopathological parameters in gastric carcinomas. However, a decrease of the mean TRF length with the age was observed except for the group aged from 50 to 59 years. The mean TRF length in the age group of 30 to 39 years was significantly longer than that in the group aged from 60 to 69 years or 70 to 79 years (P <0.05) (Table 2).

We compared the TRF length with MSI and frameshift mutation status. Table 3 shows the association of MSI and frameshift status to the TRF length of 35 gastric cancers. No relationship was observed between TRF length and MSI or frameshift mutation in gastric cancer.

Tissues from 35 patients were also studied for LOH of APC, MCC and DCC. In order to increase the assay sensitivity, three different sites, i.e., M2, M3 and VNTR were used for analysis of LOH at DCC genetic locus. LOH of APC, MCC and DCC were observed in 6 (27.3%) of 22, 7 (36.8%) of 19 and 18(56.3%) of 32 of informative cases, respectively (Figure 4). The relationship between LOH at various loci and the alteration in TRF length in primary tumors is shown in Table 4. LOH at the DCC locus were associated with telomere shortening (P<0.01). This tendency was also observed in APC and MCC genes, although there was no statistical significance.

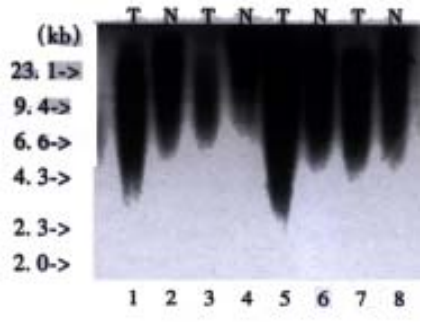

Figure 3 Southern blot analysis of telomere repeat arrays in DNA samples of patients with gastric cancer. DNA was digested with-*Hin* fI and hybridized with α-^{32} P APT end-labeled $(TTAGGG)_4$ probe.T =Tumour; N =Corresponding normal tissues.

Table 2 The relationship between the mean TRF length and clinicopathological pararmeters

Parameters	No. of cases	Mean TRF length ($\bar{x}$±s)
Gender		
Male	23	3.99±0.63
Female	12	4.32±1.00
Age(yrs)		
30-39	5	4.54±0.54
40-49	7	4.07±0.60
50-59	11	4.39±0.96
60-69	10	3.69±0.66[a]
70-79	2	3.40±0.07[a]
Size		
<5cm	12	3.80±1.20
>5cm	23	4.06±0.86
Differentiation		
Well and moderate	7	3.91±0.49
Poor	19	4.24±0.64
Mucinous	9	4.02±1.10
Clinical stage		
I and II	21	4.26±0.82
III and IV	14	3.84±0.68

[a]P<0.05 *vs* the age group of 30 to 39 years.

Table 3 Relationship between alterations of TRF length and MSI status or frameshift mutation

	No. of cases	Alterations in TRF length		
		Shortened (n=20)	Normal (n=12)	Elongated (n=3)
MSI status				
MSI-H	8	4	4	0
MSI-L	9	4	4	1
MSS	18	9	7	2
Frameshift mutation				
MSH3	35	1	1	0
TGF β RII	35	3	2	1
BAX	35	2	1	0

Table 4 Relationship between alterations of TRF length and loss of heterozygosity of APC, MCC and DCC genes

LOH	Alterations in TRF engh (LOH / Informative)		
	Shortened (n=20)	Normal (n=12)	Elongated (n=3)
APC	5 / 13	1 / 7	0 / 2
MCC	6 / 11	1 / 6	0 / 2
DCC	14 / 18[a]	3 / 11	1 / 3

[a]P<0.01.

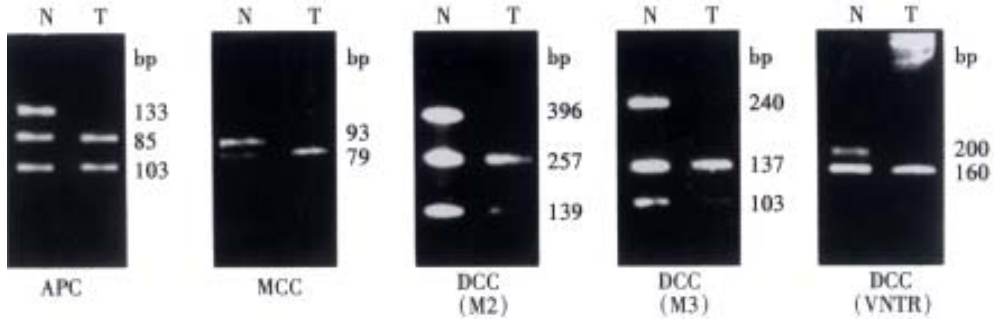

Figure 4 Representative LOH analysis of APC, MCC and DCC genes. (APC: Rsa1 RFLP in APC exon 11, loss of 133bp allele is seen in tumor DNA, MCC: A 14bp insertion / deletion polymorphism in MCC exon 10 gives rise to a 93 or 79 allele. Loss of the 93bp allele is seen in the tumor. DCC: Losses of 396bp, 240bp and 200bp alleles are seen at M2, M3 and VNTR polymorphic sequences in the tumor DNA)

DISCUSSION

In this study, 17(25%) of 68 sporadic gastric cancers had MSI in at least one locus. This finding is similar to previous studies[23,39]. There is now evidence that MSI cancer comprises distinctive high MSI and low MSI categories[40,41]. High MSI cancers are distinguished clinicopathologically and in their spectrum of genetic alterations from cancers showing low MSI and MSS cancers[40,41]. Our previous studies indicated that high MSI gastric cancers often show lower frequency of LOH of APC, MCC and DCC genes than low MSI and MSS cancers[41]. In our series of 68 gastric cancers, 8 were classified as high MSI, 9 as low MSI and 51 as MSS. Frameshift mutations of hMSH6, TGF-β RII and BAX were detected in gastric carcinomas with high MSI, but no mutation of these genes was found in those showing low MSI or MSS. These results indicate that these genes are mutational targets in high MSI tumor cells and support the notion that high MIS tumors identify an alterative pathway of tumorigenesis that has been proposed by Vogelstein and co-workers[42].

In the present study, 35 gastric carcinomas were examined for TRF length using the Southern blot. Telomere shortening was detected in 57.1% of the gastric carcinomas, indicating that telomere erosion may play a role in the gastric carcinoma development. It has been reported that telomere shortening was associated with a short survival and disease recurrent in prostate cancer[43]. In the current study, subdivision of the tumors according to telomere length did not reveal an obvious relationship between the shortening of telomere and tumor size, depth of invasion, node metastasis or clinical stages, indicating a limited role of the telomere shortening in predicting prognosis of gastric carcinomas. This finding is in agreement with the recently published data on renal cell carcinoma and colorectal carcinomas[44,45].

TRF reduction was demonstrated in various human malignant tumors[38,45-47]. In contrast to these results, it was also found that telomeres in some tumors are similar to, even elongated, as compared with normal tissues[36,48]. In this study, the mean TRF length was reduced in 20 of 35 tumors, similar in 12 cases and elongated in 3 cases, as compared with the corresponding normal mucosa. The discrepancy in TRF length may be related for several reasons. For tumor samples with TRF of normal length, there are at least three possibilities: ① The majority of cells in such tumors have not experienced many cell divisions nor experienced critical shortening of telomeric repeats; ② In most tumor cells, telomerase already activated, elongated the once shortened telomeric repeats back to normal length, as observed in HPV-immortalized epithelial cells *in vitro*[49]; and ③ The amount of tumor cells also affects TRF analysis. Therefore, with the predominance of normal cells in tumor specimens, telomere loss may be underestimated[48]. We found that the TRF length in tumors was progressively reduced with age, suggesting that patients' age should be considered in evaluating the TRF lengths of tumor. Finally, end-to-end chromosome fusion observed in some tumors could lead to telomere elongation[50].

It has been reported that telomerase activity and microsatellite instability are independent events in colorectal carcinogenesis[25]. To our knowledge, this is the first report on the relationship between MSI and telomere length. We did not find any correlation between telomere erosion and MSI, suggesting that the MSI pathway is independent of telomere erosion.

It has been found that chromosomal instability is correlated with telomere erosion and inactivation of G2 checkpoint function in human fibroblasts[12]. Alterations in the TRF length were often associated with LOH of the p53 gene and Rb gene, but less often with mutation of k-ras and p53 genes in lung cancer[38]. We found that LOH at the DCC locus was associated with telomere shortening. Although not statistically significant, LOH at the APC and MCC genetic loci tends to occur in telomere shortened gastric cancers, indicating that telomere erosion may be involved in the LOH pathway.

In summary, our data indicate that telomere shortening may play a role in gastric carcinogenesis. There are at least two distinct genetic instabilities in gastric tumorigenesis: one is the chromosomal instability (or suppressor pathway) and the other is microsatellite instability (or MSI pathway). The former, may include tumors with low MIS as well as MSS and accumulation of loss of tumor suppressor genes such as p53, Rb, APC, MCC and DCC plays an important role in their carcinogenesis; whereas the latter consists of a small subset of gastric cancer with high MSI, defective repair of mismatched bases results in an increased mutation rate at the nucleotide level, and consequent widespread MSI. Telomere shortening may be involved in the LOH pathway but independent of the MSI pathway. Our analysis of TRFs should further provide some clues to the molecular mechanisms underlying the profound genomic instability in the MSI and LOH pathway for gastric carcinoma.

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• ORIGINAL RESEARCH •

Extraction and purification of TGFβ and its effect on the induction of apoptosis of hepatocytes

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Abstract

AIM **To extract and purify the transforming growth factor β (TGF β), and to demonstrate its biological activity *in vivo* and induction of apoptosis of hepatocytes *in vitro*.**

METHODS **TGF β was isolated from fresh bovine platelets by acid/ethanol extraction method and purified with ion exchange and gel chromatography. The extracted TGF β as injected subcutaneously to mice, and its biological activity *in vivo* was observed** 72 **hrs post-injection by HE staining. The morphological changes were observed by HE staining and the occurrence of apoptosis was detected by TUNEL method after the human normal hepatic cell line QZG was treated with** 8μg·L^{-1}**TGFβ for** 12 **hrs *in vitro*.**

RESULTS **The molecular mass** 25 **ku TGF β protein was successfully extracted. It was able to induce localized granulation tissue formation *in vivo*. TGF β-treated hepatocytes showed obvious apoptotic morphological changes, including the pyknosis and dense-stained nuclei and cytoplasm, the fragmentary, annular or crescent nuclei, and the "bubbling" cytoplasm. Moreover, its apoptotic rate was significantly higher than that of the control group** (***P***<0.05)**.**

CONCLUSION **Biological active TGF β protein is extracted and purified successfully from bovine platelets, and it is able to induce the apoptosis of hepatocytes.**

Subject headings transforming growth factor beta/ isolation & purification; transforming growth factor beta/ pharmacology; liver/cytology; apoptosis

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INTRODUCTION

Liver diseases are very common in China[1-10], and apoptosis is the research focus in recent years[11-20]. Transforming growth factor β (TGFβ) is a kind of polypeptide growth factors that is extensively present in most tissues and cells. A variety of cell types, both nonneoplastic and neoplastic, synthesize TGF β and most of these cells have specific high-affinity receptors for TGF β[21-24]. It is a multifunctional molecule which is known to play an important regulatory role in cell growth, migration and differentiation[25], embryogenesis[26,27], tumorigenesis[28], wound healing[29-31], bone formation[32-35] and immunomodulation[36], acting by both autocrine and paracrine mechanisms. It is also suggested that TGF β may be responsible for some pathological process, such as scarring and fibrosis, renal diseases and immunosuppressant[37-39]. Furthermore, TGF β is able to induce many kinds of cells, including hepatic and hepatoma cells to undergo apoptosis[40,41]. The molecular mechanisms underlying TGF β induction of the apoptosis of hepatocytes is still unclear. In this study, TGF β was extracted from the fresh bovine platelets by acid/ethanol procedure and purified by ion exchange and gel chromatography, then its biological activity was detected *in vivo* and its induction of apoptosis of cultured hepatocytes was observed *in vitro*, and to provide the basis for the study of the relationship between TGFβ and the signal transduction of hepatocellular-apoptosis.

MATERIALS AND METHODS

Isolation of platelets

The fresh anticoagulant bovine blood was collected in bags containing 0.1 volume of 8.78g·L^{-1} NaCl and 22.50g·L^{-1} EDTA (pH 7.4). The blood was centrifuged at 2000r·min^{-1} for 15 min and the supernatant was recentrifuged at 4000r·min^{-1} for 15 min. Then the supernatant was discarded and the precipitated platelets was washed twice with PBS (pH 7.4) by centrifugation at 5000r·min^{-1} for 30 min[42,43]. All the centrifugations were carried out at 0℃.

Extraction procedure

TGF β was extracted by a modified acid/ethanol procedure of Roberts and others[44,45]. The platelets were suspended in acid/ethanol extraction solution containing 375mL of 950mL·L^{-1} ethanol and 7.5mL of concentrated HCl, plus 33mg phenylmethylsulfonyl fluoride(PMSF) and 1.9mg pepstatin A as protease inhibitors. The mixture was sonicated in ice-bath, extracted overnight at 4℃, and centrifuged at 15000r·min^{-1} for 40 min at 0℃. The supernatant was adjusted to pH 3.0 with concentrated ammonium hydroxide. Then 2 volumes of cold anhydrous ethanol (-20℃) and 4 volumes of cold anhydrous ether (-20℃) were immediately added. After the mixture stood at -20℃ for 48 hrs, the resulting precipitate was collected by centrifugation at 20000r·min^{-1} for 30 min at 0℃ and redissolved in 1mol·L^{-1} acetic acid. After extensive dialysis at 4℃ against 0.17mol·L^{-1} acetic acid in a dialyzing tube (molecular mass cutoff, 10000ku), the sample was then subjected to the next purification.

Ion-exchange chromatography

The above crude sample was centrifuged at 20000r·min^{-1} for 40 min at 0℃ to remove the small precipitate and the supernatant was then applied to a CM-Sepharose column

(1.6cm×11cm, Phamacia Biotech, Uppsala, Sweden) equilibrated with 0.17mol·L^{-1} acetic acid. The column was eluted successively with 0.17 mol·L^{-1} acetic acid, 41.02g·L^{-1} NaOAc, 82.04g·L^{-1} NaOAc and 4g·L^{-1} NaOH at a flow rate of 90mL·h^{-1} at room temperature. The fraction that eluted by 4g·L^{-1} NaOH was immediately neutralized by adding 100mL·L^{-1} acetic acid. The eluted fractions were collected respectively and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE).

Gel chromatography

The above fractions containing 25ku component were collected, concentrated with glycol polyethylene and extensive dialyzed at 4℃ against tridistilled water and then 1 mol·L^{-1} acetic acid. After being centrifuged at 20000r·min^{-1} for 10 min, the supernatant was applied to a Superdex 75 column (1.6cm×70cm, Phamacia Biotech, Uppsala, Sweden) equilibrated with 1mol·L^{-1} acetic acid. The elution was carried out at a flow rate of 36mL·h^{-1} with 1mol·L^{-1} acetic acid containing 11.69g·L^{-1} NaCl at room temperature. The eluted fractions were collected and analyzed by SDS-PAGE. The fraction containing the 25ku component was concentrated using glycol polyethylene. After extensive dialysis at 4℃ against tridistilled water and 0.17mol·L^{-1} acetic acid, the samples were stored at -20℃ for the following assays. The protein content was determined by Coomassie brilliant blue G-250 method and bovine serum albumin (BSA) was used as control.

Detection of TGF β activity in vivo[46]

Nine male Balb/c mice (10 days old) were injected subcutaneously each day in the back with the purified TGF β (0.5g·L^{-1}). After 72 hrs, the tissues at the injection sites were removed and fixed in 100mL·L^{-1} formalin and paraffin sections were then subjected to the routine HE staining. The other 3 mice were injected with equivalent BSA as controls.

TGF β induction of hepatocellular apoptosis

Human normal hepatic cell line QZG (purchased from the Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured routinely on cover slides. After having grown to logarithmic phase, 8μg·L^{-1} of TGF β was added and then for a further 12 hrs culture. BSA (8μg·L^{-1}) was added as control. The cells were subjected to HE staining. In addition, the cells were stained by terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL) method to detect apoptosis[47]. A random field of cells was chosen with a magnification of ×400, and adjacent non overlapping fields were counted until the minimum 500 limits for each slide was obtained. TUNEL index was expressed as the number of positive cells/the total number of cells.

RESULTS

Purification of TGF β

The crude TGFβ was purified by ion exchange chromatography on a CM-Sepharose column, as shown in Figure 1. SDS-PAGE analysis showed that peaks 2 and 3 contained the 25ku component. The peaks 2 and 3 fractions were collected and then purified by gel chromatography on a Superdex-75 column (Figure 2). SDS PAGE analysis showed that peak 2 predominantly contained 25ku component (Figure 3) . The protein content was 1.1g·L^{-1}.

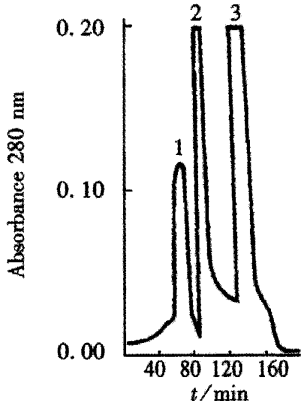

Figure 1 Ion-exchange chromatography of acid / ethanol extract on a CM Sepharose column.

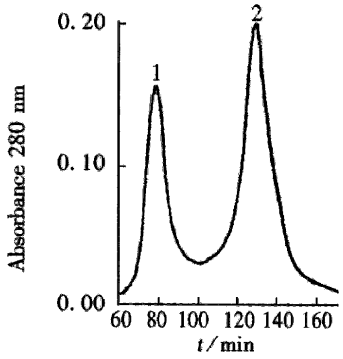

Figure 2 Gel chromatography of the CM-Sepharose peak 2 and peak 3 fractions on a Superdex-75 column.

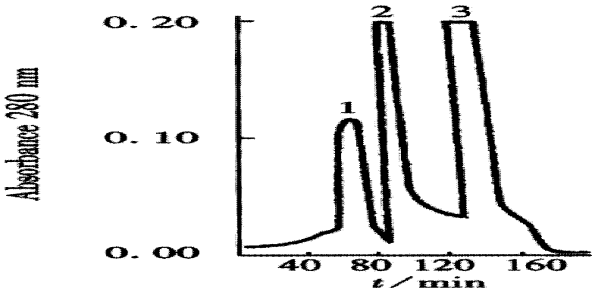

Figure 3 Molecular mass analysis of the extracted TGF β (SDS PAGE). Right Lane: marker proteins.

TGFβ activity in vivo

Seventy-two hrs after subcutaneous injection of TGF β, there was evident proliferation of fibroblasts and a few infiltration of inflammatory cells at the injection sites. The newly formed blood capillaries were also seen at the injection sites (Figure 4). The granulation tissue was confined at the injection sites. No obvious tissue change was observed in the control group.

Detection of apoptosis of hepatocytes

HE staining revealed that apoptotic hepatocytes exhibited the pyknosis and dense-stained nuclei and cytoplasm. Moreover, the nuclei demonstrated fragment, annular or crescent body, and the cytoplasm showed "bubbling" (Figure 5). TUNEL positive signals were olivine or yellow florescence in the nuclei. The TUNEL index of the TGF β treated group was 0.31 and distinctly higher than that of control group (0.09), (P<0.05)

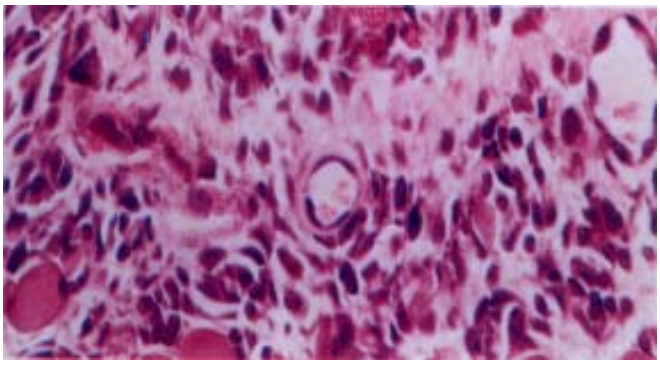

Figure 4 The granulation tissue formation after 72 hrs subcutaneous injection of TGF β. HE×400

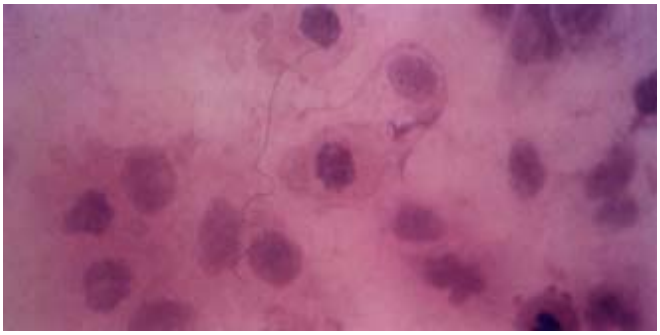

Figure 5 Hepatocytes QZG displayed apoptosis after being treated with 8μg/L TGF β for 12 hrs. HE×400

DISCUSSION

TGF β is a disulfide-linked homodimeric 25ku protein that consists of two identical 112 amino acid subunits, and only the dimer is biologically active. It is acid and heat stable and the presence of 18 half cystine residues in each dimeric molecular contributes to this stability[48-50]. There are at least five described subtypes of TGF β, encoded by distinct but closely related genes. TGF β 1, 2 and 3 have been found in many species, including humans; TGF β 4 has been found in chickens and TGF β 5 in amphibians[51] . Bone is the richest source of TGF β in the body, as it contains more than 200μg per kg of wet weight, whereas blood platelets represent the most concentrated source of TGF β (up to 20mg per kg of wet weight) and is released from β granules of platelets when blood clots[52,53]. Therefore, TGF β is generally extracted from the fresh platelets. It is suggested that TGF β is highly stable under acidic condition and can be activated by heating in boiling water for 5 min, or treatment with 1N acetic acid or 6M urea. The acid/ ethanol procedure is a practical way and previously used to extract biologically active polypeptides such as insulin, insulin like growth factor and platelet-derived growth factor[54,55]. Some other studies verify the effectiveness of this extraction procedure for isolation of TGF β from many tissues, including platelets, placenta and kidney[53,56,57]. Isolation of TGF β from platelets includes five steps: collection and washing of platelets, acid/ethanol extraction, ethanol/ether precipitation, ion exchange chromatography and gel chromatography. In the present study, crude TGF β was isolated from the fresh bovine platelets and then purified by ion exchange and gel chromatography. The SDS-PAGE analysis showed that the molecular weight of its main component was 25ku,which corresponded to that of standard TGF β protein.

Wound healing and tissue repair involve a complex series of biological events which include inflammation, cellular migration, fibroblasts proliferation, production of collagen and tissue remodeling. Growth factors have been reported to enhance the repair process in animal models by increasing the degree of cellularity, the rate of angiogenesis, and the amount of collagen accumulated[58,59]. TGF β has been studied in association with wound healing and the ability of TGF β to initiate a cascade of events leading to enhanced wound healing has been clearly demonstrated by many reports. Pierce and co-workers[60-65] have reported that TGF β significantly accelerate soft tissue repair by attracting fibroblasts into the wound and stimulating rapid synthesis, deposition and maturation of collagen *in vitro* and *in vivo*. Other major activities of TGF β are its abilities to promote the synthesis and deposition of various extracellular matrix (ECM) proteins and increase the expression of integrins and fibronectin, receptors that mediate cellular interactions with ECM proteins[66,67]. Different assay systems have been developed to measure the TGF β activities, including cell proliferation and inhibition assays, radio receptor assays, immunoassays and matrix formation or cell surface antigens expression assays[68,69]. In the present study, the analysis of activity *in vivo* demonstrated that there were fibroblast proliferation and blood capillaries formation after subcutaneous injection of the extracted TGF β. Moreover, the granulation tissue was only located in the injection sites and had no tendency to diffuse. That the action of the extracted TGF β *in vivo* led to granulation tissue formation suggested the successful extraction of TGF β and its participation in repair of tissue injury.

TGF β has been shown to either stimulate or inhibit proliferation in different cell types, and within same cell types, depending upon the stage of cell differentiation, *in vitro* condition and the presence of other growth factors. TGF β has a stimulatory effect on the proliferation of cells of mesenchymal origin, such as fibroblasts, osteoblasts and Schwann cells, yet is a growth inhibitor for cells of epithelial or neuroectodermal origin, including epithelial cells, osteoclasts, keratinocytes, T and B lymphocytes, endothelial cells and hepatocytes[70-72].

Apoptosis is a genetically and highly conserved process. Regulation of the balance between cell proliferation and apoptosis is essential for development and maintenance of multicellular organisms[73-77]. Previous studies suggest that TGF β is able to induce evidently apoptosis of hepatocytes and hepatoma cells *in vitro*. The animal experiments *in vivo* also manifest that the hepatocytes undergoing apoptosis have obviously elevated level of TGF β expression[78,79]. Furthermore, hepatoma cells which have a high apoptotic incidence rate, simultaneously demonstrate a high level of TGF β expression[80]. These studies suggest an involvement of TGF β in the initiation of apoptosis of hepatocytes. In our HE staining, normal hepatic cell line QZG showed remarkable morphological changes of apoptosis, including the pyknotic and hyperchromic cytoplasm and nuclei, and the fragmentary, crescent form or annular nuclei, and the “bubbling” cytoplasm, after being treated with exogenous TGF β. TUNEL staining also showed that the incidence rate of apoptosis was distincly higher in the TGF β treated group than that of the control group. The present study further supported the apoptotic induction of hepatocytes by TGF β, and verified the good biological activity of the extracted TGF β as well. Better understanding of the relationship between TGF β and the signal transduction of hepatocellular apoptosis requires further investigations.

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• ORIGINAL RESEARCH •

Molecular mechanism about lymphogenous metastasis of hepatocarcinoma cells in mice

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Abstract

AIM **To investigate the correlation between lymphogenous metastasis and matrix metalloproteinases (MMPs) activity and the expression of Fas ligand of tumor cells in lymph nodes.**

METHODES **Fifty-six inbred 615-mice were equally divided into 2 groups and inoculated with Hca-F and Hca-P cells. Their lymph node metastatic rates were examined. Growth fraction of lymphocytes in host lymph nodes was detected by flow cytometry. The Hca-F and Hca-P cells were cultured with extract of lymph node, liver or spleen. The quantity of MMPs in these supernatants was examined by zymographic analysis. The expression of Fas ligand, PCNA, Bcl-2 protein of Hca-F and Hca-P cells in the mice were examined by immunohistochemistry. The apoptosis signals of macrophages in lymph nodes were observed with *in situ* DNA fragmentation.**

RESULTS **On the 28th day post-inoculation, the lymph node metastatic rate of Hca-F was 80%(16/20), whereas that of Hca-P was 25%(5/20). The growth fraction of lymphocytes was as follows: in the Hca-F cells, the proliferating peak of lymphocytes appeared on the 14th day post-inoculation and then decreased rapidly, while in Hca-P cells, the peak appeared on the 7th day post inoculation and then kept at a high level. With the extract of lymph node, the quantity of the MMP-9 activity increased (P<0.01) and active MMP-9 and MMP-2 were produced by both Hca-F and Hca-P tumor cells, which did not produce MMPs without the extract of lymph node or with the extracts of the liver and spleen. The expression of Fas Ligand of Hca-F cells was stronger than that of Hca-P cells (P<0.01). The expressions of PCNA and Bcl-2 protein of Hca-F cells in the tumors of inoculated area were the same as that of Hca-P cells. *In situ* DNA fragmentation showed that the positive signals of macrophages were around Hca-F cells.**

CONCLUSION **Secretion of MMPs which was associated with metastatic ability of Hca-F and Hca-P tumor cells depends on the environment of lymph nodes. The increased expression of Fas ligand protein of Hca-F tumor cells with high lymphogenous metastatic potential in lymph nodes may help tumor cells escape from being killed by host lymphocytes.**

Subject headings liver neoplasms/pathology; lymphatic metastasis; tumor cells, cultured; metalloproteinases/secretion

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INTRODUCTION

Metastasis is the most lethal attribute of a cancer[1,2]. Metastasis is a complex process which is made up of several steps[3]. Lymph nodes are often the first organ to develop metastasis[4,5]. Whether lymph nodes or other sites first develop metastases remain poorly understood. Lymph node metastases form a bridgehead for further metastatic spread. But its molecular mechanism is still unclear because of its complicated course. Anchorage on lymph node and escape from being killed by host immune cells in lymph node were two important steps. A mouse hepatocarcinoma cell line (Hca-F) with high lymphogenous metastatic potential and its syngeneic cell line (Hca-P) with low one were separated from hepatocarcinoma (HCC)[6] in mice. Matrix metalloproteinases (MMPs)[7] are a class of proteinases with variable substrate such as collagen, fibronectin and are related to the invasion-metastasis of hepatocellular cancer (HCC)[8]. Therefore, MMPs can be used as a marker of tumor cell infiltration in lymph node. The potential of tumor cells to induce apoptosis of host immune cells is to escape from being killed by immune cells. In this study, we detected the differences in the MMPs productions of Hca-F from Hca-P cells under various conditions, and different potentials of the carcinoma cells with different lymphogenous potentials to inhibit host immune reaction.

MATERIALS AND METHODS

Animals, cell lines and flow cytometry

Fifty-six inbred 615-mice maintained in our laboratory were equally divided into two groups. The Hca-F and Hca-P tumor cell lines preserved in our laboratory were inoculated at 2×10^6 in 28 mice subcutaneously in each group. On the 7th, 14th, 21st, and 28th day post-inoculation, two mice from each group were killed, and their lymphocytes were collected and detected for growth fraction with flow cytometry. The process of flow cytometry is as follows[9]: the lymph nodes were minced and centrifuged at 3000×g, and the supernatant was discarded. After repeated washing, cells were suspended in PBS. The lymphocytes at 10^5/100μL suspension were stained for 30 minutes by Propidum Iodide. Flow cytometry

was performed on a FACScan cytometer with LYSYII software. The fluorescence of 10^4 cells was analyzed for each sample. The other 40 mice were terminated on the 28th day post-inoculation, and their lymph nodes were H.E. stained and examined under microscope by paraffin sections. Therefore, the lymph node metastatic rates of Hca-F and Hca-P tumor cells were calculated.

Cell culture and zymographic analysis

The Hca-F and Hca-P cells cultured were put into different wells at 5×10^5, and then added 50mg extract of lymph node, liver or spleen respectively. The RPMI 1640 medium without fetal calf serum was placed into each well up to 1mL. The wells containing only Hca-F or Hca-P cells, and RPMI 1640 medium added only extracts of lymph node, liver or spleen up to 1mL served as controls. These cells were cultured at 37℃ for 24h. The supernatant of cultured cells was collected by centrifugation at 3000×*g*. MMP-2 and MMP-9 and their active type, and MMP-8 contained in supernatants of Hca-F and Hca-P with or without extracts of lymph node, liver or spleen were detected by zymographic analysis, according to the method described by Fridman *et al*[10]. The density and area of each band were measured using QuantiScan Software (Biosoft, USA).

Immunohistochemistry

The expressions of Fas-L (Santa Cruz, USA), proliferating cell nuclear antigen (Santa Cruz, USA, PCNA) and Bcl-2 (Santa Cruz, USA) protein in the tumor cells of inoculated area of Hca-F and Hca-P tumor cells, and metastatic tumor cells of lymph nodes of Hca-F were detected by standard immunohistochemistry[11]. The semiquantitative estimation of cancer cells stained was classified into 4 categories by assessing the percentage of stained tumor cells: 0, <2%; 1, 2%-25%; 2, 26%-50%; 3, 51%-75%; and 4, >75% cells.

In situ DNA fragmentation

We examined DNA fragmentation of the tumor cells of inoculated area of Hca-F and Hca-P cells, and metastatic tumor cells of Hca-F cells in lymph node, by the method of Zhu *et al*[12]. After desparaffin, these slides were pre-treated with $20mg\cdot L^{-1}$ proteinase K for 30 minutes, and then incubated with terminal deoxynucleotidy transferase and fluorescein labeled dUTP containing nucleotide mixture (TUNEL reaction mixture, *in situ* Cell Death Detection Kit/ POD, Boehringer Mannheim, Germany) in a humid atmosphere at 37℃ for 30 minutes. Each experiment set up by TUNEL reaction mixture without terminal transferase served as negative control.

RESULTS

Lymph node metastatic rate and flow cytometry

On the 28th day post-inoculation, the lymph node metastatic rate of Hca-F was 80%(16/20), whereas that of Hca-P was 25%(5/20). The growth fraction of lymphocytes from lymph nodes of mice transplanted with Hca-F and Hca-P tumor cells was examined using flow cytometry (Figure 1). For the Hca-F cells, the proliferating peak of lymphocytes appeared on the 14th day post-inoculation and then decreased rapidly, and for the Hca-P cells, the peak appeared on the 7th day post-inoculation and then kept at the high level.

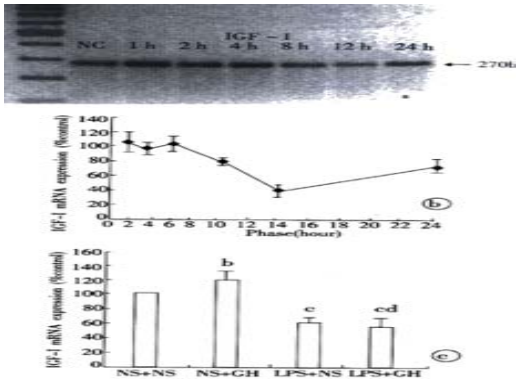

Figure 1 Changes of growth fraction of lmphocytes (G_2+S/G_1+G_2 +S+M) in draining lymph nodes of Hca-F/Hca-P burden mice.

Zymographic analysis

Both Hca-F and Hca-P cells produced a small account of MMP-9, but did not produce MMP-2 and MMP-8 (Table 1, Figure 2). Both Hca-F and Hca-P cells with extract of lymph node produced higher amounts of MMP-9 than Hca-F and Hca-P cells without extract of lymph node ($P<0.01$), and produced active MMP-9 and MMP-2. However, the Hca-F cells produce much greater quantities of activity of MMP-9, active MMP-9 and MMP-2 than Hca-P cells ($P<0.05$). The extract of lymph node did not contain any MMPs (Table 1, Figure 2). There was activity of MMP-8 in both Hca-F and Hca-P cells with extract of liver (Hca-F: 1767, Hca-P: 1564). The extract of liver contained activity of MMP-8 (1837). There was activity of MMP-8 in both Hca-F and Hca-P cells with extract of spleen (Hca-F: 2036, Hca-P: 1993). The extract of spleen contained the same quantity of activity of MMP-8 (1784). Therefore, we think that both Hca-F and Hca-P cells in the environment of liver and spleen did not produce activity of MMP-8 (Figures 3, 4).

Table 1 Activities of MMPs secreted from Hca-F and Hca-P cells under different conditions ($\bar{x}\pm s$)

Condition	MMP-2	Active MMP-2	MMP-9	Active MMP-9
Hca-F				
RPMI1640 medium	0	0	1256±157	0
Medium with lymph node extract	7364±2001	2009±901	12403±894	7297±1657
Hca-P				
RPMI1640 medium	0	0	2642±385	0
Medium with lymph node extract	2997±1990	1237±905	9086±686	3914±1253
Lmyph node extract	0	0	0	0

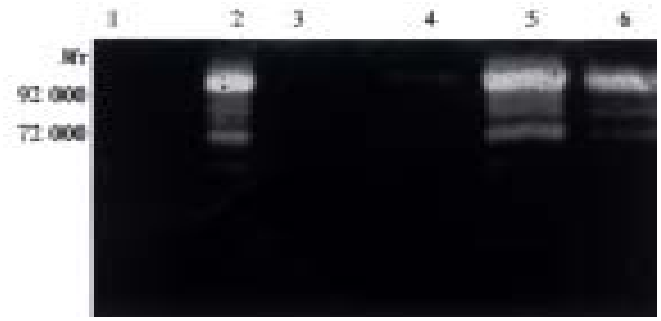

Figure 2 MMPs activity of Hca-F and Hca-P cells in RPMI 1640 with or without lymph node extract using zymographic analysis.
1. lymph node extract; 2. Type-four collagenase; 3. Hca-F cells; 4. Hca-P cells; 5. Hca-F cells in lymph node extract; 6. Hca-P cells in lymph node extract.

Figure 3 MMPs activity of Hca-F and Hca-P cells in liver extract by using zymographic analysis.
1. Type-four collagenase; 2. Hca-F cells in liver extract;
3. Hca-P cells in liver extract; 4. Liver extract.

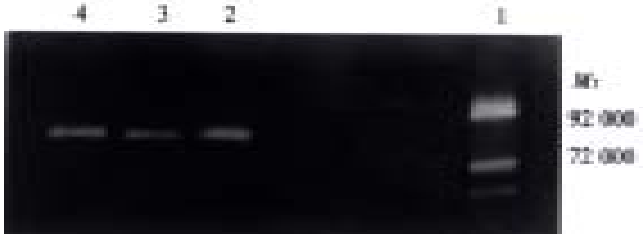

Figure 4 MMPs activity of Hca-F and Hca-P cells in spleen extract by using zymographic analysis.
1. Type-four collagenase; 2. Hca-F cells in spleen extract;
3. Hca-P cells in spleen extract; 4. Spleen extract.

Table 2 Analysis of Fas-L expression in Hca-F and Hca-P cells

Tumor	Grade				
	0	1	2	3	4
Primary tumor of Hca-F	0	0	0	8	12
Primary tumor of Hca-P	2	10	8	0	0
Metastatic tumor of Hca-F	0	5	9	6	0

Immunohistochemistry

The expression of Fas ligand protein of Hca-F cells was significantly higher than that of Hca-P cells ($P<0.01$, Table 2, Figure 5). The expressions of PCNA and Bcl-2 protein of Hca-F cells were as strong as those of Hca-P cells.

In situ DNA fragmentation

Few positive Hca-F and Hca-P cells were observed. Positive signals appeared in the macrophages around Hca-F cells (Figure 6).

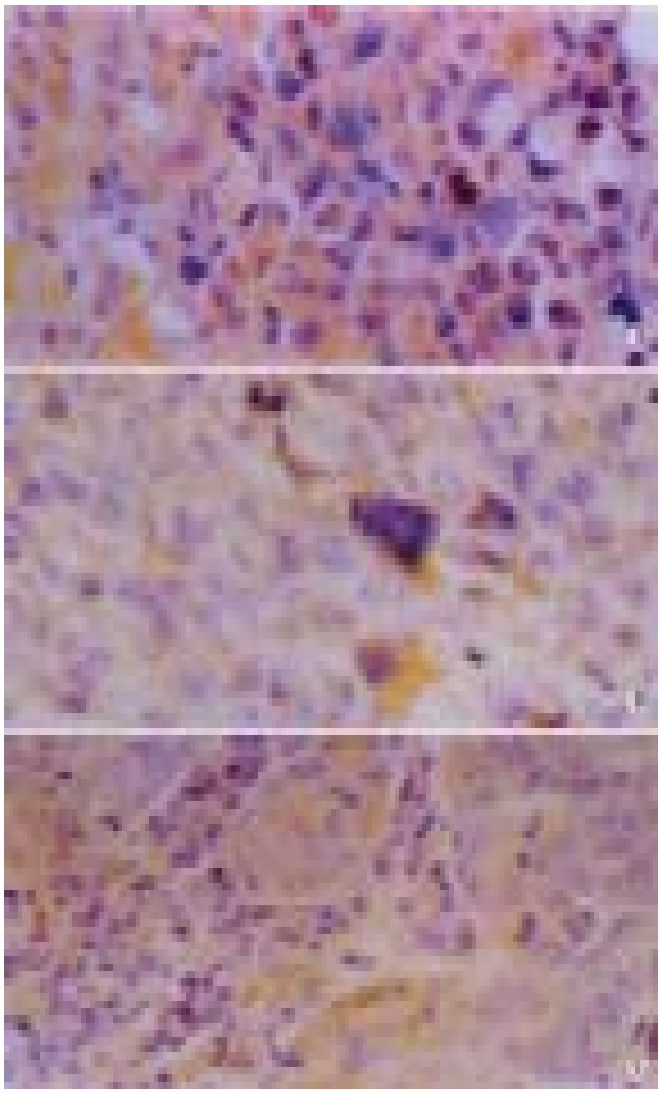

Figure 5 Fas-L expressed in Hca-F and Hca-P cells.
A: Fas-L expressed in Hca-F cells; B: in Hca-P cells;
C: in metastatic Hca-F cells.

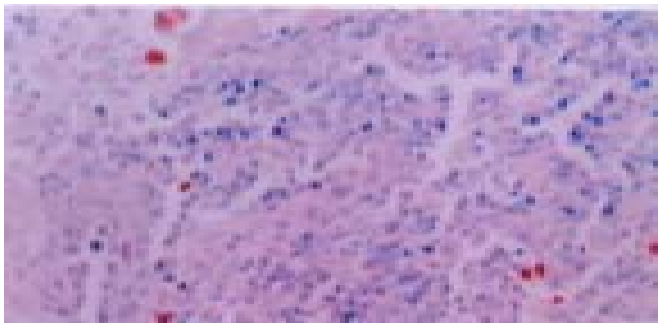

Figure 6 TUNEL of metastatic lymph node of Hca-F cells.

DISCUSSION

As early as a hundred years ago, the fact that tumor cells had organ-specific metastasis had attracted scientists' attention. Recent major discoveries concerning invasion and metastasis are identification of certain molecular mechanisms leading to organ-selective metastatization[13]. Cancer cell gene expression is regulated by interactions of tumor cells with host microenvironment, both in primary and secondary

lesions[14,15]. Whether the anchorage of carcinoma cells in lymph nodes is influenced by the specific environment of the lymph nodes remains unclear. Hca-F and Hca-P tumor cells have the potentials of specific lymphogenous metastasis. The matrix metalloproteinases[16] (MMPs) are a family of proteolytic enzymes, and the importance of MMPs in the processes of tumor invasion is now widely acknowledged in gastrointestinal cancer[17], breast cancer[18], colorectal cancer[19], and melanoma[20]. Inhibit the activity of MMPs can reduce the metastatic potential of cancer cells[21]. MMps digest collagen-containing structural barriers that cancer cells must pass in the step of cancer cell's anchoring to lymph node[22]. In this paper when the extract of lymph node was added, the quantity of the MMP-9 activity increased and active MMP-9 and MMP-2 were produced by both Hca-F and Hca-P tumor cells, which did not produce MMPs without the extract of lymph node or with extracts of liver and spleen. These results indicated that the secretion of MMPs of these tumor cells depend on the lymph node environment. In the environment with extract of lymph node, Hca-F cells with high lymphogenous metastatic potential produced much more MMPs than Hca-P cells with low one. So we can conjecture that Hca-F cells with high lymphogenous metastatic potential can easily receive the signal from lymph node and then start to infiltrate in lymph node to form metastatic focus.

The macrometastases were more proliferative than dormant micrometastases[23]. PCNA[24] functions as a cofactor of DNA-polymerase and is an important mark for evaluating the proliferation of colon cancer[25,26], gastric adenocarcinoma[27], lung cancer[28], ovarian cancer[29], thyroid carcinoma[30], and large intestine polyps[31]. We can use PCNA as an index of cellular proliferative status[32]. Bcl-2 proteins can extend cell survival by suppressing apoptosis[33] and are up-regulated in squamous cell carcinoma[34], breast cancer[35], lung cancer[36]. Bcl-2 proteins may promote metastasis in breast cancer[37] and melanoma[38]. The expressions of PCNA and Bcl-2 proteins could reflect accurately the status of cancer cells's growth. The expressions of PCNA and Bcl-2 protein of Hca-F cells in the tumors of inoculated area are the same as those of Hca-P cells. Therefore, the proliferating ability of Hca-F was equal to Hca-P, although their lymph node metastatic potentials were different.

A metastatic tumor in lymph node may form as long as the tumor cells escape the killing of lymphocytes. Fas/Apo-1, together with its protein-binding partner (Fas ligand), is a key regulator of programmed cell death and induces apoptosis when it binds FasL[39-42]. In this study, we found that growth fraction of lymphocytes in host lymph nodes was lower with Hca-F cells stimulation than with Hca-P cells. The result suggests that Hca-F cells may inhibit the growth and function of lymphocytes in lymph nodes. Tumor cell survives only by evasion of the immune system[43]. The Fas/FasL system is involved in the induction of apoptosis and mediates T-cell cytotoxicyty[44]. The expression of Fas ligand in many cancers plays an important role in establishing immunologically privileged environments that allow tumors to escape the host's immune surveillance, such as in esophageal carcinomas[45,46], lung cancer[47], melanoma[48], gastric carcinoma[49], intrahepatic cholangiocellular carcinoma[50] and promotes these cancers' metastasis. The expression of Fas ligand protein of Hca-F cells was stronger than that of Hca-P cells. Hca-F cells also produced Fos ligand in lymph node. Macrophages in lymph node are one of important antigen-presenting cells, and Fas ligand in tumor cells can combine with Fas in the membrane of macrophages to induce apoptosis and decrease their function. Because lymphocytes can not receive the signals from the macrophages, tumor cells in lymph nodes can escape the suppression of lymphocytes, then accomplish the metastatic process in the lymph node.

In a word, secretion of MMPs, which was associated with metastatic ability, of Hca-F and Hca-P tumor cells depend on the environment of lymph nodes. The increased expression of Fas ligand protein of Hca-F tumor cells with a high lymphogenous metastatic potential in lymph nodes may help tumor cells escape from the killing of host lymphocytes and shape up metastatic focus in lymph nodes.

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• ORIGINAL RESEARCH •

Dysfunction of peripheral blood dendritic cells from patients with chronic hepatitis B virus infection

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Abstract

AIM **To identify the property of dendritic cells (DCs) of peripheral blood monocytes (PBMC) in patients with chronic HBV infection.**

METHODS **Twenty patients with persistent HBV infection were included in this study,** 10 **healthy subjects being used as a control group. The peripheral blood mononuclear cells (PBMC) of T cell-depleted populations were incubated and induced into mature dendritic cells in the RPMI-**1640 **medium in the presence of cytokines GM-CSF, IL-**4**, FLt-**3**, TNF-α and** 100**mL·L^{-1} of fetal calf serum for a total of** 10-12 **days. The expressions of surface markers on DCs were evaluated using flow cytometric analysis. ELISA method was used to determine the cytokine levels of interleukin-**12 **(IL-**12**) and IL-**10 **in the supernatant produced by DCs. For detection of the stimulatory capacity of DCs to T cell proliferation, mytomycin C-treated DC were incubated with allogenic T cells.**

RESULTS **A typical morphology of mature DCs from healthy subjects and HBV-infected patients was induced in *in vitro* incubation, but the proliferation ability and cellular number of DCs from HBV-infected patients significantly decreased compared with healthy individuals. In particular, the expression levels of HLA-DR, CD**80 **(B**7-1**) and CD**86 **(B**7-2**) on DC surface from patients were also lower than that from healthy individuals** (0.46 ***vs*** 0.92 **for HLA-DR,** 0.44 ***vs*** 0.88 **for CD**80 **and** 0.44 ***vs*** 0.84 **for CD**86**, *P*<**0.05**). The stimulatory capacity and production of IL-**12 **of DCs from patients in allogenic mixed lymphocyte reaction (AMLR) significantly decreased, but the production level of nitric oxide (NO) by DCs simultaneously increased compared with healthy subjects** (86±15 ***vs*** 170±22 **μmol·L^{-1}, *P*<**0.05**).

CONCLUSION **The patients with chronic HBV infection have the defective function and immature phenotype of dendritic cells, which may be associated with the inability of efficient presentation of HBV antigens to host immune system for the clearance of HBV.**

Subject headings dendritic cells/pathology; hepatitis B, chronic/pathology; nitric oxide/analysis; interleukin-10/ analysis; interleuki-12/analysis

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INTRODUCTION

The infection of hepatitis B virus (HBV) causes 130 million HBV carriers, of them 23 million were patients with chronic hepatitis B in mainland China. As a result, a considerable number of the patients are developing progressive liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC) each year[1]. The pathogenesis of developing chronic HBV infection is complex and unclear[2]. The dominant reason responsible for viral persistence within host is mainly lack of efficient antiviral immune response to the viral antigens, in particular, inability to produce specific CTL response, which is critical for complete elimination of virus within hepatocytes[3,4]. The mechanism for weak antiviral immune reaction in patients is not well understood. Dendritic cells (DCs) are one of the most potent antigen-presenting cells (APC) and play crucial roles in the enhancement or regulation of antiviral immune reactions. The previous reports showed the defects of DCs function in HBV-transgenic mice and in patients with HCC and chronic HCV infections[5-13], but there is little evidence of defect DCs function in chronic HBV-infected patients. In order to have more insights in this regard, we induced peripheral blood monocytes (PBMC) from HBV-infected patients and uninfected healthy donors into maturation of DCs, compared their morphologic characterization, expression of surface antigens, and their proliferation capability to stimulate AMLR for evaluation of the functions of DCs.

MATERIALS AND METHODS

Patients

Blood was obtained from each of the twenty patients who were positive for HBV antigens and serum HBV-DNA in this study after written consent had been obtained from them. All patients were negative for HCV infection and had no histories of other types of liver diseases. The diagnosis of chronic HBV infection is made in accordance with the standards for chronic viral hepatitis issued in the Fifth National Conference on Infectious Diseases and Parasitosis in China (Beijing, China, 1995)[14]. Ten age-matched healthy donors from the Blood Center of Chinese PLA 307 Hospital were assigned as controls. The clinical background of patients and donors are shown in Table 1.

Table 1 Clinical background of HBV-infected patients and healthy donors

Group	*n*	Gender M/F	Age (years)	ALT (nkat·L^{-1})	AST (nkat·L^{-1})	HBV DNA	HBsAg	Anti-HBs	HBeAg	Anti-HBe	Anti-HBc
Patients	20	17/3	29.5(12-47)	215±218	186±172	19	18	—	16	4	20
Donors	10	8/2	27.5(20-35)	20-40	20-40	—	—	6	—	—	—

—: Nagative results

Reagents

RPMI1640 medium, recombinant human IL-4, GM-CSF, fetal calf serum and TNF-α were all purchased from Gibco Co. (Jingmei Biological Co, Beijing). The Flt-3 ligand, mouse anti-human FITC-conjugated HLA-DR-FITC, CD-86-FITC, PE-conjugated CD80-PE, CD1α-PE monoclonal antibodies were obtained from BD-Pharmingen (Jingmei Biological Co, Beijing).

Culture of DCs

Fifteen milliliters of peripheral blood was drawn from each study subject in heparin-coated tube, PBMC were obtained by Ficoll Hypaque separation, washed twice, resuspended in RPMI1640 medium and incubated in 6-well culture plates at the concentration of 3×10^9 cells·L^{-1} in medium at 37℃ in 50mL·L^{-1} CO_2 atmosphere for 2h. Non-adherent lymphocytes were collected by careful rinsing in 37℃ warm and cryopreserved for their future use in antigen presentation assays. The adherent aggregates were kept for incubation in complete medium with 100mL·L^{-1} fetal calf serum (FCS) in the presence of 1×10^6 IU·L^{-1} GM-CSF, 1×10^6 IU·L^{-1} IL-4, and 50μg·L^{-1} Flt3-L respectively, and expanded over the next 7. At d7, 1×10^6 IU·L^{-1} TNF-α cytokine was added into the medium for 10-12 days incubation. The wells were fed every other day by aspirating 0.3mL medium and adding back 0.5mL fresh medium with cytokines. The protocol has been proven reproducible in 20 HBV-infected patients and 10 healthy donors[15].

AMLR stimulated by DCs

To evaluate the allo-stimulatory activity of DC, AMLR (allogeneic-mixed lymphocyte reaction) was performed[16]. After DCs had been treated with 50μg mytomycin C for 45 min at 37℃ and washed three times with 37℃ warm PBS (pH 7.0). The DCs were placed at 0.5×10^4, 1.0×10^4, 1.5×10^4 and 5.0×10^4 per well on 96-well flat-bottom culture plates, incubated in complete medium. T-cells were from the PBMC of a healthy donor by removing the CD8-positive cells, monocytes, B cells and NK cells with magnetic bead-tagged mouse monoclonal anti-human CD8, CD14, CD19, and CD56 Abs (BD-PharMingen). After the separation, the degrees of positivity of these cells in the samples were all less than 5% and those of CD4-positive cells were more than 95%, respectively. CD4 T cells at 2×10^5/well were mixed with DC and cultured for 4d at 37℃, 50mL·L^{-1} CO_2 atmosphere. The MTT method was used according to the manufacturer instructions.

Detection of NO IL-10 and IL-12

The levels of nitric oxide (NO) and cytokine production by DCs were determined according to a previous report with slight modification (Kakumu *et al*, 2000). In short, DC (1×10^9·L^{-1}), enriched from HBV-infected patients and uninfected healthy donors by cytokines, were cultured in RPMI1640 plus 100mL·L^{-1} FCS for 48h in the presence of *Staphylococcus aureus Cowan* I strain (SAC). After the end of the culture, supernatant was collected and centrifuged. The production of NO and cytokines in the culture supernatants was determined[16,17]. The levels of NO production in the sample was determined by assaying the stable end product NO^{2-} by Greiss reaction using a commercial kit (Griess Assay Kit NO kit-C; Wako), as described by Lu[18]. Aliquots of culture supernatants were incubated with Griess reagent (10g·L^{-1} sulfanilamide, 1g·L^{-1} nephthylethlenediamine, dihydrochloride, and 25g·L^{-1} H_3PO_4) 100 μL at room temperature for 10min. The color developed due to enzymatic reaction was determined with an ELISA reader at 540nm. Concentrations of NO in the sample were caliberated with a reference standard of sodium nitrite supplied with the kit and the levels of NO were expressed as μmol·L^{-1}.

IL-10 and IL-12 in the supernatants were estimated by an ELISA method using commercial kit (PharMingen, San Diego, CA) according to the instructions of the manufacturer. Samples were incubated on microtitre plates coated with the respective monoclonal antibodies (mAbs), followed by addition of a biotinylated second antibody. After removal of excess antibody, color development was finished by enzymatic reaction of strepavidin peroxidase, the intensity of which was directly proportional to the concentration of the respective cytokines in the samples. The amounts of cytokines in the samples were estimated by caliberating the absorbance (optical density, OD) values of the samples with A values of the standards, supplied with kits using an ELISA reader (Labsystems Multiskan MS, USA). The lowest levels of cytokines detectable by these kits were IL-10>15.0ng·L^{-1}, and IL-12>7.8ng·L^{-1}.

Flow cytometry

After 12d incubation, the mature DCs from patients and healthy donors were washed in PBS and directly stained with an optimum dilution of FITC conjugated specific antibody (PharMingen, San Diego, CA) or stained with an optimum dilution of primary antibody followed by FITC-conjugated antibody against HLA-DR, CD86 and PE-labelled antibody against CD80 and CD1a. Finally, FITC+ and PE-cells were counted in an FACScaliber (Becton Dickinson)[19,20].Subclass matched FITC-conjugated mouse IgG and FITC-conjugated secondary antibody alone served as controls.

Statistical analysis

The Student's *t* test was used to determine significant differences in mean values between two groups. Statistical significance was established at the $P<0.05$ level.

RESULTS

Proliferation and Morphology of DCs

The adherent cells, the progenitor cells of DCs were incubated in complete medium containing 100mL·L^{-1} FCS, Flt-3-L, GM-CSF and IL-4 at 37℃ in 50mL·L^{-1} CO_2 atmosphere. The proliferation of DCs could be observed from the third day

incubation. The number of proliferative DCs increased following the extension of incubation time, and reached top value at the 12th day, then gradually went down as shown in Figure 1. Under phase-contrast microscopy, the DCs display a typical morphology with many fine dendrites. These cells extend large, delicate processes or veils in many directions from the body, and display typical morphology of DCs. The yield of proliferation of DC populations increased around 45 times for the healthy donors and 30 times for HBV-infected patients after 12d incubation.

Surface markers on DC

Mature DCs 1×10^6 after 12d incubation were defined by the expression of dendritic cell-restricted markers. The analyses of flow cytometry showed that positive cells constituted between 56% and 80% of all cells recovered at the end of the culture; the purity was not different between patients and controls. The frequencies of $CD86^+$ cells in the dendritic cell population were 84%±10% for control donors (n = 10) and 44% ± 8% for patients with chronic HBV infection (n = 20), respectively, indicating a significant difference between the groups. In addition, the average ratio of $HLA\text{-}DR^+$, and $CD80^+$ surface molecules expressed in the dendritic cell population are shown in detail in Table 2. Figure 2 shows the fluorescence intensity of representative DCs from one patient and one healthy donor, and there was a high expression of surface markers on DCs from healthy donors, reflecting a significant statistical difference from the patient group.

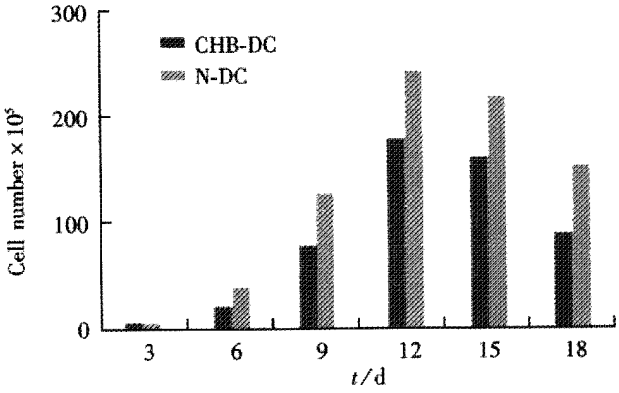

Figure 1 Proliferation of dendritic cell population in *in vitro* culture.

Table 2 Comparison of expression of DC surface markers from CHB patients and healthy donors (number fraction, $\bar{x}\pm s$)

Group	*n*	B7-1(CD80)	B7-2(CD86)	CD1[a]	HLA-DR
CHB-DC	20	0.44±0.08	0.44±0.08	0.25±0.07	0.46±0.09
Normal-DC	10	0.88±0.10[b]	0.84±0.09[b]	0.89±0.09[b]	0.92±0.09[b]

[b]P<0.001 *vs* CHB-DC.

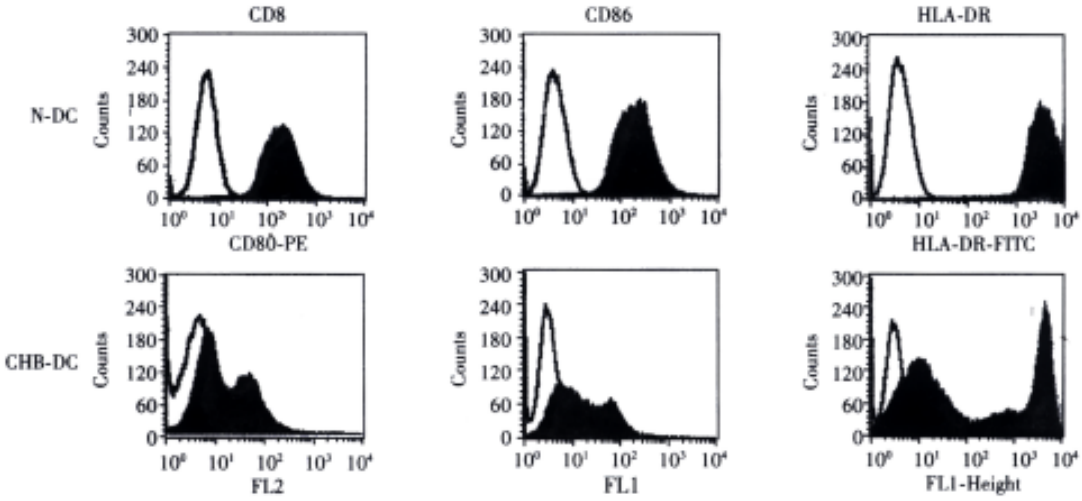

Figure 2 Comparison of fluorescent intensities of flow cytometric analyses on surface of dendritic cells (DCs) from a representative of HBV-infected patients (HB-DC) and a representative of uninfected healthy donors (N-DC). The green line is for signals from controls (DCs stained with mouse IgG_2 instead of specific antibody against surface marker of DCs). The green line with purple color represents the signals obtained from surface of DCs stained with corresponding specific antibody. Note the shift to the left in the peak with increasing expression of surface markers on DCs. Two other independent experiments gave similar results.

AMLR from HBV-infected patients

The T-cell stimulatory activity of DC populations in AMLR was expressed as stimulation index (SI) value, which is the ratio between the proliferative response (optical absorbance, OD) of T cells in the presence and the absence of DCs in the cultures at a T cell:DC ratio of 100:2.5, 100:5, and 100:25, respectively. Although the number of T cells was the same (2×10^5 cells/well) in all AMLR cultures, the SI went up significantly according to the increase of DCs number in AMLR. The SI values were between 2.1 and 4.8 for HBV-infected patients as compared with the values between 6.8 and 12.8 for healthy donors. The SI for HBV-infected patients was the lowest (SI=2.1) when the AMLR system contained 0.5×10^4 $well^{-1}$ of DCs. The results showed that HBV-infected patients tended to have significantly decreased T cell-stimulatory activity (P<0.01) as compared with the values for healthy donors.

Cytokine production by DC in AMLR

IL-12 is known to induce T cell proliferation and is the only

vital cytokine produced by DCs. Spontaneous IL-12 production of dendritic cells in the supernatants of dendritic cell population and AMLR are shown in Table 3. IL-12 produced by DC from patients (27±4ng·L^{-1}) was significantly lower than that from healthy donors (86±32ng·L^{-1}), indicating the statistical difference. Although the IL-12 in this AMLR was most likely produced entirely by the DCs, we decided to confirm this by culturing DCs for 48h and measuring the spontaneous production of IL-12 in culture by pure populations of DCs. Pure DCs from patients produced significantly lower amounts of IL-12 as against healthy donors. In addition, there was no statistical difference in IL-10 levels in AMLR supernatants of DCs from HBV-infected patients (18 ± 7 ng·L^{-1}) (Table 3) and normal controls (25±9ng·L^{-1}).

Table 3 Spontaneous cytokine production in pure DCs population and AMLR ($\bar{x}$±s, ng·L^{-1})

Dendritic cell	Supernatant from MLR culture		Supernatant from pure DCs population(ng·L^{-1})
	IL-12(ng·L^{-1})	IL-10(ng·L^{-1})	
CH-B	27±4	18±7	99±51
Normal	86±32[b]	25±9	218±104[b]

[b]P<0.01, *vs* Normal.

Increased NO production by DC from HBV-infected patients

As shown in Figure 3, the levels of NO in the AMLR supernatant of DCs from HBV-infected patients (168±35 μmol·L^{-1}) were significantly higher than that produced in AMLR of DCs from normal controls (90 ± 43 μmol·L^{-1}) (P<0.05). There was no correlation between the level of NO and the levels of transaminase (data not shown). NO produced by DCs is supposed to be one of the main products in AMLR supernatant. To have direct evidence of increased NO production by DCs, we cultured DC with SAC, a known inducer of NO. Pure DCs from HBV-infected patients produced significantly higher amounts of NO (170 ± 22 μmol·L^{-1}) than the normal controls (86 ± 15 μmol·L^{-1}, P<0.05, Figure 3). The significance of increased production of NO by DC in HBV infection might find the clinical implications of these observations.

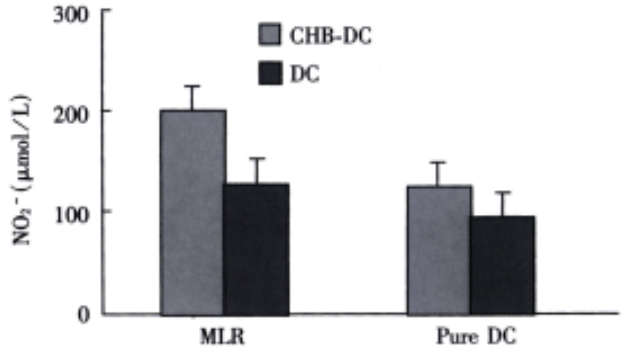

Figure 3 Increased NO production by DCs from HBV-infected patients. All the T cells (2×10^5/ well) were donated from a healthy volunteer in AMLR DC (1×10^4 well^{-1}) from patients with chronic hepatitis type B (CHB) and healthy donors were cultured for 5d. Pure population of DCs (1×10^9·L^{-1}) isolated by the cytokine enrichment method were cultured with SAC I strain 75mg·L^{-1} for 48h.

DISCUSSION

Though B and T lymphocytes are the main mediators of immunity, their function is under the control of DCs which is the sentinels of host immune system[21]. In this way, the property and its implications of DCs have become the focus of immunity against tumor and infectious diseases. Interestingly, the present culture techniques allowing the generation of large numbers of immunostimulatory DCs *in vitro* from human peripheral blood have made it possible to analyse the DC function in human diseases[7,10]. Though depletion and dysfunction of DCs were reported in some infectious diseases induced by HCV, HIV-1 and measles virus[6,20,22], dendritic cell function still remains uncertain in chronically HBV-infected patients. In particular, there appears to have few reports in the published literature about the effects of chronic HBV infection on DC function. These make us attempt to compare the stimulatory potential against allogenic CD4 T cells, levels of surface antigens, levels of interleukin-12, and degree of maturation of DCs from HBV-infected individuals with uninfected healthy donors in this study.

Flow cytometry showed that the expressions of cellular surface markers such as the co-stimulatory factors B7-1, B7-2 and CD1a and MHC II molecules HLA-DR on DCs significantly decreased in chronic HBV-infected patients compared to those in healthy donors. Furthermore, the T-cell stimulatory activities of DCs from patients were much lower than the DCs from uninfected healthy donors in AMLR.Simultaneously, there were notably low levels of cytokine IL-12 and high levels of NO produced by DCs from patients, in particular, NO was considered to have the suppressive activity on cellular enzymes and produce damage to normal cells. Taken together, our present study indicated that there is an immature phenotype and dysfunction of DC population in chronic HBV-infected patients in comparison with uninfected controls. It is necessary to study whether the dysfunction of DC population in patients is associated with the failure to mount an effective immune response for clearance of HBV.

Since IL-12 is an important cytokine to stimulate the proliferation of T lymphocytes, the decrease of IL-12 production was directly attributed to the low T-cell stimulatory of DCs from HBV-infected patients in AMLR[23]. The following reasons probably resulted in the low level of IL-12 production: ① IL-10 could inhibit the expression of class II and B7 molecules and suppress the IL-12 production of DCs[8]; ② there is a high level type I interferon in patients with HBV infection, which induces the down-regulation of IL-12 secretion of DCs[24]; ③ the previous study reported that the HBV itself and some cytokines induced by HBV infection may produce direct or indirect influences on transcriptions of IL-12 gene[25,26]; and ④ host genetic factors, such as the polymorphisms of both antigen-capturing receptor alleles and cytokine genes in DCs might explain HBV-infected patients mount dysfunction of DCs[27].

The maturation and efficient antigen-presentation of DCs are crucial for the initiation of immunity against viral infection[4]. The impairment of dendritic cell function suggested a role in the pathogenesis of chronic HBV infection, which has been probably attributed to the inability of the host to eradicate viruses[1]. It is unknown whether the HBV-infected DCs were eliminated by specific cytotoxic T lymphocytes (CTLs) if the DC is infected itself, which induced the dysfunction of DCs in these patients with chronic HBV infection or the HBV-infected DCs directly induce the T cell tolerance to HBV infection[27].

Decreased function of DC may allow the development of HBV infection, so that modulating the function of dendritic cells is considered to be beneficial to the production of

efficient immunity against viruses[28], but it must be made clear whether the dysfunction of DCs in patients is the result of direct injury of DCs from chronic HBV infection, or a protection from host itself to avoid the destruction of a large amount of HBV-infected hepatocytes induced by itself-inducing immune reaction, and or both In the early stage of HBV-infected patients, efficient antigen-presentation of DCs help the host to clear the virus[29], but in the late stage of chronic HBV-infected patients, induction of dysfunction of DCs or immune tolerance to HBV infection might also be helpful to host survival. Further studies are needed to clarify the mechanisms of depression of DCs function in HBV-infected patients[30,31].

The development of methods to generate a large number of DCs has facilitated their application for immunotherapy. Recent studies have demonstrated the safety and immunogenicity of DCs in humans and have begun to outline the durability, kinetics, and nature of the elicited T-cell responses. However, DC-based immunotherapy remains a challenge and several parameters need to be examined to optimize immune responses, in order to maximize the clinical efficacy against cancer and infectious diseases.

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• ORIGINAL RESEARCH •

Abnormal β-catenin gene expression with invasiveness of primary hepatocellular carcinoma in China

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Abstract

AIM **To study the abnormal expression of β-catenin gene and its relationship with invasiveness of primary hepatocellular carcinoma among Chinese people.**

METHODS **Thirty-four hepatocellular carcinoma (HCC) specimens and adjacent paracancerous tissues,** 4 **normal liver tissues were immunohistochemically stained to study subcellular distribution of β-catenin. Semiquantitive analysis of expression of β-catenin gene exon** 3 **mRNA was examined by RT-PCR and *in situ* hybridization. The relationship between expressions of both β-catenin protein, mRNA and clinicopathological characteristics of HCC was also analyzed.**

RESULTS **Immunohistochemistry showed that all normal liver tissues and para-cancerous tissues examined displayed membranous type staining for β-catenin protein, occasionally with weak expression in the cytoplasm. While** 21 **cases** (61.8**%**) **of HCC examined showed accumulated type in cytoplasms or nuclei. The accumuled type Labling Index** (**LI**) **of cancer tissue and para-cancarous tissue was** (59.9±26.3) **and** (18.3±9.7) **respectively** (***P***<0.01)**. Higher accumulated type LI was closely related with invasiveness of HCC. Results of RT-PCR showed the β-catenin gene exon** 3 **mRNA Expression Index** (**EI**) **of** 34 **HCCs was higher than that of para-cancerous tissue and normal liver tissue. Using *in situ* hybridization, the signal corresponding to β catenin gene exon** 3 **mRNA was particularly strong in cytoplasm of HCC when compared with those of para-cancerous and normal liver tissues. Over expression of β-catenin exon** 3 **was also found to be correlated with high metastatic potential of HCC.**

CONCLUSION **Abnormal expression of β-catenin gene may contribute importantly to the invasiveness of HCC among Chinese people.**

Subject headings hepatocellular carcinoma; wnt pathway; β-catenin gene; metastasis

Cui J, Zhou XD, Liu YK, Tang ZY, Zile MH. Abnormal β-catenin gene expression with invasiveness of primary hepatocellular carcinoma in China. *World J Gastroenterol*, 2001;7(4):542-546

INTRODUCTION

Hepatocellular carcinoma (HCC) is quite common in China. In recent years, great progresses have been made in the treatment of HCC, but the major problem is the high malignancy of HCC, that is, more than 50% of the patients receiving grossly radical treatment will suffer from recurrence within two years. So much effort has been put to investigate the molecular biological characteristics of HCC in order to lower the recurrence rate[1-20]. β-catenin is a ubiquitous intracellular protein which is important in both intercellular adhesion and Wingless/Wnt developmental signaling transduction pathway[21]. β-catenin plays an important role in the interactions between cadherins and other transmembrane receptor proteins, such as the epidermal growth factor receptor. In addition, it is also a signaling molecule and can activate gene transcription by forming a heterodimer with the T-cell factor/lymphoid enhancer-binding factor family of DNA binging proteins[22]. Previous studies have shown that β-catenin is involved in pathways that regulate cellular differentiation and proliferation. In the absence of growth or differentiation signals, cytoplasm β-catenin is rapidly turned over under the control of the APC protein and the GSK-3β, resulting in low level of cytoplasm β-catenin level in normal cells[23,24]. The presence of a wingless-Wnt signal in normal embryonic cells stabilizes β-catenin, which accumulates in the cytoplasm, where it binds to Tcf-lymphoid enhancer factor and triggers gene transcription. Abnormal expression and/or structural abnormalities of catenins are closely associated with tumor development for human esophageal, gastric and colon cancers[25,26]. Previous study has shown that E-cadherin expression was significantly lowed and is closely related with the metastatic potential of HCC[27], and abnormal β-catenin expression has been observed by immunohistochemistry in many malignant human tumors including HCC[28], so it is our logical thoughts whether abnormality of β-catenin gene existed and what its relationship with malignancy in HCC among Chinese people is because of the close relationship between E-cadherin and β-catenin.

MATERIALS AND METHODS

Tissue

Thirty-four HCC specimens and adjacent para-cancerous tissues, four normal liver tissues obtained from patients who underwent surgery in Liver Cancer Institute, Zhongshan Hospital, Shanghai Medical University were analyzed. The tissues were each cut into three parts: one was fixed in formalin, and then embedded in paraffin. Paraffin sections were stained with HE for histological examination of HCC and were also used for immunohistochemistry. One was immediately frozen by liquid nitrogen and stored at -80℃, which was to be used for DNA and RNA extraction. Genomic DNA was purified from all samples using standard proteinase K digestion and phenol/chloroform extraction. Total RNA was extracted using a Trizol reagent (Promega) according to

the protocol recommended by the manufacturer. And the last was rinsed in cold PBS, placed in OCT compound, and immediately frozen in liquid nitrogen, which was to be used for *in situ* hybridization.

Immunohistochemical staining

Immunohistochemical analysis was carried out with the avidin-biotin complex immunoperoxidase technique as described previously[29]. As the primary antibody, polyclonal human anti-β-catenin antibody (Sigma) was used at 500×dilution. As the secondary antibody, biotinylated anti-rabbit IgG (Dako) was used at 100× dilution. Staining was performed using avidin-biotin reagents, 3, 3'-diaminobenzidine, and hydrogen peroxide. As a negative control, duplicate sections were immunostained without exposure to the primary antibodies. All cases were divided into two groups according to immunostaining pattern. Cases with a membrancous staining pattern similar to that in normal hepatic cell were classified as membraneous or normal and cases with marked cytoplasmic and nuclear staining in addition to the membranous staining were defined as accumulated or abnormal. Cells from five randomized views were counted and the cell labeling index (LI) was arbitrarily defined as: (positive cells counted/all cells counted)×100.

RT-PCR

Primers for PCR were designed to amplify the consensus sequence for GSK-3 β phosphorylation in exon 3 of β-catenin gene, based on the published cDNA sequence of human β-catenin gene. To verify the validity of amplification, the primers were designed within the region of exon 3 of β-catenin gene, and the amplification was performed by direct PCR and RT-PCR respectively. Primers, F: AAAGCGGCTGTTA-GTCACTGG R: GACTTGGGAGGTATCCACATCC. PCR: PCR mixture, containing 100pM of primer A and B each, deoxyribonucleotide triphosphates at 200μmol·L^{-1} each, 1.5mmol·L^{-1} $MgCl_2$, 2U *Taq* polymerase (Promega) and 2μL DNA template was adjusted to 50μL by adding double distilled water. Then the mixture was overlaid with 50μL mineral oil and subjected to amplification for 40 cycles. Each cycle consisted of 95℃ for 60s, 55℃ for 45s, 72℃ for 45s. RT-PCR: Total RNAs were reverse-transcribed to obtain the cDNA that was going to be amplified. PCR was also performed under the above same condition except for adding 1μL cDNA to the PCR mixture. A 450bp fragment of β-actin mRNA was also amplified by RT-PCR as the internal control. The PCR products were identified first onto 20g·L^{-1} agrose gel and photographed. The photos of RT-PCR were scanned by optical density scanner (Shimadzu C-9000) and the gene expression index (EI) was arbitrarily defined as density Lum of β-catenin/density Lum of β-actin.

In situ hybridization

Cryostat sections (6μm) were obtained, dried for 2h at RT, and delipidated in chloroform for 5min. Sections were fixed in 40g·L^{-1} paraformaldehyde/PBS for 7min, rinsed in PBS for 3min, rinsed twice in 2× SSC for 5min, and prehybridized at 42℃ for 60min in 4× SSC/100g·L^{-1} dextran sulfate/1× Denhardt's solution/2mM EDTA/500g·L^{-1} deionized formamide/ 500mg·L^{-1} salmon sperm DNA. Hybridization was for 16h in 100μL of prehybridization solution and 20μg·L^{-1} digoxin labeled oligonucleotides (TGTTCC-CACTCATACAGGACTTGGGAGGTATCCACATCCTCTT CCTCAGGA). After hybridization, sections were rinsed twice in 2×SSC for 5min at 37℃, 3 times for 5min each in 60g·L^{-1} formamide and 0.2×SSC at 37℃ and twice for 5min each in 2×SSC at RT. Sections were then rinsed in 100 mol·L^{-1} Tris · HCl, pH 7.5/150 mol·L^{-1} NaCl for 5min, and treated with the same solution saturated with blocking mix for 30min, and then reacted with a 1:2000 dilution of alkaline phosphatase-conjugated sheep antidigoxigenin Fab fragments (750×10^3·L^{-1}) in the same solution. They were rinsed twice in 100mol·L^{-1} Tris · HCl, pH 7.5 and 150mol·L^{-1} NaCl for 5min each, then in 100 mol·L^{-1} Tris · HCl, pH 9.5/100 mol·L^{-1} NaCl/ 50mol·L^{-1} $MgCl_2$ for 10 min, and then reacted with 0.18g·L^{-1} 5-bromo-4-chloro-3-indolyl phosphate, 0.34g·L^{-1} nitroblue tetrazolium, and 240mg·L^{-1} levamisole (Sigma) in the same solution for 6h in the dark at RT. The reaction was stopped with 10mol·L^{-1} Tris · HCl (pH 8.0) and 1mol·L^{-1} EDTA. Sections were counterstained in nuclear methyl green, mounted with aqueous solution, and the final results of average density area and density lum of 500 signal positive cells were analyzed by a multifunctional true digital system (MTDS) using a computer. Albumin oligonucleotide probe and hybridization solution without probe were used as positive and negative control respectively.

RESULT

Immunohistochemical analysis

Immunostaining with polyclonal antibody was performed to evaluate the significance of β-catenin accumulation in HCC. Immunohistochemistry showed that all normal liver tissues and para-cancerous tissues examined showed membranous type, occasionally with weak expression of β-catenin in the cytoplasm, but no β-catenin accumulation in nuclei was found. While for HCC, 21 cases (61.8%) showed accumulated type (Figure 1). The LI of accumulated type for tumor tissue and paracancerous tissue were 59.9±26.3 and 18.3±9.7 respectively (P<0.01), while the LI of membraneous type for tumor tissue and paracancerous tissue were 24.6±8.5 and 91.8±10.6 respectively (P<0.01, Table 1). When LI of accumulated type was analyzed according to the clinicopathological characteristics of HCC, close relationship could be seen with capsule, portal vein tumor thrombus, pathological grade, intrahepatic metastasis (Table 2) and postoperative recurrence (Figure 2).

β-catenin exon 3 mRNA expression

Since the primers were designed in such a way that the product was within β-catenin gene exon 3, direct PCR and RT-PCR were used separately to verify the amplification. Agrose gel electrophoresis showed that PCR and RT-PCR amplification products were both 132bp, which were the same as those of normal liver tissues, para-cancerous tissues and HCC tissues. None of amplification products showed fragment that was shorter. RT-PCR results showed the β-catenin exon 3 mRNA EI were (0.77±0.16) and (0.50±0.05) for HCC tissues and para-cancerous tissues respectively (P<0.05, Figure 3). In HCC, higher EI of β-catenin mRNA attempted to be seen in cancer with incomplete capsule, intrahepatic metastasis and portal vein thrombus (Table 2). Using *in situ* hybridization, we also found the signal corresponding to β-catenin exon 3 mRNA was particularly strong in cytoplasm of HCC when compared with those of para-cancerous tissues and normal liver tissues (Figure 4) and stronger signal of β-catenin mRNA was also closely related to incomplete capsule, intrahepatic metastasis and portal vein thrombus.

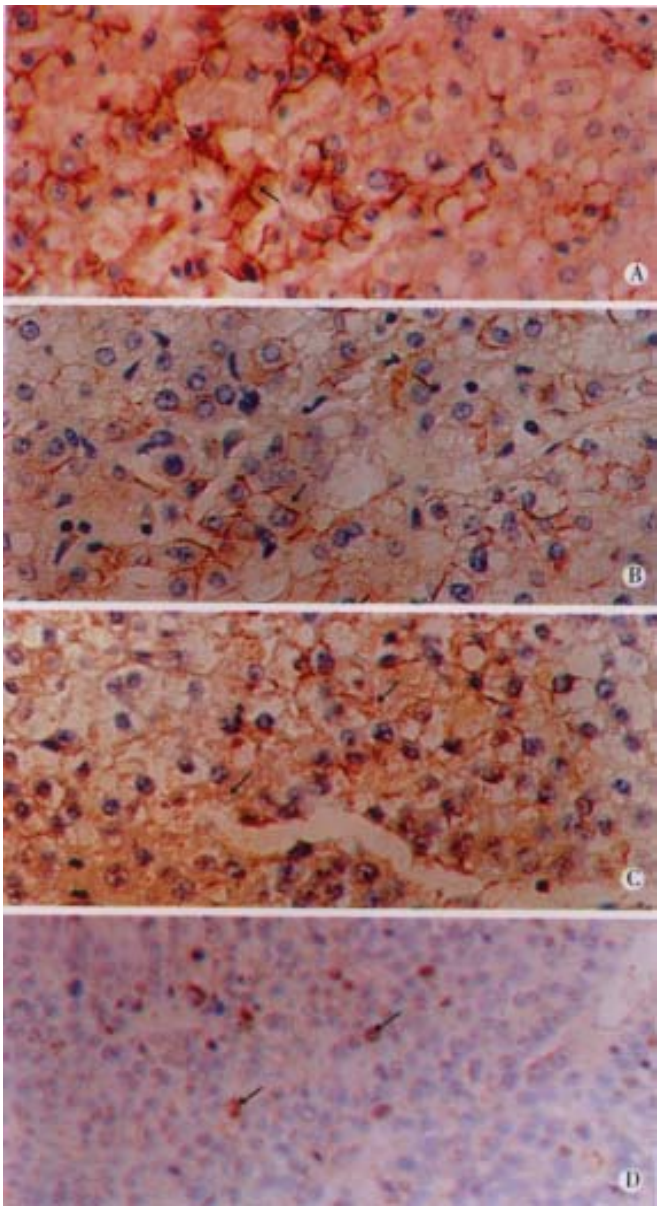

Figure 1 Immunohistochemistry of β-catenin.
A: In normal liver tissue, the staining was mainly positive on the cellular membrane (arrowpoint), with very weak cytoplasmic staining. ×200 B: Para-cancerous cirrhotic liver tissue showed membrane staining (arrowpoint) like normal liver tissue. C,D: For HCC, cytoplasmic and nuclear staining was dominant (arrowpoints), whereas membrane staining was rare. ×200

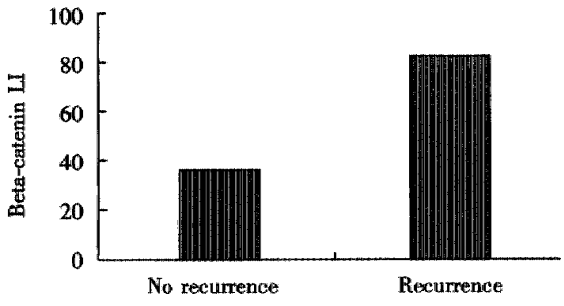

Figure 2 Labeling index (LI) of β-catenin.
Recurrent patient (n = 15) was much higher than that of non-recurrent patient (n=19) (84.9±17.4) *vs* (39.1±14.3).

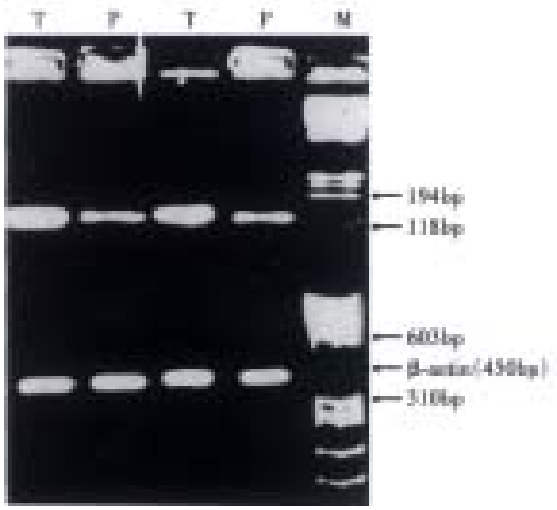

Figure 3 β-catenin mRNA expression index (EI).
HCC was higher *vs* para-cancerous tissue (P<0.05). P: para-cancerous tissue; T: HCC; M: nucleic acid molecular mass marker.

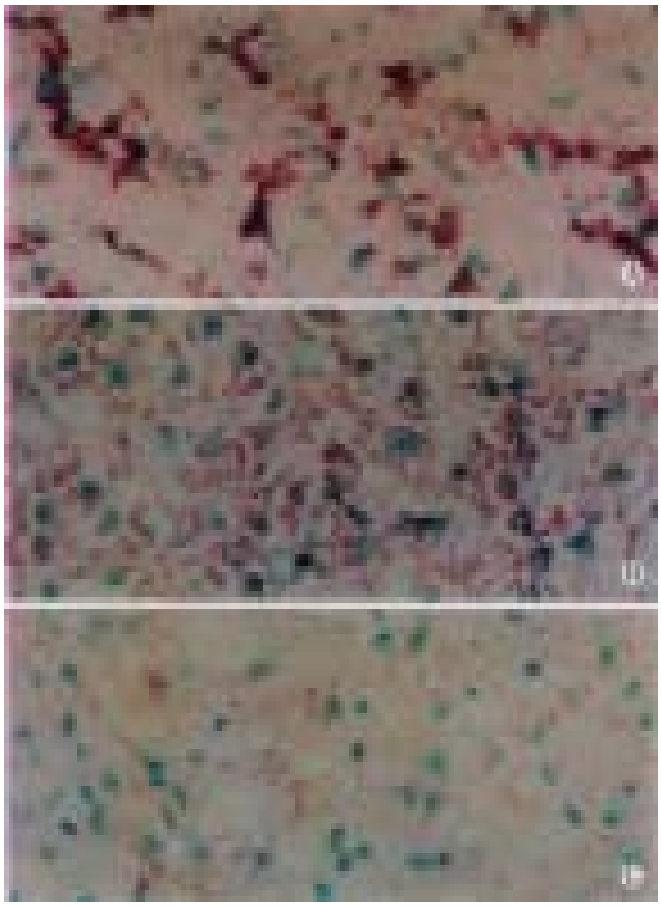

Figure 4 *In situ* hybridization of β-catenin gene mRNA. Stronger in HCC (A) *vs* para-cancerous cirrhotic liver tissue and (B) normal liver tissue (C).

Table 1 Labeling index for β-catenin accumulated type and membraneous type in HCC and para-cancerous tissues (n=34, $\bar{x}$±s)

Tissue	Membraneous	Accumulated
HCC	59.9±26.3	24.6±8.5
Para-cancerous tissue	18.3±9.7[b]	91.8±10.6[b]

[b]P<0.01 *vs* HCC.

Table 2 Relationship between labeling index of β-catenin accumulated type, expression index of β-catenin mRNA and clinicopathological characteristics of HCC

	n	LI of β-catenin accumulated type	EI of β-catenin mRNA
Male	31	58.4±14.2	0.8±0.2
Female	3	54.1±15.3	0.9±0.1
AFP ≤20ng/mL	9	49.3±17.2	0.8±0.1
AFP>20ng/mL	25	54.3±13.7	0.8±0.1
Tumor size			
≤5cm	15	58.7±20.4	0.8±0.2
5cm~10cm	7	54.4±21.3	0.8±0.2
>10cm	12	55.9±17.9	0.8±0.1
Capsule			
Complete	15	72.2±23.4	0.7±0.1
Incomplete	19	44.4±21.1[b]	0.9±0.1[a]
Intrahepatic Metastasis Yes	14	77.2±25.5	0.9±0.2
Intrahepatic Metastasis No	20	41.3±19.6[b]	0.7±0.1[a]
Portal vein thrombus Yes	19	79.8±14.9	0.9±0.2
Portal vein thrombus No	15	52.8±25.9[a]	0.6±0.2[a]
Edmondson's Grade II	19	39.7±20.0	0.7±0.4
Edmondson's Grade III	15	75.9±18.7[b]	0.8±0.2
Cirrhotic nodule ≤ 0.5cm	23	54.3±12.5	0.8±0.2
Cirrhotic nodule >0.5cm	11	62.2±16.6	0.8±0.1

[a]$P<0.05$, [b]$P<0.01$.

DISCUSSION

Previous studies have shown that activation of the wnt pathway results in up-regulation of cytoplasmic β-catenin and its translocation to the nucleus, presumably via the binding of β-catenin to T-cell factor/lymphoid-enhancing factor family members[25,26,30]. Thus, as a first assessment, we examined the subcellular localization of β-catenin in 34 HCC specimens with the result that 61.8% of HCC specimens showed to be accumulated type, suggesting cytoplasmic stabilization of the protein. This showed that activation of Wnt pathway maybe of importance in the carcinogenesis of HCC among Chinese people. Although either β-catenin mutations involving the GSK-3 β phophorylation sites or inactivation of APC and some other factors are related to activation of the Wnt pathway in colon cancer and melanomas[31,32], loss of heterozygosity at the APC locus on chromosome 5 has been detected only at low frequency in human HCC, indicating that inactivation of APC may be infrequent[33]. So mutation of exon 3 of β-catenin gene is probably one of the most important factors activating Wnt pathway and thus causing β-catenin protein accumulated in the cytoplasms in HCC.

Although some studies have been made to investigate β-catenin mutation and abnormal Wnt pathway in HCC[34-41], no previous results have been reported concerning about the relationship between expression abnormality of β-catenin and clinocopathological features of HCC. Furthermore, research reports about the relationship between β-catenin abnormal expression and clinicopathological features of tumors such as colon cancer[42,43], melanoma[44,45], breast carcinoma[46,47], gastric carcinoma[48,49], and lung carcinoma[50,51] are rather various and some of the results were even totally contradictory. That is partly due to most of the previous immunohistochemical studies on β-catenin did not differentiate between membrane-associated type and intracellular accumulated type. Most tumors showed reduced β-catenin in the cytoskeletal fraction but increased β-catenin in the cytosolic fraction and truncated β-catenin protein which was encoded by mutational β-catenin gene was found bound weakly to β-catenin monoclonal antibody when compared with non-truncated β-catenin[52]. This is the reason why we chose polyclonal antibody instead of monoclonal antibody in our study. In this study we aimed to determine which type of expression abnormalities for β-catenin correlate with clinicopathological features and postoperative recurrence in HCC. Our results demonstrated that although great difference existed between cancer tissue and non-cancer tissue, we failed to show the LI of membraneous type to be correlated with the invasiveness of HCC (data not shown here). But, the LI of accumulated type was discovered closely related with the invasive characteristics of HCC, higher EI would predict high ability of invasiveness of HCC and thus a worse prognosis. This was different from another article about gastric carcinoma, which showed that membraneous type, instead of accumulated type, was related to the invasiveness and prognosis of the tumor[47].

Since abnormal expression of β-catenin protein can be caused by both β-catenin gene mutation and over expression, and in some HCCs, both strong membraneous type and accumulated type of staining could be observed, it is our logical thoughts to figure out whether over expression of β-catenin gene existed and what its relationship with the invasiveness of HCC was. This article is the first one to study the β-catenin gene expression in HCC at mRNA level. First we used RT-PCR to examine the expression of β-catenin gene exon 3 mRNA. Since RT-PCR was not very accurate in semi-quantitive analysis of gene expression, we chose *in situ* hybridization to reconfirm the results of RT-PCR. The results of them are the same, that is over expression did exist in HCC and it showed relationship with the invasiveness of HCC (data of relationship between *in situ* hybridization and HCC clinicopathological characteristics not shown). This could give some explanation why strong membranous and cytoplasmic distribution of β-catenin was observed on immunohistochemistry in some HCC while β-catenin gene exon 3 mutation was not observed. It was the accumulation of β-catenin, though apparently normal, that exceeded the capacity of E-cadherin combination and GSK-3 βdegradation, resulting in increase and stabilization of this protein in the cytoplasm.

Although we found that LI of β-catenin accumulated type was related with HCC recurrence, we were unable to find there was such relationship between β-catenin gene EI and HCC recurrence, either by RT-PCR or *in situ* hybridization. This implies that the LI of β-catenin accumulated type would be of greater value in predicting recurrence of HCC. From above we can see that abnormal expression of β-catenin protein, especially the accumulated type, which is closely related to the invasiveness of HCC among Chinese people. Further study should be carried out to confirm this and investigate what the other mechanism causing abnormal expression of β-catenin gene is.

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• ORIGINAL RESEARCH •

A study on pathogenicity of hepatitis G virus

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Abstract

AIM **To study the pathogenicity of hepatitis G virus (HGV) and observe the genesis and pathological process of hepatitis G.**

METHODS **HGV-RNA in serum was detected by RT-PCR assay. The immunohistochemical assays of liver tissue were performed with HGV monocoloned antibody (McAb) expressed from the region of HGV NS5 nucleic acid sequence. The clinical and pathological data of 52 patients with hepatitis G were discussed. In animal experiment, the Chinese Rhesus monkeys were infected with the serum of a patient with HGV infection. And the dynamic changes in serology and liver histology of animals were observed.**

RESULTS **One hundred and fifty-four patients with HGV-RNA positive were selected from 1552 patients with various kinds of hepatitis. Of 154 patients with HGV infection, 52 were infected with HGV only, which accounted for 33.8% (52/154) and 102 with positive HGV-RNA were super-infected with other hepatitis viruses, which accounted for 66.2% (102/154). The clinical and pathological observation showed that the acute and chronic hepatitis could be induced by HGV. The slight abnormality of transaminases ALT and AST in serum of monkeys lasted nearly 12 months and histological results showed a series of pathological changes.**

CONCLUSION **HGV is a hepatotropic virus and has pathogenicty.**

Subject headings hepatitis virus G; pathogenicity

Xu JZ, Yang ZG, Le MZ, Wang MR, He CL, Sui YH. A study on patogenicity of hepatitis G virus. *World J Gastroenterol*, 2001;7 (4):547-550

INTRODUCTION

Hepatitis G virus (HGV/GBV) is a new type of hepatitis virus which was first identified by Simons and Linnen between 1995 and 1996[1,2]. It has been shown that HGV is a single-stranded, positive chain DNA virus which has world-wide distribution, and spread by blood circulation. HGV infection is mostly mixed with infections caused by other types of hepatitis viruses. The patients with single hepatitis G infection were rare. It makes the investigation difficult. Currently, the pathogenicity of hepatitis G virus is controversial. We report the results of our study below.

MATERIALS AND METHODS

Patients

One hundred and fifty-four patients with hepatitis G infection were collected from 1552 patients with different types of hepatitis admitted consecutively to our hospital during recent 4 years. The serum level of alanine trasaminase (ALT) in all investigated patients was twice that of the normal subjects. Fifty-two of these cases were simply HGV infected and 102 were super-infected with other types of hepatitis virus. The liver biopsies were performed in 42 of the 52 patients with single HGV infection.

Etiological assays for serum specimens

Serum specimens were tested for HGV-RNA by retro-polymerase chain reaction (RT-PCR), for HBV-M, anti-HAV IgM, anti-HEV IgG, anti-HCV IgG by enzyme-linked immunosorbent assay (ELISA), for HBV-DNA by PCR, and for HCV-RNA by RT-PCR. Reagents were obtained from Shanghai Meihua Company, DA-AN Genotype-Diagnostic Center of Zhongshan Medical University, Medical Institute of Nanjing Military Area, respectively. The primers of HGV were obtained from the Institute of Microbiology and Epidemiology of Chinese Military Academy of Medical Sciences.

Histological and immunohistochemical assays

The biopsied liver tissues from the patients were sent to 3 pathologists for histological examinations under light and electron microscopy to determine the degree of necrosis of the liver cells, inflammatory cellular infiltration and fibroplastic proliferation. As for immunohistological examinations, monoclonal antibodies (McAb) were obtained from HGV NS5 gene antigen, the labeled antigen and antibody were presented by the Institute of Microbiology and Epidemiology of Chinese Military Academy of Medical Sciences. The histochemical reagents with HCVAg (NS3) and HBsAg were obtained from Beijing Zhongshan Biotechnical Limited Company and Fuzhou Maixin Biotechnical Company Limited, respectively. The technical procedures were followed according to the requirements for use.

RESULTS

The relationship between HG and the other different types of viral hepatitis

One hundred and fifty-four cases of HG in our study were collected from 1552 cases of different types of viral hepatitis

and evidence of an HGV infection were confirmed by positive HGV-RNA twice in all 154 cases. The relationship between them is shown Table 1.

There were 52 patients with the simple HG infection (33.8%), and 103 of HG super-infected with other type of HV. Most of the cases were complicated with HCV and/or HBV infection (66.2%).

The clinical and pathological features of HGV infection

Of 52 cases of HG, the biopsies were performed in 42. The clinical and/or pathological diagnosis of 154 cases is shown in Table 2.

Table 1 The relationship between HG and the other viral hepatitis

Group	No. of cases	No. of positive HGV-RNA	%
HNA-E	583	52	8.9
HA	38	2	5.3
HB	713	69	9.1
HC	125	18	14.4
HB+HC	17	5	29.4
HA+HB	22	2	13.6
HB+HE	20	1	5.0
The others *	7	0	0
Total	1552	154	9.9

*The others include: HA+HC 2, HA+HE 1, HC+HE 3 and HA+HB+HE 1.

Table 2 The clinical and/or pathological diagnosis of 154 cases

Group	No.of cases	Acute hepatitis	Chronic hepatitis	Severe	Cirrhosis hepatitis
Sigle HG	52	2	49		1
Super-infected HG	102	80	4		18

It is difficult to identify what damages of the liver are predominant clinically and pathologically in the patients with HG super-infected with other HV infection. We had intensively observed the clinical manifestations and pathological lesions in single-infected cases of HG so as to better clarify the pathogenicity of HGV. Of the 52 cases, only 2 cases were acute hepatitis (confirmed by clinical and pathological evidence). Most of single-infected patients with HG had chronic hepatitis.

Case 1 A 46-year-old female with a history of surgery in 1988 was hospitalized with endometriosis. During the operation, blood transfusion was taken. Fifty days after transfusion, she felt weak and had a poor appetite, abnormal liver functions with ALT 180U/L. After treatment with biphenyl dimethyl dicaboxylate, the ALT level was lowered. But her liver function was continued to be abnormal (ALT: 66-120, AST: 57-108U/L) for 9 years. Serum examinations of pathogens performed in many large hospitals were negative. She was admitted to our hospital on Sept. 27, 1997. Laboratory examinations showed ALT 106U/L, AST-192U/L, normal level of TSB, A/G 1.17, and r- GP 22%. Serum tests for HA -E were negative. The test for HGV-RNA was positive. Liver biopsy was taken on October 18. Histological examinations showed hepatocyte swelling, acidophilic degeneration, piecemeal necrosis, obvious fibroplastic proliferation in the portal area and P-P bridging hepatic necrosis. The pathologic diagnosis showed chronic hepatitis: G 2-3, S 1-2. CT-displayed splenomegaly and occupied lesions in the liver. The patient was treated with α-Interferon at a dose of 3 million IU three times per week, for 6 months. Thirty days later, HGV-RNA became negative. Two months after cessation of the treatment, the HGV-RNA again became positive. The ALT continued to be abnormal. The patient had faint jaundice and symptoms of liver cirrhosis (Figure 1).

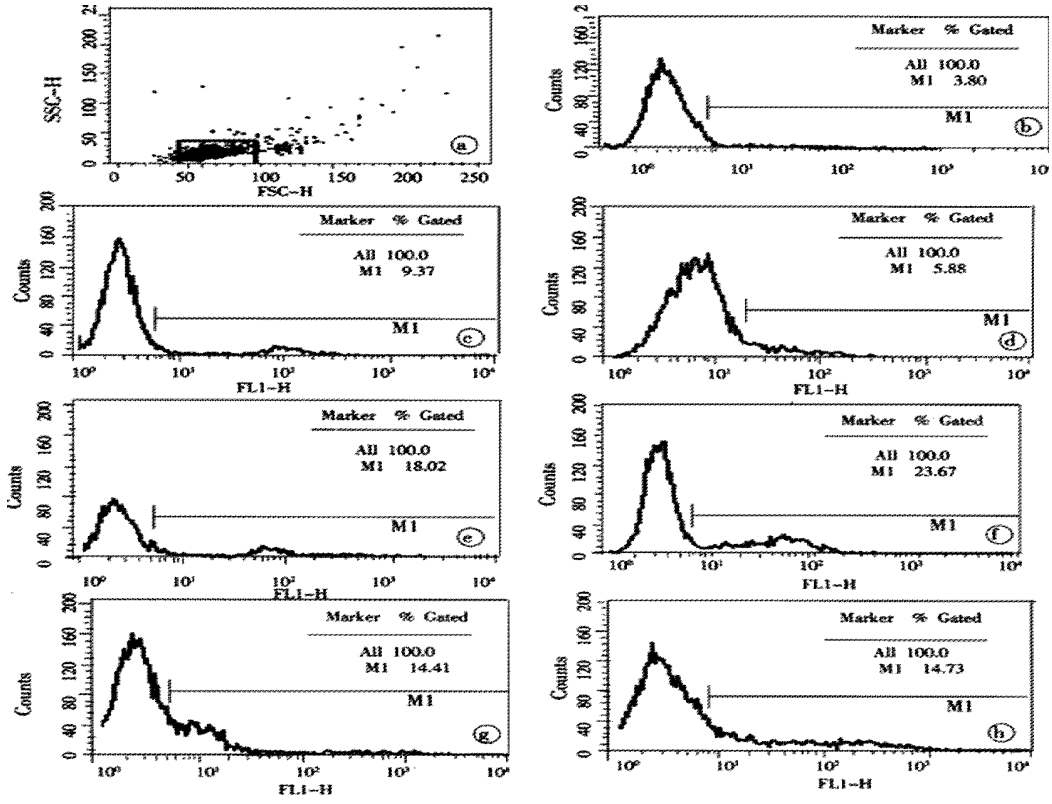

Figure 1 Dynamic changes of serum ALT, AST and HGV-RNA in patient Hu.

Case 2 An 8-year-old girl was admitted on July 9,1998 because of abdominal distension. Splenomegaly and a small amount of ascites were displayed by B super-sound examination. Repeated examinations showed positive HGV-RNA, ALT 60U, AST 120U/L, A/G 0.98, and r-GP 28%. CT showed uneven liver density with small nodular lesions, splenomegaly extended in an area of 8 costae. The clinical diagnosis was chronic hepatitis G infected from liver cirrhosis (Figure 2).

In our group, there were 2 patients with acute hepatitis G having following histological findings: cloudy hepatocyte swelling, partial vacuolation (Figure 3), punctate necrosis of liver cells, focal lymphocyte infiltration, and accidophilic degeneration of partial liver cells. In patients with chronic hepatitis G, the histological examinations of the liver tissue showed extended portal area, a moderate degree of lymphocyte infiltration, and piecemeal necrosis (Figure 4). A tendency of P-P bridge necrosis in the portal area could be found. In a case of acute hepatitits G, the electron micrographs showed shrinkage of liver cells with zigzag edges, extension of rough surfaced endoplasmic reticulum in hepatocytes, and proliferation of collagen fibrils extended into the cytoplasm in the damaged hepatocytes (Figure 5). In immunohistochemical assays, brown-yellow granules were found in the cytoplasm of hepatocytes stained by specific HGV McAb in either acute hepatitis G or chronic hepatitis G patients. The positive-stained granules were also seen in the nuclei of a few hepatocytes (Figure 6). Negative results were obtained by histochemical analysis with anti-HBs McAb and anti-HCV (McAb) expressed from HCV N3. All these results suggested that HGV could cause a series of histological damages of the liver.

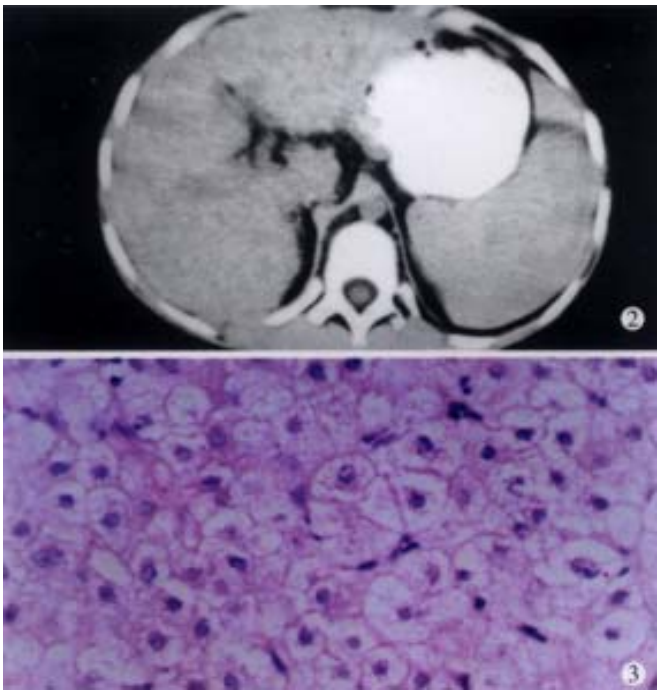

Figure 2 CT picture displays uneven liver density, small nodular changes and splenomegaly extended in an area of eight costae. EM× 15000

Figure 3 Histological changes of the liver showing cloudy swelling of hepatocytes.

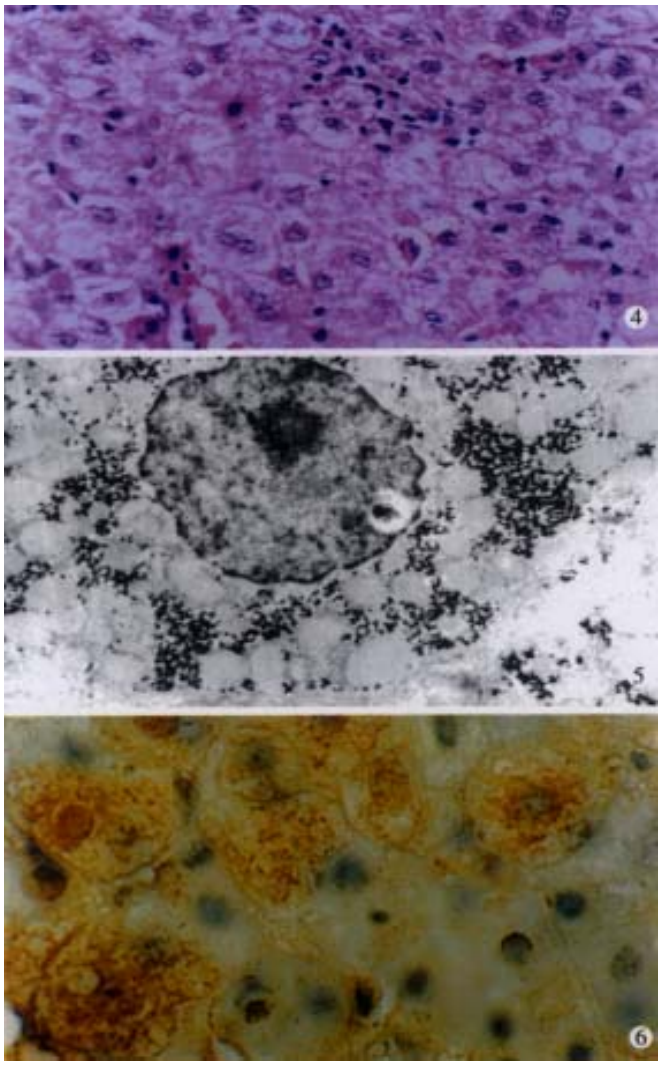

Figure 4 Histological changes of the liver in a patient with acute single-infected hepatitis G showing punctate necrosis, focal lymphocyte infiltration, and acidophilic degeneration of hepatocytes.

Figure 5 Ultrastructure of the liver tissues in a patient with acute simple HGV infection showing shirinkage of liver cells with zigzag edges, extension of rough surfaced endoplastic reticulum of hepatocytes, proliferation of collagen fibrils extended into the cytoplasm of hepatocytes. EM×15000

Figure 6 Immunohistochemical preperation stained by specific HGV McAb for HGVNS5 in a patient with single HG infection showing brown-yellow granules presented mostly in cytoplasm of hepatocytes, and partially in the nuclei. DAB staining, hematoxylin staining, BA staining. (oil)×1000

DISCUSSION

In recent years, the pathogenicity of hepatitis G virus has caused much controversies, because of the following aspects. ① HGV infection is usually mixed with the infections caused by other hepatitis viruses. The patient with single hepatitis G infection are rare; ② A body of evidence has been limited to serologic studies without sufficient histological evidence; ③ Lack of animal modes for the study of hepatitis G. A study on hepatitis G in our hospital was started earliest in China. In 1994, at "World Chinese Symposium on Hepatopathy" our report on clinical and pathological study in chronic hepatitis produced by "non-A, non-B and non-C agents" aroused great interests[3]. In 1996, we reported the paper "Clone of partial

HGV genes in Nanjing, China and analysis of its cDNA sequence", and established the method for determination of HGV-RNA by RT-PCR. The clinical and pathological characteristics of viral hepatitis G were described[4,5]. Of the 154 cases of HG collected from 1552 cases of different kinds of hepatitis in this paper, 52 were single HGV infection and 102 were super-infected with other hepatitis viruses, which accounted for 33.8% (52/154) and 66.2% (102/154) respectively. The clinical and pathological data from the simple HG patients in this study showed that: ① the clinical symptoms of HG patients vary in degree. It was mostly- sub-clinical; ② majority of patients had no jaundice, less hepatic damage than HB and a mild to moderate elevated ALT and AST level; ③ most of the patients were chronic. Of 52 patients with HG, there were 49 chronic cases, 1 cirrhosis and only 2 acute cases in our study. One of the two patients with acute HG had jaundice (TSB 106.7μmol/L) 30 days after blood transfusion, with the ALT level 535U/L and AST 116U/L; ④ histological changes were local necrosis and piecemeal necrosis in most patients, P-P bridge necrosis and a tendency of cirrhosis in a few patients. Electron micrographs of the two acute patients displayed proliferation of collagen fibrils extended into the cytoplasm of hepatocytes; ⑤ HGV antigen was generally present in cytoplasm and sometimes in the nuclei of the hepatocytes by immuohistochemical assay. We studied a Chinese Rhesus monkey intravenously attacked with the serum from a chronic hepatitis G patient only with positive HGV-RNA for one year. The dynamic changes of the serology and histology of the liver in the animal were observed before and after infection. It was found that the serological and histological changes of the animal were similar to that of the patients in our study[6]. Eighteen months after the infection, the monkey was dissected and its internal organs were taken for histological examination. The internal organs were normal except the liver tissue in which there were focal necrosis and slight piecemeal necrosis. The pathological diagnosis was confirmed to be chronic hepatitis. It is suggested that HGV would be a hepatotropic virus and had the pathogenicity to human.

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• ORIGINAL RESEARCH •

Differential expression of a novel colorectal cancer differentiation-related gene in colorectal cancer

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Abstract

AIM **To investigate SBA2 expression in CRC cell lines and surgical specimens of CRC and autologous healthy mucosa.**

METHODS **Reverse transcription-polymerase chain reaction (RT-PCR) was used for relative quantification of SBA2 mRNA levels in 4 human CRC cell lines with different grades of differentiation and 30 clinical samples. Normalization of the results was achieved by simultaneous amplification of** β-actin as an internal control.

RESULTS **In the exponential range of amplification, fairly good linearity demonstrated identical amplification efficiency for SBA2 and β-actin (82%). Markedly lower levels of SBA2 mRNA were detectable in tumors, as compared with the coupled normal counterparts ($P<0.01$). SBA2 expression was significantly ($0.01<P<0.05$) correlated with the grade of differentiation in CRC, with relatively higher levels in well-differentiated samples and lower in poorly-differentiated cases. Of the 9 cases with lymph nodes affected, 78% (7/9) had reduced SBA2 mRNA expression in contrast to 24% (5/21) in non-metastasis samples ($0.01<P<0.05$).**

CONCLUSION **SBA2 gene might be a promising novel biomarker of cell differentiation in colorectal cancer and its biological features need further studies.**

Subject headings colorectal neoplasms/genetics; colorectal neoplasms/pathology; DNA, complementary; cell differentiation; gene expression; polymerase chain reaction

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INTRODUCTION

Colorectal cancer (CRC) is increasing in China[1-10], and the treatment is still difficult in advanced stage[11-20]. Butyrate is an important colonic fuel and induces differentiation in colonic cell lines[21]. cDNA for a novel CRC differentiation-related gene, designated SBA2 (GenBank accession No: AF229181), has been identified in human CRC cell line CloneA after modulation by sodium butyrate. We investigated the use of reverse transcription-polymerase chain reaction (RT-PCR) for the relative quantification of SBA2 expression in 4 human CRC cell lines with different grades of differentiation and in 30 surgical specimens of CRC and autologous healthy mucosa.

MATERIALS AND METHODS

Materials

The poorly-differentiated human CRC cell line CloneA, moderately-differentiated human CRC cell line CX1, and well-differentiated human CRC cell line CCL187 were obtained from Dana-Farber Cancer Institute, Harvard Medical School. The moderately-differentiated human CRC cell line LS174T was bought from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. These cells were cultured in Dulbecco's modified Eagle's medium containing $50mL \cdot L^{-1}$ calf serum at 37℃ with 100% humidity and $50mL \cdot L^{-1}$ CO_2. Tumor tissues were obtained from 30 patients (15 men and 15 women) at the time of surgery for removal of CRC. All cases were assessed by histopathology. Both tumor tissues and autologous healthy mucosa (at the distance >5cm from the neoplastic focus) were sampled. All specimens were snap frozen and stored in liquid nitrogen. Total cellular RNA was isolated with the TRIzol reagents (Gibco-BRL). The yield and quality of RNA preparation were determined by spectrophotometry.

Quantification of gene expression by semi-quantitative RT-PCR

Reverse transcription of 2μg of total RNA using 0.5μg oligo (dT)-15 primer was performed for 1h at 42℃ in 20μL of a reaction mixture containing 15U AMV reverse transcriptase (Promega), 5 mmol/L $MgCl_2$, 1 × RT buffer (10 mmol/L Tris-HCl, pH 9.0 at 25℃, 50 mmol/L KCl, 1 g/L Triton X 100), 1mmol/L dNTP mixture and 25U of recombinant RNasin ribonuclease inhibitor. The samples were then heated at 99℃ for 5 min to terminate the reverse transcription.

Primers used for amplification of β-actin specific sequence were residues 2217-2238 (TGTATGCCTCTG-GTCGTACCAC; sense-strand) and residues 2009-2030 (ACAGAGTACTTGCGCTCAGGAG; antisense-strand). PCR using these primers yields a 592 bp product. SBA2-specific sequence was amplified by the sense-strand primer (residues 51 - 72: GCTTGTCACGGCTTCTTACGAT) and the antisense-strand primer (residues 390 - 411: GCATAAGTGCTTCAGTGAGGAC), which yield a 361bp product.

Unless otherwise specified, 2.5μL of the reversely-

transcribed mixture was used as template DNA and amplified in a reaction volume of 25 μL containing 1 mmol/L $MgCl_2$, 1 ×RT buffer (10mmol/L Tris-HCl, pH 9.0 at 25℃, 50mmol/L KCl,1g/L Triton X100), 0.2μmo/L each of 5' and 3'primers for β-actin, 0.5μmol/L each of 5' and 3' primers for SBA2. After heat denaturation at 95℃ for 5min, 1.25U of thermus aquaticus DNA polymerase (Promega) was added to the mixture. Amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus) in sequential cycles at 94℃ 30s; 68℃ 45s and 72℃ 1 min. After the last cycle, all samples were incubated for an additional 10 min at 72℃. Ten μL of PCR product were separated on 15g/L agarose gels, stained with ethidium bromide, and loaded onto 80g/L polyacrylamide gels, stained with DNA silver staining system (Promega). The gel was analyzed with the Electrophoresis Documentation and Analysis System 120 (Kodak 1D).

RESULTS

Linearity of PCR amplification

The yield of PCR product is proportional to the starting amount of the template only under conditions in which PCR amplification proceeds exponentially at a constant efficiency. To establish the optimal conditions for detection and quantification of SBA2 expression, the relative yield of PCR products was determined by terminating aliquots of starting reaction solution at sequential PCR cycles as shown in Figure 1. The resultant amplified sequences were analyzed and the signal intensities of the bands were plotted on a semilogarithmic scale against the cycle number to obtain amplification curves. Figure 1 shows that at higher cycle numbers (>28), the yield of the SBA2-specific product approaches the plateau while β-actin shows a tendency of saturation beyond 26 cycles. Before saturation at the plateau, however, good linearity is observed for both SBA2 and β-actin amplification over the range of cycles examined. Figure 2 compares the results obtained with successive dilutions of cDNA. The serial 1:2 dilutions were performed beginning with 5μL of reversely-transcribed mixture, as shown in the graph, and amplified 24 cycles. Thus the number of 24 cycles and the volume of 2.5μL of cDNA were chosen as the optimal parameters for the semi-quantitative RT-PCR.

If the efficiency of amplification is the same for each cycle (as indicated by the good linearity of the curves), the amount of cDNA produced can be predicted from the equation $cDNAn = (cDNA_0) \times (1+R)^n$, where cDNAn stands for the amount of product after n cycles, $cDNA_0$ is the initial amount of cDNA, and R is the efficiency of amplification. The efficiency of the amplification can be assessed from the following equation: $\log cDNAn = \log cDNA_0 + n \times \log (1+R)$. Within the exponential range, the linear regress equation for SBA2 is $y = 0.2612 \times x - 0.6179$ ($r = 0.9496$), for β-actin: $y = 0.2594 \times x - 0.4468$ ($r = 0.9754$), where y is the logarithm of cDNAn, x is the number of PCR cycles (n), r is correlation coeficient. The slope of the curve should be $\log (1+R)$ in the semi-logarithmic plot. Thus an R- value of 82% and 82% can be determined for SBA2 and β-actin respectively. Since the same amplification efficiency is observed for both the target and the internal control within the exponential range, the relative amounts of SBA2 mRNA can be determined by comparison with β-actin.

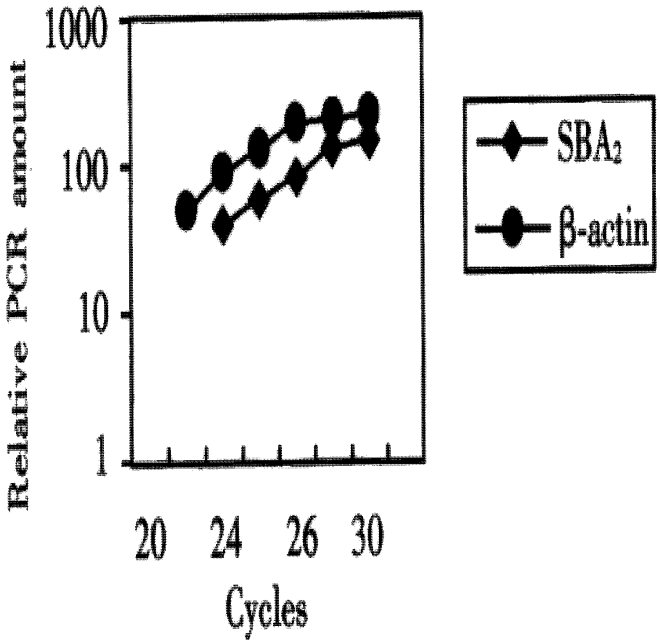

Figure 1 Detection of the exponential range by termination at sequential PCR cycles.
1-7: Cycles of 20, 22, 24, 25, 26, 28 and 30, respectively. M: Marker (100 bp DNA ladder).

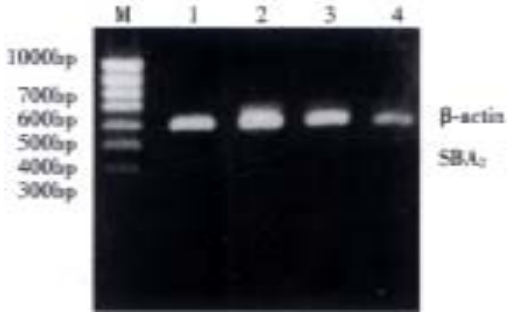

Figure 2 Detection of the exponential range by serial dilutions of cDNA.
1-4: cDNA of volume of 5, 2.5, 1.25, and 0.625μL, respectively. M: Marker (100 bp DNA ladder).

Patterns of SBA2 expression

In cultured cell lines and clinical specimens of CRC, the assay described above was used to investigate the levels of SBA2 mRNA in 4 human CRC cell lines and in 30 surgical samples of CRC and autologous healthy colonic mucosa. In the 30 samples analyzed, significantly lower levels of SBA2 mRNA were detectable in tumors, as compared with the coupled normal counterparts (Wilcoxon test, $T = 6$, $P<0.01$).

Differential expression of SBA2 in clinical samples of CRC with different grades of differentiation was similar to that observed in 4 cell lines of CRC, as shown in Figures 3 and 4, respectively. Thirty clinical samples were assigned into 3 groups, depending on the grade of differentiation. The levels of SBA2 mRNA were significantly (q test, $0.01<P<0.05$) correlated with the degree of differentiation in CRC, with relatively higher levels in well-differentiated samples and lower in poorly-differentiated ones. Mean levels of SBA2 mRNA was calculated for each group. There was a significant difference between well-differentiated group (0.384 ± 0.024) and poorly-differentiated group (0.158 ± 0.014) ($q = 3.9564$, $0.01<P<0.05$), between moderately-differentiated (0.297

±0.015) and poorly-differentiated ($q = 3.2767$, $0.01<P<0.05$), in spite of no significant difference ($q = 0.0798$, $P>0.05$) between well-differentiated and moderately-differentiated.

We also analyzed the relationship between SBA2 expression and lymph nodes metastasis. Of the 9 patients with lymph nodes affected, 78% (7/9) had reduced SBA2 mRNA expression in contrast to 24% (5/21) in non-metastasis samples ($\chi^2=5.5622$, $0.01<P<0.05$).

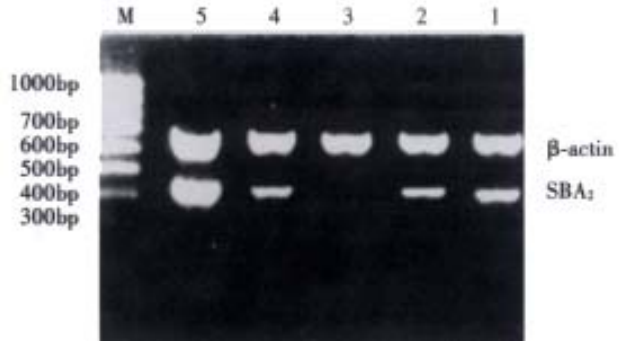

Figure 3 Relative quantification of SBA2 mRNA in colorectal tissues. 1-4: Moderately, moderately, poorly, and well differentiated CRC, respectively. 5: Healthy colorectal mucosa. M: Marker (100 bp DNA ladder).

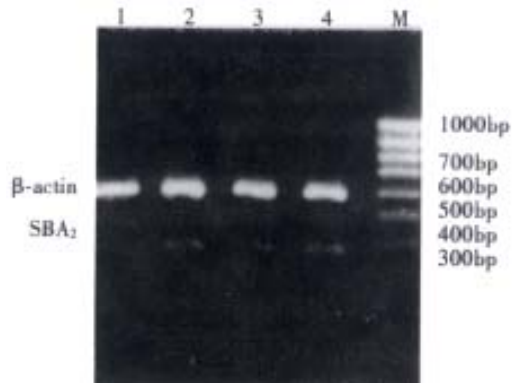

Figure 4 Relative quantification of SBA2 mRNA in cell lines of CRC. 1-4: Human CRC cell line CloneA, CX1, CCL187, and LS174T, respectively. M: Marker (100 bp DNA ladder).

DISCUSSION

We have developed an efficient protocol for relative quantification of gene expression in both clinical samples and cell lines of CRC by semi-quantitative RT-PCR. Linear evaluation of gene expression over a wide range was achieved by plotting the amplification curves of the sequence of interest and the β-actin sequence that served as an internal control. Determination of the optimal parameters of an exponential range was carried out by terminating the PCR at sequential cycles and by serially diluting the amount of cDNA. The amplification efficiency of PCR and the amount of sample cDNA have been verified by the simultaneous amplification of a sequence of a gene that serves as an internal control, such as, aldolaseA, β2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (G_3PD), dihydrofolate reductase[22]. However, the simultaneous amplification of a large amount of a sequence of an internal control can affect the efficiency of amplification of the sequence of interest[23]. A possible explanation of the competition is that the effect of an internal control sequence on amplification efficiency may depend on the gene sequences amplified[24]. For instance, the simultaneous amplification of a β2-microglobulin mRNA sequence suppressed the amplification efficiency of an *mdr* mRNA sequence, while amplification of β-actin sequence did not affect the amplification efficiency of a tax/rex sequence[25]. In our study, in the exponential range of amplification, fairly good linearity demonstrated identical amplification efficiency for SBA2 and β-actin (82% for both). Thus β-ctin can be used as an internal control to normalize the relative levels of SBA2 mRNA in this experiment.

Histologic types reflect the biologic nature of the cancer, exercising the most decisive influence upon the diagnosis and prognosis. A variety of pathologic changes are all closely associated with the cellular differentiation in CRC, including gross typing, growth pattern, depth of infiltration and metastasis into lymph nodes[26,27]. Classic grading categorized CRC into three discrete classes: well, moderately and poorly differentiated. Such grading, however, is largely dependent on the subjective assessment of the histopathologist. Heterogeneity of differentiation in the same cancer specimen often leads to considerable inter-and intra-observer variation in grading[28]. Colorectal neoplasia develops in a mucosa that has alterations in proliferation, maturation, and differentiation[29]. Normally, the major zone of cell proliferation is at the base of the crypts (the lowest one third) with little extension to the surface of proliferating cells. As cells migrate from the crypts to the luminal surface, they become increasingly differentiated and mature so that by the time they have reached the surface they have lost their proliferative capabilities, finally leading to apoptosis (i.e. programmed cell death)[30].

Butyrate is an important colonic fuel and induces differentiation in colonic cell lines[21]. cDNA for a novel colorectal cancer (CRC) differentiation-related gene, designated SBA2 (GenBank accession No: AF229181), has been identified in human colorectal cancer cell line CloneA after modulation by sodium butyrate. It consisted of 2470 nucleotides and an open reading frame (ORF) encoded 404 amino acid residues with a M_r of 44400. The deduced amino acid sequences showed significant homology to mouse SWiP-2 (96%), mouse WSB-2 (95%) and human WSB-1 (52%), which belong to a new family of the suppressor of cytokine signaling (SOCS). The SBA2 protein product may be a new member of SOCS protein family negatively regulating cytokine signal transduction. We have used RT-PCR for relative quantification of SBA2 mRNA levels in 4 CRC cell lines with different grades of differentiation and 30 surgical specimens of CRC and autologous healthy mucosa. Our assay showed significantly lower levels of SBA2 mRNA in tumors, as compared with coupled normal counterparts. Furthermore, differential expression of SBA2 in CRC with different grades of differentiation was observed both in clinical tissues and in cell lines SBA2 mRNA levels were correlated with the degree of differentiation in CRC, being relatively higher in well-differentiated samples and lower in poorly-differentiated ones. There was also a significant difference in the frequency of lower-level SBA2-expressing samples between patients with lymph nodes metastasis and those without metastasis. It was indicative of the close association of SBA2 with metastasis in CRC. The reduced levels of SBA2 mRNA in CRC and the correlation between SBA2 mRNA expression and the grade of differentiation in CRC suggest that SBA2 might be a

promising novel biomarker of cell differentiation of CRC, and that the biological features of SBA2 and its product in CRC and other tumors need further studies.

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• ORIGINAL RESEARCH •

Relationship between plasma D(-)-lactate and intestinal damage after severe injuries in rats

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Abstract

AIM **To explore the kinetic changes in plasma D(-)-lactate and lipopolysaccharide (LPS) levels, and investigate whether D(-)-lactate could be used as a marker of intestinal injury in rats following gut ischemia/reperfusion, burn, and acute necrotizing pancreatitis (ANP).**

METHODS **Three models were developed in rats: ① gut ischemia/reperfusion obtained by one hour of superior mesenteric artery occlusion followed by reperfusion; ② severe burn injury created by 30% of total body surface area (TBSA) full-thickness scald burn; and ③ ANP induced by continuous inverse infusion of sodium taurocholate and trypsin into main pancreatic duct. Plasma levels of D(-)-lactate in systemic circulation and LPS in portal circulation were measured by enzymatic-spectrophotometric method and limulus amebocyte lysate (LAL) test kit, respectively. Tissue samples of intestine were taken for histological analysis.**

RESULTS **One hour gut ischemia followed by reperfusion injuries resulted in a significant elevation in plasma D(-)-lactate and LPS levels, and there was a significant correlation between the plasma D(-)-lactate and LPS (r = 0.719, P<0.05). The plasma concentrations of D(-)-lactate and LPS increased significantly at 6h postburn, and there was also a remarkable correlation between them (r = 0.877, P<0.01). D(-)-lactate and LPS levels elevated significantly at 2h after ANP, with a similar significant correlation between the two levels (r =0.798, P<0.01). The desquamation of intestine villi and infiltration of inflammatory cells in the lamina propria were observed in all groups.**

CONCLUSION **The changes of plasma D(-)-lactate levels in systemic blood paralleled with LPS levels in the portal vein blood. The measurement of plasma D(-)-lactate level may be a useful marker to assess the intestinal injury and to monitor an increase of intestinal permeability and endotoxemia following severe injuries in early stage.**

Subject headings gut/injury; ischemia reperfusion/blood; burn/blood; acute necrotizing pancreatitis/blood; D(-)-lactate/blood; lipopolysaccharide/blood; intestinal permeability

Sun XQ, Fu XB, Zhang R, Lü Y, Deng Q, Jiang XG, Sheng ZY. Relationship between plasma D(-)-lactate and intestinal damage after severe injuries in rats. *World J Gastroenterol*, 2001;7(4):555-558

INTRODUCTION

Apart from their major functions of digestion and absorption of nutrients, the intestines also act as a barrier to prevent micro-organisms and toxins contained within the lumen from spreading to distant tissues and organs[1-7]. Failure of intestinal barrier function often occurs in many clinical conditions, including hemorrhage shock, severe burn injury, and the surgically critical illness, resulting in the increased intestinal permeability and subsequent translocation of bacteria or/and endotoxin from gut[8-12]. It is clear that increased gut permeability and bacteria with or without endotoxin translocation play a key role in the development of severe complications such as systemic inflammatory response syndrome (SIRS), sepsis, multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF)[13-20]. Therefore, it is important to know the intestinal injuries following a variety of insults (shock, burn injury, sepsis, and some critically surgical illness)[21,22]. D(-)-lactate is a product of bacterial fermentation. It is produced by many of the bacteria found in the human gastrointestinal tract[23]. Tissues in mammalian do not produce it and its metabolism is very slow[24]. In this study, we investigated the changes of plasma D(-)-lactate and lipopolysaccharide (LPS) levels and their correlation in gut ischemia/reperfusion, burn injury and acute necrotizing pancreatitis (ANP); and explored whether the changes of D(-)-lactate levels could be used as a predictor of increased intestinal permeability and endotoxemia following severe injuries.

MATERIALS AND METHODS

Animals

Male Wistar rats were used in this serial studies. They were housed in individual cages. The room temperature was maintained at 22℃ - 24℃ with a 12h light-dark cycle, and free access to a commercial laboratory rodent chow and fresh water were allowed. Twelve hours prior to experiment, the rats were fasted, but allowed free access to water.

Rat models of gut ischemia/reperfusion

Rats weighing 190g - 250g were divided into three groups. Gut ischemic group (n = 20): Animals were anesthetized with an intraperitoneal injection of 0.3mL 30g·L^{-1} pentobarbital sodium. Through a middle abdominal incision, intestinal

ischemia was produced by occluding the superior mesenteric artery for 1 and 1.5h with an automatic microvascular clamp. Animals were sacrificed at the end of gut is chemia. Gut ischemia/reperfusion group (n=50): superior mesenteric artery was occluded for 1h and then the vascular clamp was removed to produce gut reperfusion. Animals were sacrificed at 0.5, 1, 2, 6 and 24h after gut reperfusion. Sham-operated control (n=10): animals were treated identically omitting the superior mesenteric artery occlusion. Blood samples were collected aseptically from cervical artery and portal vein for D (-)-lactate and LPS assay before animals were killed at each time point.

Rat models of burn

Male Wistar rats weighing 190g - 250g were used. Animals were divided into two groups. In thermal group, they were subjected to a 30% total body surface area (TBSA) full-thickness scald burn injury (n=40). They were anesthetized with an intraperitoneal injection of 30g·L^{-1} pentobarbital sodium (60mg·kg^{-1}) and then the dorsal hair was shaved. A 30% TBSA full-thickness burn was created on the back of the rats in boiling water at 98℃-100℃ for 12s. Rats were resuscitated immediately after thermal injury with 50g·L^{-1} glucose saline solution (50mL·kg^{-1}) intraperitoneally. In the control group (n=10), rats were exposed to the room-temperature water. Animals in thermal group were killed at 3, 6 12 and 24h after burn. Blood samples were collected aseptically from cervical artery and portal vein before the rats were killed at each time point.

Rat models of acute necrotizing pancretitis (ANP)

Male Wistar rats weighing 270g-330g were randomly divided into two groups. In the ANP group (n=27), animals were anesthetized with 30g·L^{-1} pentobarbital sodium (60mg·kg^{-1}, ip). After medium laparotomy, the duodenum was mobilized and the pancreatic duct was identified at its duodenal junction. ANP was induced by a continuous inverse infusion of sodium taurocholate (50g·L^{-1}, 1mL·kg^{-1}) and trypsin (1.67×10^{5}U·kg^{-1}) into the main pancreatic duct. Animals were immediately given saline (50mL·kg^{-1}) subcutaneously after injury. In control group (n=6), animals were treated identically with infusion saline. Blood samples were taken aseptically from cervical artery and portal vein at 2, 8, 24 and 48h after injury.

D(-)-lactate determination

The plasma from systemic blood samples was obtained and subjected to a deproteination and neutralization process by acid/base precipitation using perchloric acid and potassium hydroxide. The protein-free plasma was then assayed for D (-)-lactate concentration by enzymatic-spectrophotometric method with minor modification[25].

Lipopolysaccharide (LPS) determination

The plasma from portal vein blood was also obtained and subjected to a deproteination and neutralization process by acid/base precipitation using perchloric acid and sodium hydroxide. The LPS levels of portal vein blood were assayed by the chromogenic limulus amebocyte lysate (LAL) test with a kinetic modification according to the test kit procedure[26].

Morphologic studies

Tissue samples of intestines were taken for morphologic study. Biospies were fixed in 100mL·L^{-1} neutral buffered formalin, embedded in paraffin, microtome sectioned at 4μm-6μm thickness, and stained with hematoxylin and eosin. Sections were examined under light microscope.

Statistical analysis

Data were expressed as means ± SD. The statistical significance of mean values between groups was evaluated by the Student's t test. The relationship between circulating systemic D(-)-lactate and portal vein LPS concentrations was determined by the calculation of Pearson correlation coefficient. P<0.05 was considered to be significant.

RESULTS

Kinetics of D(-)-lactate and lipopolysaccharide concentrations in plasma after gut ischemia/reperfusion in rats

One hour of gut ischemia alone induced a slight increase in systemic blood D(-)-lactate and portal vein blood LPS concentrations (Table 1). Either D(-)-lactate or LPS concentrations had a further significant increase at 0.5h-2h after gut reperfusion (P<0.05-0.01), and decreased to normal at 6h. Meanwhile, correlation analysis revealed a significant correlation between systemic blood D(-)-lactate levels and portal vein blood LPS concentrations (r = 0.719, P<0.05).

Table 1 The plasma contents of D(-)-lactate and lipopolysac charide in rats after gut ischemia / reperfusion insults (mean±SD)

Groups	Time (h)	No.	D(-)-lactate (mmol/L)	LPS(EU/L)
Sham-operated control		10	0.234±0.072	380±84
Gut ischemia	1	10	0.260±0.086	407±41
	1.5	10	0.269±0.092	453±129
Gut ischemia / reperfusion	0.5	10	0.489±0.179[b]	576±244[a]
	1	10	0.373±0.179[a]	611±278[a]
	2	10	0.253±0.062	562±167[a]
	6	10	0.237±0.044	335±73
	24	10	0.228±0.025	283±81

Compared with sham-operated control, respectively: [a]P<0.05; [b]P<0.01.

Alterations in plasma D(-)-lactate and LPS levels in thermal rats

Results presented in Table 2 indicated that there was a significant increase both in circulating blood D(-)-lactate and portal vein blood LPS concentrations at 6h after injury, and kept significantly increasing to the end of our observation period (72h, P<0.01). In addition, correlation analysis revealed that there was a strong positive correlation between plasma levels of D(-)-lactate and LPS after injury (r = 0.877, P<0.01).

Table 2 Changes in systemic blood D(-)-lactate levels and portal blood LPS content in thermal rats (mean±SD)

Groups	Time(h)	No.	D(-)-lactate (mmol/L)	LPS (EU/L)
Control group		10	0.275±0.175	118±37
Thermal group	3	10	0.371±0.123	159±83
	6	10	0.517±0.162[a]	347±111[a]
	12	10	0.619±0.208[a]	670±139[a]
	24	10	0.638±0.198[a]	396±57[a]

Compared with control group, respectively: [a]P<0.01.

Changes in plasma D(-)-lactate and LPS levels in ANP rats

In rats subjected to ANP, the levels of D(-)-lactate in systemic blood and LPS in portal vein blood began to increase at 2h after ANP (P<0.01) (Table 3), and peaked at 24h after injury. Furthermore, a marked correlation was noted between the changes in contents of plasma D(-)-lactate and LPS (r=0.798, P<0.01).

Table 3 Alterations in systemic blood D(-)-lactate levels and portal blood LPS content in ANP rats (mean±SD)

Groups	Time(h)	No.	D(-)-lactate (mmol/L)	LPS (EU/L)
Control group		6	0.157±0.044	105±7
ANP group	2	6	0.328±0.063[a]	301±131[a]
	8	7	0.507±0.157[a]	449±164[a]
	24	7	0.653±0.216[a]	611±210[a]
	48	7	0.448±0.112[a]	422±136[a]

Compared with control group, respectively:[a]P<0.01.

Gut pathology

Mucosal edema, necrosis, and the loss of the epithelium in mucosa, as well as vascular dilution, congestion, edema and inflammatory cell infiltration in the lamina propria were observed in small intestinal biopsies in three groups. The intestinal injury paralleled with the changes of plasma D(-)-lactate levels.

DISCUSSION

The present study showed that the intestinal damage caused by gut ischemia caused a slight increase in plasma concentrations of D(-)-lactate in systemic blood and LPS in portal vein blood. After gut ischemia followed by reperfusion, the plasma levels of D(-)-lactate and LPS significantly elevated, but declined to normal rapidly at 6h after reperfusion[27-29]. The intestinal damage mediated by burn injury or ANP displayed a more severe damage than that in gut ischemia/reperfusion. A remarked increase of plasma D(-)-lactate and LPS concentrations occurred at 6h, and 3h after insult, respectively, and persisting to the end of our observation. Moreover, the elevation of plasma D(-)-lactate levels in systemic blood was associated with increased plasma LPS contents in portal vein blood, and histological examination also exhibited intestinal injury in those three rodent models.

D(-)-lactate is produced by some bacteria including *Klebsilla*, *Escherichia coli*, *Lactobacillus species*, and *Bacteroides species*. It is an indigenous products in gut[24]. Normally, serum levels of D(-)-lactate in mammals are quite low. During the event that an ischemia/reperfusion insults, the mucosa is injured and intestinal permeability is increased, leading to an efflux of bacteria and the products of their metabolism[30-33], including D (-)-lactate into the circulation. Otherwise, the gut ischemic insult leads to a loss in normal host defenses against bacterial overgrowth, resulting in increased numbers of bacteria within the lumen of the infected intestine[34-36]. This bacterial proliferation would be expected to cause an increased bacterial metabolism with increased production of D(-)-lactate. Mammals do not possess the enzyme system to rapidly metabolize D (-)-lactate, thus, it passes through the liver with unchanged way and enters the peripheral blood early in the disease process. Thus, D(-)-lactate accumulation in the systemic circulation can generally be considered as a result of bacterial over growth and increase in gut permeability induced by some gastrointestinal disorders. Therefore, D(-)-lactate levels could be used as a predictor of intestinal injury. In fact, the elevation of plasma D(-)-lactate levels has been used as the predictor of bacterial infection in patients with short-bowel syndrome[37]. In rat model of acute mesenteric ischemia, D (-)-lactate was significantly elevated after gut ischemia, and the histopathological evaluation scores of intestinal injury were remarkably correlated to the plasma D(-)-lactate levels[38,39]. Recently, in clinical study, it has also been demonstrated that patients with mesenteric ischemia at laparotomy had significantly elevated D(-)-lactate levels in systemic circulation as compared with patients operated on for an acute abdomen or normal abdomen[40].

In conclusion, our data in these rat models suggest that the changes in D(-)-lactate concentrations paralleled with LPS concentrations, and correlated similarly with the intestinal histopathological alterations as well. Therefore, plasma D(-)-lactate in systemic circulation measurement would be a useful marker to evaluate intestinal injury and endoxemia following severe injuries.

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• ORIGINAL RESEARCH •

Killing effect of TNF-related apoptosis inducing ligand regulated by tetracycline on gastric cancer cell line NCI-N87

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Abstract

AIM **To clone the cDNA fragment of human TRAIL (TNF-related apoptosis inducing ligand) into a tetracycline-regulated gene expression system, the RevTet-On system, transduce expression vectors into a gastric carcinoma cell line-NCI-N87 and examine the effects of controlled expression of TRAIL *in vitro* on the gastric carcinoma cells.**

METHODS **The full-length cDNA of TRAIL was inserted into a vector under the control of the tetracycline-responsive element (TRE) to obtain the plasmid pRevTRE-TRAIL, which was transfected into a packaging cell line PT67. In addition, vector pRev-Tet On and pRevTRE were also transfected into PT67 separately. After hygromycin and G418 selection, the viral titer was determined. The medium containing retroviral vectors was collected and used to transduce a gastric carcinoma cell line NCI-N87. The resulting cell line NCI-N87-Tet On TRE-TRAIL and a control cell line, NCI-N87 Tet On-TRE, were established. TRAIL expression in the cell line was induced by incubating cells with doxycycline (Dox), which is a tetracycline analogue. The killing effect on gastric carcinoma cells was analyzed after induction.**

RESULTS **The recombinant plasmid pRev-TRE-TRAIL was constructed. After hygromycin or G418 selection, the producer cell lines PT67-TRE, PT67-TRE-TRAIL and PT67-Tet On were obtained, with titers of about 10^8 CFU·L^{-1}. By transducing NCI-N87 cells with retroviral vectors from these cell lines, stable cell lines NCI-N87-Tet On TRE-TRAIL (NN3T) and control cell line NCI-N87-Tet On TRE (NN2T) were established. The growth curves of the selected cell lines were the same with the wild type NCI-N87. When Dox was added, cell death was obvious in the test groups (29%-77%), whereas no difference was observed in control and wild type cell lines. With the addition of a medium from the test group, human leukemia cell line Jurkat was activated till death (83%), indicating the secretion of active TRAIL proteins from the test cells to the medium.**

CONCLUSION **With the use of the RevTet-On system, a regulated expression system for TRAIL was constructed. Using this system, the selected killing effect of TRAIL on gastric carcinoma cell line NCI-N87 could be observed.**

Subject headings TRAIL; Tet gene expression system; gastric carcinoma; stomach neoplasms/pathology; tumor cells, cultured; tumor necrosis factor; tetracycline; apoptosis; gene expression

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INTRODUCTION

TRAIL (TNF-related apoptosis inducing ligand, Apo2 ligand, Apo 2L), which belongs to the tumor necrosis factor (TNF) cytokine family, can induce rapid apoptosis in a wide variety of tumor cell lines[1-3]. TRAIL protein consists of 281 (human TRAIL) or 291 (mouse TRAIL) amino acids.The human TRAIL gene is located in chromosome 3(3q26). The mRNA distribution of TRAIL is broad[1,4-8]. Although the *in vivo* role of the TRAIL is not currently known, *in vitro* studies have found that TRAIL is capable of inducing apoptosis in a wide range of human tumor cells, but generally not normal cells[9-16]. TRAIL induces apoptosis by binding and cross-linking death-domain containing receptors[17-21], TRAIL-R1[22,23] (also known as DR4) and TRAIL-R2[24-26] (DR5). Apoptotic signaling occurs via recruitment of adapter proteins, which results in the activation of caspases[24,27]. Because of its selective killing effect on tumor, TRAIL is likely to be a drug in cancer treatment[28-30]. Therefore, we tested the effect of TRAIL expression in human gastric carcinoma cells, most commonly found in oriental population. In addition, the study may form a basis for the safety and effectiveness of TRAIL expression in gene therapy of cancer.

In gene therapy, it is important to regulate gene expression effectively. The tetracycline-controlled gene expression system (Tet system) is a tight control system[31]. It is based on the Tn10 specified tetracycline resistance operon of *E. coli*. In the Tet-Off system, the regulator unit, encodes a hybrid tTA (tetracycline-controlled transactivator) protein composed of the tetracycline repressor (tetR) fused to the herpes simplex virus (HSV) transactivator protein, VP16. The response unit, tetracycline-responsive element (TRE), is composed of the tetracycline resistance operon regulatory elements (tetO) embedded within a minimal cytomegalovirus (CMV) promoter. The expression of a gene inserted downstream of the promoter is highly dependent on tTA, which binds tetO sequences. With the addition of tetracycline, tTA protein dissociates from the TRE and the gene expression is inhibited. Conversely, the Tet-On system allows gene expression to be activated by the addition of

tetracycline. It is based on the reverse tTA (rtTA) in the regulator unit.

The RevTet-Off/On system uses the retroviral vectors. They have the characteristic of Tet system, and their gene transfer is more rapid and efficient, which can be used in gene therapy. In this study, TRAIL gene was cloned into the RevTet-On system, to achieve tight control of the expression of TRAIL protein in mammalian cells. The gene expression system on gastric carcinoma cell line NCI-N87 was constructed, and the killing effect of controlled-gene expression in the resulting cells was observed.

MATERIALS AND METHODS

Construction of retroviral plasmids

The RevTet-On System, which include pRevTet-On and pRev-TRE vectors and the RetroPack PT67 cell line, were purchased from Clontech, Palo Alto, California, USA. TRAIL gene was obtained by using PCR method from a human placenta cDNA library (Clontech). The PCR primers are: 5'-AAGCTTATGGCTATGATGGAGGTCCAGGGGGG-AC-3'and 5'-AAGCTTTTAGCCAACTAAAAAGGCCCCG-AAAAAACTGGC-3'. The resulting PCR product was cloned into an intermediate vector PCR-2 (Invitrogen, Carlsbad, California, USA) and then cut with *Hin* dIII, which was cloned into the same site in vector pLNCX, resulting in vector pLNCX-TRAIL. The Tet-regulated vector pRev-TRE-TRAIL was cloned in the same manner.

Cell culture

The retroviral packaging cell line PT67 and the murine fibroblast cell line NIH3T3 were maintained in Dulbecco's modified essential medium (DMEM) containing 100mL·L^{-1} fetal bovine serum. The human gastric carcinoma cell line, NCI-N87, and the human leukemia cell line, Jurkat, were maintained in RPMI 1640 medium containing 100mL·L^{-1} or 150mL·L^{-1} fetal bovine serum respectively.

Transfections, infections and determination of viral titer

The packaging cells PT67 were plated in a 60 mm plate at a density of 50%-80% 24h before transfection. Cells were washed with DMEM twice and incubated with 2mL DMEM before transfected with 10μg of plasmid DNA by lipofectin method (Life Technology, Gibco BRL). Four h-6h after transfection, 2mL DMEM (containing 200mL·L^{-1} FBS) was added. To obtain stable virus-producing cell lines, the packaging cells transfected with retroviral plasmids were plated in selection medium 48h later. The regulatory vector Tet-On carries the neomycin gene as a selectable marker. For G418 selection, cells were cultured in the presence of G418 (0.4g·L^{-1}, Gibco) for two- weeks. The vectors pRev-TRE and pRev-TRE-TRAIL carry the gene for hygromycin selection. These cells were selected in the presence of hygromycin B (0.06g·L^{-1}, Sigma) for two weeks. For transduction, NCI-N87 cells were plated 24h before infection. The medium from packaging cells containing virus were collected, filtered through a 0.45μm filter, and added to the NCI-N87 cells in the presence of Polybrene (4g·L^{-1}, Sigma). The medium was replaced 4h later. Three to six serial infections were performed to increase the efficiency of infection. Forty-eight hours after infection, the cells were subjected to G418 or hygromycin selection. For determination of viral titer, NIH3T3 cells were plated 24h before transduction in 6-well plates (2×10^5 cells per well). The cells were infected with filtered virus-containing medium (six 10-fold serial dilutions, 1 - 10^5). The cells were then incubated in G418 or Hygromycin 48h later, and the selection lasted about weeks until clear colonies appears.

Growth curve

The cells were plated in 96-well plates (5×10^4 cells per well), and counted by Typan blue dye exclusion method over a 24h period.

Induction of gene expression

The cells were added with the medium containing Dox (0.01 -10mg·L^{-1}, Sigma). After a period of time, the fraction of dead cell was calculated by Typan blue dye counting. The medium after induction was added in Jurkat cells (100mL·L^{-1}), the fraction of dead cell was calculated by Trypan blue dye.

RESULTS

Construction of retroviral vectors

To clone the TRAIL gene into the Tet system, we inserted the cDNA of TRAIL into the Tet response vector pRev-TRE, which was named pRevTRE-TRAIL. The desired recombinant plasmid orientation was confirmed by Hin dIII and *Ssp* I digestion. In this plasmid, the TRAIL gene is the downstream of the TRE, which can bind rtTA expressed by pRevTet-On. So, in combination with the pRevTet-On regulatory vector, the TRAIL gene can be inductively expressed at high levels in response to varying concentrations of tetracycline or doxycycline. Because of their retrovirus-mediated gene transfer, we can incorporate the Tet-controlled TRAIL protein expression system into mammalian cells.

Establishment of Tet-controlled TRAIL system

We transfected vectors into packaging cells PT67. Using G418 (pRevTet-On) and hygromycin selection (pRev-TRE and pRevTRE-TRAIL), we obtained the stable virus-producing cell lines, PT67-TetOn, PT67-TRE and PT67-TRE-TRAIL. Viral titers were 1-2×10^{8}CFU·L^{-1}, determined by titering NIH3T3 cells. After serial infections of NCI-N87 cells with viral-containing supernatants and antibiotic selections, two kinds of cell lines were established. One is the test group NCI-N87-TetOn-TRE-TRAIL (NN3T, Figure 1A), which is a Tet-controlled TRAIL expression cell line. The second is the control cell line NCI-N87-TetOn-TRE (NN2T, Figure 1B), which is the same with NN3T but without TRAIL expression.

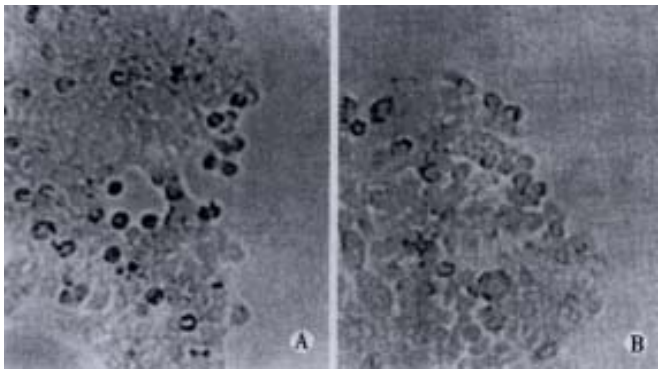

Figure 1 Morphology of cells. A: NN3T; B: NN2T

Killing effect of TRAIL induced by doxycycline

When we added doxcycline to cells, some cells died in the test group NN3T 48h later (Figure 2), whereas no clear death effect can be detected in cells in the control group (NN2T)

and wild type NCI-N87. Under normal conditions without Dox, the growth curves of these three types of cells showed no obvious differences (Figure 3). In order to test the possible secretion of TRAIL protein into the medium, the media from the test group and control group were removed and inoculated into the human leukemia cells Jurkat (sensitive to TRAIL-induced apoptosis). Obvious cell death was observed after one day culturing in the test group as compared with the control and the normal groups. From these results, we concluded that the regulated expression of TRAIL in the Tet system has killing effect on the gastric carcinoma cell NCI-N87.

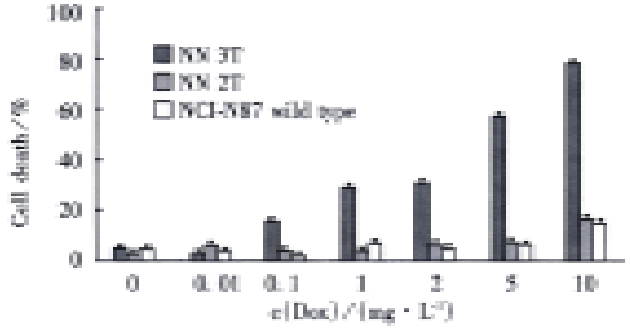

Figure 2 Killing effect of TRAIL regulated by doxcycline.

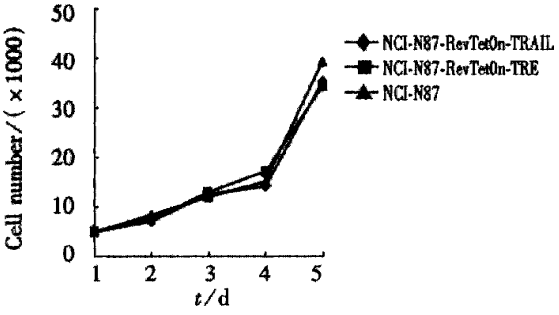

Figure 3 Growth curves of cells.

DISCUSSION

Tumor necrosis factor (TNF) is a prototypic member of the family of cytokines that interact with a corresponding set of receptors that form the TNF receptor (TNFR) family. Three of these ligands, CD95L, TNF and LTα, have received particular attention because they can induce apoptosis in transformed cells and activated lymphocytes[32-34]. The potential utility of systemically administered ligands is limited by their acute toxic effects on normal tissues *in vivo*, thereby limiting their potential widespread use in the treatment of cancer[35]. Previous studies have shown that TRAIL can induce apoptosis in a variety of tumor cell lines. In contrast to other members of TNF family, TRAIL mRNA is expressed constitutively in many tissues including peripheral blood lymphocytes, spleen, thymus, prostate, ovary, small intestine, colon and placenta[1,7,17,36-38], which suggests the existence of physiological mechanisms that can protect many normal cell types from induction of apoptosis specifically by TRAIL. A relatively high proportion (approximately two-thirds) of tumor cell lines tested so far are sensitive to the cytotoxic effects of TRAIL *in vitro*[1,7,11,39], indicating that TRAIL may prove to be a powerful cancer therapeutic factor.

Although TRAIL potently induces apoptosis in tumor cells and some virally infected cells, it has little or no detectable cytotoxic effects on normal cells, whereas this was first thought to be due to regulated expression of the TRAIL receptors[3,24,40-42], the fact that mRNA for both TRAIL and TRAIL receptors is often expressed in the same cells makes this explanation untenable[17,43]. Indeed, the identification of four distinct TRAIL receptors (TRAIL-R1, R2, R3, R4) has significantly increased the potential complexity of this receptor/ligand system[17,43,44]. Based on current information it seems mikely that multiple factors, both intra and extra-cellular, may function together to protect normal cells from the cytotoxic effects of TRAIL[17,43,45-48], but many questions remain unexplained.

Although the reason for determining the sensitivity of cells to TRAIL-induced apoptosis has not been understood, we still try to use TRAIL in cancer treatment to achieve the effectiveness and the safety of the therapy. In our laboratory, the cDNA sequence of TRAIL has been cloned from human placenta cDNA library. We have expressed the TRAIL in the *E. coli* by the pET system[49], after purification and refolding the antitumor activity of the protein has been examined[50]. On the other hand, a chief concern with the potential use of TRAIL protein in the treatment of tumors *in vivo* is the potential undesirable toxicities. Because of the recent report that some normal human cells were sensitive for the apoptosis induced by TRAIL[51], we inserted the gene into a mammalian expression vector to study its effect further in this experiment.

In most inducible mammalian gene expression systems (heavy metals, steroid hormones, or heat shock), induction is nonspecific and expression levels cannot be precisely regulated. In addition, these systems are generally leaky in the "off" state, and the inducing agent itself may be cytotoxic or have pleiotropic effects. In contrast, regulation of gene expression by the Tet system is very specific[31]. Furthermore, the levels of tetracycline or doxycycline required for the full range of gene expression are not cytotoxic and have no significant effect on cell proliferation or animal growth. Using retroviral vectors instead of DNA transfection to transduce the complete inducible system greatly expands the target cell types in which gene functions can be studied. This strategy may be useful for clinical applications in human gene therapy trials.

In this study, the effectiveness of TRAIL in killing human gastric cells was evident. It showed that the gastric carcinoma cell line was sensitized to TRAIL-induced cell death. In addition, the concentrations of doxycyline that constituted the effect were within a range of clinical security. We can believe that this effect will be safe to normal tissues with the specificity of TRAIL and regulation of the Tet system.

At the moment, malignant gastric carcinoma remains one of the difficult types of cancer to treat successfully, and with the incidence of gastric carcinoma increasing at China, it continues to be a leading cause of death in Japan and other countries of Asia. In our experiment, the expression system of TRAIL gene controlled by tetracycline was established in gastric carcinoma cell line NCI-N87, and its killing effect to the tumor cell was observed. This study clearly demonstrates the possible usefulness of Tet-controlled TRAIL expression system in gastric cancer gene therapy, and further studies of the mechanisms of TRAIL-mediated cytotoxicity are required to assess the potential use of TRAIL as an anti-cancer therapeutic *in vivo*.

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• BRIEF REPORT •

Barrett's- metaplasia: clinical implications

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Abstract

The incidence of Barrett's metaplasia (BM) as well as Barrett's adenocarcinoma (BA) has been increasing in western populations. The prognosis of BA is worse because individuals present at a late stage. Attempts have been made to intervene at early stage using surveillance programmes, although proof of efficacy of endoscopic surveillance is lacking, particularly outside the specialist centres. The management of BM needs to be evidence-based as there is a lack clarity about how best to treat this condition. The role of proton pump inhibitors and antireflux surgery to control reflux symptoms is justified. Whether adequate control of gastroesophageal reflux early in the disease alters the natural history of Barrett's change once it has developed and or prevents it in patients with gastroesophageal reflux disease but with no Barrett's change remains unanswered. There is much to be learned about BM. Thus there is great need for carefully designed large randomised controlled trials to address these issues in order to determine how best to manage patients with BM.

Subject headings Barrett's esophagus/complications; metaplasia/complications; gastroesophageal reflux

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INTRODUCTION

The recent rise in the incidence of esophageal adenocarcinoma in parallel with an increased incidence of Barrett's metaplasia (BM) favours the theory that BM is a pre-cancerous lesion for adenocarcinoma. This BM is thought to occur as a result of prolonged and severe gastroesophageal reflux that can lead to chronic inflammation resulting in replacement of normal squamous epithelium in the distal oesophagus with specialised intestinal-type columnar epithelium containing goblet cells. Attempts have been made to treat acid reflux with medical treatment as well as anti-reflux surgery in the hope that it might prevent progression of BM. Surveillance strategies for established BM to detect early cancer have not been proven cost effective[1,2], although lesions identified during surveillance program have better prognosis. Despite extensive research, the natural history of BM is poorly understood. Many questions remain regarding the diagnosis of BM, its treatment, and the impact of surveillance strategies on early detection of cancer. It is important to realise the sheer scale of the problem as oesophagitis secondary to gastroesophageal reflux disease is one of most common conditions in the western world with up to 30% of adults complaining of heartburn at least once per month, a third of whom will develop oesophagitis. About 10% of patients with oesophagitis will progress to BM, of whom up to 5% will progress to cancer.

Using pooled data, the cancer risk in BM is about 1% (range 0.5% - 2%)[3]. Proposed clinical risk factors for cancer progression including chronicity of symptoms, length of Barrett's segment, gastroesophageal reflux and mucosal damage. For example gastroesophageal reflux symptoms are considered an independent risk predictor for cancer risk but may not discriminate low from high risk BM. Molecular changes in p53, p16, and *cyclin* D1 overexpression, decreased E-cadherin expression, and loss of heterozygosity of the adenomatosis polyposis coli (APC) gene have been detected[3]. These molecular changes have been evaluated in a clinical research setting but are not routinely used in clinical practice, although such genetic changes may become useful screening markers to monitor progression of BM and to identify individuals at risk of developing malignant transformation in the future. Thus, there is a pressing need for better understanding of BM and to develop strategies not only to prevent this pre-cancerous condition but also to identify high-risk individuals with this condition who are at risk of developing Barrett's adenocarcinoma. This article discusses issues that concern clinicians in the management of BM including definition, diagnosis, screening and surveillance of BM, as well as management of uncomplicated BM.

DEFINITION AND DIAGNOSIS OF BM

BM may be defined as visible columnar-lined oesophagus of any length above the esophago-gastric junction (OGJ) confirmed on biopsies with the presence of specialised intestinal-type columnar epithelium containing goblet cells. However, there is considerable disagreement in defining BM. One technical problem in this regard is the precise identification of the OGJ. Suggested endoscopic criteria for identifying OGJ include the point of flaring of the stomach from the tubular oesophagus[4] and confluence of the proximal margin of longitudinal gastric folds[5]. One has to realise that this location point of flare can shift during breathing as well as peristaltic activity in the oesophagus and prolapse of the gastric folds into the oesophagus could further confuse the situation. Distinction between long and short Barrett's segment is irrelevant as the tendency to develop high-grade dysplasia may be similar in short and long segment Barrett's[6].

To avoid confusion endoscopists should avoid such terminology and instead describe only what they see i.e. if columnar-lined oesophagus is present or not and if present whether it is continuous or tongues and islands are observed. The histopathologist often finds it difficult to differentiate between intestinal metaplasia occurring in the distal oesophagus and in the cardia, and thus should avoid the term Barrett's and instead report whether columnar epithelium is present and the presence or absence of intestinal metaplasia

and identification of squamo-columnar junction. It is essential that precise site of biopsies with reference to OGJ should be documented to help the histopathologist make an accurate diagnosis. Thus the diagnosis of BM becomes clinicopathological integrating both endoscopic and histopathological information to increase diagnostic accuracy. Occasionally (1% - 5% of cases), however, the pathologists see esophageal glands submerged under columnar-lined tissue corroborating the biopsy as being esophageal in origin (personal correspondence Prof N Shepherd, Gloucester Royal Hospital, UK) (Figure 1).

EPIDEMIOLOGY OF BM

The true prevalence of BM in the general public is unknown. Reported lifetime risk in adult general populations in western countries is 1%[7] based on post-mortem studies. In addition, the incidence of new cases is about 0.5%-2.0%/year[3] and increasing[8]. It mainly occurs in white people, with a male predominance (male: female 2.3:1)[9]. Recently its prevalence has been shown to be rising all over the world, including in the Far East[10]. There is no significant relationship between smoking, alcohol consumption and high body mass index in the genesis of this metaplastic change.

SCREENING AND SURVEILLANCE OF BM

It is not clear why only 10% of patients with oesophagitis progress to BM and why only a tiny fraction of these patients will go on to develop adenocarcinoma. In view of the low prevalence of BM in patients with gastroesophageal reflux as well as uncertainties regarding diagnosis and treatment, it is difficult to justify screening for Barrett's in patients with gastroesophageal reflux, although patients aged >45 with longstanding severe reflux symptoms (5 - 10 years) should have a one off screening endoscopy.Barrett's adenocarcinoma develops through stages of increasing dysplasia (Sandic 1998). If this theory is correct, then surveillance endoscopies biopsies of BM should permit early cancer detection and reduce mortality from Barrett's adenocarcinoma[11], as prognosis in early Barrett's adenocarcinoma is more favourable as compared to advanced disease[12]. Attempts to identify tumours at early stage with surveillance programmes has not been cost effective in few studies[1,2]. Surveillance of BM with no dysplasia at 2.5 years has been recommended, although its usefulness has never been validated in a randomised study. Using the Markov model based on UK NHS cost per life saved if the surveillance programme is based on two yearly endoscopy, surveillance is comparable to other health disciplines in terms of cost per cancer detected and cost per life saved. It is fair to say that for an expense of this order we should first have some convincing evidence of effectiveness of surveillance programmes. Until results of such studies are available we face the dilemma of telling patients that they have a pre-cancerous condition with 5% life-time risk of cancer and that we could offer lifelong surveillance endoscopy programme that cannot guarantee to detect every cancer that may develop.

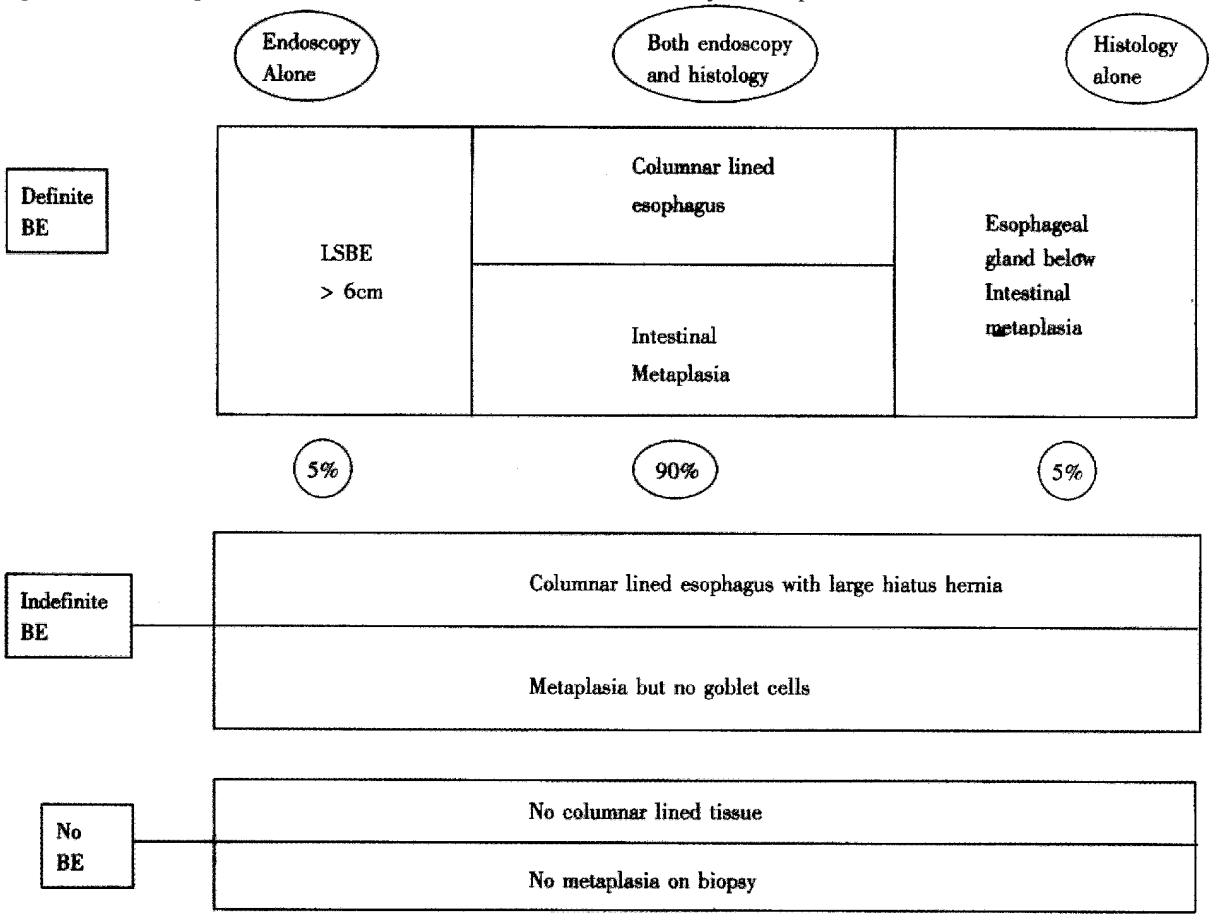

Figure 1 Diagnostic criteria for Barrett's esophagus (BE), based on endoscopic, histological criteria or both. LSBE (long segment Barrett's esophagus). Definite diagnosis can be made with endoscopy in 5%, histology alone in 5%, but in about 90% both endoscopy and histology is required.

MANAGEMENT OF UNCOMPLICATED BM (NO DYSPLASIA)

The symptoms of GORD, a presumed cause of BM, can be effectively eliminated with medical treatment or with anti-reflux surgery; however, regression of established BM does not occur with either intervention. On the other hand, many patients with BM will either have reduced or absent symptoms due to reduced sensation of Barrett's epithelium. The role of PPI's in BM is unclear. High dose PPI's are still proposed regardless of symptoms by many who justify that such an approach may be necessary to achieve regression of BM[13]. Opponents of this approach advocate no treatment of asymptomatic BM and are less convinced that acid suppression prevents complications. Until there is more clarity in the scientific evidence, PPI's remain an attractive treatment for the BM, especially if there is endoscopic evidence of esophagitis above the BM segment as is the case in about 40% -60% cases.

Competent fundoplication for BM has been advocated in patients with complications or intractable symptoms unresponsive to conservative medical treatment. If performed early, before the development of Barrett's changes, fundoplication is slightly more effective than medical treatment to prevent this metaplastic change. The effect of this anti-reflux surgery on the natural history of BM once it has developed is less clear. Until we have more data to determine the role of anti-reflux surgery in the setting of BM or GORD, it would be sensible to perform these procedures once medical treatment fails or on patients fully informed of the choice. In addition, because there is a 0.1%-0.2% mortality rate for fundoplication surgery, this detracts from its efficacy. In particular, if there is an estimated 5% lifetime risk of cancer in BM, only half will die of the cancer because co-morbidity such as cardiac diseases will usually kill them. Secondly, no treatment is ever 100% effective in preventing cancer, so if 50% of individuals could be prevented from getting cancer only 1.25% of the 5% at risk population would benefit from surgery. Therefore for every 9 patients benefiting from long term advantages of surgery, 1 or perhaps even 2 would die prematurely from the surgery itself.

Because of the inability to effect regression of Barrett's mucosa with medical or anti-reflux surgery, there has been renewed interest in the development of new modalities to eliminate this metaplastic change and hence reduce the cancer risk by destruction of Barrett's mucosa by endoscopic ablation with thermal (Laser, Argon Plasma Coagulator), chemical (photodynamic) or mechanical (surgical ultrasound) in a reflux free environment to prevent further damage. These techniques have shown healing by squamous epithelium regeneration in 66% - 100% patients[14], although nests of glandular epithelium may remain beneath the neo-squamous epithelium in up to 60% of patients that may progress to cancerous change. In view of this and high complication rates[14], many authors have debated the usefulness of these potentially hazardous therapies. Using NNT calculations, a reduction of absolute risk has not been established for any of these therapies. Efficacy of such treatment should thus be verified in controlled trials before their widespread use.

CONCLUSIONS

In spite of extensive research in this area it appears that we have more questions than answers. Our understanding of the natural history of BM is limited, as available data is not only insufficient but also either contradictory or subject to variable interpretation. Longstanding gastroesophageal reflux has been proposed to contribute towards BM. Whether adequate control of gastroesophageal reflux early in the disease alters the natural history of Barrett's change once it has developed, or prevents it in patients with GORD, remains unanswered. To date, we simply cannot estimate or eliminate either the cancer risk or BM itself. On the basis of evidence available, it is difficult to promote or reject Barrett's surveillance programmes on economic grounds alone. We must not forget that only 25% Barrett's adenocarcinoma patients are known to have BM before they develop cancer and that 75% of cancer patients present for the first time with this disease without any prior knowledge of GORD or BM. Thus there is a pressing need for more work to be done, in large randomised controlled trials, to unravel these issues before we will be able to treat this condition effectively.

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• BRIEF REPORT •

Liver transplantation and artificial liver support in fulminant hepatic failure

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INTRODUCTION

Fulminant hepatic failure (FHF) is a severe disease with devastating consequences; the incidence is high in China. Before the availability of liver transplantation, the mortality rate was more than 80%[1,2]. The advent of liver transplantation revolutionized the outcome of FHF[3,4]. However, many patients were unwilling to accept liver transplantation until very late, hence most of them died because of donor shortage and urgency of the disease[5-7]. To overcome the problems, we performed orthotopic liver transplantation (OLT) in combination with artificial liver support (ALS) in the treatment of FHF in the past 2 years with satisfactory results. Our experience was reported below.

PATIENTS AND METHODS

Patients

All eight patients were male with a mean age of 40 (range 32 -49) all had hepatitis B with acute absolute liver failure on admission. These patients had a history of hepatitis for 7 days to 12 weeks, with acute onset of severe hepatic disfunction, rapidly progressive jaundice, abdominal distention, asthenia, ascites, coagulopathy and encephalopathy. Two of them were complicated with acute hepatorenal syndrome and acute necrotizing pancreatitis respectively. Seven patients had stage II-IV coma. All 8 patients received artificial liver support for 2-20 times before transplantation.

Artificial liver support therapy

All patients received artificial liver support treatment with Plasmaflo KM8800 (Kuraray Co. Japan). Plasma exchange (PE), hemodiafiltration (HDF) or bilirubin absorption were singly or jointly selected to treat the patients respectively[8]. On the first treatment, anconeus venous or femorali catheterization was established. Plasma exchange given was 2000mL - 4000mL, plasma transfusion or plasma substitute of 2500 mL - 4500 mL and albumin infusion of 20 g - 40 g each time. The rate of plasma seperation and the flow rate of plasma exchange were controlled at the speed of 20-30mL/ min and 60 - 100mL/min, respectively. The whole course took 3-5 hours.

Procedure of liver transplantation

The 8 patients underwent orthotopic liver transplantation under veno-venous bypass[9,10], of whom 7 underwent standard orthotopic liver transplantation[11] and 1 modified Piggyback liver transplantation[12]. The transplantation was successfully performed with a median anhepatic phase of 78 minutes and operation lasted averagely 5 hours and 30 minutes, but the 2 patients with preoperative renal failure had oliguria during operation. An average bleeding of 5600mL (2000mL-10000mL) was recorded during the operation and the blood lost was collected with Cellsaver.

RESULT

Recipient's survival

All patients became conscious soon after liver transplantation and one in stage IV coma also awoke 2 hours postoperatively. Six of 8 recipients have survived for 2-20 months with good hepatic function, of whom 3 had returned to normal work for more than 18 months. Three days after transplantation, 2 patients died of multi-organ failure (MOF), one with acute necrotic pancreatitis that was unnoticed. Acute rejection occurred in one patient who recovered after anti-rejection treatment of methylprednisolone.

Effect of artificial liver support on FHF

After treatment with artificial liver support the ascites and coagulopathy, decreased serum bilirubin declined, encephalopathy relieved and hepatic function improved (Table 1).

Table 1 Changes of hepatic function before and after ALS

Treatment	N	ALT(U/L)	AST(U/L)	TBil(μmol/L)	IBil(μmol/L)	PT(second)
Before	8	125.4+55.9	132.0+42.9	559.2+209.3	310.8+151.8	40.9+6.7
After	8	120.3+35.5	119.0+29.6	423.7+157.0	252.3+118.5	36.7+6.2
t		0.574	1.336	6.187	4.206	1.959
P		>0.50	>0.20	<0.001	<0.005	>0.05

DISCUSSION

Fulminant hepatic failure progresses rapidly with high mortality and liver transplantation has emerged as an effective therapy for whom do not responded to the standard treatment[13-16]. All patients with FHF must be considered as potential transplantation candidates[17,18]. At King's College Hospital in England, the criteria used for liver transplantation are dependent on the cause of FHF[19,20]. In patients with paracetamol-induced FHF, a pH of less than 7.3 at 24 hours or more after overdose, with concurrent presence of a serum creatinine level greater than 300 μmol/L (>3.4 mg/dL), hepatic encephalopathy of grade III or IV, and a prothrombin time greater than 100 seconds are considered indications for liver transplantation. In non-paracetamol-induced FHF, the decision is based on the occurrence of three of the following: a prothrombin time greater than 50 seconds; jaundice proceeding to encephalopathy more than 7 days; non-A, non-B hepatitis or drug-induced hepatitis; age younger than 10 years or older than 40 years; bilirubin level greater than 300 μmol/L (>17.5 mg/dL); or an isolated finding of prothrombin time of greater than 100 seconds. The indications of 8 patients in our group met with the criteria of King's College Hospital. But in China, where brain death has not been accepted as a criterion for human death, most of the patients with FHF died before the organ became available because not only of the organ donor shortage but also of the rapidity of the course. Thus, it is necessary to develop artificial liver support system as a bridge to cross over to liver transplantation[21,22]. In our study, 8 patients with FHF underwent artificial liver support, then 6 of them survived the most critical period and returned to normal life. Liver transplantation plus artificial liver support creates a new avenue for treatment of FHF.

Artificial liver support system can remove the toxic substances by way of plasma exchange[23], hemodialysis[24], hemo-infiltration[25] and absorption[26], in order to substitute the hepatic function of detoxication[27-31]. Perhaps ALS can remove the toxic substances causing encephalopathy, improve the patients' consciousness, prevent and treat multi-organ failure[32,33]. Why ALS is effective in preventing brain edema is also unelucidated, perhaps by change in permeability of the blood-brain barrier and by raising the osmolality of neural cells[34-36]. ALS, particular the hemodialysis, may prevent the brain edema[37-41]. Our experience showed that pretransplant ALS may not only help the patients to tide over critical period but also increase the chance for liver the transplantation

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• BRIEF REPORT •

Protective effects of cyclosporine A on T-cell dependent ConA-induced liver injury in Kunming mice

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INTRODUCTION

The T-cell dependent specific liver injury in mice induced by concanavalin A (ConA) is a newly established experimental liver injury model, which is considered more eligible for the study of pathophysiology of several human liver diseases, such as viral hepatitis and autoimmune hepatitis[1-9]. T cell activation and several cytokines release had been proven to play a critical role in ConA-induced liver injury[10-19]. Cyclosprine A (CsA), an effective in hibitor of activation of T lymphocyte, has been used widely in clinical treatment, especially in autoimmune diseases and organ transplantation[20-25]. In this study, we investigated the possible effect of CsA on ConA-induced liver injury in Kunming mice.

MATERIALS AND METHODS

Materials

Male Kunming mice were purchased from the animal experimental center of the Second Military Medical University, weight range 17g-21g, free access to water and food prior to the experiment. ConA and CsA were purchased from Dongfeng Ltd Shanghai and Sandoz Ltd respectively.

Methods

All the fifteen Kunming mice were divided into three groups randomly. ConA at a dose of 40mg·kg^{-1} was administered through the tail vein as a solution in pyrogen-free PBS at a volume of 300μL, which was the ConA group. CsA was injected subcutaneously twice at a dose of 130mg·kg^{-1} 15 and 1h before ConA challenge, which was used as the CsA group. PBS only in the corresponding volume served as controls.

Eight hours after ConA administration, the Kunming mice were sacrificed by cervical dislocation. Blood samples were obtained by puncture of heart with 25g·L^{-1} heparin. Liver specimen was fixed immediately in 100 mL·L^{-1} formalin/PBS for histological examination with HE stain. The degree of liver injury was assessed by determination of serum alanine aminotransferase (ALT) activity, serum TNF-α was determined by radioimmunoassay.

Statistics

The results were analyzed by Student's *t* test. The data were expressed as $\bar{x} \pm s$, and $P<0.05$ was considered to be significant.

RESULTS

ConA-induced liver injury in Kunming mice

Eight hours after ConA administration, two out of the five experimental mice were found dead in ConA only group, with elevated serum ALT 22 261 ± 2 523 nkat·L^{-1}. The concentration of serum TNF-α also increased significantly in ConA only group, increased more significantly than that of PBS only group 647±183ng·L^{-1} (Table 1).

Histological examination of liver specimen from ConA-treated mice showed diffuse cloudy swelling of the cytoplasm, spotty and necrotic foci were frequently present, severe agglutination of erythrocytes in the sinusoids of the liver were also observed. Lots of infiltrated lymphocytes in the portal area were the characteristic of this new liver injury model (Figure 1), indicating that lymphocyte may play an important role in the pathogenesis of ConA-induced liver injury, whereas no obvious tissue damage was found in the lung or kidney.

CsA protection

When pretreated with CsA (CsA group), serum ALT activity declined significantly (730 ± 266) nkat·L^{-1}, and the serum TNF-α was below the detective level (Table 1). No obvious hepatic necrosis or lymphocytes infiltration in the portal area was observed under light microscopy in the CsA group (Figure 2).

Table 1 ConA-induced liver injury in Kunming mice (n=5, $\bar{x}$±s)

	ALT- nkat·L^{-1}	TNF-α ng·L^{-1}	Dead
Con A only	22 261±2 523[b]	1230±240[b]	2
PBS only	647±183		0
CsA	730±266		0

[b]$P<0.05$, *vs* PBS only or CsA.

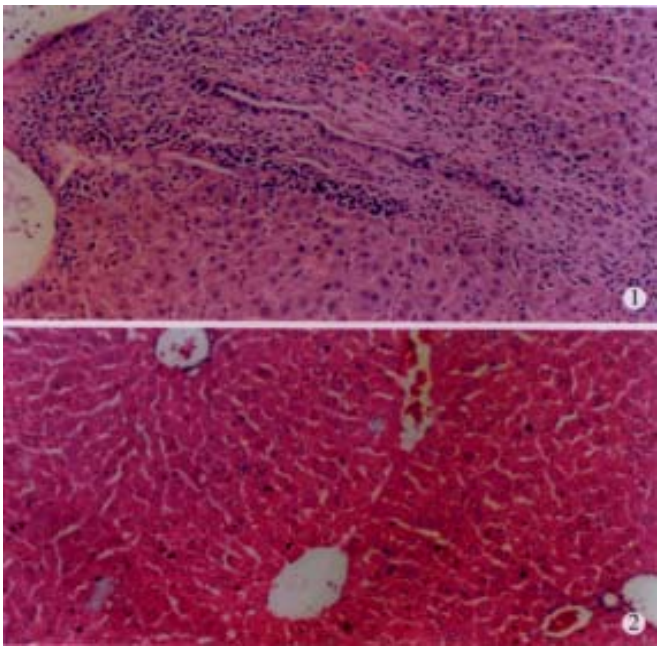

Figure 1 ConA (40 mg·kg^{-1}) induced liver injury. Hepatocyte necrosis and infiltration of lymphocytes in the portal area. HE×66
Figure 2 CsA pretreated, no obvious hepatocyte necrosis or infiltration of lymphocytes was observed. HE×66

DISCUSSION

ConA-induced specific liver injury in mice is a newly developed experimental animal model, which has been closely studied in the pathogenesis of the liver injury in recent years. T lymphocyte activation, cytokines release such as TNF-α, interferon-γ, and interleukines have been discovered to be involved in the pathogenesis of this liver injury model, especially the activation of T lymphocyte and the subsequent release of TNF-α are considered to play a much more important role in this experimental liver injury. The pathological process of ConA-induced liver injury was similar to what seen in several human liver diseases, such as viral hepatitis, and at least three types of autoimmune hepatitis. CsA is a specific inhibitor of T lymphocyte by inhibiting the transcription[21,23], and whether CsA has any protective effect on ConA-induced Kunming mice liver injury by inactivation of T lymphocytes has to be studied In our experiment, the ConA-induced specific liver injury was successfully duplicated in Kunming mice. The results (Table 1) showed that eight hours after ConA administration, the serum ALT activity was significantly increased compared with that in the control group (PBS only group). At the same time, two mice (2/5) died within eight hours. When pretreated with CsA, no death occurred, and the serum ALT level also declined significantly as compared with that of ConA group 730 ± 266 *vs* 22 261± 2 523 nkat·L^{-1}, P<0.01. The experimental results showed that CsA had potential protective effect on the ConA-induced liver injury in Kunming mice.

TNF-α has been proven to be the key cytokine in the destruction of hepatocyte in human liver diseases or liver injury animal model, such as acute and chronic viral hepatitis, especially in fulminant liver failure[26-41]. Anti-TNF antibody resulted in complete protection of ConA-induced liver injury in Balb/C mice[13]. We found that TNF-α increased significantly within eight hours when treated with ConA, but when pretreated with CsA before ConA administration, serum TNF-α became undetectable, hence the reduction of TNF-α might be due to the partial protective effects of CsA. Besides destruction of hepatocytes seen in the liver specimen of ConA group, lots of infiltrating lymphocytes in the portal area were also observed (Figure 1). When pretreated with CsA (CsA group), there was absence of lymphocytes infiltration in the portal area (Figure 2). The histological results gave a direct evidence that the protective effect of CsA in ConA-induced Kunming mice liver injury is through abrogating the activation of the T lymphocytes. The decline of serum TNF-α in CsA group may be a subsequent to T lymphocyte inactivation. $CD4^+$ lymphocyte was identified as the effector cells in the ConA-induced liver injury[3], however, in view of the fact that TNF-α synthesis is substantially higher in macrophages than in T lymphocytes, it seems likely that activated T lymphocytes might stimulate the macrophages to release TNF-α[15], but details of the concrete process worth further studies.

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• BRIEF REPORT •

Prevention of grafted liver from reperfusive injury

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INTRODUCTION

The incidence of primary non-function (PNF) of grafted liver in the early postoperative stage is 2% - 23%[1-4], its main cause is the ischemic-reperfusive injury[5,6]. In this experiment, anisodamine was added into the preserving fluid and the grafted liver was rewarmed at different temperatures to protect the cell membrane and prevent ischemic-reperfusive injury.

METERIALS AND METHODS

Selection and grouping

Twenty male Wistar rats (270g - 330g in weight, 10 - 12 weeks in age) were used in the experiment. The rats were divided into 2 groups, 10 in each group, and the action of anisodamine was studied. In the experimental group, 40mg anisodamine was added into 1 liter of preserving fluid, no anisodamine was used in the control group. The rats were divided into 4 groups, 5 in each group, and the action of rewarming was studied. Before reperfusion, the 12℃, 20℃, 28℃ and 36℃ of gelofusine were injected into the portal vein respectively to rewarm the grafted liver.

Establishment of animal model[7-9]

Make a midline epigastric incision, dissociate the liver fully, incise the infrahepatic inferior vana cava (IVC), input a shaped three way stopcock, make its upper end 5mm higher than liver, ligate the both ends of IVC incision, and obstruct hepatic artery[10]. Cut off the portal vein, connect its distal end with one opening of the three-way stopcock, shunt the portal-cava vein provisionally, and reverse the blood of IVC and portal vein to the heart through the duct. Inject proximately 5 mL saline mixed with 1 mL heparin. Ligate the suprahepatic inferior vana cava provisionally, release the ligature above the IVC incision, wash the liver through portal vein, make the preserving fluid flow outside the duct. Wash the liver at low temperature for 4 hours within the body, maintain the pressure at 90 - 100 cm H_2O, and velocity at 8-12mL/min. The preserving fluid was the lactic Linger's fluid composed of 10mL dethomaxone, 100mg ATP and 100U/L insulin. After managing the experimental factors, take out the three-way stopcock, connect with the portal vein, release the occlusion of hepatic artery, repair the IVC incision, and restore the hepatic reperfusion[11].

Collection and test of samples

Liver tissue of 500mg was resected before the obstruction of blood and reperfusion, and 30min and 60min after reperfusion respectively. Superoxide dismutase (SOD)[12,13] and lipid peroxidase (LPO) were tested[14], and the morphologic changes were observed under microscopy and electric microscopy synchronically[15,16].

Statistical analysis

Data were presented as the mean ± SE. The *t* test was applied between two groups and variance analysis between multi-groups. $P<0.05$ values were regarded as significant.

RESULTS

Effect of anisodamine on the changes of oxygen-derived radicals (Table 1)

Table 1 Effect of anisodamine on change of oxygen-derived radicals

Groups	LPO(nmol/100mg)		SOD(nu/mg pr)	
	EG(10)	CG(10)	EG(10)	CG(10)
Pre-obstruction of blood	48.50±2.53	53.80±2.19	109.70±4.23	105.00±7.33
Pre-reperfusion	61.10±5.12	72.30±2.44	100.20±5.66	97.60±6.35
30' post-reperfusion	164.40±10.55	273.30±14.61[b]	72.50±5.60	55.10±6.47[b]
60' post-reperfusion	142.40±11.35[b]	242.40±11.92[b]	61.50±6.99[b]	43.10±6.61[b]

[b]$P<0.01$ *vs* control group.

Effect of rewarming on LPO and SOD of grafted liver (Tables 2 and 3)

Table 2 Effect of rewarming on LPO of grafted liver

Temperature of rewarming	*n*	Pre-obstruction	Post-rewarming	30'post-reperfusion[b]	60'post-reperfusion
12℃	5	51.25±536	71.00±14.72	245.00±44.63	195.25±38.14
20℃	5	51.00±6.92	68.00±11.95	211.25±37.49	192.25±10.08
28℃	5	55.50±11.24	70.00±13.01	206.25±38.80	180.25±38.54
36℃[a]	5	50.00±7.22	1.75±7.55	190.50±25.34	175.50±18.65

[a]$P<0.05$ *vs* the other groups; [b]$P<0.01$ *vs* the post-rewarming group.

Table 3 Effect of rewarming on SOD of grafted liver

Temperature of rewarming	*n*	Pre-obstruction	Post-rewarming	30'post-reperfusion[b]	60'post-reperfusion
12℃	5	105.00±10.02	87.25±14.00	52.75±13.90	44.50±10.74
20℃	5	103.20±13.64	90.75±10.46	64.50±8.21	55.50±7.35
28℃	5	108.23±6.89	92.50±5.98	65.50±4.50	56.50±4.65
36℃[a]	5	112.50±8.24	90.25±9.64	72.50±10.44	64.50±10.10

[a]$P<0.05$ *vs* the other groups; [b]$P<0.01$ *vs* the post-rewarming group.

Morphologic change of liver cells

Observation under microscopy No obvious changes in HE stain between the post-rewarming groups, the hepatic tissue swelled when rewarmed at 4℃ at 30min and 60min post-reperfusion. Light red granules could be seen in the cellular plasm, no obvious changes after the rewarming at 28℃ and 36℃. At 60min post-reperfusion, the effect was better in anisodamine group than in the other groups.

Observation under electric microscopy The chrodrosome of hepatic cells swelled slightly after rewarming and the structure was roughly normal. At 30min post-reperfusion, the chrodrosomes of hepatic cells swelled, being destroyed partially and impaired in structure and the endoplasmic reticulums dilated in the 4℃, 12℃ and 20℃ rewarming groups. The injury was more serious at 60min post-reperfusion. Accasionally, the chrodrosomes swelled slightly and the ridges decreased. At 60min post-reperfusion, the chrodrosomes of hepatic cells swelled, and were impaired obviously, and the endoplasmic reticulums dilated in the non-anisodamine group. The results of anisodamine group were better evidently than the other groups. The injury of hepatic cells was the most slight in the 36℃ rewarming group.

DISCUSSION

Oxygen-derived radical and malmicrocirculation were the main causes of postoperative primary nonfunction of grafted liver[15,17]. Resent studies found that anisodamine can stabilize cell membrane and resist oxygen-derived radical[18-21], thus protecting cells from injury. Up to now, there has been no report about application of anisodamine in liver transplantation. This study deals with the protective action of anisodamine during the low temperature preserving period. The results showed that anisodamine had no obvious influence on LPO and SOD during the low temperature preserving period, yet it may reduce the production of LPO and stop the decrease of SOD after reperfusion[22]. At the time of ischemia-reperfusion, the increase of intracellular Ca^{2+} activates Ca^{2+} dependent proteinase, which can change xanthine dehydrogenase into xanthine oxidase (XOD). Rich oxygen supply accompanying with reperfusion oxidates xanthine and hypoxanthine into uric acid under the action of XOD, meanwhile produces- lots of oxygen-derived radicals[23,24]. Anisodamine is the antagonist of Ca^{2+}, it may inhibit the change of xanthine dehydrogenase into xanthine oxidase, thereby the anti-oxygen-derived radical action of anisodamine may reduce the peroxide injury of lipid of cell membrane, and relieve the reperfusive injury of grafted liver[25].

The study found that the production of LPO and decrease of SOD occurred chiefly after reperfusion. With the increase of LPO, SOD decreased gradually, indicating that SOD may antagonize LPO[26]. Pathologic observation verified that the injury of hepatic cells became more serious with the lasting of reperfusion, indicating that peroxide action of lipid caused by oxygen-derived radicals mainly occurred after reperfusion. Oxygen-derived radicals may lead to peroxide reaction of lipid, and the lipid radicals cava cause further decrease of mobility and increase of the permeability of cell membrane, swelling of the chrodrosome, release of lysosome, and serious injury of tissues[27]. We reckoned that the oxygen-derived radicals after reperfusion may damage the grafted liver, which is a chief cause of post-operative primary non-function of grafted liver.

In 36℃ rewarming group, the level of LPO was obviously lower and the activity of SOD higher than that in other groups. There was no evident morphologic change under microscopy in the 28℃ and 36℃ rewarming groups, and the change under electric microscopy was slight. It indicated that rewarming to grafted liver preserved in the low temperature fluid reduced the production of oxygen-derived radicals, and relieved the injury of grafted liver. Low temperature may decrease the activity of ATPase and the function of K^+ Na^+ and Ca^{2+} pumps in cell membrane, impair the electrolytes[28,29]. Reperfusion may lead to anomaly of Ca^{2+} and production of oxygen-derived radicals. Rewarming may improve the activity of ATPase and restore the function of pumps, therefore decreasing the intracellular concentration of Ca^{2+} and inhibiting the production of oxygen-derived radicals[30], and protecting the cells of grafted liver[31]. This study showed that morphologic change of hepatic cells was slighter in the 28℃ and 36℃ rewarming groups than in other groups. There was no significant difference between the 28℃ and 36℃ groups. Less oxygen-derived radical was produced in the 36℃ group than in other groups. Therefore, we think that it is a favorable choice for liver transplantation to apply anisodamine during the low temperature preserving period and

rewarm the grafted liver before reperfusion at 36℃[32].

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• BRIEF REPORT •

Differentially expressed genes in hepatocellular carcinoma induced by woodchuck hepatitis B virus in mice

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major causes of death in the world. The mechanism of carcinogenesis is unknown, although it is widely accepted that HBV and HCV are closely related to liver cancer[1-5]. Previously, a variety of studies have described the differences in gene expression which distinguished tumor from nontumor[6-11]. Cloning of the genes, especially the genes associated with HBV and HCV, is still very important to account for the development of liver cancer.

Traditionally, several methods were used to clone the new genes, which means to compare two population of mRNA and obtain clones of genes that expressed in one population but not in the other. Although these methods have been successful in some cases, they require many rounds of hybridization and are not well suited for the identification of rare messages. The suppression subtractive hybridization is a latest method employed in the gene cloning, which is a unique method based on selective amplification of differentially expressed sequences and overcomes technical limitation of traditional subtraction methods[12-14]. Hence, the purpose of our study is to find the differentially expressed genes in liver tumor and nontumor tissues induced by woodchuck hepatitis B virus using suppression subtractive hybridization.

MATERIALS AND METHODS

Patient samples

The tumor and nontumor tissues induced by woodchuck hepatitis B virus were obtained from Department of Pathology & Cell Biology, Thomas Jefferson University, Philadelphia, USA. The other HCC and surrounding nontumor liver tissues used for analysis were obtained from the patients who had undergone surgery for the removal of their tumors in Xijing Hospital. Fresh frozen blocks and -80℃ snap frozen paired liver and tumor samples from individual patients were collected, and were then made available for RNA extraction and *in situ* hybridization.

Total RNA and mRNA extraction

Total RNA and mRNA were extracted separately from tumor and nontumor tissues by using the Qiagen RNeasy Kit (Qiagen, Inc. Valencea, CA, USA) and the quality of extraction was determined by assaying 18S and 28S rRNA with agarose gel electrophoresis and ethidium bromide staining.

RT-PCR and adaptor ligation

The reverse transcriptase PCR was started with 2μg poly-(A) + RNA isolated from tumor and nontumor tissues. Two adaptors were ligated to the fraction of *Rsa* I digested cDNA generated by RT-PCR. The sequence of two adaptors is as follows:

Ad1: 5'-CTAATACGACTCAC-TATAGGGCTCGAGCGGCC-GCCCGGGCAGGT-3'
Ad2: 5'-TGTAGCGTGAAGACGACAGAAAGGGCGTGG-TGCGGAGGGCGGT-3'

cDNA subtraction and suppression PCR

The cDNA from tumor was referred to as tester, and the reference cDNA from nontumor as driver. The tester and driver cDNA were digested with *Rsa* I to obtain shorter, blunt-ended moleclules. The tester cDNA was then subdivided into two portions and each ligated with different cDNA adaptors. The driver cDNA had no adaptor. Two hybridizations were then performed. In the first hybridization, an excess of driver cDNA was added to each sample of tester for equalization and enrichment of differentially expressed gene. During the second hybridization, templates for PCR amplification were generated from differentially expressed sequence. The entire population of molecules was then subjected into PCR to amplify the desired differentially expressed genes. In the first PCR, only differentially expressed genes were amplified exponentially because of using suppression PCR. The second PCR was performed using nested primer which matched the sequence of adaptors to reduce the background and further enrich the differentially expressed genes.

Sequencing and GeneBank search of cloned genes

Following agarose gel electrophoresis, the unique fragments were eluted from the gels (using Qiagen gel extraction kit,

Qiagen, Inc. Valencea, CA, USA) and cloned into pT7Blue (R) T vector (Novagen, Medison, WI, USA). Positive clones were selected by blue-white phenotype. Recombinant DNAs were isolated from minipreps of individual clones, and digested by *Rsa* I to check insert size, and then both strands were individually analysed by sequence analysis in the DNA sequence facility at the Kimmel Cancer Institute of Thomas Jefferson University in USA. The sequences obtained were compared with those in GeneBank using the FASTA command in the GCG software package for homology to known genes.

In situ hybridization (ISH)

The gene fragments obtained from PCR select cDNA subtraction were used as probes for *in situ* hybridization (ISH). ISH was conducted to verify that the subtraction hybridization procedure yielded probes whose expression was different between tumor and normal tissues. ISH was carried out using the Oncor ISH and digoxigenenin/biotin detection kits according the instruction provided by the manufacturer (Oncor, Gaithersburg, MD, USA).

RESULTS

PCR selected cDNA subtraction, cloning, sequencing and GeneBank search

PCR select cDNA subtraction generated totally 14 differentially expressed genes in tumors as compared with nontumors. Among them, 8 cDNA fragments from both tumor and nontumors had considerable homology with known genes in GeneBank (Table 1). Five genes from tumor and one gene from normal liver tissues had no homology as compared to those in the GeneBank, which implied that these may be new genes. PCR select cDNA subtraction was also performed with HBV virus X gene transfected HepG2 cells and control HepG2 cells. Ten genes were differentially expressed in HepG2X compared with HepG2 cells(data not shown). Interestingly, three genes cloned from the tumor tissue of woodchuck mouse liver shared considerable homology with sequences independently found to be upregulated in HBV-X [+] cells, suggesting that the different expressions of HBxAg effector can be independently observed in the tumor and nontumor tissues induced by woodchuck hepatitis B virus.

Table 1 Differentially expressed genes in tumor and nontumor liver induced by woodchuck hepatitis B virus

Clone	GeneBank search	
	Match	% homology
Tumor[a]		
T8[b]	Human chromosome 1(UT751,L1637).	54% in 280bp overlap
T18	Unknown protein, uterine endometrium(x7723)	60% in 151bp overlap
T19	Ribosomal protein L35A(x03475)	88% in 91bp overlap
T22	Human T cell receptor beta chain(L166059)	61% in 97 bp overlap
T6	None	
T7[b]	None	
T11[b]	None	
T24	None	
T25	None	
Nontumor[a]		
N7	Human aminopeptidase N(x13276)	93% in 54 bp overlap
N10	Human IFN receptor gene (U10360)	79% in 271 bp overlap
N11	Human glutathione S-transferase(L02321)	75% in 248 bp overlap
N13	Beta-2 glycoprotein 1 from HepG2(S80305)	79% in 159bp overlap
N8	None	

[a]The clones represent fragments of genes whose expression is activated (T6,T7, T11, T18, T19, T22, T24, T25) or suppressed (N7, N8, N10, N11, N13) in HCC compared to nontumor cells.
[b]Probes whose sequences share considerable homology with sequences independently found to be upregulated in HbxAg[+] cells.

Validation and in vivo expression patterns of these genes

The cDNA fragments obtained from subtraction hybridization of tumor and nontumor tissue were then used as probes for *in situ* hybridization.In all cases, the probes from tumor showed transcripts that were preferentially expressed in tumor tissue compared with nontumor tissues. In contrast, the genes from nontumor tissue demonstrated strong hybridization in normal tissues, but little or no signal in tumor tissues.

DISCUSSION

Hepatocellular carcinoma is one of the major causes of the death in the world[15-20]. Although many researchers worked on HCC, the mechanism is still unclear[21-46]. It is widely accepted that HBV is closely associated with HCC, especially HBxAg. A common feature of HBV infection is the integration of HBV DNA, in whole or in part, into host chromatin[47-49]. The site of HBV integration is scattered throughout the host genome[50], making it unlikely that HBV brings about hepatocellular transformation by cis acting mechanisms in most cases. With regard to virus sequences, integration commonly occurs within a small region near the end of the virus genome[51], which is consistent with the hypothesis that transformation may be associated with the expression of one or more virus proteins from the integrated templates acting in trans. Integrated fragments of HBV DNA have been shown to make a truncated preS/S and or HBX polypeptides, both of which have trans-activting activities[52-56]. However only HBxAg transforms a mouse hepatocyte cell line in culture[57,58], and gives rise to liver tumors in at least one strain of transgenic mice[59-61]. Independent work has also shown that HBxAg stimulates the cell cycle, perhaps by the activation of a number of signal transduction pathways[62-66]. HBxAg is more consistently expressed than preS in the liver of infected patients. In addition, the findings that HBxAg binds to and inactivates the tumor suppressor p53 both *in vitro* and *in vivo*[67-69], and that it may bind to and alter the function of other transcriptional factors in the cells[70], implied that HBxAg

function is important to the pathogenesis of HCC. There is some evidence that HBxAg naturally trans activates the insulin-like growth factor-1 (IGF-1) receptor[71], and may also stimulate the production of IGF-11[72], both of which may help sustain the survival and/or growth of tumor cells.

Because lots of factors are involved in the development of HCC induced by HBV and the mechanism need to be further elucidated, the new genes, especially the functional genes directly related with tumor are still worth being found in the liver tissues infected by HBV. Using the newly created method, which is the suppression subtractive hybridization, we identified the difference in gene expression which distinguished tumor from nontumor induced by woodchuck hepatitis B virus. The use of these fragments as probes for *in situ* hybridization of tumor and nontumor tissues verified that the PCR-selected cDNA subtraction actually yielded differences in the gene expression that distinguished tumor from nontumor, and that its differential expression may be relevant to the pathogenesis of HCC. Because of hepatitis B virus is closely associated with the development of chronic liver diseases, such as hepatitis and cirrhosis, as well as with the development of HCC, it is not known whether these differences are associated with HBxAg associated trans-activation[73-77], its inhibition of protesome function[60], its ribo/deoxy APTase[78], or AMP kinase activation[79], and/or its ability to alter signal transduction pathways[80]. However, experiments are in progress to firmly address these issues.

The results of this study showed that the up-regulation of multiple genes in tumor had considerable homology with known products from GeneBank, suggesting that the function of these genes is likely to positively regulate cell growth, while several genes generated from normal tissues suggests that these genes may be the negative regulators for cell growth. In addition, five genes from tumor and one gene from normal liver tissues had no homology as compared with entries in GeneBank, which implied that these may be new genes, and that it is very important to clone the full-length genes of these cDNA fragments to do the functional analysis. This kind of experiments are already on the way.

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• BRIEF REPORT •

Cloning of differentially expressed genes in human hepatocellular carcinoma and nontumor liver

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INTRODUCTION

The mechanism of hepatocellular carcinoma (HCC) is still unclear, although some genes have been found to play a role in the transformation of liver cells, and a variety of studies have described differences in gene expression which distinguished tumor from nontumor[1-6]. The new genes, especially the functional genes directly related with tumor are still worth being found.

The purpose of our study is to find the different genes between human liver tumor and normal tissues using suppression subtractive hybridization.

MATERIALS AND METHODS

Patients samples

HCC and surrounding nontumor liver tissues used for analysis were obtained from the patients who had undergone surgery for the removal of their tumors in Xijing Hospital. Fresh frozen blocks and -80℃ snap frozen paired liver and tumor samples from individual patients were collected, and were then made available for RNA extraction and *in situ* hybridization.

PCR selected cDNA subtraction, cloning, sequencing and identification of cloned gene fragments

The difference in gene expression between human tumor and nontumor tissues were evaluated by a commercially available subtraction hybridization approach (the PCR selected cDNA subtraction kit from Clontech, Palo Alto, CA, USA) according to the instruction provided by the manufacturer. Briefly, we got total RNA and mRNA from tumor and nontumor tissues using the Qiagen RNeasy Kit (Qiagen, Inc. Valencea, CA, USA), and then both mRNA (2 μg each) were converted into cDNA. We refer to the cDNA from tumor as tester, and the reference cDNA from nontumor as driver. The tester and driver cDNA were digested with *Rsa* I to obtain shorter, blunt-ended moleclule. The tester cDNA was then subdivided into two portions and each ligated with different cDNA adapters. The driver cDNA had no adaptor. Two hybridization was then performed. In the first hybridization, an excess of driver cDNA was added to each sample of tester for equalization and enrichment of differentially expressed gene. During the second hybridization, templates for PCR amplification were generated from differentially expressed sequence. The entire population of molecules was then subjected into PCR to amplify the desired differentially expressed genes. In the first PCR, only differentially expressed genes were amplified exponentially because of using suppression PCR. The second PCR was performed using nested primer to reduce any background and to further enrich differentially expressed genes. The cDNA fragments were directly inserted into a T/A cloning vector(Novagen, Medison, WI, USA), and homology analysis was undertaken within GeneBank. On the other hand, we used normal tissues as the tester and tumor as the driver to do PCR select cDNA hybridization. The procedure was as above.

In situ hybridization (ISH)

The gene fragments obtained from PCR select cDNA subtraction were used as probes for *in situ* hybridization (ISH). ISH was conducted to verify that the subtraction hybridization procedure yielded probes whose expression differed in tumor compared to normal tissue. ISH was carried out using the Oncor ISH and digoxigenenin/biotin detection kits according to the instruction provided by the manufacturer (Oncor, Gaithersburg, MD, USA).

RESULTS

PCR selected cDNA subtraction, cloning, sequencing and GeneBank search

PCR select cDNA subtraction generated totally 19 differentially expressed genes in tumors and nontumors. Among them, 14 cDNA fragments had considerable homology with known genes in GeneBank (Table 1). For example, T2 and T3 had homology with ribosomal protein and elongation factor EF-1α, suggesting that these genes may stimulate cell growth. N1 from normal tissues had homology with interferon gamma gene, suggesting that this gene may be a negative regulator for cell growth. Interestingly, one gene from tumor

and three genes from normal liver tissues had no homology as compared with those in GeneBank, which implied that these may be new genes.

Validation and in vivo expression patterns of these genes

The cDNA fragments obtained from subtraction hybridization of tumor and nontumor tissues were then used as probes for *in situ* hybridization. In all cases, the probes from tumor showed transcripts that were preferentially expressed in tumor tissues as compared with nontumors. In contrast, the genes from nontumor tissues demonstrated strong hybridization in normal tissues, but little or no signal in tumor tissues.

Table 1 Differentially expressed genes in human tumor and nontumor liver

Clone	GeneBank search	
	Match	% homology
Tumor		
T1	Retinoblastoma gene (L11910)	75% in 193 bp overlap
T2	Ribosomal protein L7(L16588)	87% in 209 bp overlap
T3	Elongation factor EF-1α (J04617)	85 % in 157 bp overlap
T4	2-oxoglutarate dehydrogenase (D10525)	89% in 258 bp overlap
T5	Proteasome activator HPA28 subunit β (D45348)	93% in 204 bp overlap
T6	Ribosomal protein S2 (X57432)	89% in 195bp overlap
T7	Rab geramylgeranyl transferase-α Subunit(Y08200)	90% in 110 bp overlap
T8	Nuclear-encoded mitochondrial NADH-ubiquitinone reductase	93% in 197 bp overlap
T9	None	
Nontumor		
N1	Interferon gamma gene (L07633)	88% in 308bp overlap
N2	None	
N3	V-fos transformation effector protein	92% in 200bp overlap
N4	Sigma-1 receptor (266537)	75% in 123bp overlap
N5	Glycoprotein gll gene (D00464)- 3'flanking region	62% in 549bp overlap
N6	None	
N7	RABAPTIN-5 protein(X91141)	86% in 110bp overlap
N8	Dishevelled-3 (DUL3) protein	89% in 72bp overlap
N9	None	
N10	None	

DISCUSSION

Hepatocellular carcinoma is one of the major causes of death in the world[7-10]. The mechanism of carcinogenesis is unknown, although it is widely accepted that hepatitis B virus (HBV) and hepatitis C virus (HCV) are closely related to liver cancer, especially hepatitis B virus X antigen[11-14]. A common feature of HBV infection is the integration of HBV DNA, in whole or in part, into host chromatin[15-17]. The sites of HBV integration are scattered throughout the host genome[18], making it unlikely that HBV brings about hepatocellular transformation by cis-acting mechanisms in most cases. With regard to virus sequences, integration commonly occurs within a small region near the end of the virus genome[19], which is consistent with the hypothesis that transformation may be associated with the expression of one or more virus proteins from the integrated templates acting in *trans*. Integrated fragments of HBV DNA have been shown to make a truncated preS/S and or HBX polypeptides, both of which have trans-activting activities[20-24]. However, only HBxAg transforms a mouse hepatocyte cell line in culture[25,26], and gives rise to liver tumors in at least one strain of transgenic mice[27-29]. Independent work has also shown that HBxAg stimulates the cell cycle, perhaps by the activation of a number of signal transduction pathways[30-34]. The expression of HBxAg is more consistent than that of preS in the liver of infected patients. In addition, the findings that HBxAg binds to and inactivates the tumor suppressor p53 both *in vitro* and *in vivo*[35-37], and that it may bind to and alter the function of other transcriptional factors in the cells[38], implied that HBxAg function is important to the pathogenesis of HCC. There is some evidence that HBxAg naturally trans-activates the insulin-like growth factor-1 (IGF-1) receptor[39], and may also stimulate the production of IGF-11[40], both of which may help sustain the survival and/or growth of tumor cells.

Because the mechanism of HCC induced by HBV still need to be elucidated, cloning of the genes, especially the genes associated with HBV and HCV, is still very important to account for the development of liver cancer. By the newly created method, which is the suppression subtractive hybridization, we identified the difference in gene expression which distinguished tumor from nontumor. The use of these fragments as probes for *in situ* hybridization of tumor and nontumor tissues verified that the PCR-selected cDNA subtraction actually yielded differences in the gene expression that distinguished tumor from nontumor, and that its differential expression may be relevant to the pathogenesis of HCC. It is not known whether these differences are associated with HBxAg associated trans-activation[41,42], its inhibition of protesome function[43] its ribo/deoxy APTase[44], or AMP kinase activation[45], and/or its ability to alter signal transduction pathways[46], because hepatitis B virus is closely associated with the development of chronic liver diseases, such as hepatitis and cirrhosis, as well as with the development of hepatocellular carcinoma (HCC)[47-60]. However, experiments are in progress to firmly address these issues.

The results of this study showed that the up-regulation of multiple genes in tumor which have considerable homology with known products from GeneBank, for example, ribasomal protein and elongation factor EF-12, suggesting that the

function of these genes is likely to positively regulate cell growth. Several genes are generated from normal tissues and one has >88% homology with interferon gamma gene, suggesting that these genes may be the negative regulators for cell growth. In addition, one gene from tumor and three genes from normal liver tissues had no homology, as compared with entries in GeneBank, which implied that these may be new genes, and that it is very important to clone the full-length genes of these cDNA fragments to do the functional analysis. This kind of experiments are already on the way.

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• BRIEF REPORT •

Identification of the epitopes on HCV core protein recognized by HLA-A2 restricted cytotoxic T lymphocytes

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Abstract

AIM **To identify hepatitis C virus(HCV) core protein epitopes recognized by HLA-A2 restricted cytotoxic T lymphocyte (CTL).**

METHODS **Utilizing the method of computer prediction followed by a 4h ^{51}Cr release assay confirmation.**

RESULTS **The results showed that peripheral blood mononuclear cells (PBMC) obtained from two HLA-A2 positive donors who were infected with HCV could lyse autologous target cells labeled with peptide "ALAHGVRAL (core 150-158)". The rates of specific lysis of the cells from the two donors were 37.5% and 15.8%, respectively. Blocking of the CTL response with anti-CD4 mAb caused no significant decrease of the specific lysis. But blocking of CTL response with anti-CD8 mAb could abolish the lysis.**

CONCLUSION **The peptide (core 150-158) is the candidate epitope recognized by HLA- A2 restricted CTL.**

Subject headings hepatitis C virus; cytotoxic T lymphocyte; HLA-A2; epitope

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INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus responsible for the majority non-A non-B hepatitis[1,2]. More than 50% - 60% of acute infection lead to chronic disease, and once chronicity is established, spontaneous recovery is exceptional. The related mechanism is still unknown[3-5]. Recent studies demonstrate that major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTL) of patients with chronic hepatitis C recognize epitopes from different regions of both structural and nonstructural HCV proteins[6-12]. Some scholars speculate that CTL-mediated cellular immune response probably plays an important role in viral clearance[13,14].

$CD8^+$ CTL interact through their polymorphic T cell receptor with HLA class I molecules containing endogenously synthesized peptides of 9-11 on the surface of infected cells. The presence of allele-specific amino acid motifs has been demonstrated by sequencing of peptides eluted from MHC molecules. Among the best studied motifs is that of HLA-A2, which is prevalent in a high percentage of population. Several reports[7,8,15-20] described the method of using HCV derived synthetic peptides containing the HLA-A2.1 binding motif to identify and characterize the HLA-A2 restricted CTL in the peripheral blood of patients with chronic HCV infection. We[21] have designed a computer programme to score the reported HCV peptides. Our results revealed that all the reported peptides were with a relative high score of 144 points or higher. Based on the previous study, we attempted to identify the epitopes recognized by the HLA-A2 restricted CTL on HCV core protein utilizing the method of computer prediction followed by 4h ^{51}Cr release assay.

MATERIALS AND METHODS

Materials

Subjects Six patients with chronic hepatitis C and 2 healthy controls were selected from among those monitored at Xijing blood center. Table 1 summarizes patient characteristics and history of treatment. All subjects had not received any antiviral treatment for at least one year.

Table 1 HLA-A and serology of patients studied for CTL response to HCV epitopes

Subjects	HLA-A	Anti-HCV	HCV-RNA
Experiment			
Li	A2A31	+	+
Zhang	A2A11	+	+
Tang	A2A33	+	+
Zhang	A2A11	+	+
Patient control			
Li	A11	+	+
+	A3A33	+	
Health control			
Zheng	A2A11	-	-
Wang	A2A24	-	-

Note: All subjects received no treatment and had been followed-up for one year.

HLA typing HLA typing of PBMC from patients and from normal donors was determined by microcytotoxicity, using trays (One lamda, Canoga Park, CA). The HLA haplotypes of subjects participating in this study are shown in Table 1.

Merthods

Prediction of candidate HLA-A2 restricted CTL epitopes Based on previous study, we use our computer programme to predict HLA-A2 restricted CTL on HCV C protein. In brief, a

computer programme with the function of finding peptides containing HLA-A2 allele-specific peptide motif was written in C language. The HCV cDNA is translated into HCV amino acid sequence from which the peptides was chosen, and the selected peptides include those with a length of 9-11 amino acids, a leucine (L), isoleucine (I) or methionine (M) at position 2 and a leucine (L) or Valine (V) at the last position. According to Nijiman's score system, we scored six points for an anchor residue, four points for a strong and two points for a weak residue. The score for a given peptide is obtained by multiplication of the scores for each amino acid position. Predicted candidate CTL epitopes with scores of 144 or higher.

Synthetic peptides Peptides YLLPRRGPRL (core35-44), NLGKVIDTL (core 118 - 126), DLMGYIPLV (core 132 - 140) and ALAHGVRAL (core 150 - 158) were selected from the predicted results and synthesized in automated multiple peptide synthesizer (American Research Genetics, Inc). All peptides were >90% pure and diluted to $1g \cdot L^{-1}$ with RPMI1640 medium before use (Gibco, Grand Ialand, N.Y.).

CTL generation PBMC from donors were separated on Ficoll-Hypaque density gradients (Shanghai Huajing, Inc), washed three times in phosphate-buffered saline (PBS), resuspended in RPMI1640 medium (Gibco, BRL.) supplemented with L-glutamine ($10g \cdot L^{-1}$), penicillin ($5 \times 10^4 U \cdot L^{-1}$), streptomycin ($50mg \cdot L^{-1}$) and Hepes ($5mol \cdot L^{-1}$) containing $100mL \cdot L^{-1}$ fetal calf serum (FCS) and plated in 24-well plates at 4×10^6 cells per well. PBMC were stimulated with concanavalin A (ConA, 20μg per well) during the first week. On d3, 1mL of complete medium supplemented with rIL-2 at 2×10^3 $U \cdot L^{-1}$ final concentration was added into each well. On d7, the cultures were restimulated with the peptides plus rIL-2 and irradiated (30Gy) autologous PBMC feeder cells, and the cultured PBMC were restimulated five days later with the original peptides plus rIL-2. On d16, the stimulated cells were used as effectors in CTL assay.

Preparation of autoguous B lymphoblastoid cell line After Ficoll-Hypaque separation, PBMC were suspended in the RPMI1640 medium containing $200 mL \cdot L^{-1}$ FCS and then plated in 24-well culture plate at a concentration of 2×10^6 cells per well. EBV-transfected B cell lines were established by culturing 2×10^6 PBMC with $100g \cdot L^{-1}$ of cyclosporin A and 1 mL B95-8 EBV culture supernatant (provided by Dr. Jin, the Fourth Military Medical University, Xi'an). After transformation, the lymphoblastoid cell lines (B-LCL) were maintained in RPMI1640 medium with $200 mL \cdot L^{-1}$ FCS, with media change twice each week. The cell lines were maintained at 37℃ in a humidified chamber with $50 mL \cdot L^{-1}$ CO_2 and used as targets.

CTL assay Target cells were incubated overnight with synthetic peptides at $200 mg \cdot L^{-1}$, and then were labeled with 3.7MBq 1-Cr for 1h and washed three times with PBS. Cytotoxity activity was determined in a standard 4h Cr release assay using U -bottom 96 welL plates containing 5000 autogenous targets per well. All assays were performed in triplicate with effector target cell (E/T) ratios of 100:1, 50: 1, and 1:1. Maximum release was determined on the basis of lysis of labeled target cells with $50g \cdot L^{-1}$ Triton X-100. We examined spontaneous release by incubating target cells in the absence of effector cells. It was less than 25% of the maximum release. Percent cytotoixity was determined by the formula: 100×[(experiment release-spontaneous release)/ (maximum release-spontaneous release)].

Blocking of CTL response by antibodies CTL responses were tested in the presence of anti-CD8 or anti-CD4 monoclonal antibody added to the 96-well plates at the indicated concentrations used for the CTL assay.

RESULTS

Prediction of HLA-A2 restricted CTL epitopes on HCV protein

Seven high-scoring peptides (≥144 points) were selected from HCV C protein using our computer programme. Among them, peptide ① and peptide ④, namely peptide YLLPRRGPRL and peptide DLMGYIPLV, have been reported to be epitopes recognized by HLA-A2.1 restricted CTL. Predicted peptide ⑦, namely FLLALLSCL (core 177-185) was almost the same as the reported peptide LLALLSCLTV (core 178-187). The rest predicted peptides have not been proved to be epitopes recognized by HLA-A2 restricted CTL. Four peptides (peptide ①,②,④,⑤), were selected randomly from the seven predicted peptides to be used in CTL assay (Table 2).

Table 2 Predicted results of epitopes recognized by HLA-A2 restricted CTL on HCV C protein

No	Peptide sequence	Peptide site	Score
①	YLLPRRGPRL	35-44	144
②	NLGKVIDTL	118-126	576
③	TLTCGFADL	125-133	144
④	DLMGYIPLV	132-140	576
⑤	ALAHGVRAL	150-158	576
⑥	NLPGCSFSIFL	168-176	288
⑦	FLLALLSCL	177-185	144

A: Reported epitopes recognized by HLA-A2.1 restricted CTL; B: Almost consistent with the reported epitope LLALLSCLTV (core 178 -187)

Screen of HCV peptide-specific response

In this experiment PBMC from 8 subjects were stimulated individually with the four peptides from HCV C protein, and cultures were tested after 16d of expansion for peptide-specific CTL activity. Two of the four donors of HLA-A2 and HCV RNA positive responded to peptide ⑤. After induced by peptide ⑤, the two donors' PBMC can lyse autologous target cells pulsed with peptide ⑤ and the specific lysis was 37.5% and 15.8%, respectively (Table 3). Treatment of the CTL specific for peptide ⑤ with anti-CD8 mAb, but not anti-CD4 mAb, plue complement markedly reduced cytotoxic activity on target cells (Figure 1).

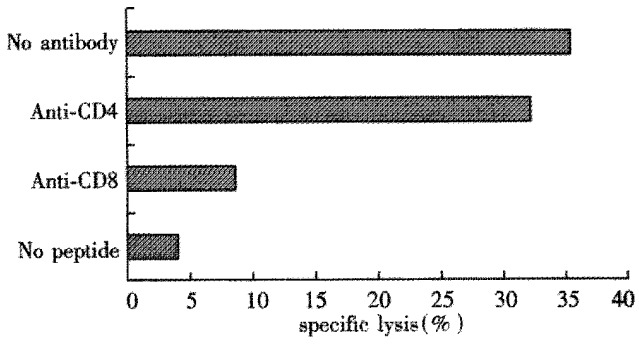

Figure 1 Blocking of CTL activity of Mr.Tang with mAb.

Table 3 CTL activity induced by predicted peptides on HCV C protein

Subject	HLA type				^{51}Cr-release (%lysis)			
	A		B		①	②	④	⑤
Experiment								
Li	2	31	51	16	-2.6	9.6	0.5	NT
Zhang	2	11	8	27	0.4	NT	2.4	6.9
Tang	2	33	44	55	-3.8	5.1	3.3	37.5
Zhang	2	11	62	35	-1.1	9.8	8.4	12.2
Healthy control								
Zheng	2	11	62	39	-2.9	8.0	6.8	-0.2
Wang	2	24	61	46	0.2	NT	3.7	10.0
Patient control								
Li	11		62	35	-2.6	2.6	2.3	-1.8
Zhu	3	33	17	35	-2.5	1.6	0	2.5

CTL activity induced by peptides at E/T ratio of 50:1; NT: No test; ①, ②, ④ and ⑤ represent YLLPRRGPRL (core35 - 44), NLGKVIDTL (core118-126), DLMGYIPLV (core132-140) and ALAHGVRAL (core150-158), respectively.

DISCUSSION

CTL mediated cellular immune response probably plays an important role in anti HCV infection. Many researchers reported[22-30] that CTL specific for HCV were discovered in PBMC and liver infiltrated lymphocytes of patients infected with HCV, and that the epitopes recognized by CTL were identified. Owing to the fact that HLA-A2 exhibits a high gene frequency in populations, Cerny *et al*[7] focused their study on the epitopes recognized by HLA-A3 restricted CTL and have determined several epitopes on every protein region recognized by CTL. However, because of the great work and high cost, it is quite difficult to manipulate in general laboratory. In this study, we attempted to identify the HCV peptides containing HLA-A2 binding motifs, and to confirm the prediction via 4h ^{51}Cr release assay.

Prediction of candidate epitopes recognized by HLA-A2 restricted CTL

According to the reference[31-32], determining epitopes recognized by CTL included two steps: synthesis of many peptides with multi-peptide overlapping method, and identification of the peptides with experimental means. Up till now, peptides YLLPRRGPRLL (core35 - 44), DLMGYIPLV (core132 - 140), and LLALLSCLTV (core178 -187) were determined by using this method. The method is direct and reliable, but difficult to manipulate. We analyzed HCV core protein using computer programme. The results demonstrated that there were only 7 peptides with scores of 144 or higher. Of those[21], two peptides, YLLPRRGPRL (core35 - 44), DLMGYIPLV (core132 - 140) were ever reported in other studies, which could be recognized by HLA-A2 restricted CTL. Another peptide, FLLALLSCL (core177 -185) was in agreement with the reported peptide LLALLSCLTV (core178 - 187). Purposeful study of the 7 peptides would simplify experimental processes and save cost. Of those, 4 peptides (Nos ①,②,④,⑤), were synthesized and applied in CTL assay.

Activation of CTL in peripheral blood of donors by synthsized peptides

Four synthesized peptides of the HCV core protein were tested using CTL assay. Four donors were positive for HLA-A2. Among donors positive for HCV RNA, 2 donors' PBMC were found to have lysed autologous target cell-labeled with peptide ⑤. The specific lysis rate was 37.5% and 15.8% respectively. The other 3 peptides didn't show obvious CTL induction action. CTL response was very weak in two healthy and HLA-A2 positive donors, and also in two HCV RNA+ HLA-A2- donors.

According to the reference[33], the lysis might be considered specific with the lysis rate $\geqslant$15%. The specific lysis rate was up to 37.5% in Tang with effector / target cell (E/T) ratio of 50:1, and 15.8% in Zhang with E/T ratio of 100:1. Blocking of the CTL response with anti-CD4 mAb did not decrease the specific lysis significantly. But blocking of the CTL response with anti-CD8 mAb could abolish the lysis. It indicated that[34-40] the lysis was mediated by CD8$^+$ T cells rather than CD4$^+$ T cells, and that the epitope ⑤ was probably the candidate epitope recognized by HLA-A2 restricted CTL.

Although 3 peptides, including 2 reported in other studies, YLLPRRGPRL (core35 - 44), DLMGYIPLV (core132-140), didn't demonstrate obvious CTL induced activity, we could not draw a conclusion that they were not HLA-A2 restricted CTL recognized epitopes. The two reported epitopes were recognized by HLA-A2.1 restricted CTL, but in this study, we did not determine the HLA-A2 subtypes. Various subtypes of HLA-A2 restricted CTL probably recognized different epitopes[41-50]. Another pssible reason is that HCV protein sequence of HCV-infected patients might not be in complete accordance with the synthesized peptides. To clarify the reasons, further study is still necessary.

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• BRIEF REPORT •

Treatment of malignant digestive tract obstruction by combined intraluminal stent installation and intra-arterial drug infusion

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Abstract

AIM **To study the palliative treatment of malignant obstrution of digestive tract with placement of intraluminal stent combined with intra-arterial infusion of chemotherapeutic drugs.**

METHODS **A total of** 281 **cases of digestive tract malignant obstruction were given per oral** (**esophagus, stomach, duodenum and jejunum**), **per anal** (**colon and rectum**) **and percutaneous transhepatic** (**biliary**) **installation of metallic stent. Among them,** 203 **cases received drug infusion by cannulation of tumor supplying artery with Seldinger's technique.**

RESULTS **Altogether** 350 **stents were installed in** 281 **cases, obstructive symptoms were relieved or ameliorated after installation. Occurrence of restenotic obstruction was** 8-43 **weeks among those with intra-arterial drug infusion, which was later than** 4-26 **weeks in the group with only stent installation. The average survival time of the former group was** 43 (3-105) **weeks, which was significantly longer than** 13 (3-24) **weeks of the latter group.**

CONCLUSION **Intraluminal placement of stent combined with intra-arterial infusion chemotherapy is one of the effective palliative therapies for malignant obstruction of the digestive tract with symptomatic as well as etiological treatment.**

Subject headings digestive tract disease; treatment; stent; therapeutic embolization chemotherapy; infusion, local

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INTRODUCTION

Intraluminal installation of stent in digestive tract is being more and more widely used[1-31]. Drug infusion via supplying artery and embolization therapy have been used in clinical practice as an effective way of suppressing the growth of malignant tumors[32-43] But up to now there have been few reports of the combined use of the two in the treatment of malignant stenotic lesions[44,45]. We have used the combined therapy in cases of malignant stenosis of digestive tract and have obtained good results in relieving symptoms, improving quality of life and prolongation of survival time. We managed 281 cases from October 1996 to May 2000 and the therapeutic results are reported below.

MATERIAL AND METHODS

Patients

A total of 281 patients with malignant obstruction of digestive tract were treated. Among them, 182 were males and 99 females, age ranged from 20 to 93 years, with an average of 65 years. The cause of obstruction was stenosis or obstruction of the digestive tract by infiltration or pressure of the malignant tumor. All cases belonged to late stages of tumor, impossible for surgical resection. One hundred and seventy-seven cases had operative histories of primary tumor resection, with reconstruction of digestive tract, biliary endostomy, bypass drainage or laparatomy, etc, 6 had histories of four operations, 83 cases of one operation . The primary disease included carcinoma of esophagus, 79 cases; stomach cancer 55 cases; carcinoma of liver, 25 cases; carcinoma of pancreas, 53 cases; colorectal cancer, 39 cases; cholangiocarcinoma, 10 cases; and other carcinomas, 20 cases. Pathological types: adenocarcinoma, 96 cases; squamous cell carcinoma, 74 cases; and others, 111 cases. Among the 281 cases, site of stenosis and obstruction at esophagus, 119 cases; at stomach (including gastrointestinal stoma), duodenum and jejunum, 76 cases; colorectal segment, 28 cases; and biliary tract, 58 cases. Some cases had more than two sites of stenosis or obstruction. They were divided in to groups A and B by patient or close relative's choice. Group A (203 cases) received treatment by installation of intraluminal stent combined with intra-arterial cannulation for drug infusion via supplying artery. Group B (78 cases) received only installation of intraluminal stent and again according to their choice supplemented with intravenous chemotherapy, radiotherapy or traditional Chinese medicinal treatment. Those with impaired liver and /or kidney function due to the cancer, or very poor general condition not suitable for intravenous chemotherapy and radiotherapy were advised to join group A. In group A, there were 132 males and 71 females, aged 20 - 93 years, averaging 61 years. Of them, 82 cases could not meet the requirements of routine radiotherapy and chemotherapy such as blood routine, liver and kidney

function or abnormalities of some other tests. In group B, there were 50 males and 28 females, age ranged from 28 - 87 years, averaging 58 years. Fourteen cases could not meet the requirements of routine radiotherapy and chemotherapy. Karnofsky quality of life evaluation in group A was 10-80 (average 36.2) score and 20 - 80 (average 40.2) score in group B.

Metallic intraluminal stent

Woven type nickel-titanium alloy stent was mainly used. The tubular meshwork, the whole some structure somewhat like wallstent was woven by single fine threads of nickel-titanium alloy of 0.20mm-0.32mm in diameter. Membranous stent was coated with polyurethane or silica gel on the mesh of the stent main body. For biliary tract, straight-neted duct stent was used without trumpet ends. The diameter of the tube is 0.8cm - 1.6cm, and the length 6cm - 10cm. The stent used for esophagus, stomach, duodenum and jejunum is of the type with trumpet opening on one end or both ends. The proximal end is basin-trumpet in shape, and the distal end cup or mushroom in shape. The tubular diameter of the stent main body is 1.1 cm - 1.5 cm at cervical segment, 1.8 cm - 2.8 cm at thoracic and abdominal segment of the esophagus. At stomach body, it is 2.0cm-2.5cm, and at duodenum and jejunum 1.8 cm - 2.0 cm; the length of the stent is 3.5 cm - 14cm. The stent for the use of colon and rectum has both ends of ball or mushroom shape trumpet opening. The tubular diameter of the stent main body is 2.0cm - 2.2cm at transverse colon, 2.5cm - 2.8cm at descending colon, and 2.5cm - 3.2cm at sigmoid colon and rectum; the length of the stent is 8cm-14cm. The width of the trumpet opening of all stents is 0.4cm-0.7cm. Some stents are specially made for specific requirements. Other stents we have used are home made"Z" shape esophageal stent, Wallstent for biliary and esophageal use (Schineder) and Memotherm biliary stent (Agmidel).

Transporter and auxilliary instruments

Wallstent and Memotherm stents were provided with a disposable coaxial two duct type transporter. Home-made "Z" shape stent was fixed with a sheath tube type pusher. Home made biliary and esophageal stents for cervical segment were used together with a sheath tube type pusher of Cook Company. For other stents we used self-made posterior positioned coaxial three ducts type transporter. Other auxilliary instruments included 6F Corber transmitting catheter, 2600mm long Torumo Radifocus guide wire, 2600mm-3000mm long soft head super-hard guide wire, 1300mm long exchange catheter, bulb dilating catheter of 25mm in diameter and double-channel imaging catheter made from used bulb catheters. Gastroscope, colonoscope, microwave or diathermic apparatus were also available.

Treatment modalities

Installation of intraluminal stent. Stent was placed perorally to esophagus, stomach, duodenum and jejunum. Radiopaque guide wire was inserted via gastroscope or perorally. The pusher attached with the stent was introduced by the guide wire so that the stent could be slowly released after passing through the stenosed segment. For the installation of stent in duodenum and jejunum, super-hard guide wire was introduced via exchange catheter, and the stent was put by the pusher introduced by the hard guide wire. Biliary stent was installed by percutaneous puncture and insertion method. The puncture needle was inserted percutaneously and transhepatically into biliary tract. Radifocus guide wire was introduced through the puncture needle along the intrahepatic biliary tract into the common bile duct, and then through the stenosed segment deep into the small intestine. Through exchange soft head hard guide wire, sheath tube or coaxial two ducts type transporter put the stent into the stenosed segment. Colonic and rectal stent was inserted through anus. Radifocus guide wire was inserted through the stenosed segment by the catheter or via colonoscope. Exchange catheter was introduced and replaced the super-hard guide wire. The hard guide wire introduced the coaxial two ducts type transporter that releases the stent.

Intra-arterial drug infusion

Two to eight days before the installation of the stent or 0d-4d after it, chemotherapeutic infusion via cannulation of the supplying artery of the tumor was given. The Seldinger's technique was used. According to the primary lesion or the infiltration field of the metastatic site, the supplying artery of the tumor was chosen as the target artery. Generally, the main trunk of the supplying artery was chosen for cannulation or multiple target points were chosen for cannulation at one time. The target arteries chosen for cannulation are: external jugular artery, inferior thyroid artery, or subclavicular artery bronchial artery, esophageal propriae artery, intercostal artery, hepatic artery (or coeliac artery trunk), superior mesenteric artery, inferior mesenteric artery, internal iliac artery, etc. The perfusing drugs used with dosage calculated according to surface area of the body were epirubicin 25mg·m^2, carboplatin 200mg·m^2, 5-Fu 500mg·m^2 forming triad drug group for use. For patients with abnormal liver and /or kidney functions (all caused by the malignant factors), dosage will be decreased accordingly. For those patients having normal routine profiles with better general condition, VM26 50-100mg·m^2 was added. For those with impaired cardiac function, adriamycin was substituted by pirarubicin 300mg·m^2. For some suitable patients emulsified 40% lipiodol and chemotherapeutics were given for superselective emolization. Patients with arterial venous fistula were first given gelfoam strips to block the fistular tract and then drug infusion. The second treatment was administered three weeks afterwards, and the third treatment was given after another 3 -5 weeks. From then on, the interval could be prolonged to 1.5-5 months.

RESULTS

Installation of stent (Table 1)

A total of 350 stents were installed in 281cases. Among them 144 were esophageal stents (23 in cervical segment, 69 in thoracic segment and 52 in abdominal segment of esophagus). Ninety-eight stents were put in stomach duodenum and jejunum (21 in gastric body and pyloric region, 22 in gastrointestinal anastomotic oriface, 49 in descending and horizontal part of duodenum and 6 in proximal jejunum); 30 in colon and rectum (9 in transverse colon, 5 in descending and 16 in sigmoid colon and rectum). Seventy-eight were biliary stents. Among the 144 esophageal stents, 142 were home-made woven type tubular mesh stents (127 with attached membrane and 15 without membrane), one was home-made "Z" type stent with attached membrane. There

was one wallstent without membrane. Among 98 stents used in stomach, duodenum and jejunum, 97 were home made (35 with attached membrane, 62 without). One Wallstent without membrane was used. Thirty stents used in colon and rectom (20 with membrane and 10 without) and 71 of 78 biliary stents were all home made. There were 4 Wallstents and 3 Memotherm stents. Biliary stents were all without membrane. The distribution of stents installed in groups A and B are shown in Table 1.

Drug infusion (Table 2)

Group A (203 cases) had received cannulation of supplying artery with infusion of chemotherapeutics (a total of 708 times). The minimum was one time (9 cases) and maximum was 14 times (one case). The average time was 3.49. Table 2 shows different patients undergoing drug infusion. Among the nine cases receiving treatment only once, which was refused by their relatives in 5 cases, and discontinued due to deterioration of their general condition in 2 cases, and the remaining 2 cases died. Three out of the 203 cases had received lipiodol emulsion embolization for 7 times, 11 cases had gelfoam embolization for 18 times. Besides occational hematoma at the puncture site, few cases complained of pain at the site of drug infusion and embolization treatment and 37% of cases had reversible lowering of blood counts, and no other arterial interventional procedure related complication was noted.

Clinical symptomatic impro ement

Among 119 cases with installation of esophageal stent, 18 had stent in the cervical segment and they all could take low residue regular diet. In 101 cases with stent in the thoracicor abdominal segment, those with simple stenosis basically restored to normal meals. Among 13 patients with esophagotracheal or esophagothoracic fistula, 11 could take normal meals after complete obliteration of the fistular tract, one still had some irritating cough while eating. Another case was a patient who developed esophageal stenosis with esophagothoracic fistula after unilateral pneumonectomy. Four days after installation of stent and obliteration of the fistula, the patient developed obvious irritating cough during eating. Imaging demonstrated proximal orifice of the stent protruded into the thoracic cavity forming another fistular tract. Among 76 patients with stent in stomach, duodenum or jejunum, 71 restored to normal meals and 5 had the obstructive symptoms improved, but could only take liquid diet. Twenty-eight cases with stent in colon or rectum had immediate relief of intestinal obstructive symptoms after installation, one of them who had colonic pelvic fistula had no more exudative leakage from the fistula tract after the installation. A case of rectal vaginal fistula had much less fecal exudation from vagina after installation. In 58 cases with installation of stent into the biliary tract, jaundice was significantly decreased, 39 cases had serum bilirubin returned to normal within a week. Nineteen cases had serum bilirubin dropped below 70mmol/L. Clinical symptomatic improvement were similar between groups A and B shortly after treatment. Among 82 cases in group A whose conditions could not reach the requirements of laboratory profiles for routine radiotherapy or chemotherapy, 46 cases fulfilled the requirements after two drug infusions. Among 14 cases of group B, only 3 cases approached the requirements after 1-2 months. As the disease progressed, the number of patients whose original profiles were normal but became abnormal when the disease got worse, was significantly larger in group B than in group A. Comparison of Karnofsky life quality evaluation between the two groups at 4 - 6 week after installation of stent showed that the average score in group A increased from 36.2 to 53.2, while in group B, from 40.2 to 46.2. There was significant difference between the two groups.

Maintenance of efficacy and survival time

In group B, 23 restenotic obstructions occurred 4-26 weeks after treatment in 15 (23.1%) cases. In group A, 29 (14.3%) had 38 restenotic obstructions 8-43 weeks after treatment (Table 3). The survival time in group A was 3-105 weeks, averaging 43 weeks, while 3-24 weeks in group B averaging only 13 weeks. The survival time of group A was more significantly prolonged than group B (Table 4).

Table 1 Installation of stents

Site	Group A		Group B		Total	
	No.of cases	No.of stents	No.of cases	No.of stents	No.of cases	No.of stents
Esophagus	81	95	38	49	119	144
Stomach, small intestine	55	64	21	34	76	98
Colon, rectum	19	20	9	10	28	30
Biliary tract	48	53	10	25	58	78
Total	203	233	78	116	281	350

Table 2 Drug infusion

Site	*N*	Infusion (*n*)	Total infusion times	Max. per case	Min. per case	Average per case
Esophagus	119	81	310	12	2	3.83
Stomach, small intestine	76	55	229	14	1	4.16
Colon, rectum	28	19	39	8	1	2.94
Biliary tract	58	48	130	9	2	3.65
Total	281	203	708			3.49

Table 3 Maintenance of efficacy (groups A / B)

Site	*n*	Restenosis		Restenosis (wk)
		n	No.of times	
Esophagus	81 / 38	15 / 10	20 / 12	12-35 / 04-17
Stomach, small intestine	55 / 21	06 / 04	07 / 05	17-42 / 07-16
Colon, rectum	19 / 09	02 / 00	02 / 00	26-43 / 0
Biliary tract	48 / 10	06 / 04	09 / 06	8-33 / 05-26
Total	203 / 78	29(14.3) / 15(23.1)	38 / 23	08-43 / 04-26

Table 4 Comparison of survival time (groups A / B)

Sites	Cases	Survival (*n*)	Death (*n*)	Survival (wk)	
				Range	Average
Esophagus	81 / 38	23 / 03	58 / 35	5-103 / 5-20	36 / 14
Stomach, small intestine	55 / 21	09 / 02	46 / 19	4-105 / 2-17	38 / 11
Colon, rectom	19 / 09	02 / 00	17 / 09	3-98 / 3-13	33 / 08
Biliary tract	48 / 10	06 / 01	42 / 09	6-54 / 3-24	30 / 18
Total	203 / 78	40 / 06	163 / 72	3-105 / 3-24	43 / 13

$P<0.05$, for all sites.

DISCUSSION

Significance and technical difficulties

Digestive tract is the necessary passage of alimentation digestion, absorption and excretion of waste metabolites. Obstruction of digestive tract interferes with food intake, bowel movement, or causes obstructive jaundice. All these seriously affect the quality of life of the patients and even accelerate death. In 1983, Frimberger first reported the use of metallic stent to treat esophageal stenosis[46]. Domschke in 1990 successfully used self-expansible woven mesh type metallic stent to treat a case of esophageal malignant obstruction[47]. Thus it was made possible the relief of obstruction of digestive tract by non-surgical procedures. Karnel *et al*[48], Goldrn *et al*[49], Keymling *et al*[50] and others had respectively tried to use metallic intraluminal stent in treating colonic, biliary and duodenal obstruction. Their success had laid the basis of the expanded use of digestive tract intraluminal stent. As intraluminal installation of stent in digestive tract is a non-surgical method with mini-invasive technique to render the stenosed digestive tract becoming patent again, thus providing a new approach in the palliative treatment of digestive tract malignant obstruction. Comparing with the conventional surgical operation, treatment by intraluminal installation of stent has the characteristics of mini-invasion, fast, effect, good clinical sutclins and repeatablity of the procedure. This avoids the damage caused by surgical operation. For those with no indication for operation or too weak to stand the operation, treatment with intraluminal installation of stent can provide palliation to the patients with symptomatic relief and improved quality of life.

Compared with vascular lumen and other non-vascular lumens, the various lumens at different parts of the digestive tract have special histologic structures and functional characteristics. For example, the cervical segment of esophagus has strong contractive force and is very sensitive to foreign body. Duodenum and jejunum are quite distant from mouth, their lumens are tortuous with frequent peristalsis. Colon has a haustral structure with a strong group contractile force. The peculiar tissue structure and functional characteristics make the procedure of installation of intraluminal stent somewhat difficult. So, up to now, the clinical use of installation of digestive tract intraluminal stent is still limitted to esophagus and biliary tract. We have chosen nickel-titanium alloy in the form of single fine threads woven longitudinally and transversely into a flexible mesh tabular stent according to the common feature of digestive tract structure and function. For the installation of stent in the high position cervical segment of esophagus, we made tolerance dilatation test and used small caliber so as to ameliorate foreign body or pain sensation, thus succeeding in putting the stent in the cervical segment of esophagus. We used high hardness, small friction and elongation coefficient, not easily restored once deformed material, polytetrafluroethylene, to make coaxial duct and put it in between the external and internal ducts made of polyethylene, which is soft, easily to be restored with big friction force. The coaxial duct type transporter was made in this way that can be introduced into tortuous intestinal tube to release the coaxial duct type transporter of the stent. We also used endoscope to help the insertion of the guide wire. Super-hard guide wire helps enlarge the turning angle of the colonic loops. All these measures solve the difficulty encounted in the remote release of the stent. This not only increased the success rate of installation of duodenal and proximal jejunal intraluminal stent orally[11], but also facilitated the installation of stent at high level transverse colon via anal route[17]. At the same time, we selected big caliber with high degree hardness double bulb shaped or mush room shaped trumpet orifice stent. This not only increased the expanding force but also increased the compliance of the connecting segment between the terminal opening of the sent and the normal intestinal tract. The therapeutic effect of colonic and rectal stent installation was thus elevated and its complication reduced.

Drug infusion via supplying artery of tumor

Intraluminal stent treatment can build up the basis for the patients for further treatment by the relief of digestive tract obstructive symptoms and improvement of life quality of late tumor cases. The advanced patients with tumor can rarely tolerate the toxic or side effect of traditional radio-and/or chemotherapy. In fact, the sensitivity toward traditional radio- and chemotherapy in absolute majority of patients with solid tumor of digestive tract is rather poor. As

chemotherapeutics have killing and injurious effect to most tumor cells, the difference of therapeutic effect is mainly determined by whether the drug in the target organ can reach effective antitumor blood drug concentration or not. The general effect of traditional chemotherapy makes it difficult to reach an effective blood drug concentration in the target organ of gastrointestinal tract at safe dosage. As a result, clinical efficacy is low, while toxic and side effects are severe. Interventional chemotherapy by drug infusion of supplying artery can make the blood drug concentration in the vascular network of the tumor area reach an effective antitumor level by relative safe dosage, thus decreasing toxic and side reactions and increasing therapeutic efficacy. Intraluminal installation of stent in combination with drug infusion of supplying artery can release obstruction, improve life quality and at the same time inhibit growth of the malignant tumor. In the present study, the average survival time of our group B patients was 13 weeks, close to that reported by Turegano-Fuentes *et al*[51], Cwikiel *et al*[52] and P Scott Mackie *et al*[53]. The average survival time was 44 weeks in group A, significantly longer than the former. The restenosis was also more significantly prolonged in group A than in group B. This showed that interventional chemotherapy by arterial cannulation can produce in certain extent inhibition of the malignant tumor growth by its therapeutic effect. In conclusion, intraluminal stent therapy in combination with intra-arterial cannulation of interventional chemotherapy can be considered as an effective therapy with regard to symptomatic and etiological treatment and should be used more widely in the near future.

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