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• REVIEW •

Chromosomal aberrations related to metastasis of human solid tumors

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Abstract

The central role of sequential accumulation of genetic alterations during the development of cancer has been firmly established since the pioneering cytogenetic studies successfully defined recurrent chromosome changes in specific types of tumor. In the course of carcinogenesis, cells experience several genetic alterations that are associated with the transition from a preneoplastic lesion to an invasive tumor and finally to the metastatic state. Tumor progression is characterized by stepwise accumulation of genetic alterations. So does the dominant metastatic clone. Modern molecular genetic analyses have clarified that genomic changes accumulate during the development and progression of cancers. In comparison with the corresponding primary tumor, additional events of chromosomal aberrations (including gains or allelic losses) are frequently found in metastases, and the incidence of combined chromosomal alterations in the primary tumor, plus the occurrence of additional aberrations in the distant metastases, correlated significantly with decreased postmetastatic survival. The deletions at 3p, 4p, 6q, 8p, 10q, 11p, 11q, 12p, 13q, 16q, 17p, 18q, 21q, and 22q, as well as the over-representations at 1q, 8q, 9q, 14q and 15q, have been found to associate preferentially with the metastatic phenotype of human cancers. Among of them, the deletions on chromosomes 8p, 17p, 11p and 13p seem to be more significant, and more detail fine regions of them, including 8p11, 8p21-12, 8p22, 8p23, 17p13.3, 11p15.5, and 13q12-13 have been suggested harboring metastasis-suppressor genes. During the past decade, several human chromosomes have been functionally tested through the use of microcell-mediated chromosome transfer (MMCT), and metastasis-suppressor activities have been reported on chromosomes 1, 6, 7, 8, 10, 11, 12, 16, and 17. However, it is not actually known at what stage of the metastatic cascade these alterations have occurred. There is still controversial with the association between the chromosomal aberrations and the metastatic phenotype of cancer. As the progression of human genome project and the establishment of more and more new techniques, it is hopeful to make clear the genetic mechanisms involved in the tumor metastasis in a not very long future, and provide new clues to predicting and controlling the metastasis.

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INTRODUCTION

The central role of sequential accumulation of genetic alterations during the development of cancer has been firmly established since the pioneering cytogenetic studies successfully defined recurrent chromosome changes in specific types of tumor. In the course of carcinogenesis, cells experience several genetic alterations (including gains and deletions) that are associated with the transition from a preneoplastic lesion to an invasive tumor and finally to the metastatic state. High frequency of chromosomal deletions elicited as losses of heterozygosity (LOH) is a hallmark of genomic instability in cancer. Functional losses of tumor suppressor genes caused by LOH at defined regions during clonal selection for growth advantage define the minimally lost regions as their likely locations on chromosomes. LOH is elicited at the molecular or cytogenetic level as a deletion, a gene conversion, single or double homologous and nonhomologous mitotic recombinations, a translocation, chromosome breakage and loss, chromosomal fusion or telomeric end-to-end fusions, or whole chromosome loss with or without accompanying duplication of the retained chromosome. Because of the high level of specificity, LOH has recently become invaluable as a marker for diagnosis and prognosis of cancer[1].

Many new molecular cytogenetic techniques, including various kinds of fluorescent in situ hybridization (FISH), comparative genomic hybridization (CGH), allelic imbalance (AI) and genotyping analyses, single nucleotide polymorphism (SNP) analysis, etc., have been established in recent years. These new techniques allow positional identification of gains and losses of DNA sequences in the entire tumor genome. These analytic approaches, particularly CGH and AI analysis, have already been applied over a wide range of tumor specimens, cell lines, and archival materials to define chromosomal imbalances. Molecular genetic analyses with these new strategies have clarified that genomic changes accumulate during the development and progression of cancers. A considerable amount of genetic alterations on solid tumors has been accumulated during the past few years.

Tumor progression is characterized by stepwise accumulation of genetic alterations. The dominant metastatic clone is also characterized by an accumulation of genetic alterations, but it is not actually known at what stage of the metastatic cascade these alterations have occurred. In comparison with the corresponding primary tumor, additional events of chromosomal aberrations (including gains or allelic losses) are frequently found in metastases and the incidence of combined chromosomal alterations in the primary tumor, plus the occurrence of additional aberrations in the distant metastases, correlated significantly with decreased postmetastatic survival. It is hypothesized that the occurrence of additional genetic aberrations is either involved in termination of dormancy of micrometastatic tumor cells at distant organ sites or acquired during further progression of metastases. However, only a few indications as to which genetic alterations among the multitude of changes might be

preferentially associated with the metastatic phenotype[2-8].

During the past decade, several human chromosomes have been functionally tested through the use of microcell-mediated chromosome transfer (MMCT), and metastasis-suppressor activities have been reported on chromosomes 1, 6, 7, 8, 10, 11, 12, 16, and 17. Such functional studies, combined with positional and expression-based gene cloning techniques, have enabled the identification of KAI1, KISS-1, MKK4/SEK1, and BRMS1 as metastasis-suppressor genes[9]. In this paper, we will review the chromosomal aberrations associated with the metastatic phenotype of solid tumor.

CHROMOSOME 8

Alterations of human chromosome 8p have been commonly detected in many tumor types[10-12]. Some studies have shown that loss of 8p is associated with the advance of tumors, and plays an important role in the tumor progression of many tumors including colorectal[13], bladder[14,15], breast[10] and liver cancers[16]. Recently, some studies have also shown that loss of 8p may be associated with the metastasis of laryngeal carcinoma[17], bladder cancer[18], renal cell carcinoma[19], colorectal carcinoma[20], lung cancer[21], mantle cell lymphoma (MCL)[22], and the poor prognosis of colorectal cancer patients [23]. Bockmuhl *et al* found that 8p23 allelic loss was an independent prognostic marker for disease-free interval, and was associated with poor prognosis in head and neck squamous cell carcinoma and could be useful in refining diagnosis of these tumors[24]. So, 8p might harbor one or more tumor suppressor genes that are important in the progression,especially in the metastasis of cancers, which was confirmed by irradiated MMCT technique[25, 26].

The presence of at least three tumor suppressor or metastasis suppressor genes loci on 8p (8p21, 8p22, and 8p23) that may be cooperative events has been suggested in HCC and ovarian cancer[12,27]. Several candidate tumor suppressor genes have been mapped to 8p including DLC-1(8p21.3-22)[28], FEZ1 gene (8p22)[29] and liver-related putative tumor suppressor (LTPS) gene (8p23)[30]. However, alterations of these genes may occur as an early event in the development of cancer, and their association with the metastasis is not confirmed[31]. Oba *et al*. found two putative tumor suppressor genes on chromosome 8p might play different roles, deletions on 8p22-p21.3 play an important role in tumor differentiation, while an 8p21.1-p21.2 deletion plays a role in the progression and metastasis of prostate cancer[32]. Allelic loss at 8p22 was associated with higher tumor grade, and no tumor that retained heterozygosity for markers at 8p22 had metastasized to distant organs, whereas a substantial portion of tumors that lost alleles in that region had done so. These imply that loss or inactivation of tumor-suppressing activity encoded on 8p contributes to malignancy and to the metastatic potential of bladder cancers[18]. Arai' s results suggest that putative tumor suppressor genes, which may be involved in the metastatic process of colorectal cancer, are located on chromosomes 8p21-22. Allelic losses in these regions are possible risk factors for early lymph node metastasis[33]. Nihei *et al*[26] used a functional positional cloning strategy to define the region harboring the metastasis suppressor gene in 8p21-12, and localized it to a 60-kb cloned region.

Chromosome 8p deletion is also one of the recurrent chromosomal aberrations in HCC that are common detected either by CGH or by microsatellite analysis[34-36]. In our previous study, we compared the differences of genomic alterations between matched primary and metastatic HCC by CGH, and found that the majority of chromosomal aberrations in both primary and metastatic lesions of HCC were consistent with those in previous reports. The most interesting finding in this study is the deletion of 8p which was detected in 8 metastatic lesions but only in 3 corresponding primary HCC, 5 cases of HCC acquired deletion on 8p as they progressed to metastatic stage, although some differences of genomic alterations between primary and its corresponding metastatic lesion were also found. These suggest that 8p may harbor one or more tumor suppressor genes that are important in the HCC progression especially in the tumor metastasis[37]. This result was confirmed in the metastatic model of HCC and its cell line[38]. Recently, in another genome-wide microsatellite analysis, deletion on chromosome 8p was further proved to be related to progression and metastasis of HCC, and 8p23.3, 8p11.2 were two likely regions harboring metastasis-related genes[39].

CHROMOSOME 17

The frequency of allele loss on 17p has been correlated significantly with prognostic features such as the number and size of liver secondaries, the depth of invasion, metastasis to the lymph nodes, and venous invasion of cancers[40-42]. Multiple regression analysis identified the numerical aberrations of chromosome 17 as independent significant determinants of lymph node metastasis. A similar result is also found in HCC. LOH on 17p13 of HCC correlates with the stage, portal invasion, intrahepatic metastasis, and nuclear morphometry of cancer cells. These suggest that the LOH on 17p13 is closely connected to the progression of HCC[43].

p53 is an important TSG on 17p13.1, and its aberration has been linked to the development and progress of human cancers including HCC[44]. However, in addition to 17p13.1, many studies showed that the frequently deleted region was 17p13.3[45-49]. The minimum region of LOH on chromosome 17p13.3 in HCC has been defined within the region between D17S643 and D17S1574. Moreover, D17S926 in the minimum region of LOH has the highest frequency of LOH, and its sequencing analysis has been accomplished. In this region, 6 novel genes have been characterized. One of them is designated HCC suppressor 1 (HCCS1)[45]. Our recent study showed that the AI ratio of chromosome 17p was as high as 74-87 % in primary lesions, and 73-87 % in metastasis lesions of HCC. Moreover, high level of AI was also identified in 17p11.2-12 (74-87 %). A 20cM segment within 17p11.2-13.1 was found related to metastasis phenotype, with the highest increased-grade AI (28-44 %) in metastatic lesions[39]. All these suggested that in addition to the p53 gene at 17p13.1, an as yet unidentified TSG(s) residing at 17p13.3 might play a role in development, progression and metastasis of HCC.

Discontinuous portions of human chromosome 17 (D17S952-D17S805, D17S930-D17S797, and D17S944-qter) that together suppress the metastatic ability of AT6.1 Dunning rat prostatic cancer cells when introduced via MMCT have been identified[50,51]. PCR and Southern blot analyses demonstrated that three of the four markers on 17p13, including HIC1 and TP53, and 12 of the 13 markers in 17q21-23, including BRCA1 and the metastasis-suppressor gene NME1 (nm23), were not retained in this region[50]. AT6.1 microcell hybrids containing this portion of chromosome 17 were tested in vivo in spontaneous metastasis assays. Spontaneous metastasis is measured by the ability of tumor cells to form a locally growing tumor at the site of injection and disseminate to and grow at secondary sites thereafter. At the experimental end point, the number of overt surface metastases observed in the lungs from mice with AT6.1-17 tumors was reduced 15- to 30-fold compared with lungs from mice bearing parental AT6.1 tumors[50]. This suppression could be due to the inhibition of any step within the metastatic cascade. A series

of in vivo experiments were conducted, and no evidence was found to suggest that there is a decrease in the number and/or viability of tumor cells colonizing the lung[51]. Development of overt metastases was associated with loss of the metastasis-suppressor region of chromosome 17[10,52,53].

CHROMOSOME 1

Frequent allelic losses on the short arm of chromosome 1 have been observed in a wide variety of human tumors. Allelic loss at 1p22-p31 was correlated with lymph node metastasis. Alterations of one or more tumor suppressor genes at 1p22-p31 may play a role at late stages of carcinogenesis, especially with regard to local progression and lymph node metastasis[54]. There may be at least two distinct tumor suppressor genes inactivated by allelic deletion on 1p36.1 and 1p36.3, respectively[55].

Amplification of 1q was also commonly detected in esophageal carcinoma, breast cancer, and colon cancer[56-58]. Gain of 1q might be one of the early genetic changes in HCC since it was one of the most commonly detected alterations in HCC[59, 60]. In most cases, the gain of 1q involved whole long arm. However, in our previous study, high copy number amplification on 1q was detected in 4 primary and 6 metastatic HCC with a minimum amplification region at 1q12-q22. Most interestingly, in two cases amplification of 1q12-q22 was only detected in metastatic HCC. This imply that overexpression of an oncogene(s) at 1q12-q22 confers a selective advantage in HCC. High copy number amplification of 1q12-22 may only occur in late stage of HCC and provide more advantage of growth selection. This suggests that 1q might harbor one or more oncogenes related to the development or progression of many cancers. Moreover, this provides us a candidate minimum amplification region on 1q12-q22 for further study to clone genes related to the development and progression of HCC[37]. A correlation of metastatic events with an increase in the copy number of genes located at 1q, in particular at 1q21-q23 is also found in renal clear cell carcinomas[61]. And more, an insertion of chromosome 13 material in the short arm of chromosome 1 has been only observed in micrometastatic cells[62].

CHROMOSOME 6

Highly frequent loss of 6q is found in many kinds of solid tumors, and is considered later events associated with tumor progression, and is thought to confer metastatic potential to the carcinomas (such as in biliary tract, etc.)[63]. Yoshida *et al*. introduced an intact chromosome 6 into the highly metastatic C8161 human melanoma cells by MMCT. Parental cells formed tumors in every mouse given an intradermal injection of 1×10^6 cells, and more than 90 % of the mice developed regional lymph node and lung metastases. In contrast, chromosome 6-C8161 hybrids (neo6/C8161) were still tumorigenic, but completely suppressed for metastasis. Intravenous injection of neo6/C8161 cells also did not produce metastases. Introduction of a version of chromosome 6 with deletions on the long arm allowed refinement of the metastasis-suppressor locus to a 40-megabase (Mb) region represented by chromosomal bands 6q16.3-q23. The neo6/C8161 cells were still locally invasive, and cells were even detected in efferent vessels. This finding implied that the step(s) in the metastatic cascade inhibited by introduction of chromosome 6 occurred subsequent to intravasation[9,64-71]. Shirasaki *et al*[72] found that inactivation of a tumor suppressor gene(s) mapping to 6q16.3-q23 by deletion or mutation coupled with LOH may lead to the down-regulation of a putative metastasis suppressor gene, KiSS1.

CHROMOSOME 10

LOH on chromosome 10q is found associated with tumour progression in SCC, SCLC, and prostate cancer. It may become a useful genetic marker in the assessment of the malignant potential and a potential genetic discriminator between progressors and nonprogressors after radical surgery of these tumour types[73,74]. Deletions on chromosome 10q25-q26 is responsible for the metastatic phenotype of squamous cell carcinomas in head and neck[75], and colorectal carcinomas[76], which may play a particular pathogenetic role in the metastatic process. One gene, LAPSER1 [an LZTS1(or FEZ1)-related gene] maps within a subregion of human chromosome 10q24.3 that has been reported to be deleted in various cancers, including prostate tumors, as frequently as the neighboring PTEN locus. This gene is involved in the regulation of cell growth, loss of its function may contribute to the development of cancer[77].

A telomerase repressor gene may be located on 10p15.1 by deletion mapping using MMCT, radiated microcell fusion (RMF), FISH and STS analysis. 10p15.1 harbors a gene involved in repression of telomerase RNA component in human somatic cells and each putative repressormay act independently [78].

CHROMOSOME 11

A strong relationship between LOH at chromosome 11q23.3 and the presence of extensive tumor plugs in lymphvascular spaces (LVS) has been demonstrated, which suggests that genes at this locus may regulate vasculoinvasion. Patients with LOH at 11q23.3 are significantly more likely to have disease recurrence than patients without LOH at 11q23.3. Although unlikely to have an impact early in carcinogenesis, tumor-suppressor genes located in the region of 11q23.3 appear to be important in tumor progression, facilitating lymphvascular space invasion and, by inference, spread to lymph nodes in squamous cell carcinoma of the cervix[79] and melanoma[80]. Deletion on 11q22-qter, together with the gains of 8q23-qter and 20q was observed in tumors metastatic to the lymph nodes. Gains of 8q23-qter and 20q and loss of 11q22-qter allow the prediction of lymph node metastasis[81]. LOH at 11q13.1-5, the region around the MEN1 locus, may be valuable in predicting the invasiveness of pituitary adenomas[82]. However, Pairwise found metastasizing tumors are characterized by overrepresentations on chromosomes 11q13 and 22q, and deletions on 18q[83].

The centromeric part of chromosome segment 11p15.5 contains a region of frequent allele loss in many adult solid malignancies. This region, called LOH11A, is lost in 75 % of lung cancers and is thought to contain a gene that may function as a metastasis suppressor. Genetic complementation studies have shown suppression of the malignant phenotype including reduction of metastasis formation. Bepler *et al*. constructed a high-resolution physical map and contig over 1.4 Mb that includes the beta-hemoglobin gene cluster and the gene for the large subunit of ribonucleotide reductase (RRM1). Through sequencing and computerized analysis, we determined that this region contains an unusually large number of transposable elements, which suggests that double-stranded DNA breaks occur frequently here. Twenty-two putative genes were identified. Because of its location at the site of maximal allele loss in the 650-kb LOH11A region and previous functional studies, RRM1 is the most likely candidate gene with metastasis suppressor function. The malignant phenotype results from a relative loss of function rather than a complete loss[84]. Two distinct tumor suppressor loci on chromosome 11p15, one is between D11S1318 and D11S4088 (approximately 500 kb)

within 11p15.5, a second, critical region of LOH spans the markers D11S1338-D11S1323 (approximately 336 kb) at 11p15.5-p15.4, may contribute to tumor progression and metastasis in breast cancer and lung cancer[85,86]. The human SRBC gene (hSRBC), a candidate tumor suppressor gene, is mapped to chromosome region 11p15.5-p15.4, close to marker D11S1323, at which frequent LOH has been observed in sporadic breast, lung, ovarian, and other types of adult cancers as well as childhood tumors[87]. Introduction of human chromosome 11 by MMCT could suppress MDA-MB-435 breast carcinoma cell metastasis[88]. One important metastasis-suppressor gene, KAI1, maps to 11p11.2-p13.

CHROMOSOME 13

LOH of 13q is a common event in oncogenesis and/or progression of oral SCC and larynx carcinoma, and significant correlation between LOH of 13q14.3 and lymph node metastasis is found. These suggest the existence of a new suppressor gene near D13S273-D13S176 loci which may play a role in these events[18,89]. Allelotype analysis of whole chromosomes showed that allelic loss at 13q12-13 of the primary ESC was closely associated with lymph node metastasis, and unidentified tumor suppressor gene(s) in this region might be involved[90]. The high LOH rate for different microsatellite markers in and around the putative TSG locus C13 on chromosome 13q13 has been found[91]. However, Hyytinen et al. found that gain of the 13q12-q13 chromosomal region as well as losses of 4, 6q24-qter, 20p and 21q were associated with androgen independence and tumorigenicity with additional changes correlating with metastasis[92].

CHROMOSOME 14

Significantly more LOH events at markers D14S62 and D14S51 in primary breast cancers from patients with lymph node-negative disease than these with lymph node-positive disease were found, suggesting the presence of a gene in this region that affects metastatic potential. Analysis of small interstitial or terminal deletions in the tumors of six especially informative patients with lymph node-negative disease places the putative metastasis-related gene in a 1490-kilobase region near D14S62. LOH in the D14S62 region may impede the process of metastasis. Therefore, the D14S62 region LOH profile may have prognostic implications, and the isolation of the metastasis-related gene(s) in this region may lead to better diagnosis and treatment of breast cancer[93]. This unusual observation suggests that, whereas the LOH of this region promotes primary breast cancer formation, some gene(s) mapping to this 1.6-Mb region is rate-limiting for breast cancer metastasis. Thus, if primary breast cancers delete this region, their ability to metastasize decreases. To identify this gene(s), Martin *et al*[94] physically mapped this area of chromosome 14q, confirmed the position of two known genes and 13 other expressed sequence tags into this 1.6-Mb region. One of these, the metastasis-associated 1 (MTA1) gene, previously identified as a metastasis-promoting gene, mapped to the center of our 1.6-Mb target region. Thus, MTA1 represents a strong candidate for this breast cancer metastasis-promoting gene.

CHROMOSOME 16

Genomic aberration at the chromosome 16q arm is one of the most consistent abnormalities observed by LOH and CGH analyses in human prostate cancer, suggesting that there are tumor suppressor or metastasis suppressor genes encoded by this chromosomal region. When the MMCT hybrid cells containing whole human chromosome 16 were injected, the number of metastatic lesions in the lung was significantly reduced as much as 99 % on average. Therefore, chromosome 16 has a strong activity to suppress the metastatic ability of AT6.1 cells while it did not affect the tumorigenesis and tumor growth rate. A PCR analysis of various microcell hybrid clones with sequence-tagged site markers indicates that the metastasis suppressor activity is located in the q24.2 region of chromosome 16. These suggest that there is a metastasis suppressor gene in this region that may play an important role in the progression of prostate cancer[95]. Deletion of chromosome 16q23-24 appears in a high frequency in metastases of prostate cancer. The strong correlations suggest that they may be important risk factors, contributing to the metastatic potential of the tumor[96-98]. The presence of putative tumor-suppressor genes on chromosome 16q23.2-24.1 has been suggested by LOH analysis in several cancer types. The candidate gene WWOX/FOR has been mapped within this region[99-101].

CHROMOSOME 18

Deletion on 18p is shown as one of the most frequent losses in metastasizing tumors in primary larynx tumor[102]. Two distinct minimum regions of AI on 18q have been identified. The first region is between the genetic markers D18S1119 and D18S64. The second region lies more distal on the long arm of the chromosome and is between the genetic markers D18S848 and D18S58. Significantly higher AI was found in the metastatic samples, which is consistent with 18q losses occurring late in tumor progression[103]. Detailed deletion mapping in these tumors identified a distinct commonly deleted region within a 5-cM interval in 18q21.1. The primary tumors had either no detectable abnormality of chromosome 18 or the region involving LOH was limited while the metastatic foci showed more frequent and extended allelic losses on this chromosome. These suggest that inactivation of one or more putative tumor suppressor genes on 18q21 other than DCC and DPC4 plays an important role in the progression of human prostate cancer[104]. Loss of 18q in metastatic and locally recurrent tumors, but not in primary tumors from the same patients, suggests that a tumor suppressor gene in this region may be important in the progression of breast cancer, squamous cell carcinoma, etc.[7,105,106].

OTHERS

In addition, losse of 9p and gains of 17q and Xq are other genomic changes which frequently occurred in metastases but not in the corresponding primary tumor[19].

The deletions at 3p12-p14, 3p21, 4p15-p16, 6q24-qter, 8p22-p23, 10q21-qter and 21q22, as well as the over-representations at 1q21-q25, 8q, 9q34, 14q12 and 15q12-q15, occurred significantly more often in the metastatic tumour group[107]. Areas of deletion predominantly or completely common to the colorectal and the metastatic tumour were detected on chromosomes 5q, 8p, 17p, 18q, and 22q. Preferential loss in metastatic tumours was observed on chromosomal arm 3p[108]. Chu *et al*[109] compared the genetic abnormalities specifically associated with varying metastatic potential of prostate cancer cell lines by CGH, and found that PC3M-LN4, the derivative line that produced significantly larger metastatic tumors in the lymph nodes and had higher incidences of distant metastases, had a specific gain of 1q21-q22 and losses of 10q23-qter and 18q12-q21. LNCaP-LN3, a derivative line that had a significantly higher incidence of

lymph node metastases and produced significantly larger metastatic tumors in the lymph nodes, had specific losses of 16q23-qter and 21q.

A strong association between 12p12-13 LOH and distant metastasis has been found, which raises the possibility that mutational inactivation of a gene at 12p12-13, possibly p27/kip1, plays a pivotal role in the development of metastatic disease[110, 111].

LOH of 19q13 was associated with overall survival in local-regional International Neuroblastoma Staging System stages 1, 2, and 3 patients and was specifically present in tumors at the site of recurrence[112].

The gain of chromosome 20 may be available as a genetic marker for the diagnosis or prediction of liver metastasis[113,114].

The Xq25 region harbors a putative tumor suppressor gene whose inactivation in breast cancer is associated with tumor progression and metastasis. LOH at this region, therefore, potentially could be used as a prognostic marker for disease development[115].

QUESTIONS AND PROSPECTS

There is still controversial with the association between the chromosomal aberrations and the metastatic phenotype of cancer. Some reports showed the frequencies of the most common aberrations were relatively similar in primary tumors and metastases, but no aberrations specific to metastases were detected[116]. And more, among of the chromosomes mentioned above, which one plays the most important role in the metastatic process. Based on the published data and the author's experience, chromosomes 8 and 17 should be paid more attention to. To date, combined analyses of the different aberrations of various chromosomal regions would be much more valuable in predicting the metastatic potential and prognosis of human solid tumors[8]. As the progression of human genome project and the establishment of more and more new techniques, it is hopeful that the genetic mechanisms involved in the tumor metastasis could be made clear in a not very long future, and provide new clues to predicting and controlling the metastasis.

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• ESOPHAGEAL CANCER •

Identification of differentially expressed proteins between human esophageal immortalized and carcinomatous cell lines by two-dimensional electrophoresis and MALDI-TOF-mass spectrometry

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Abstract

AIM: To identify the differentially expressed proteins between the human immortalized esophageal epithelial cell line (SHEE) and the malignant transformed esophageal carcinoma cell line (SHEEC), and to explore new ways for studying esophageal carcinoma associated genes.

METHODS: SHEE and SHEEC cell lines were used to separate differentially expressed proteins by two-dimensional electrophoresis. The silver-stained 2-D gels was scanned with EDAS290 digital camera system and analyzed with the PDQuest 6.2 Software. Six spots in which the differentially expressed protein was more obvious were selected and analyzed with matrix-assisted laser desorption/ionization time of flying mass spectrometry (MALDI-TOF-MS).

RESULTS: There were 107±4.58 and 115±9.91 protein spots observed in SHEE and SHEEC respectively, and the majority of these spots between the two cell lines matched each other (r=0.772), only a few were expressed differentially. After analyzed by MALDI-TOF-MS and database search for the six differentially expressed proteins, One new protein as well as other five sequence-known proteins including RNPEP-like protein, human rRNA gene upstream sequence binding transcription factor, uracil DNA glycosylase, Annexin A2 and p300/CBP-associated factor were preliminarily identified.

CONCLUSION: These differentially expressed proteins might play an importance role during malignant transformation of SHEEC from SHEE. The identification of these proteins may serve as a new way for studying esophageal carcinoma associated genes.

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INTRODUCTION

Since Wilkins and Williams first proposed the concept of "Proteome" in 1994, the studies on tumor proteome have been made mighty advances[1]. It is expected to find new special tumor markers and clone their associated genes via separating and identifying the tumor differentially expressed proteins by the proteomic approach to reveal the tumor pathogenesis and carry out the gene therapy[2-6].

Esophageal carcinoma is one of the most common malignant tumors in China[7-19], and its etiology and pathogenesis remain to be determined[20-23]. Recent studies are mainly focused on the relationship between the change of oncogenes/suppressor oncogenes and esophageal carcinoma. However, there is no strong evidence to indicate that these oncogenes and suppressor oncogenes, including myc, ras, EGFR, int-2, cyclin D1, p53, Rb, p16, MCC, APC which are cloned originally from other kinds of tumors, are closely related to the esophageal carcinoma[24-28]. Therefore, it is necessary to clone the new oncogenes or suppressor oncogenes, which might have an more intimate relationship with esophageal tumor pathogenesis, directly from esophageal carcinoma tissues or cells.

In recent years, it has been increasingly concerned about the roles of the human papilloma virus (HPV) played in the esophageal carcinogenesis[29-32]. In our previous work, we transfected human embryonic esophageal mucosa cells with HPV18 E6E7 genes, and established an immortalized epithelial cell line SHEE[33,34]. The SHEE cells were further exposed to the tumor promoter (12-O-tetradecanoyl-phorbol-13-acetate, TPA) to be induced malignant transformation, and from which a human embryonic esophageal epithelial carcinoma cell line SHEEC was then established[35,36]. These studies not only provided the evidence for the close relationship between HPV and the esophageal carcinogenesis, but also established a reliable model for studying the molecular mechanisms of esophageal carcinogenesis, and cloning new esophageal carcinoma associated genes. In the present study, the differential expression of proteins between SHEE and SHEEC was investigated by the proteomic approach including two-

dimensional electrophoresis and MALDI-TOF-MS, which might serve as a new way for studying esophageal carcinoma associated genes.

MATERIALS AND METHODS

Cell culture

SHEE and SHEEC were cultured in MEM medium (Gibco) supplemented with 100 ml/L fetal borine serum (100 u/ml penicillin, 100 u/ml streptomycin) and incubated at 37 ℃ in humidified atmosphere of 50 ml/L CO_2 incubator.

Whole soluble protein extraction and pre-treatment

To obtain whole soluble protein, the experimental procedures in *Molecular cloning* (2nd ed.) were employed[37]. Briefly, when the cultured cells grew into a full monolayer, they were washed with ice-cold phosphate-buffer saline (PBS) three times and then treated with cold buffer containing 50 mmol/L Tris-HCl, pH8.0, 150 mmol/L NaCl, 1 %Triton X-100, 100 μg/ml Phenylmethylsulfonyl fluoride (PMSF) for 20 min at 4 ℃. The broken cells were collected with a scraper and centrifuged at 12 000 g for 5 min. The supernatant, which contained the whole soluble proteins, was added to Micro Bio-Spin® chromatography columns, and the purified sample was obtained after centrifugation at 1 000g for 4 min. Protein concentrations were determined by Bradford method (BIOPhotometer, Eppendorf). The sample aliquots were stored at -20 ℃ until used.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out by using the Mini-PROTEAN II 2-D apparatus (Bio-Rad). 70 μg of the whole soluble proteins were mixed with the rehydration solution containing 8 mol/LUrea, 4 %CHAPS,10 mmol/L DTT, 0.2 %(w/v) IPG buffer (pH3-10,liner) to a total volume of 125 μl. The mixture was pipetted into IPG strip tray channels. Both the rehydration and focusing were performed in the same focusing tray. IPG dry strips (pH3-10, 7 cm) were lowered onto the mixture with the gel side down, and then covered with mineral oil. The rehydration and isoelectric focusing (IEF) were done as follows: 1) rehydration for 12-14h, 0V; 2) 250V, 30 min; 3) 250V to 4 000V, 2 h; 4) 4 000V, 5 h. All the procedures above were performed at 20 ℃. After IEF separation, the strips were immediately equilibrated for 2×10 min with 6 mol/L Urea, 0.375 mol/L Tris-HCl (pH8.8), 2 % SDS and 20 % glycerol. In the first equilibration solution, 2 % (w/v) DTT was included, and 2.5 %(w/v) iodoacetamide was added in the second equilibration. Then the IPG strips were placed on a 1.0 mm thick, 12 % SDS-PAGE gel and sealed with 1 % LowMelt agarose. Electrophoresis was carried out at a constant voltage (40 mV/gel) until the bromophenol blue frontier reached the bottom of the gels about 0.5 cm. After electrophoresis, the SDS-PAGE gels were stained with silver stain plus kit (Bio-Bad).

Image acquisition and analysis

Image scanning for the silver-stained 2-D gels was performed with EDAS290 digital camera system (Kodak) and image analysis with the PDQuest 6.2 Software (Bio-Rad). To get reliable results, three gels were employed for each cell line. After the background subtraction, spot detection and match, one standard gel for each cell line was obtained. These standard gels were then matched to yield information about the spots of differentially expressed proteins.

Protein identification by MALDI-TOF-MS

Six spots in which the differentially expressed protein was more obvious in each cell line were cut out from the gel. After washed with 300 μl milliQ water for 15 min, each protein spot was decolorized with the successive action of 50 μl of 15 mmol/L Potassium ferricyanide and 50 mmol/L sodium thiosulphate for 5-10 min. The faded gel pieces were dried in a vacuum centrifuge tube for 5 min. The cysteine reduction and alkylation were performed as incubated with 10 mmol/L DTT, 100 mmol/LNH_4HCO_3 at 56 ℃ for 1 h in the dark. The gel pieces were then dried again and incubated with 50 mmol/L fresh iodoacetamide in 100 mmol/L NH_4HCO_3 at room temperature for 30 min. Thereafter the gel pieces were rehydrated in digestion buffer containing 20 μl of 12.5 μg/ml modified trypsin and 20 mmol/L NH_4HCO_3 for 30 min in ice. The excess liquid was removed and the gel pieces were digested continuously at 30 ℃ overnight(>16 h). The resulting peptide mixture was extracted from the digested solution by centrifugation and then resuspended in 10 μl of 50 % CH_3CN and 0.1 % trifluoroacetic acid (TFA) for 10min at 30 ℃ on a shaking platform. Peptide mass maps were generated by Applied Biosystems Voyager System 6192 MALDI-TOF-mass spectrometry (ABI,USA). Peptide masses were analyzed using the MS-Fit search program (http://prospector.ucsf.edu/ucsfhtml4.0u/msfit.htm).

RESULTS

2-D map and image analysis

The whole soluble proteins of SHEE and SHEEC were extracted in one step and desalted with Micro Bio-Spin® chromatography columns, which made the 2-D electrophoretic patterns a much higher quality (to be published). Three pairs of gels from different batches of SHEE and SHEEC were analyzed for the purpose of quantitative and qualitative comparison with the software PDQuest6.2. There were 107± 4.58 and 115±9.91 protein spots observed in SHEE and SHEEC respectively, and the majority of these spots between the two cell lines matched each other (r=0.772), only a few were expressed differentially. Six spots in which the differentially expressed protein was more obvious were selected and analyzed with MALDI-TOF-MS. The spot 1 was only expressed in the samples of SHEEC and absent in that of SHEE. In contrast, the spots 2 to 6 were merely observed in the SHEE samples. These six spots were marked with arrows at the corresponding site in Figure 1.

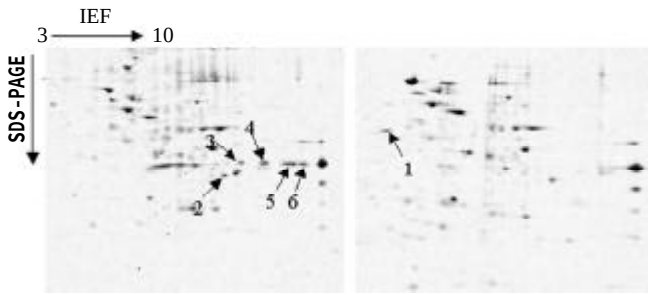


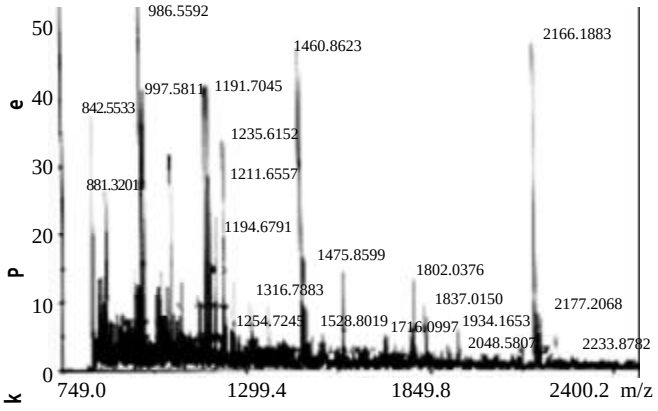
Figure 1 The differentially expressed protein spots observed in SHEE (left) and SHEEC (right) two-dimensional gels samples

Table 1 Protein identified by mass spectrometry

Spot No.	Accession No (NCBInr)	Theoretic M_r	Theoretic p*I*	Intensity Matched(%)	Length(AA)	expression	Protein name
1	10719660	55549	4.8	10%	494	↑	RNPEP-like protein
2	1916615	75940	8.8	44%	654	↓	ribosomal RNA gene upstream sequence binding transcription factor
3	35053	35493	8.2	27%	331	↓	uracil DNA glycosylase
4	16306978	38618	7.6	47%	339	↓	annexin A2
5	7428977	92928	9.2	44%	832	↓	p300/CBP-associated factor
6	NEW PROTEIN	-	-	-	-	↓	-

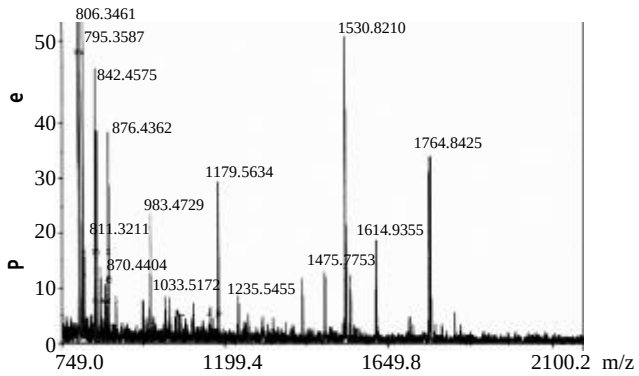
MALDI-TOF-MS analysis and protein identification

The proteins contained in the six spots were identified respectively by MALDI-TOF-MS on the basis of peptide mass matching; In this way, the peptide mass fingerprinting map for each protein spot was obtained as show in Figure 2. The experimental data revealed that the protein in spot 6 was with an unknown sequence (Figure 3), and its characteristics remains to be investigated. The identified protein names, accession numbers, as well as the number of the matching peptide, the theoretical *M*r and p*I* values, i.e. for each protein spot were listed in Table 1.



Mode of operation: Reflector Extraction mode: delayed Accelerating voltage: 20000 V
Acquisition mass range: 750-3500Da Number of laser shots: 150/spectrum Laser intensity: 2233

Figure 2 The MALDI-TOF mass spectrum map of protein spot 1



Mode of operation: Reflector Extraction mode: delayed Accelerating voltage: 20000 V
Acquisition mass range: 750-3000Da Number of laser shots: 200/spectrum Laser intensity: 2224

Figure 3 The MALDI-TOF mass spectrum map of protein spot 6

DISCUSSION

The investigation of differentially expressed proteins that occured during the generation and development of tumors is a new effective way to study tumor associated genes[38,39]. In the present study, we preliminarily studied several of the differentially expressed proteins between SHEE and SHEEC with two-dimensional electrophoresis and MALDI-TOF-mass spectrometry. By comparing the reference proteins or peptides, five sequence-known proteins and a novel sequence-unknown protein, which expressed more differentially in the course of malignant transformation of esophageal epithelial cells, were identified for the first time. It is therefore further indicated that the methods adopted in the present study could provide a new way to study esophageal carcinoma associated genes.

RNPEP-like protein being composed of 494 amino acid residues has 49 % identity with aminopeptidase B in amino acid sequence[40]. As judged by the parameters such as the numbers and intensity of peptide matching peak, the sequence coverage of matching peptide, as well as the theoretical and approximate values of *M*r and p*I*, RNPEP-like protein was considered as the much higher expression in SHEEC cells. However, the significance of its overexpression in SHEEC remains unknown.

Human rRNA gene upstream sequence binding transcription factor (hUBF) is a critical element in the regulation of rRNA transcription, which performs its function by binding to the rRNA gene upstream regulator sequence (-200 to -107 and -45 to 20)[41]. In our present work, it has been shown that the down-regulation of hUBF expression was obvious in the course of malignant transformation of SHEE, which might be the results of alterations in the regulation mechanism of rRNA gene transcription occurred in the malignant transformation of the human immortalized esophageal epithelial cells. In addition, whether or not the fact that diminished hUBF expression has been found in the well-differentiated teratocarcinoma cells[42] related hUBF to the neoplastic differentiation, remain to be investigated.

Uracil DNA glycosylase (UDG) is an enzyme for the DNA repairment. It can hydrolyse the N-glycosidic bond connecting the base to the deoxyribose and release free uracil base and DNA with an abasic site as its products[43]. Moon and his associates[44] found that the uracil DNA glycosylase gene (UNG) in sporadic glioblastomas had a point mutation in exon 3, and concluded that the genetic alterations of UNG might play an important role in the development of primary glioblastomas. In our study, the UDG expression shows absent or too low to be detected after SHEE malignantly transformed into SHEEC, which means that there might exist repairing deficiencies for the damaged DNA in the course of malignant transformation of the human immortalized esophageal epithelial cells.

Annexin A2 belongs to the family of annexins that bind to phospholipids in a calcium-dependent manner. So far at least 13 annexin family members have been found. There are four repeats of a 70 amino acid motif and a variable N-terminal end contained in all these annexin family members. According to lots of investigations, the annexins seem to be involved in various biological processes including endocytosis, exocytosis, the phospholipase A2 inhibition as well as ion channel and protein kinase C activity[45,46]. In recent years, it has been found that the annexin I was overexpressed in some kinds of malignant tumors such as human hepatocellular[47] and pulmonary carcinomas[48], which suggests that some of the annexin family members might relate to the carcinogenesis. Moreover, Chetcuti and his associates[49] found that the annexin II was expressed in the nomal and benign hyperplastic prostate tissue, and absent in all prostate cancer specimens. These results indicated that different members of the annexin family may have varied roles in the development and progression of tumors. The fact that the annexin A2 was expressed in SHEE but absent in SHEEC in our research indicates that annexin A2 might play a role of suppressor oncogene during malignant transformation of the human immortalized esophageal epithelial cells.

The p300/CBP-associated factor (P/CAF) that possessed intrinsic histone acetylase activity could regulate the gene expression of various sequence-specific factors that are involved in cell growth and/or differentiation including CREB, c-Jun, Fos and c-Myb through promoter-specific histone acetylation. Yang *et al*[50] found that the expression of P/CAF in HeLa cells could block the cell-cycle progression from G1 to S phase, and counteract the mitogenic activity of adenoviral oncoprotein E1A. The P/CAF expression was absent in the esophageal carcinoma cells found in our study suggests that the P/CAF might play a role in the suppressing of the esophageal carcinoma development.

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• GASTRIC CANCER •

The effects of vitamin E succinate on the expression of *c-jun* gene and protein in human gastric cancer SGC-7901 cells

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Abstract

AIM: To investigate the effects of vitamin E succinate (VES) on the expression of *c-jun* gene and protein in human gastric cancer SGC-7901 cells.

METHODS: After SGC-7901 cells were treated with VES at different doses (5,10,20 mg·L^{-1}) at different time, reverse transcription-PCR technique was used to detect the level of *c-jun* mRNA; Western Blot was applied to measure the expression of *c-jun* protein.

RESULTS: After the cells were treated with VES at 20 mg·L^{-1} for 3 h, the expression rapidly reached its maximum that was 3.5 times of UT control (P<0.01). The level of *c-jun* mRNA was also increased following treatment of VES for 6 h. However,the expression after treatment of VES at 5 mg·L^{-1} for 24 h was 1.6 times compared with UT control (P<0.01). Western blot analysis showed that the level of *c-jun* protein was obviously elevated in VES-treated SGC-7901 cells at 20 mg·L^{-1} for 3 h. The expression of *c-jun* protein was gradually increased after treatment of VES at 20 mg·L^{-1} for 3, 6, 12 and 24 h, respectively, with an evident time-effect relationship.

CONCLUSION: The levels of *c-jun* mRNA and protein in VES-treated SGC-7901 cells were increased in a dose- and time-dependent manner; the expression of *c-jun* was prolonged by VES, indicating that *c-jun* is involved in VES-induced apoptosis in SGC-7901 cells.

Zhao Y, Wu K, Xia W, Shan YJ, Wu LJ, Yu WP. The effects of vitamin E succinate on the expression of *c-jun* gene and protein in human gastric cancer SGC-7901 cells. *World J Gastroenterol* 2002; 8(5):782-786

INTRODUCTION

RRR-α-tocopheryl succinate (vitamin E succinate, VES), a derivative of natural vitamin E, has been shown to be a potent growth inhibitor of various cancer cell types *in vitro* and *in vivo*[1-7]. Growth inhibition by VES is attributed to cell cycle blockage[4,8-10], induced cellular differentiation[11,12], increased expression of biologically active transforming growth factor-βs (TGF-βs) and their type Ⅱ cell surface receptors[1,13,14] and the induction of apoptosis[15-18]. VES is noteworthy not only for its antiproliferative effects on tumor cells, but also for its non-toxic effect on normal cell types.

Gastric cancer is one of the most common tumors in China[19-28]. Up to date, the exact mechanisms of tumorigenesis is still unclear, but our previous studies showed that VES can block cell cycle, arrest DNA synthesis and induce apoptosis in human gastric cancer SGC-7901 cells, therefore inhibiting the cell growth[29-32]. In addition, our *in vivo* research in our laboratory demonstrated that VES inhibited benzo(a)pyrene (B(a)P)-induced forestomach carcinogenesis in female mice[33]. The exact mechanisms of apoptosis are not clearly known, but we found that VES can secrete and activate biologically active TGF-β and then TGF-β increases the kinase activity of *c-jun* N-terminal kinase (JNK) followed by phosphorylation of *c-jun*, and finally activated *c-jun* triggers apoptosis in human gastric cancer SGC-7901 cells[34]. In this study, the expression of *c-jun* mRNA was detected using reverse-transcription polymerase chain reaction (RT-PCR) technique and the level of *c-jun* protein was measured using western blot in order to further investigate the mechanisms of VES-triggered apoptosis.

MATERIALS AND METHODS

Materials

VES was purchased from Sigma Co. Ltd. RPMI 1640 media and TRIzol total RNA isolation kit were obtained from Gibco BRL, TITANIUM™ one-step RT-PCR kit from Clontech. Inc. *c-jun* (H79) rabbit polyclonal antibody was from Santa Cruz Biotechnologies.

Methods

Cell culture Human gastric cancer cell lines SGC-7901 were maintained in RPMI 1640 medium supplemented with 100mL·L^{-1} fetal calf serum (FCS), 100 kU·L^{-1} penicillin, 100 mg·L^{-1} streptomycin and 2 mmol·L^{-1} L-glutamine under 50 mL·L^{-1} CO_2 in a humidified incubator at 37 ℃. SGC-7901 cells were incubated for different time periods in the presence of VES at 5, 10 and 20 mg·L^{-1} (VES was dissolved in absolute ethanol and diluted in RPMI 1640 complete condition media correspondingly to a final concentration of VES and 1 mL·L^{-1} ethanol), succinic acid, vitamin E and ethanol equivalents as vehicle (VEH) control and condition media only as untreated (UT) control.

RT-PCR After SGC-7901 cells were treated with VES for 3, 6 and 24 h, respectively, total cellular RNA was isolated by using TRIzol Reagent according to the manufacturer's instructions. The concentration and purity of total RNA were determined by DU^R 640 nucleic acid and protein analyzer (Beckman, USA). One-step RT-PCR was carried out following the manufacturer's instructions. RT-PCR mixture was heated 1h at 50 ℃ for reverse transcription and 5 min at 95 ℃ for pre-denaturation, then into 34 PCR cycles of 30 s at 94 ℃ for

denaturation, 30 s at 60 ℃ for annealing, 30 s at 72 ℃ for extension in PTC-100 programmable thermal controller (MJ Research, USA). The corresponding fragment of *c-jun* gene was amplified with specific primers synthesized[35]. β-actin gene was designed as an internal standard with purpose to remove false negative outcome (Table 1).

Table 1 Nucleotide sequence and size of the expected PCR products for oligonucleotide primers used for RT-PCR

Genes		Sequence	Size (bp)
c-jun	Upstream:	5'-GGA AAC GAC CTT CTA TGA CGA GCC C-3'	315
	Downstream:	5'-GAA CCC CTC CTG CTC ATC TGT CAG G-3'	
β-actin	Upstream:	5'-GTG GGC CGC TCT AGG CAC CAA-3'	540
	Downstream:	5'-CTC TTT GAT GTC ACG CAC GAT TTC-3'	

The amplified products were seperated in 20 g·L^{-1} agorose gel stained with ethidium bromide. After electrophoresis, the gel was observed and photographed under ultraviolet reflector. The density and area of each band were analyzed using ChemiImager™ 4000 Digital System (Alpha Innotech Corporation, USA).

Western blot SGC-7901 cells treated with VES were harvested, washed in PBS and lyzed in lysis buffer containing 150 mmol·L^{-1} NaCl, 1 mL·L^{-1} NP-40, 5 mg·L^{-1} sodium deoxycholate, 1 g·L^{-1} SDS, 50 mmol·L^{-1} Tris (pH 7.4), 1 mmol·L^{-1} DTT, 0.5 mmol·L^{-1} Na_3VO_4, 10 mmol·L^{-1} phenylmethylsulfonyl fluoride (PMSF), 10 mg·L^{-1} trypsin, 10 mg·L^{-1} aprotinin and 5 mg·L^{-1} leupeptin. Following the centrifugation of 12 000×g for 30 min at 4 ℃, the amount of protein in the supernatant was determined using Biorad DC protein assay. Equal amount of protein was separated on 10 % SDS-PAGE and transferred to nitrocellulose filter (Gibco BRL, USA) overnight. Blocked with 50 g·L^{-1} defatty milk, the filter was incubated with *c-jun* (H79) rabbit polyclonal antibody and horseradish peroxidase-conjugated IgG, finally developed with DAB.

Statistical analysis

The data were expressed as $\bar{x}\pm s$. Statistical analysis was performed using student's *t*-test. $P<0.05$ was considered significant.

RESULTS

Effect of VES on the expression of c-jun mRNA in SGC-7901 cells

1 μg of total cellular RNA from groups of control, succinate, vitamin E, VES at 5, 10 and 20 mg·L^{-1} was added to amplify *c-jun* and β-actin genes by RT-PCR. Baseline expression of *c-jun* mRNA was observed in SGC-7901 cells (Figure 1). After the cells were treated with VES at 20 mg·L^{-1} for 3 h, the expression rapidly reached its maximum that was 3.5 times of UT control ($P<0.01$). The level of *c-jun* mRNA was also increased following treatment of VES for 6 h. However, the expression after treatment of VES at 5 mg·L^{-1} for 24 h was 1.6-fold increase compared with UT control ($P<0.01$), while there was no significant difference between 10 and 20 mg·L^{-1} VES groups and UT control group (Table 2).

Effect of VES on the expression of c-Jun protein in SGC-7901 cells

Western blot analysis showed that the level of c-Jun protein was obviously elevated in VES-treated SGC-7901 cells at 20 mg·L^{-1} for 3 h in a significant dose-dependent manner (Figure 2A, 2B). Meanwhile, compared with the cells in UT control group, the VES-treated cells at 20 mg·L^{-1} exhibited 1.8-, 2.0-, 2.3- and 2.8-fold increases in the expression of *c-jun* protein for 3, 6, 12 and 24h, respectively, with an evident time-effect relationship (Figure 3A,3B).

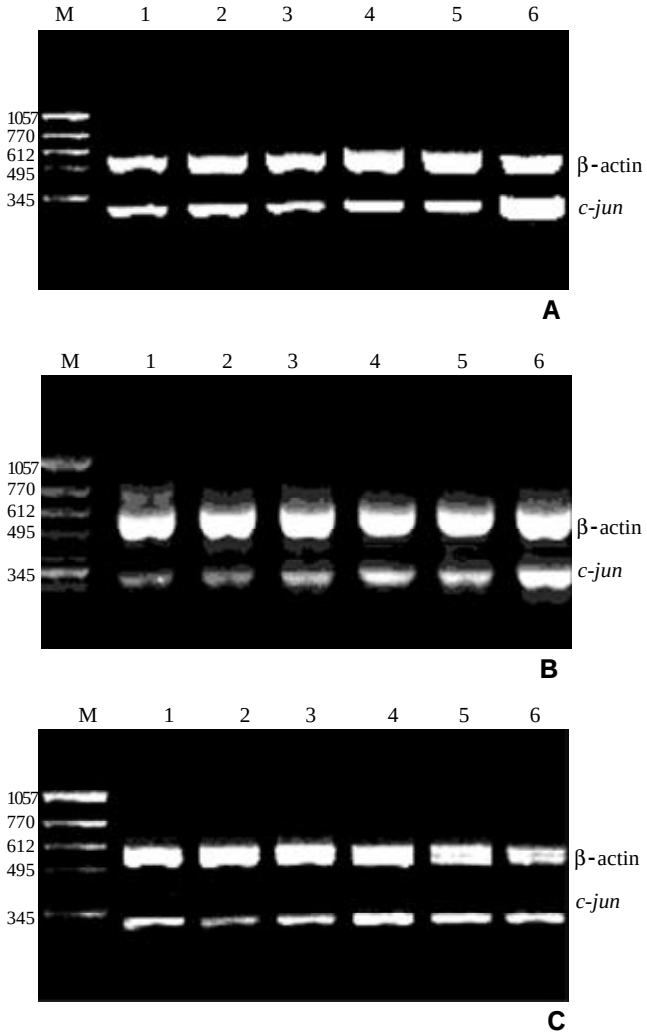

Figure 1 Effect of VES on the expression of *c-jun* mRNA in SGC-7901 cells for different time points by RT-PCR. A: treatment of VES for 3 h; B: treatment of VES for 6 h; C: treatment of VES for 24 h. 1: UT control; 2: succinate; 3: vitamin E; 4: VES at 5 mg·L^{-1}; 5: VES at 10 mg·L^{-1}; 6: VES at 20 mg·L^{-1}; M: molecular weight marker.

Table 2 The relative expression of *c-jun* mRNA in SGC-7901 cells (n=6,$\bar{x}\pm s$)

Groups	Ratio of *c-jun* / β-actin		
	3h	6h	24h
UT control	0.469±0.092	0.432±0.095	0.368±0.104
succinate	0.426±0.082	0.408±0.078	0.361±0.083
vitamin E	0.514±0.101	0.430±0.081	0.367±0.075
5mg·L^{-1} VES	0.550±0.115	0.621±0.086^b	0.584±0.097^b
10mg·L^{-1} VES	0.471±0.086	0.584±0.101^a	0.421±0.077
20mg·L^{-1} VES	1.663±0.109^b	0.905±0.099^b	0.411±0.094

$^aP<0.05$, $^bP<0.01$, *vs* UT control.

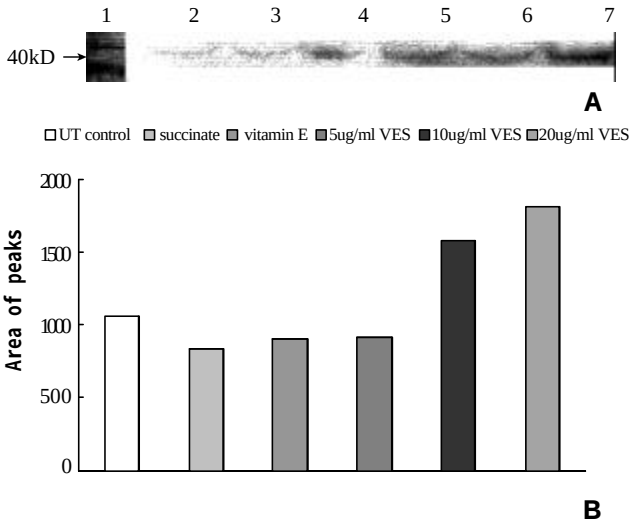

Figure 2 The expression of *c-jun* protein in SGC-7901 cells following treatment of VES for 3h. Lane1: Molecular weight marker; Lane2: UT control; Lane3: succinate; Lane4: vitamin E; Lane5: VES at 5 mg·L^{-1}; Lane6: VES at 10 mg·L^{-1}; Lane7: VES at 20 mg·L^{-1}.

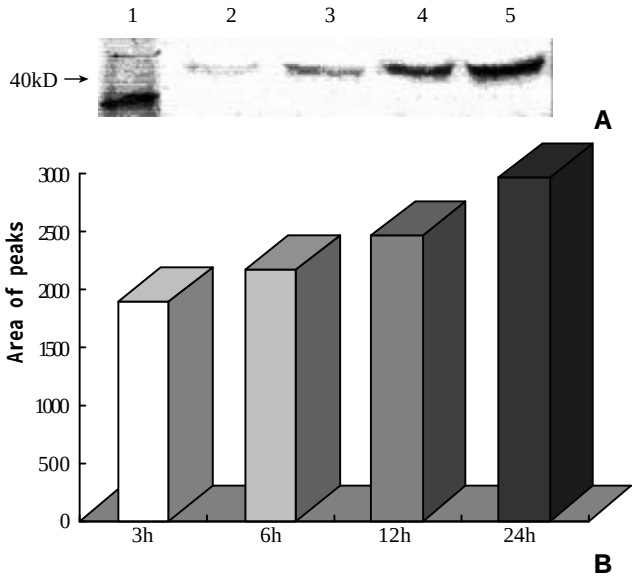

Figure 3 The expression of *c-jun* protein in SGC-7901 cells following treatment of VES at 20 mg·L^{-1} for different time poits. Lane1: Molecular weight marker; Lane2: 3 h; Lane3: 6 h; Lane4: 12 h; Lane5: 24 h

DISCUSSION

The oncogene, *c-jun*, belongs to an immediate early gene and can be rapidly and transiently induced in response to multiple extracellular stimuli[36-38]. The product of *c-jun* gene is a nuclear transcription factor, an important composition of activation protein 1 (AP-1) dimmers, involved in signal transduction and regulation of many kinds of genes[39-41].

Transcription of *c-jun* mRNA rises after exposure of cells to a number of treatment including ultraviolate, irradition, heat shock,H_2O_2,TNF-α and other apoptosis-associated factors[42-46]. In addition to this transcriptional mode of regulation, *c-jun* activity can also be modulated directly at the protein level. In certain cell types, induction of *c-jun* is observed during apoptosis. There is some evidence that prolonged expression of *c-jun* in selected vulnerable cells suggests neuronal cell death[47].

Apoptosis is an innate program of cell suicide that is required for removal of unnecessary or damaged cells from bodily structures. Apoptosis is complex and regulated by a variety of factors[48-58]. Previous studies showed that the induction of apoptosis in tumor cells is one of the important mechanisms of VES-induced cell growth inhibition[59-61]. In the present study, the expression of *c-jun* mRNA and protein was measured in human gastric cancer SGC-7901 cells treated with VES at different doses for different time points. We found that the expression of *c-jun* mRNA was evidently promoted after 3 h of VES treatment at 20 mg·L^{-1} and reduced to the normal level after 24 h of treatment; whereas in the case of VES treatment at 5 mg·L^{-1}, that was also increased after 3 h and remained a high level after 24 h. The data above showed that the *c-jun* activation was enhanced and prolonged by VES, therefore indicating that *c-jun* is involved in VES-triggered apoptosis in SGC-7901 cells. The results from western blot ananlysis showed that the level of *c-jun* protein was elevated following SGC-7901 cells were treated with VES at different doses for 3 h and with VES at 20 mg·L^{-1} for different time in a dose- and time-dependent manner.

The diversity of signals and signaling pathways that are directed toward *c-jun* is also reflected in the biological responses, in which the transcription factors have been implicated. It is reported that the mainly biological functions of *c-jun* are blockage of cell cycle and induction of apoptosis[62-64]. The study presented here demonstrated that VES can obviously increase the expression of *c-jun* mRNA and protein in human gastric cancer SGC-7901 cells, implicating that *c-jun* is involved in VES-induced apoptosis.

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• GASTRIC CANCER •

Mutation analysis of APC gene in gastric cancer with microsatellite instability

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Abstract

AIM: To evaluate the role of APC mutation in gastric carcinogenesis and to correlate APC mutation with microsatellite instability (MSI) in gastric carcinomas.

METHODS: APC mutation was measured with multiplex PCR, denaturing gradient gel electrophoresis and DNA sequencing; and MSI was analyzed by PCR-based methods.

RESULTS: Sixty-eight cases of sporadic gastric carcinoma were studied for APC mutation at exon 15 and MSI. APC mutaions were detected in 15(22.1 %) gastric cancers. Frequence of APC mutation (33.3 %) in intestinal type of gastric cancer was significantly higher than that in diffuse type (13.1 %, $P<0.05$). On the contrary, no association was observed between APC mutation and tumor size, differentiation, depth of invasion, metastasis or clinical stages. Using five microsatellite markers, MSI in at least one locus was detected in 17 of 68 (25 %) of the tumors analyzed. APC mutations were all detected in MSI-L (only one locus, $n=9$) or MSS(tumor lacking MSI or stable, $n=51$), but no mutation was found in MSI-H (⩾2 loci, $n=8$).

CONCLUSION: APC mutation is involved in carcinogenesis of intestinal type of gastric cancer and is independent of MSI phenotype but related to the LOH pathway in gastric cancer.

Fang DC, Luo YH, Yang SM, Li XA, Ling XL, Fang L. Mutation analysis of APC gene in gastric cancer with microsatellite instability. *World J Gastroenterol* 2002;8(5):787-791

INTRODUCTION

The mechanisms of carcinogenesis in the gastric mucosa remain unclear. Adenomatous polyposis *coli* or APC, has been characterized as a tumor suppressor gene and considered a "gatekeeper" because alterations in this gene occur as an early event in the neoplastic transformation[1-3]. The APC gene was first identified in the germline of individuals with the condition known as familial adenomatous polyposis or FAP[4] and has been shown to play a role in the developmemt of sporadic colorectal cancer[5,6]. Studies have attempted to characterize the mutation of APC in sporadic gastric cancer[7-10] and in a limited number of familial gastric cancer[11,12]. It has been found that inactivation of APC plays a role in the development of some gastric cancers, particularly very well differentiated adenocarcinomas and signet-ring cell carcinomas[13,14], and that the mutations of the APC gene, similar to those in colorectal tumorigenesis, occur during the early stages of gastric adenoma development[15,16].

Genetic instability is strongly involved in neoplastic transformation and progression[17]. In gastrointestinal carcinomas, such genetic instability may be classified into two different forms in which hypermutability occurs either by means of chromosomal instability or microsatellite instability (MSI)[18-23]. 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)[24,25] in which germ-line mutation occurs within the mismatch repair genes hMSH2, hMLH1, hPMS2 or hMSH6[26-28]. Mutations of the transforming growth factor type β receptor gene (TGF-βRⅡ), and BAX gene are strongly correlated with MSI[29-31]. 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[32-34]. Although mutations of APC have been reported in gastric cancer, less clear, however, is the relevance of APC mutation as a potential factor in MSI-positive gastric cancer. The aim of the present study is to correlate APC mutation with MSI in gastric carcinomas.

MATERIALS AND METHODS

Tissue samples

Sixty-eight cancer and corresponding normal tissues were obtained from surgically resected gastric carcinoma in Southwest 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 confirm 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.

Multiplex PCR

Multiplex PCR was utilized in order to visualize the product of several regions simultaneously. Singleplex PCR was also used to help confirm cases and for regions that were difficult to amplify. Multiplex PCR analysis of APC exon 15 was performed using primers with a GC clamp (Table 1). Genomic DNA(100-400 ng), 0.25 μM primer for multi-primer reaction,

1×buffer, 1.5 mM $MgCl_2$, 0.25 mM of each dNTPs, and 0.1 U/μl Amplitaq Gold were combined in 10 μl reaction. The PCR reaction consisted of a denaturation step at 95 ℃ for 12 minutes followed by 32 cycles of 40 seconds at 94 ℃, 60 seconds at 49 ℃, and 90 seconds at 72 ℃. Elongation occurred through 10 minutes at 72 ℃, 10minutes at 98 ℃, 30 minutes at 50 ℃ and 30 minutes at 37 ℃.

Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed for scanning mutations at 15 exon of APC gene. 8 μl of each sample was loaded on the gels consisting of 10 % polyacrylamide and 10-60 % urea-formamide. The gels were run at 90V for 17 hours in tanks of buffer at 50 ℃. Gels were stained with 0.5×Sybr Green I and Sybr GreenⅡ for 20 minutes and visualized by UV transillumination. Heteroduplex samples were further analyzed by manual sequencing.

Manual sequencing

Samples were amplified for sequencing with PCR primers that did not include the GC clamp (Appendix A). The PCR reaction included 200-400 ng of genomic DNA, 0.25 μM primer, 1×buffer, 1.5mM $MgCl_2$, 0.25mM of each dNTPs, and 2.5U Amplitaq Gold were combined in a 10 μl reaction. The PCR reaction consisted of a denaturation step at 94 ℃ for 3 minutes followed by 35 cycles of 60 seconds at 94 ℃, 60 seconds at 55 ℃, and 60 seconds at 72 ℃. A final extension step included 10 minutes at 72 ℃. 1.5 % agarose gels were run at 150V to confirm the existence of PCR product. The product was purified following the instructions of the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Following the purification process, the product was run on a 1.5 % agarose gel at 150V to verify the product. Sequencing was performed following the instruction of the system. The film was read independently by two individuals according to the APC sequence.

Table 1 Primer Sequences used in APC mutation analysis

Exon	GC Clamp	Primer Sequences	Size (bp)
15.1	2/0	F-TTCAGGCAAATCCTAAGAGA/R-TTGAGCCAGGAGACATAATA	329
15.2	0/2	F-GGAATCTCATGGCAAATAGG/R-TCATCATGTCGATTGGTGTC	284
15.3	2/0	F-AGCAAAGTCTCTATGGTGAT/R-TGACACTTCTTCCATGACTT	387
15.4	0/2	F-CTACCATCCAGCAACAGAAA/R-TGGCATAAGGCATAGAACAT	312
15.5	2/0	F-AAGCTCTGCTGCCCATACAC/R-CTAGGTCGGCTGGGTATTGA	282
15.6	2/5	F-CTTATGCCAAATTAGAATAC/R-ACTGCTCATCTGAATATTTA	306
15.7	7/5	F-ATGATGGAGAACTAGATACA/R-ATCAGTGCTCTCAGTATAAA	253
15.8	2/5	F-AAGACCCAAACACATAATAG/R-TAATTGGTAGGCTTATCATC	327
15.9	2/0	F-AAATCGAGTGGGTTCTAATC/R-TTCTCACTGCTTGAAGACAT	355
15.10	2/6	F-CAGCCTATTGATTATAGTTT/R-AACATATTGGAGTATCTTCT	344
15.11	2/0	F-TTAACCAAGAAACAATACAG/R-CTTCGCTCACAGGATCTT	320
15.12	2/0	F-GGAAGCAGATTCTGCTAATA/R-ATAGTGTTCAGGTGGACTTT	287
15.13	2/5	F-AGAATCAGCCAGGCACAAAG/R-CATGGTTTGTCCAGGGCTAT	310
15.14	2/6	F-TGGTAAGTGGCATTATAAGC/R-AGTATCAGCATCTGGAAGAA	279
15.15	5/2	F-AAACCAAGCCAGAAGTACCT/R-AGGCTGCTCTGATTCTGTTT	335
15.16	2/5	F-CGATGAGCCATTTATACA/R-TTTGCTTTACGTGATGAC	291
15.17	4/2	F-ATTATTTCTGCCATGCCAAC/R-TTCGATTGTTAGATCACTTA	300
15.18	2/6	F-TGCCACGGGTGTATTGTGTT/R-TTTCCCTTTGGGCATAGCAG	351
15.19	4/2	F-GGTGATATTCTTGCAGAATG/R-CACGTGTCCTATATTCAGTA	250
15.20	2/0	F-GAAACCAACTTCACCAGTAA/R-AATAGGCGTGTAATGATGAG	378
15.21	2/0	F-AGCTCCCAAATAATGAAGAT/R-GATTGTTGGTTGGAGGTTAG	342
15.22	2/6	F-GCTAAAGTTACCAGCCACAC/R-TTGGTCAATGTCACTGAGAG	
15.23	2/0	F-CCAGTCATCCAAAGACATAC/R-CAACAGGTCATCTTCAGAGT	
15.24	2/5	F-TCACAGGGAGAACCAAGTAA/R-TTTGCACCTTCCTGAATAGC	
15.25	2/3	F-ATGGGTGGCATATTAGGTGA/R-TCGTGGGCCTTTATTACTTG	

GC clamp: 1=CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG;
2=CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG;
3=CG; 4=GCGCG; 5=CGTCCCGC; 6=GCGCCCGTCCCGCCC;
7=CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGCCCG

MSI detection

MSI analyses included five microsatellite markers: BAT25, BAT26, BAT40, D2S123, and D5S346. PCR was performed as previously described[35]. 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 MSI-H if they manifested instability at two or more markers, MSI-L if unstable at only one marker, and MSS if they showed no instability at any marker.

Statistical analysis

Chi-square test with Yates' correction was used. A P value <0.05 was considered significant.

RESULTS

Multiplex PCR and DGGE were used to scan the mutation at the exon 15 of APC in 68 gastric cancers (Figure 1). The samples that showed alterations by DGGE were further analyzed through munual sequencing (Figure 2). APC mutaions were detected in 15 samples (22.1%) held APC mutations that lead to premature protein truncation (Table 2). The truncation of the protein occurred as a result of a stop codon created through a base pair change, an insertion, or a deletion.

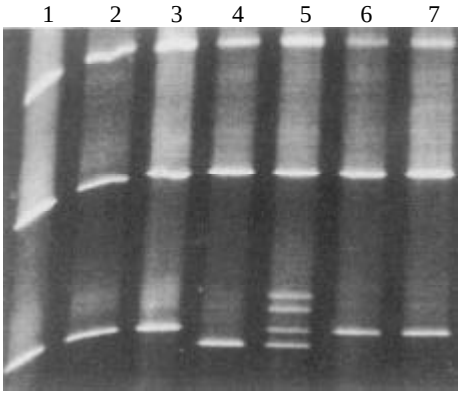

Figure 1 Abnormal four-band patterns in lane 5 after multiplex PCR and denaturing gradient gel

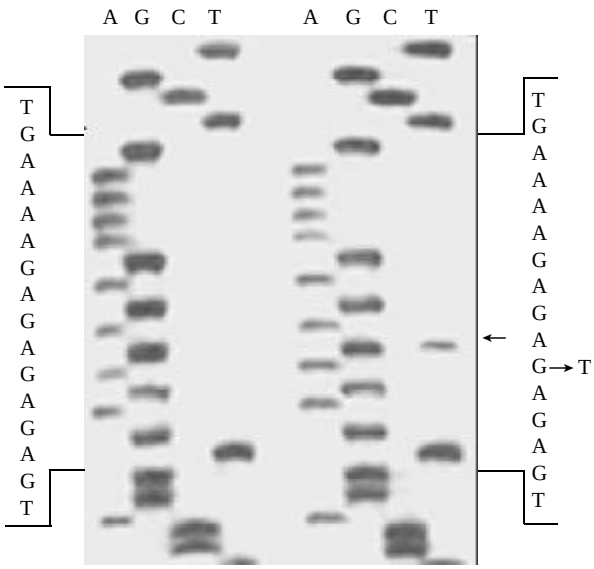

Figure 2 DNA sequencing shows a G→T change at 1464 coden

We compared mutation of exon 15 of APC gene in gastric cancer with various clinicopathological parameters including tumor size, differentiation, TNM status, clinical stage and Lauren's types. Table 3 shows the association of mutations of APC to the clinicopathological parameters of 68 gastric carcinomas. Mutations of APC gene were observed more common in intestinal type than in diffuse type tumors (P<0.05).

Alterations of electrophoretic patterns of PCR products of five microsatellite markers were compared between tumor and normal DNA in each patient. MSI affecting at least one locus was observed in 17 (25 %) of 68 tumors, among which eight (11.8 %) were MSI-H, nine (13.2 %) were MSI-L, and fifty-one (75 %) were MSS. Table 4 shows the association of mutations of APC to MSI of 68 gastric carcinomas. Three mutations were found in 9 tumors with MSI-L and 12 were found in 51 tumors with MSS, and no mutation was found in 8 tumors with MSI-H.

Table 2 APC mutation in 12 gastric carcinomas

Exon region	Nucleotide alteration	Nucleotide location	Codon number
15.4	C→A	2883	940
15.5	insA	2866	935
15.10	del A	3835 or 3836	1258
15.11	del AAAA	3981	1307
15.12	del AAAGA	3988	1309
15.12	G→T	4010	1317
15.13	delT	4146	1362
15.13	delGT	4254	1398
15.13	delT	4282	1407
15.14	C→T	4410	1450
15.14	G→T	4452	1464
15.15	delAG	4455	1465
15.19	insCT	5326	1755
15.21	insT	5596	1845
15.22	delG	5940	1960

Table 3 The relationship between the APC mutation and clinicopathological parameters

Parameters	No. of cases	APC mutation
Size		
<5cm	31	7
>5cm	37	8
Differentiation		
Well	14	4
Moderate	20	6
Poor	28	3
Mucinous	6	2
Lauren classification		
Intestinal type	30	10[a]
Diffuse type	38	5
Serosal invasion		
Absent	30	6
Present	38	9
Metastasis		
Absent	33	8
Present	35	7
Clinical stage		
Ⅰ and Ⅱ	36	9
Ⅲ and Ⅳ	32	6

[a]P<0.05 *vs* diffuse type

Table 4 The relationship between MSI and APC mutation

MSI status	No. of cases	APC mutation
MSI-H	8	0
MSI-L	9	2
MSS	51	13

DISCUSSION

Mutations of APC gene has been shown to play an important role in colorectal tumorigenesis. A germline mutation in APC contributes significantly to the development of colitis-associated neoplasia[36]. In the current study, we investigated mutations of the APC gene in 68 gastric carcinomas obtained surgically. Mutations at exon 15 of APC gene were detected in 22.1 % of gastric cancers. This finding is similar to the previous studies[14,15]. This proportion may be an underestimate because the mutation of exons 1 through 14 were not detected in this study. Previous investigations have found that the 5' half of APC holds the majority of the germline mutations[3,37]. The mutations identified in the study were spread throughout the exon 15 of APC sequence. Sequencing analysis confirmed that the mutations resulted in truncation of the gene products or in an amino acid change. The APC gene encodes a large protein with multiple cellular functions and interactions. Mutations in this gene would lead to alterations in signal transduction of cell, differentiation, mediation of intercellular adhesion, stabilization of the cytoskeleton and possibly regulation of the cell cycle and apoptosis[38,39]. Our results imply that APC plays a crucial role in gastric carcinogenesis, as was observed in colorectal carcinogenesis.

In the current study, we did not find an obvious relationship between the APC mutation and tumor size, depth of invasion, node metastasis or clinical stages, indicating a limited role of the APC mutation in predicting prognosis of gastric carcinomas. This finding is in agreement with the recently published data on cholangiocarcinoma and breast cancer[40,41]. Gastric carcinomas can be divided into "intestinal" type and "duffuse" type. The intestinal type of gastric cancer is the predominant type in elderly population at high risk and preceded by well-defined precancerous lesions, such as intestinal metaplasia and atrophic gastritis. The diffuse type is relatively more frequent in low risk populations and is not as often preceded by intestinal metaplasia. A distinct genetic pathway exists in gastric carcinogenesis of different histological subtypes and their tumor progression[42-45]. Increased beta-catenin mRNA levels and mutational alterations of the APC and beta-catenin gene were present in intestinal type gastric cancer[46,47], whereas epigenetic inactivation of E-cadherin via promoter hypermethylation may be an early critical event in the development of undifferentiated tumors[48-51]. In this study, marked difference in APC mutation was noted in gastric cancer by histological type. APC mutations were significantly more frequent in intestinaltype gastric cancers as compared with diffusetype gastric cancers, suggesting that APC gene is not only a predisposing gene in colorectal cancer but also a predisposing gene in intestinal type of gastric cancer. The mutation of APC gene may be considered makers for intestinal or colonic differentiation. The mutation of APC in the majority of intestinal type cancers also supports the theory that these cancers result from transformation of intestinal metaplasia.

There is evidence that MSI cancer comprises distinctive MSI-H and MSI-L categories[14]. MSI-H cancers are distingushed clinicopathologically and in their spectrum of genetic alterations from cancers showing MSI-L and MSS cancers[14]. Our previous studies indicated that MSI-H gastric cancers often show lower frequency of LOH of APC, MCC and DCC genes than do MSI-L and MSS cancers[1]. In present study, 15 APC mutations were all detected in MSI-L and MSS, but no mutation was found in those showing MSI-H. This result indicates that APC is mutational target in MSI-L and MSS tumor cells and support the notion that APC mutation-positive tumors may identify an alternative pathway which is probably different from MSI-related phenomenon observed in HNPCC. Our analysis of APC mutation 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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• GASTRIC CANCER •

Effects of Chinese Jianpi herbs on cell apoptosis and related gene expression in human gastric cancer grafted onto nude mice

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Abstract

AIM: To explore the mechanism of the Sijunzi decoction and another Chinese herbal recipe (SRRS) based mainly on the Sijunzi decoction in treatment of gastric cancer.

METHODS: A human gastric adenocarcinoma cell line SGC-7901 grafted onto nude mouse was used as the animal model. The mice were divided into 3 groups, one control and the two representative experimental conditions. Animals in the two experimental groups received either Sijunzi decoction or SRRS over a 40-day period starting at 1st day after grafting. Control animals received saline on an identical schedule. Animals were killed 41 days after being grafted. The effect of therapy was assessed by two ways: (1) tumor size was periodically measured during the life of the animals; (2) tumor weight was determined by a electron balance immediately after the animals killed. For detection of apoptotic cells, apoptotic indices(AI) were examined by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) method. Morphological alterations were observed with electron microscopy. S-P immunohistochemical method was used to detect the expression of Ki-67 in xenografts. Expression of bcl-2 and p53 was semiquantitatively detected using a reverse transcriptase-polymerase chain reaction (RT-PCR) technique.

RESULTS: When compared with controls, tumor growth (size and weight) was significantly inhibited by treatment with the Sijunzi decoction ($P<0.05$) or SRRS ($P<0.01$). The tumor inhibitory rate in the Sijunzi decoction group was 34.33 % and SRRS group 46.53 %. AI of human gastric cancer xenografts in nude mice was significantly increased to 16.24 ±3.21 % using TUNEL method and 11.38±6.46 % by FACScan in the Sijunzi decoction group compared with the controls (TUNEL: 2.63±1.03 %, $P<0.01$; FACScan: 7.15± 1.32 %, $P<0.05$). SRRS group was also found a significantly increased AI by using TUNEL method and flow cytometry analysis compared with the controls (TUNEL: 13.18±3.05 %, $P<0.05$; FACScan: 11.58±5.71 % ($P<0.05$). Under electron microscope, cell shrinkage, nuclear chromatin condensation, formation of membrane blebs and apoptotic bodies were frequently observed in Sijunzi decoction group and SRRS group. The average labeling index (LI) for Ki-67 in SRRS group was significantly decreased to 8.43±2.22 % compared with the control group (10.37±4.91 %) ($P<0.05$). The average labeling index for Ki-67 in sijunzi decoction group was 7.95± 2.54 % which was lower than that of the control group, but showed no significance ($P=0.07$). The expression level of p53 mRNA was lower in both Sijunzi decoction group and SRRS group than that in control group ($P<0.05$; $P<0.01$). The expression of bcl-2 mRNA was also decreased in SRRS group compared with the control ($P<0.01$).

CONCLUSION: The inhibition of gastric cancer cell growth *in vivo* by Chinese Jianpi herbs and SRRS is related to induction of the cell apoptosis which may be involved in aberrant expression of p53 and bcl-2 genes

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INTRODUCTION

Apoptosis plays a crucial role in the proliferation and turnover of cells in various tumors. It has been clear that its extent is often enhanced in tumor by many anticancer drugs[1-5], such as cytotoxic drugs[6], hormone[1], or some Chinese herbal medicine[7-10]. In clinic studies, some Chinese Jianpi herbs had been proved to have effect on malignant tumors, especially on gastric and colorectal tumors[11-13]. Among these herbs we found that Codonopsis pilosula (Franch) Nannf., Atractylodes macrocephala koidz. and the Sijunzi Decoction might suppress gastric carcinoma cell proliferation and cause tumor cell loss and the nuclear condensation *in vitro*[14]. The Sijunzi Decoction and another Chinese herbal recipe SRRS of deheat-toxin, softening hard lumps and dissolving phlegm enhance apoptosis of human gastric cancer xenografts in nude mice[15]. Based on previous studies, we examined the apoptotic indices of human gastric cancer grafted onto nude mice after the treatment with Sijunzi Decoction and SRRS and investigate the underlying mechanism of the tumor suppressive effect of these Chinese Jianpi herbs.

MATERIALS AND METHODS

Materials

Animal models Thirty 6-7 weeks old female BALB/C-nu/nu mice (weight 18-22 g) and a human gastric carcinoma cell line SGC-7901 were obtained from Shanghai Tumor Institute (No.01842). The animals were subcutaneously grafted with the SGC-7901 cell. The tumor transplantation procedure was described previously[15].

Drugs The Sijunzi Decoction is composed of Codonopsis pilosula (Franch) Nannf., Atractylodes macrocephala koidz, Poria cocos (Schw.) Wolf, Glycyrrhiza uralensis Fisch. The concentration of the Sijunzi Decoction was 160 g/L; SRRS is composed of Atractylodes macrocephala koidz., Poria cocos(Schw.) Wolf, Sargentodoxa cuneata(Oliv.) Rehd. Et Wils., Prunella vulgaris L. And etc. The concentration of the SRRS decoction was 240 g/L.

Experimental schedule After grafting the mice were randomly divided into 3 groups, one control and the two experimental groups assigned to receive the Sijunzi Decoction or SRRS. Each animal in the two experimental groups was given 0.5 mL of the Sijunzi Decoction or SRRS by gastric perfusion every day over a 40-day period beginning at 1st day after grafting. The control animals received normal saline according to the same schedule. Animals were killed 41 days after being grafted.

Methods

Tumor growth The effect of therapy was assessed by two ways: **(1)** tumor size was measured twice a week by multiplying two perpendicular diameters. **(2)** tumor weight was determined immediately by electron balance after the animals were killed.

Apoptosis For detection of apoptotic cells, apoptotic indices were examined by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) method[16-18] and flow cytometry analysis. Morphological alterations were observed with electron microscope. **(1)** TUNEL: *In situ cell death detection Kit POD* (ISCDD, BOEHRINGER MANNHEIN) was used to detect the apoptotic cell. The procedures was according to protocol of the kit and the other references. The positive cells were identified, counted and analyzed under the light microscope. Non-necrotic zone was selected in the tissue section and images were sent to computer by AEC camera (Grundig Electronic Co. Ltd., Germany). 10 image at least 1000 cells were selected on the screen, positive ratio analyzed by KS400 Video Image Digital Analysis System (ZEISS, Germany). (2)Electron microscopy: Some of specimens in each group were fixed with 2.5 % glutaraldehyde. Semi-thin and ultra-thin sections were cut and viewed with scanning electron microscope. The characteristics of cell apoptosis showed nuclear chromatin condensation, peripheral masses of condensed chromatin with enclosed membrane or crescent. The nuclear membrane is complete. There is little or no swelling of mitochondria or other organelles. (3) Flow cytometry analysis: Propidium iodide (PI) staining[19-21] was used for flow cytometric detection of apoptosis. 10^6 cells from each of the sample were treated with RNase and stained with PI. The apoptotic cells having DNA strand breaks that had been labeled were measured on a flow cytometer (FACSCalibur, Becton Dickinson, USA). The data from 10^6 cells/sample were collected, stored, and analyzed using CELLQUEST (Becton Dickinson USA) and ModFIT LT for mac V1.01 software (Becton Dickinson, USA).

Cell proliferation The level of expression of Ki-67 was used as a marker of cell proliferation. In present study Ki-67 was measured by S-P immunohistochemical method.

Expression of bcl-2 and p53 mRNA The Expression of p53 (5-8 exons) and bcl-2 was semiquantitatively detected using RT-PCR technique[22-24]. A β-actin was used as an internal standard. Sequences of the primers used for RT-PCR analysis are described in Table 1. Total cellular RNA was isolated from tumor tissues using the acid-guanidium-phenol-chloroform technique. The RNA extracted from each sample was qualified by agarose gel electrophoresis and ethidium bromide staining and the amount of RNA was determined by electrophotometry *A*:260/280. The extracted RNA was converted to first strand cDNA with AMV reverse transcriptase (Promega). p53 and β-actin were amplified by polymerase chain reaction consisting of stage one: 2 minutes of denaturation at 94 ℃ before additon of Taq DNA polymerase; stage two: 1 minute of denaturation at 94 ℃, 1 minute of primer annealing at 56 ℃, and 1 minute of extension at 72 ℃ (Taq DNA polymerase, Shanghai Sangon Biology Engineering Technique Service Co. Ltd.). Gene expression of bcl-2 was also analyzed by RT-PCR, in the same manner except for annealing at 55 ℃. The cycler of PCR gene amplification is from Omn-E (Hybaid). Each reaction tube contained: 1.5 μl 25mM $MgCl_2$ +2.5 μl 10×PCR buffer +0.5 μl 10mM dNTP +1 μl×2 20 pmol/μl p53 or bcl-2 primers or β-actin primers (primer concentration: 0.8 μM) +2 μl cDNA +0.5 μl Taq (2.5-5U/μl) +16 μl ddH_2O. Quantitative analysis: After the amplification step was completed, equal amounts (5 μl) of PCR produces were loaded onto each lane of 1.7 % agarose gels and electrophoresed. FR-200UV/WHITE ANALYSIS (Shanghai Fu Ri Science & Technology Co. Ltd., China) took the image of electrophoresis with β-actin as internal standard, and FR-980 biological electrophoresis analysis system (Shanghai Fu Ri Science & Technology Co. Ltd., China) was used to analyze the quantity of nuclear acid.

Statistical analysis

The results were expressed as $\bar{x}\pm s$ and significant difference was assessed by Student' s *t* test.

Table 1 Sequences of primers for amplifed cDNA of the p53,bcl-2 and β-actin

Primers		Sequences	Combining sites	Amplifiers
p53	Sense	5'-GGAGGTTGTGAGGCGCTGC-3	645bp-663bp	311bp
	Antisense	5'-CACGCACCTCAAAGCTGTTC-3	936bp-955bp	
bcl-2	Sense	5'-CAGCTGCACCTGACGCCCTT-3'	1810bp-1829bp	191bp
	Antisense	5'-GCCTCCGTTATCCTGGATCC-3'	2021bp-2040bp	
β-actin	Sense	5'-AGCGGGAAATCGTGCGTGAC-3	665bp-674bp	471bp
	Antisense	5'-ACTCCTGCTTGCTGATCCACATC-3	1103bp-1125bp	

Table 2 Chinese Jianpi herbs-induced effects on gastric cancer cell SGC-7901 ($\bar{x}\pm s$)

Treatment	Tumor weight / g (*n*)	Tumor size / mm^3 (*n*)	Percentage of control / %	Apoptotic index(AI) / %		ki-67 / %
				TUNEL (*n*)	FACScan (*n*)	
Sijunzi Decoction	0.66±0.16(9)[a]	371.81±52.51(9)[a]	34.33	16.24±3.21(9)[b]	11.38±6.46(9)[a]	7.95±2.54(9)[c]
SRRS	0.54±0.23(9)[b]	322.66±126.14(9)[b]	46.53	13.18±3.05(8)[a]	11.58±5.71(8)[a]	8.43±2.22(8)[b]
Control	1.01±0.32(10)	603.61±263.39(10)		2.63±1.03(10)	7.51±1.32(10)	10.37±4.91(10)

[a]$P<0.05$, [b]$P<0.01$, [c]$P=0.070$ *vs* control *t* test.

RESULTS

Chinese Jianpi herbs-induced effects on tumor growth

Each one case that the xenograft emerged later was eliminated in Sijunzi decoction group and SRRS group. Compared with the control group, tumor growth (size and weight) was significantly inhibited by treatment with the Sijunzi decoction ($P<0.05$) or SRRS ($P<0.01$). The tumor inhibitory rate of the sijunzi decoction group was 34.33 % and that in the SRRS group was 46.53 % (Table 2).

Chinese Jianpi herbs-induced effects on tumor cell apoptosis

Apoptotic index (AI) of xenografts in nude mice was significantly increased to 16.24±3.21 % using TUNEL method and 11.38± 6.46 % FACScan in the Sijunzi decoction treatment group, compared with the controls (TUNEL: 2.63±1.03 %, $P<0.01$; FACScan: 7.15±1.32%, $P<0.05$). SRRS group was also found a significantly increased AI by using TUNEL method and flow cytometry analysis compared with the controls (TUNEL: 13.18±3.05 %, $P<0.05$; FACScan: 11.58±5.71%; $P<0.05$). But there was no significant difference between Sijunzi decoction group and SRRS group by using either TUNEL method or flow cytometry analysis. Under electron microscope cell shrinkage, nuclear chromatin condensation, formation of membrane blebs and apoptotic bodies were frequently observed in Sijunzi decoction group and SRRS group (Table 2).

Chinese Jianpi herbs-induced effects on tumor cell proliferation

The average labeling index for Ki-67 (LI) in SRRS treatment group (8.43±2.22 %) was significantly lower than that in the control group (10.37±4.91 %) ($P<0.01$). The average labeling index for Ki-67 in sijunzi decoction group was 7.95±2.54 % which was lower than that of the control group, but showed no significance as the P value was 0.07 (Table 2).

Chinese Jianpi herbs-induced genetic effects

Expression of p53 (exons 5-8) and bcl-2 was semiquantitatively detected with β-actin used as an internal standard. The expression level of p53 mRNAs was 0.36±0.27, significantly lower in Sijunzi decoction group than that in control group (0.69±0.20) ($P<0.05$), in SRRS group (0. 19±0.18) also significantly lower than that in control group ($P<0.01$). The expression of bcl-2 mRNA in SRRS group (0.33±0.23) was decreased, compared with the control (0. 81±0.40) ($P<0.01$). In Sijunzi decoction group the expression of bcl-2 (0.41±0.15) slightly decreased, compared with the control, but no statistics difference existed ($P=0.071$) (Table 3, Figure 1).

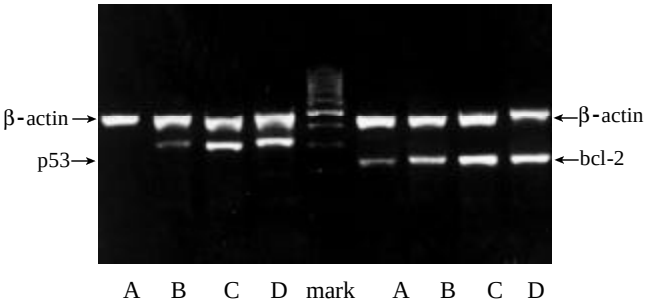

Figure 1 p53,bcl-2 mRNA expression in SGC-7901 grafted onto nude mice after treatment of Chinese Jianpi herbs by RT-PCR analysis. Lane A: SRRS group, Lane B: Sijunzi decoction group, Lane C: control group, Lane D: control group.

Table 3 Chinese Jianpi herbs-induced genetic effects ($\bar{x}\pm s$)

Treatment	p53(exons 5-8)	bcl-2
Sijunzi Decoction	0.36±0.27(8)[a]	0.41±0.15(8)[c]
SRRS	0.19±0.18(9)[b]	0.33±0.23(9)[b]
Control	0.69±0.20(9)	0.81±0.40(9)

[a]$P<0.05$, [b]$P<0.01$, [c]$P=0.071$ *vs* control t test.

DISCUSSION

Despite its declining incidence, the gastric carcinoma remains one of the most common cause of cancer-related death in the world[25-27]. At present gastric carcinoma is still detected later in most patients throughout the world, and even with curative resection, they remain at a high risk of relapse. Thus, there is a great need for effective adjuvant therapy for patients with gastric carcinoma[28-30]. Our previous clinic paired comparative studies suggested that Chinese herbal recipe *SRRS* have therapeutic effects on advanced gastric cancer, with increasing the surviving period of the patients, improving the life quality, and decreasing the metastasis and recurrence rates after operation[12,14]. Because of its lower toxic side-effect compared with chemical therapy, it is worth to make a further research on its anti-cancer mechanism.

As the other malignant tumor, gastric carcinoma is not only a disease with abnormal cell proliferation and differentiation, but also a disease with abnormal apoptosis[5, 31-35]. The enhanced induction of apoptosis in human gastric carcinoma cells can be observed after treatment with 5-Fluorouracil[36],Cisplatin[37], arsenous oxide[38], etc. These data suggest that inducing cancer cell apoptosis may be a therapeutic method for gastric carcinoma. The present study indicated that tumor growth was significantly inhibited by treatment with the Sijunzi decoction or SRRS. TUNEL method and cytometry analysis clarified that Sijunzi decoction and SRRS enhanced apoptosis. The results suggest that the inhibition of gastric cancer cells *in vivo* by Jianpi herbs described here is related to inducing apoptosis. Immunohistochemical staining for Ki-67 showed that SRRS inhibited cell proliferation. So the inhibition of gastric cancer by SRRS is also related to suppressing the proliferation.

Apoptosis is a complex, tightly regulated, and active cellular process whereby individual cells are triggered to undergo self-destruction in a manner that will neither injure neighboring cells nor elicit any inflammatory reaction[1, 39-42]. Various triggering factor initiate corresponding proteo-lysis cascade reaction depending on mitochondrion or APO-1/FAS/ CD95 receptor mediate apoptotic pathways[40,43]. There are many oncogenes and tumor suppressor gene products in the regulation and execution of apoptosis. Among them are p53, Rb, myc, ras, raf, etc[23, 40, 41, 44, 45]. p53, because of its role in apoptosis , has earned the name “guardian of the genome”. It monitors the state of DNA, and in case of DNA damage , stalls the cell cycle. This takes place through the induction of CIP/ WAF1/p21. In the absence of phosphorylated, active cyclin-dependent kinases, also another regulator of the cell cycle, Rb, remains inactive(unphosphorylated), and , hence, the cell cycle halts. This then leads to activation of DNA repair machinery. If the DNA repair fails, p53 takes over again and triggers apoptosis in a process that involves upregulation of the apoptosis-inducing bax and down-regulation of the apoptotic bcl-2[23]. p53 also upregulates KILLER/DR5, a 45-kd apoptosis-inducing member of the tumor necrosis factor receptor family. Analogous to the APO-1//FAS/CD95 receptor

system, its activation also lead to a caspase activation[37]. Thus p53 is known as one of the essential genes for cells to undergo apoptosis. In gastric cancer, mutant p53 expression decreased cancer cell apoptosis, p53 mutant provided selective growth superiority to the tumor cell. In our investigation we adopt the human gastric cancer cell line SGC-7901 which has point mutation in exon 6 codon 204 GAG→GCG coding Glu→Ala, which expresses mutant p53[46, 47]. RT-PCR detected p53 mRNA level was significantly lower in Sijunzi decoction group than that in control group. The expression level of p53 mRNA in SRRS group was also significantly lower than that in control group. The data suggest that these two recipes composed of Jianpi herbs induce SGC-7901 cancer cell apoptosis by down-regulation of mutant p53 mRNA expression.

We also detected apoptosis-inhibiting member of the bcl-2 family[41, 44]: bcl-2 mRNA. The expression of bcl-2 mRNA was decreased in SRRS group, compared with the control. In Sijunzi decoction group the expression of bcl-2 was slightly decreased compared with the control, but there was no statistics difference. It suggested that SRRS could suppress bcl-2 expression, but it was not stronger for than that of Sijunzi decoction. bcl-2 is the epitome of an antiapoptotic or survival gene. Attesting to its role in an apoptosis checkpoint, it counteracts apoptosis initiated by quite disparate signals, such as chemotherapeutic drugs, oxidative stress, viral infections, and p53. In gastric carcinoma, bcl-2 over-expressed at both protein and mRNA level in many cases[48].

In our study SRRS and Sijunzi decoction down-regulated the expression of p53 mRNA and SRRS also decrease the expression of bcl-2 mRNA in SGC-7901 cancer cell undergoing apoptosis. We inferred that p53 and bcl-2 may be involved in the regulation of these Chinese Jianpi herbs inducing gastric cancer cell SGC-7901 apoptosis. The interaction contact of p53 and bcl-2 in SRRS or Sijunzi decoction inducing apoptosis needs further investigation.

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• LIVER CANCER •

DNA-PKcs subunits in radiosensitization by hyperthermia on hepatocellular carcinoma hepG_2 cell line

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Abstract

AIM: To investigate the role of DNA-PKcs subunits in radiosensitization by hyperthermia on hepatocellular carcinoma HepG_2 cell lines.

METHODS: Hep G_2 cells were exposed to hyperthermia and irradiation. Hyperthermia was given at 45.5 ℃. Cell survival was determined by an *in vitro* clonogenic assay for the cells treated with or without hyperthermia at various time points. DNA DSB rejoining was measured using asymmetric field inversion gel electrophoresis (AFIGE). The DNA-PKcs activities were measured using DNA-PKcs enzyme assay system.

RESULTS: Hyperthermia can significantly enhance irradiation-killing cells. Thermal enhancement ratio as calculated at 10 % survival was 2.02. The difference in radiosensitivity between two treatment modes manifested as a difference in the α components and the almost same β components, which α value was considerably higher in the cells of combined radiation and hyperthermia as compared with irradiating cells (1.07 Gy^{-1} versus 0.44 Gy^{-1}). Survival fraction showed 1 logarithm increase after an 8-hour interval between heat and irradiation, whereas DNA-PKcs activity did not show any recovery. The cells were exposed to heat 5 minutes only, DNA-PKcs activity was inhibited at the nadir, even though the exposure time was lengthened. Whereas the ability of DNA DSB rejoining was inhibited with the increase of the length of hyperthermic time. The repair kinetics of DNA DSB rejoining after treatment with Wortmannin is different from the hyperthermic group due to the striking high slow rejoining component.

CONCLUSION: Determination with the cell extracts and the peptide phosphorylation assay, DNA-PKcs activity was inactivated by heat treatment at 45.5 ℃, and could not restore. Cell survival is not associated with the DNA-PKcs inactivity after heat. DNA-PKcs is not a unique factor affecting the DNA DSB repair. This suggests that DNA-PKcs do not play a crucial role in the enhancement of cellular radiosensitivity by hyperthermia.

Zeng ZC, Jiang GL, Wang GM, Tang ZY, Curran WJ, Iliakis G. DNA-PKcs subunits in radiosensitization by hyperthermia on hepatocellular carcinoma hepG_2 cell line. *World J Gastroenterol* 2002; 8(5):797-803

INTRODUCTION

Hepatocellular carcinoma (HCC) remains one of the most difficult tumors to treat[1-15]. About 90 % of patients are unresectable at presentation because of tumor size, location, or underlying parechymal disease[16-20]. Those patients are sometimes recommended to receive non-surgical therapies, including radiotherapy[21-28], radiofrequency hyperthermia[29,30], or the hyperthermia as an adjuvant to radiation in the treatment of local and regional disease[31]. Thermoradiotherapy currently offers the most significant advantages in the treatment of certain types of cancer[32]. Numerous uncontrolled studies have been performed in which comparable lesions were treated with either radiation alone or combined with hyperthermia[33]. Although many of these studies are difficult to evaluate, they give strong evidence that adjuvant heat treatment increases the probability of complete response and, consequently, tumor control. The cause of this radiosensitization has not been firmly established, however, in part this sensitization is thought to be through inhibition of repair of radiation induced DNA damage[34-36]. The mode of this repair inhibition is still unclear. Protein denaturation and aggregation appear to be the most relevant process underlying the biological effects of hyperthermia.

Several studies have shown that hyperthermia could inhibit both recovery of radiation induced potentially lethal radiation damage (PLD) and sublethal damage (SLD)[37]. Such inhibition was dependent on the time, temperature, and sequence of hyperthermia treatment. It was shown that polymerase β may be one of the mechanisms involved in thermo-radiosensitization[38]. In addition, DNA-dependent protein kinase (DNA-PKcs) plays a central role in the repair of DSB[39]. DNA-PKcs is a complex consisting of three proteins: Ku70 and Ku80 and the catalytic subunit, DNA-PKcs[39]. The Ku70 and Ku80 proteins are involved in binding to the DNA ends at DSB and this binding activates the DNA-PKcs[39]. A possible mechanism for hyperthermic radiosensitization is mediated through the heat lability of Ku subunits of DNA-PKcs[40]. To support this mechanism, we have used Hep G_2 cells to study the relationship of DNA-PKcs activity in thermal radiosensitization and the kinetics of DNA DSB rejoining with the time after irradiation, addressing the main question that the role of DNA-PKcs subunits in thermal radiosensitization.

MATERIALS AND METHODS

Cell culture

$HepG_2$ cell line was obtained from the American Type Culture Collection (ATCC) and was grown in MEM medium supplemented with 100×10^3 U· L^{-1} penicillin, 100 mg· L^{-1} streptomycin, and 100 ml· L^{-1} fetal calf serum at 37 ℃ in a humidified incubator, at an atmosphere of 50 ml· L^{-1} CO_2 and 950 ml· L^{-1} air. Cells were maintained in a phase of nearly logarithmic growth by subculturing every 4 days at an initial concentration of 2×10^5 cells in T-25 tissue culture flasks for both clonogenic assay and DNA DSB rejoining studies, 2×10^6 cells in T-75 tissue culture flasks for determination of DNA-PKcs activity. The cells were passed several times through a 20-gauge needle in syringe to make the clamp cells single in each subculturing.

Hyperthermia treatment

Hyperthermia was carried out by sealing cell cultures grown in tissue culture flasks with parafilm and immersing the flasks into a temperature control waterbath (±0.05 ℃). The continuous heating experiments ranged from 5 to 30 minutes at an interval of 5 minutes. After heating at 45.5 ℃, flasks were put into ice for 10 minutes for the DNA DSB rejoining and DNA-PKcs activity studies, or a 37 ℃ waterbath for 5 minutes to equilibrate to 37 ℃ for clonogenic assay. At this point, if required, the flasks were irradiated on the ice.

Radiation treatment

Cells in flasks were irradiated using a Pantak X-ray machine operated at 320 kV, 10 mA with a 2 mm Al filter (effective photon energy about 90 kV), at a dose rate of 2.7 Gy· Min^{-1}. Dosimetry was performed with a Victoreen dosimeter which was used to calibrate an in-field ionization monitor.

Clonogenic survival

Cells were trypsinized at 37 ℃ for 10 minutes, and pipetted 7 times to keep the clamp cells to be single cell suspension using 20-gauge needle and 5 ml syringe in 5 ml medium. The single cell suspension was adjusted and seeded into 60-mm tissue culture dishes at various densities aiming at 20-200 colonies per dish. Cells were irradiated at room temperature in 5 mL medium and were immediately kept at 37 ℃, 50 ml· L^{-1} CO_2 incubator for 13 days. Cells were stained with crystal violet and colonies of more than 50 cells were counted. The radiation results presented for heat plus X-rays were corrected for the cell killing caused by heat alone.

Induction and repair of DNA DSB

Cells for DNA DSB repair experiments were labeled with 3.7MBq· L^{-1} ^{14}C-thymidine plus 2.5 μmol· L^{-1} cold thymidine for the entire period of growth. The cells were used 3 days later as the concentration reached 1×10^6 cells/T-25 flask. When indicated by the experimental protocol, cells were treated with 20 μmol· L^{-1} Wortmannin for 1 hour or hyperthermia at 45.5 ℃ for various times before irradiation. Cells were cooled to 4 ℃ prior to irradiation and were irradiated on ice. After irradiation, the medium was replaced with fresh growth medium pre-warm at 42 ℃ to rapidly restore to 37 ℃, and then cells were quickly returned to the incubator at 37 ℃ to allow for repair. Cells were prepared for DNA DSB analysis at various time intervals thereafter.

After completion of the repair time interval, cells were trypsinized for 90 minutes in ice for the first 4 hours, and 10 minutes at 37 ℃ at later points. The cells were collected with 5 ml cold medium, centrifuged at 4 ℃, and washed with 5 ml cold serum-free medium. The cells were resuspended in 165 μl cold serum-free medium. This cell suspension was mixed with an equal volume of 10 g· L^{-1} agarose(InCert agarose, FMC) to reach a concentration of 3×10^9 cells· L^{-1}. The cell-agarose suspension was then pipetted into a 3 mm diameter glass tubes and placed into ice to allow for solidification. The solidified cell-agarose suspension was extruded from the glass tubes and cut into 3×5 mm cylindrical blocks containing approximately 1.5×10^5 cells/block[41]. Blocks were then placed in lysis buffer containing 10 mmol· L^{-1} Tris, pH 8.0, 50 mmol· L^{-1} NaCl, 0.5 mol· L^{-1} EDTA, 2 g· L^{-1} N-Lauryl Sarcosyl (NLS), A0.1 g· L^{-1} proteinase E & O, and incubated first at 4 ℃ for 45 minutes and then at 50 ℃ for 16-18 hours. Following lysis, agarose blocks were washed for 1 hour at 37 ℃ in a buffer containing 10 mmol· L^{-1} Tris, pH 8.0 and 0.1 mol· L^{-1} EDTA, and were then treated for 1 hour at 37 ℃ in the same buffer, at pH 7.5, with 0.1 g· L^{-1} RNAase A. Cells from identically treated non-irradiated cultures were also processed at pre-defined times to determine the signal generated by non-irradiated cells as background. For dose response, a similar protocol was also employed to determine the induction of DNA DSB except that in this case cells were embedded in agarose prior to irradiation with various doses on the ice, and were lysed immediately thereafter.

Pulsed-field gel electrophoresis

Asymmetric field inversion gel electrophoresis (AFIGE) was carried out in 5 g· L^{-1} Seakem agarose (FMC), cast in the presence of 0.5 mg· L^{-1} ethidium bromide, in 0.5×TBE (45 mmol· L^{-1} Tris, pH 8.2, 45 mmol· L^{-1} Boric Acid, 1 mmol· L^{-1} EDTA) at 10 ℃ for 40 hours. During this time, cycles of 1.25 V· cm^{-1} for 900 seconds in the direction of DNA migration alternated with cycles of 5.0 V· cm^{-1} for 75 seconds in the reverse direction. The agarose gels were quantified to estimate DNA damage by means of a PhosphorImager (Molecular Dynamics). Gels were dried and exposed to radiation-sensitive screens for 48-96 hours. DNA DSB was quantitated by calculating the fraction of activity released (FAR) from the well into the lane in irradiated and non-irradiated samples. The FAR measured in non-irradiated cells (background) was subtracted from the results shown with irradiated cells. Gel images were obtained either by photographing ethidium bromide-stained gels under UV light, or from the PhosphorImager.

Repair kinetics were fitted assuming two exponential components of rejoining according to the equation $FAR=Ae^{-bt}+Ce^{-dt}$[42]. The first term in the equation was fitted to the slow and the second to the fast component of rejoining. Fitting was achieved using the non-linear regression analysis routines of a commercially available software package (SAS). Parameters A and C describe the amplitudes, and parameter b and d and the rate constants of the slow and the fast components of rejoining, respectively. From these parameters the half-time for the rejoining of the slow and the fast components were calculated as $t_{50,fast}=\ln2/b$, and $t_{50,slow}=\ln2/d$, respectively. The fraction of DSB rejoined by fast kinetics was calculated as $F_{fast}=A/A+C$ and $F_{slow}=C/A+C$.

Determination of DNA-PKcs activity

Cell extract preparation: Cells (2×10^6) were grown in the T-75 tissue culture flask for 5 days. After treatment, about 30×10^6 cells were collected in cold PBS after being trypsinized at 37 ℃ for 10 minutes, centrifuged at 4 ℃, and resuspended with 1 mL cold PBS and transferred to Eppendorf tube. After spun 1 500 r· min^{-1} for 5 minutes at 4 ℃, PBS was replaced with 0.5 ml (about 4 volumes of cells) hypotonic buffer containing 10 mmol· L^{-1} HEPES KOH pH 7.9 at 4 ℃, 5 mmol· L^{-1}

KCl, 1.5 mmol· L^{-1} $MgCl_2$, 20 mmol· L^{-1} b-Glu, 0.2 mmol· L^{-1} phenylmethylsulfonyl fluoride (PMSF), 0.5 mmol· L^{-1} dithiothreitol (DTT). The cells were put on ice for 10 minutes, frozen in liquid nitrogen and thawed for 3 cycles, adjusted salt being 50 mmol· L^{-1} KCl in hypotonic buffer (16 μl, 1.6 mol· L^{-1} KCl), and allowed to stay for 10 minutes on ice. After centrifugation (40 minutes at 14 000 r· min^{-1} at 4 ℃), cytoplasm extract was obtained from the collection of supernatants. Nuclei were resuspended with 50 mmol· L^{-1} KCl hypotonic buffer (100 ml), the salt was adjusted to 400 mmol· L^{-1} KCl with 3 mol· L^{-1} KCl in the buffer (13 μl), mixed in the cold room (4 ℃) for 30 minutes. After centrifuged at 14 000 r· min^{-1} for 15 minutes at 4 ℃, nucleic extract was obtained from the collection of supernatants. Protein concentration of both cytoplasm and nucleic extracts were determined using Bio-Rad protein II assay.

Activity assay of DNA-PKcs: In 1.5 ml microfuge tube, the reaction buffer was set up on ice in a mixture containing 4 μl 5X kinase buffer (250 mmol· L^{-1} HEPES, pH 7.5, 50 mmol· L^{-1} $MgCl_2$, 1 mmol· L^{-1} EGTA, 5 mmol· L^{-1} DTT), 2 μl 10X substrate peptide (2 mmol· L^{-1}), 2 μl 10X sonacated calf thymus DNA (100 mg· L^{-1}), 2 μl(7.4 MBq · L^{-1}) of (γ-^{32}P) ATP. In this reaction buffer mixture, an equal volume (5 μg in 10 μl) of nucleic extract was added and mixed quickly. The optimal incorporation time was found to be 15 minutes at 30 ℃. After incubation, the reaction was terminated with 20 μl stop solution (300 g· L^{-1} acetic acid, 1 mmol· L^{-1} ATP). Out of 40 μml reaction mixture, 20 μl was spotted on Watermann P81 phosphocellulose paper, and washed 4 times with 150 g· L^{-1} acetic acid for 15 minutes each time. The filters were placed in scintillation vials, and the adsorbed radioactivity was quantitated. To calculate the specific activity of (γ-^{32}P) ATP, we removed 5 μl from any two reaction tubes, and added to scintillation vial to count. Calculation of the specific activity of (γ-^{32}P) ATP in cpm/pmol is shown as follows (40/5)×X/10 000=X/1 250 [40 is the sum of the reaction volume (20 μL) + stop buffer (20 μl), 5 is the volume (μl) used for the specific activity of (γ-^{32}P) ATP, X is the average counts, and 10 000 is the number of pmoles of ATP in the reaction]. Calculation of incorporated ATP (pmol) is ($CPM_{reaction\ with\ DNA}$-$CPM_{reaction\ without\ DNA}$) / The specific activity of [γ-^{32}P] ATP in 10^{12}cpm· mol^{-1}.

RESULTS

The survival curves of $HepG_2$ cell exposure to X-ray combined with or without hyperthermia are shown in Figure1A and B. The mean dose of survival fraction at 2 Gy (SF_2) was 0.230±0.033 for the group of radiation alone, and 0.148±0.043 for the radiation combined with hyperthermia group. The linear-quadratic model $S=e^{-\alpha D-\beta D2}$ was applied to describe the survival data in Figure 1, where S is the fraction of cells surviving a dose D, and α and β are constants. The α and β values are 0.805±0.037 Gy^{-1} and 0.072 ± 0.012 Gy^{-2} for the group of radiation alone, and 0.950 ± 0.018 Gy^{-1} and 0.010 ± 0.001 Gy^{-2} for the group of radiation combined with hyperthermia. The α/β ratios are 11.2 Gy and 97 Gy for the groups of radiation alone and combination modes, respectively. Thermal enhancement ratio as calculated at 10 % survival (TER_{10}) was 2.02. The difference in radiosensitivity between these two groups can be interpreted as being due to some phenomena, which manifest as a difference in the a components.

Figure 1B shows the survival curves for cells exposed for various periods of time to 45.5 ℃. The combination of hyperthermia with 3 Gy X-ray significantly improved the killing effects in comparison of hyperthermia alone. Figure 1C shows a combination of hyperthermia at 45.5 ℃ for 15 minutes first and then 3 Gy of X-rays. When the time intervals between hyperthermia and radiation in existence, the cell survival increased with the time interval increase within 8 hours, but no significant change was observed 8 hours later.

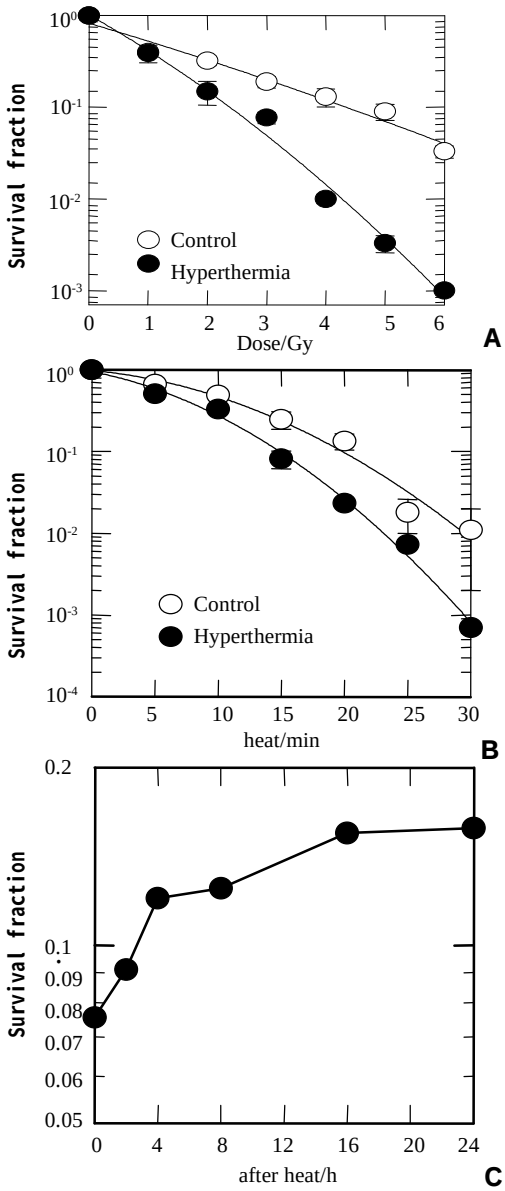

Figure 1 Survival fraction of $HepG_2$. A: Exposure to X-rays combined with (close circles) or without (open circles) 45.5 ℃ for 15 minutes; B: Heat-induced clonogenic cell death as a function of time combined with (close circles) or without (open circles) 3 Gy of X-rays; C: Irradiated with 3 Gy of X-rays at different hours after heat of 15 minutes at 45.5 ℃.

The DNA-PKcs activity was measured before and after irradiation with 40 Gy X-ray, or hyperthermia for 20 minutes at 45.5 ℃, or both. All of DNA-PKcs values were expressed with both relative percentage at left side and pmol at right of figures in this paper. Figure 2A shows that DNA-PKcs activity was inhibited by about 70 % at heating. Radiation stimulated DNA-PKcs activity increased about 30 % in the cells treated with or without hyperthermia at the 2nd hour. In order to determine whether the level of DNA-PKcs activity recovers its activity after hyperthermia, the time point was extended to 24 hours to correspond to clonogenic survival in Figure 1C.

As shown in Figure 2B, no restore was found in DNA-PKcs activity up to 24 hours. The DNA-PKcs activity was inhibited after the cells exposed to heat at 45.5 ℃ for 5 minutes (Figure 2C). The DNA-PKcs activity remained at almost the same level despite the hyperthermic time extending.

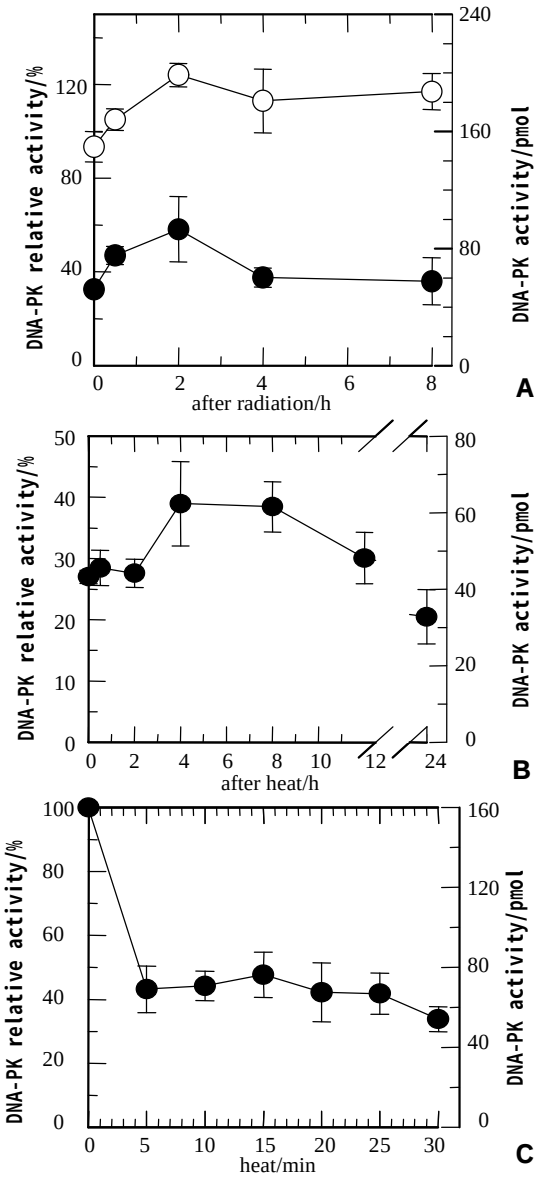

Figure 2 DNA-PKcs activity of HepG2 cells. A: Cells were heated at 45.5 ℃ for 20 minutes and then received 40 Gy of X-rays (closed circles) or irradiated with 40 Gy of X-rays only (open circles). At various periods, DNA-PKcs activity in heated cells was inhibited, and kept at low level about 30 %. The DNA-PKcs activity in both groups showed slight increase; B: After heated at 45.5 ℃ for 20 minutes, DNA-PKcs activity was still inhibited at various periods; C: DNA-PKcs activity was inhibited after exposure to heat at 45.5 ℃ for 5 minutes, even though the heat time was prolonged, DNA-PKcs activity levels still remained unchanged.

To understand the role of DNA-PKcs subunits in radiosensitization by hyperthermia, induction of X-ray induced DNA DSB was measured by AFIGE. Figure 3 shows the dose response curves for Hep G_2 cells receiving radiation combined with or without hyperthermia. The upper panel shows a typical gel scanned with ^{14}C-TdR, while the lower panel shows the quantitative data as described in the Methods. The FAR, a measure of DNA DSB presence, increased almost linearly with dose up to 30 Gy but bended downward at higher doses. Similar increases in FAR as a function of dose were observed in radiation alone or combined with hyperthermia, suggesting similar yields of DNA DSB.

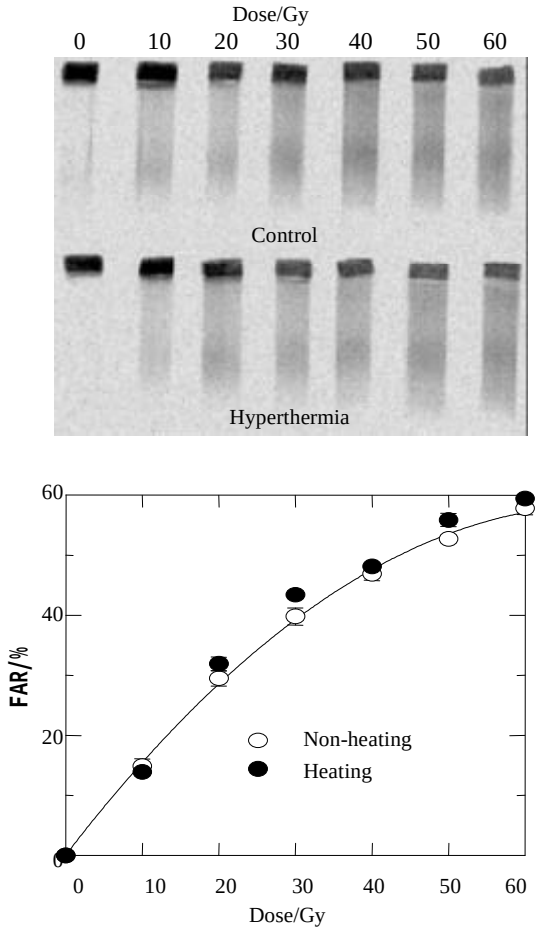

Figure 3 Dose response curves for Hep G2 cells received radiation combined with or without hyperthermia. The upper panel shows a typical gel scanned with ^{14}C-TdR, while the lower panel shows quantitative data as described in the Methods. The FAR increases almost linearly with dose up to 30 Gy but bends downward at higher doses. Similar increases in FAR as a function of dose are observed in radiation alone or combined with hyperthermia, suggesting similar yields of DNA DSBs.

The rate of rejoining of radiation-induced DNA DSB was subsequently examined to determine whether differences in repair between radiation alone and combined with hyperthermia for different periods of time account for the results shown in Figure 2A that the DNA-PKcs activity was inhibited after heat. Figure 4 shows that DNA DSB repairs kinetics in Hep G_2 cells. Cells were irradiated with 40 Gy and prepared for AFIGE after various periods of incubation at 37 ℃ to allow for repair. The upper panel in the Figure shows a typical AFIGE gel, while the lower panel shows its quantification as described in the Methods. The rejoining of DNA DSB decreased with increasing the lengths of hyperthermic time at 45.5 ℃. Table 1 shows that the half-time for rejoining of the fast components was shorter in the control group or exposure to heat for 5 minutes than the exposure to longer time (15 and 20 minutes). However, the half-time of slow components in both control and hyperthermic groups were almost the same which ranged between 7 and 9 hours.

Wortmannin inhibits the entire family of PI-3 kinases and probably also other cellular kinases. To evaluate the

contribution of DNA-PKcs to Wortmannin-induced inhibition of DNA DSB rejoining, we searched for Wortmannin treatment, which is able to compare heat-induced inhibition of DNA DSB rejoining. Figure 5 indicates the kinetics of DNA DSB rejoining after treatment with Wortmannin. We found that the slow rejoining component is strikingly high. The deficiency of DNA DSB rejoining was found in Wortmannin treatment cells after 2 hours. These results are different from those in hyperthermic groups.

Table 1 The half-time and fraction of DNA DSB rejoined by fast and slow kinetics

	$T_{50,fast}$	$T_{50,slow}$	F_{fast}	F_{slow}
Control	0.49	7.07	0.80	0.20
5 min	0.33	4.15	0.44	0.56
15 min	0.92	9.37	0.49	0.51
20 min	1.25	9.24	0.30	0.70
20 mmol·L^{-1} Wort.	0.87	7×10^{8}	0.44	0.56

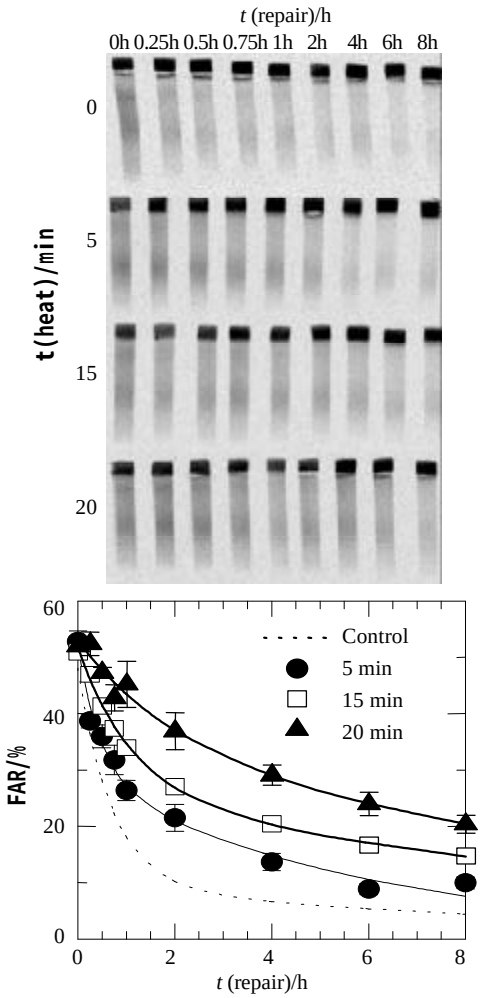

Figure 4 DNA DSBs repair kinetics in Hep G2 cells. Cells were irradiated with 40 Gy and prepared for AFIGE after various periods of incubation at 37 ℃ to allow for repair. The upper panel in the Figure shows a typical AFIGE gel, while the lower panel shows its quantification as described in the materials and Methods. The rejoining of DNA DSBs was decreasing with the increase of the lengths of hyperthermic time at 45.5 ℃.

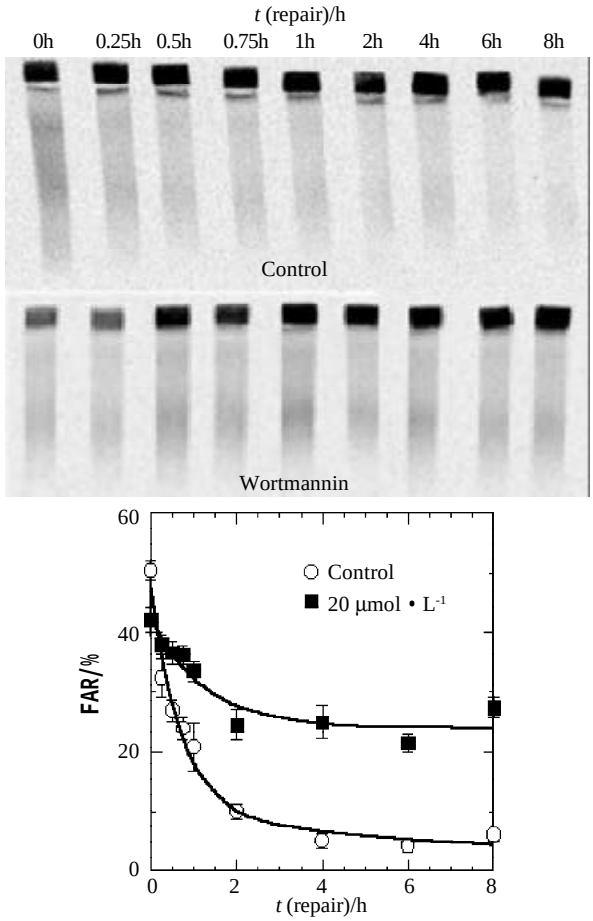

Figure 5 The kinetics of DNA DSBs rejoining after treatment with Wortmannin. The slow rejoining component is strikingly high. The deficiency of DNA DSBs rejoining was found in Wortmannin treatment cells after 2 hours.

DISCUSSION

Tumors of liver are among the most common malignancies in the world. Primary hepatocellular carcinoma was the second most common cancer and the leading cause of cancer deaths behind gastric cancer in China. Surgical resection has been accepted as the only curative therapy for primary liver cancer. Unfortunately, most patients were surgically unresectable[1-28]. Hyperthermia[29,30], radiation therapy[21-28], or combination of both[31,32] were introduced as an alternative therapeutic approach. The cause of this radiosensitization has not been firmly established.

The inhibition of DNA-PKcs by hyperthermia has been demonstrated in several studies[40, 43,44]. In our series, DNA-PKcs activity was inhibited by about 60 % after hyperthermia at 45.5 ℃ for 5 minutes, and no significant change was found after increasing hyperthermia time. This result is similar to other reports[40, 43]. Interestingly, the rejoining of DNA DSB was decreasing with the increase of the hyperthermic time at 45.5 ℃. The decrease of DNA DSB repair did not correspond proportionally to the heat inhibited DNA-PKcs activity which was kept at a same low activity level between 5 and 30 minutes. This means DNA-PKcs is not critical for DNA DSB repair in heat.

The activity of DNA-PKcs depends upon the presence of double-stranded DNA ends, and is based on the similarity of their DNA-binding properties, and Ku was identified as the DNA-targeting component of this protein complex[45-51]. This can be used to explain the fact that DNA-PKcs activity increased by about 30 % at the 2nd hour after 40 Gy X-ray irradiation, which produced plenty of DNA DSB. After

hyperthermia at 45.5 ℃ for 15 minutes, DNA-PKcs activity was stable at a low level (30 %) up to 24 hours. We are eager to know whether the low level DNA-PKcs affects the clonogenic survival. The results showed that the effects of thermal radiosensitization were lowered, even though DNA-PKcs activity did not restore. This indicated that there was no correlation between thermal inactivation of DNA-PKcs and radiosensitization by heat.

Wortmannin is a fungal metabolite originally characterized as an irreversible inhibitor of PI3K-like family including DNA-PKcs[52]. The inhibition of DNA-PKcs by Wortmannin could increase the slow rejoining component, resulting in deficiency of DNA DSB repair. The kinetics of DNA DSB rejoining in hyperthermia groups different from that treated with wortmannin, inhibited the DNA-PKcs activity. This confirms that DNA DSB repair is not completely affected by DNA-PKcs, other factors might also involve in the repair.

DNA-PKcs is not critical for radiosensitization by heat. What might explain the synergistic interaction between heat and radiation? One possibility is that homologous recombination may impair after heat shock, but the contribution of this repair pathway in mammalian cells seems limited[53]. Another possibility is that heat-induced loss of activity of DNA repair enzymes other than DNA-PKcs has been observed and proposed previously as the mechanism that underlies DNA repair inhibition and radiosensitization. These enzymes include DNA polymerases[37], ATM system[54], ATR system[38], etc. Motsumoto found recently that heating up to 90 minutes affected only marginally DNA-PKcs activity in four different human cell lines (results not published). The contradictory results are due to the methods of measurement, which can not distinguish between inhibition of ATM and DNA-PKcs as the substrate, and peptide is good for both kinases.

In clonogenic assay, the difference in radiosensitivity between radiation and thermal radiation groups manifested as α difference in the a components. The dose range over which the linear component dominates in a linear-quadratic (LQ) survival relationship depends on the relative values of α and β: the higher the relative value of α, the more linear response at low doses and the less sensitive it is to dose fraction[55]. In hyperthermia group, the α/β ratio is high, with no detectable influence of the quadratic function over the first two decades of reduction in cell survival, implying that accumulation of sublethal injury plays a negligible role in cell killing by thermal radiosensitization clinically[56]. From these results, we can deduce that heat inhibits the repair of radiation-induced single-strand breaks and radiation-induced chromosome aberrations. This inability to repair molecular damage translates into the inability to repair both sublethal damage and potentially lethal damage produced by radiation.

In conclusion, when the DNA-PKcs activity was determinated using the cell extracts and the peptide phosphorylation assay, DNA-PKcs activity was inactivated by heat treatment at 45.5 ℃, and could not restore. Cell survival is not associated with the DNA-PKcs inactivity after heat. DNA-PKcs is not a unique factor affecting DNA DSB repair. This suggests DNA-PKcs does not play a crucial role in the enhancement of cellular radiosensitivity by hyperthermia.

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• LIVER CANCER •

Association of hTcf-4 gene expression and mutation with clinicopathological characteristics of hepatocellular carcinoma

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Abstract

AIM: Hepatocellular carcinoma(HCC) is a significant health problem in China. But the molecular mechanisms of HCC remains unclear. APC/β-Catenin/Tcf signaling pathway, also known as Wnt pathway, plays a critical role in the development and oncogenesis. As little is known about the alteration of human T-cell transcription factor-4 (hTcf-4) gene in HCC, it is of interest to study the expression and mutation of hTcf-4 gene in HCC and the relationship between hTcf-4 gene and progression of HCC.

METHODS: Reverse transcription-polymerase chain reaction (RT-PCR) method was used to detect the expression of hTcf-4 mRNA in 32 HCC and para-cancerous tissues and 5 normal liver tissues. PCR-single strand conformation polymorphism (PCR-SSCP) method was used to detect the mutation of hTcf-4 exons 1, 4, 9 and 15 in HCC. The correlation of expression and mutation of the hTcf-4 gene with clinicopathological characteristics of HCC was also analyzed.

RESULTS: RT-PCR showed that the expression rate of hTcf-4 mRNA in HCC, para-cancerous tissues and normal liver tissues was 90.6 %, 71.9 % and 80 %, respectively. The gene expression level in tumor was 0.71±0.13, much higher than that in para-cancerous liver 0.29±0.05 and normal liver 0.26±0.05 ($P<0.001$), although there was no significant difference in gene expression level between para-cancerous tissues and normal liver ($P>0.05$). Furthermore, hTcf-4 gene expression was closely associated with tumor capsule status and intrahepatic metastasis of HCC. On SSCP, 2 of 32 cases of HCC (6.25 %) displayed characteristic mutational mobility shifts in exon 15 of the hTcf-4 gene. No abnormal shifting bands were observed in para-cancerous tissues.

CONCLUSION: The high expression level of hTcf-4 in HCC, especially in tumors with metastasis, suggests that the over-expression of hTcf-4 gene may be closely associated with development and progression of HCC, but the mutation of this gene seemed to play less important role in this respect.

Jiang Y, Zhou XD, Liu YK,Wu X, Huang XW. Association of hTcf-4 gene expression and mutation with clinicopathological characteristics of hepatocellular carcinoma. *World J Gastroenterol* 2002;8(5):804-807

INTRODUCTION

Recent work has shown that APC/β-Catenin/Tcf pathway, also known as Wnt signaling pathway, plays a key role in regulation of development and growth of the cells[1-3]. The alteration of APC/β-Catenin/Tcf pathway leading to cancer has been described. In differentiated cells, the cytoplasmic level of β-catenin is maintained very low through degradation by the ubiquitin-proteasome pathway, whereby serine and threonine residues in exon 3 are phosphorylated by GSK3β[4-6] and ubiquitinylated by binding to proteins such as APC, axin and conductin[7-11]. In experiments with colorectal cancer and melanoma cell lines, dysfunction of APC resulted in stabilization of β-catenin and binding of excess β-catenin to Tcf/Lef to activate transcription in the nucleus[12-16]. Furthermore, in cell lines having no APC mutations, mutations of the β-catenin gene that altered amino acid residues representing potential GSK3β phosphorylation sites could confer resistance to degradation and lead to intracellular accumulation of β-catenin[12,17,18]. Activated cytoplasmic β-catenin, probably bound to Tcf/Lef, is thought to migrate into the nucleus and stimulate transcription of downstream genes in a constitutive manner[12,13]. However, recent studies focused mainly on the relationship between cancers and mutations of APC and β-catenin gene, and little is known about the change of human T-cell transcription factor-4 (hTcf-4) gene in tumors, especially about its expression and mutation in hepatocellular carcinoma (HCC). To further understanding the role of the APC/β-Catenin/Tcf pathway in HCC, the present study examined expression and mutation of the hTcf-4 gene in HCC by reverse transcription-polymerase chain reaction (RT-PCR) and PCR-single strand conformation polymorphism (PCR-SSCP).

MATERIALS AND METHODS

Patients

Thirty-two fresh HCC specimens and the para-cancerous tissues and 5 normal liver tissues were analyzed. All specimens were obtained from patients who underwent surgery for HCC or hemangioma between 1999 and 2000 at Liver Cancer Institute, Fudan University, and stored frozen at -70 ℃ until use. The diagnosis was confirmed by pathological examination. The patients with HCC consisted of 12 women and 20 men with the mean age of 56 years and range from 16 to 75 years. Of the 32 patients, 16 showed abnormal serum concentration of alpha-fetoprotein (AFP) (>20 μg/L), 18 had macronodular cirrhosis (cirrhotic nodules measuried at least 0.3 cm in greatest dimension) and 14 had micronodular cirrhosis (cirrhotic nodules measured <0.3 cm). HCC was large in 17 patients (>5 cm in greatest dimension) and small in 15 patients (≤5 cm). Macroscopically poorly encapsulated tumors were found in 18 patients (56 %) and cancerous thrombi in portal vein or intrahepatic metastasis were found in 15 patients (47 %).

RNA Extraction and RT-PCR

Total RNA was isolated from tissues using Trizol Reagent (Life

Technologies, Inc.) according to the manufacturer's protocol. A 3-μg aliquot of total RNA from each specimens was reverse-transcribed into single-strand cDNA using oligo $(dT)_{15}$ primer and Superscript II (Life Technologies, Inc.). Each single-strand cDNA was diluted for subsequent PCR amplification of hTcf-4 and β-actin with the latter used as an internal quantitative control. The PCR was carried out in a reaction volume of 25 μl for 5 min at 95 °C for initial denaturing, followed by 25 (for β-actin) or 30 (for hTcf-4) cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 40 s on the Gene Amp PCR system 9 600 (Perkin-Elmer Corp.). The primer sequences used for amplification were 5'-CTTCCTTCCTGGGCATGGAG-3' and 5'-TGGAGGGGCCGGA CTCGTCA-3' for β-actin and 5'-TGTACCCAATCACGACAGGA-3' and 5'-GCCAGCT CGTAGTATTTCGC -3' for hTcf-4. PCR products were resolved in 2 % agarose gels and visualized by staining with ethidium bromide. To quantify PCR products, the bands representing amplified products were analyzed by Pharmacia Biotect Image MASTER VDS.

PCR-SSCP-silver staining

DNA was isolated from tissues using a DNA extraction kit (BBST Corporation) according to the manufacturer's protocol. The primers of PCR amplification of exons 1, 4, 9 and 15 of hTcf-4 were as follows: 5'-AATTGCTGCTGGTGGGTGA-3' and 5'-CCCGAGGGGCTTTT CCTA-3' for exon 1 (234bp); 5'-GAACGCTTTGATTTGGTTTC-3' and 5'-GCTTCAGAATCTCTTGCGT-3' for exon 4 (124bp); 5'-GATTCTGACGATTTACACAG-3' and 5'-GCTACGAAGAAGGTGAGAA-3' for exon 9 (196bp) and 5'-CGACCCACCATTGTGTTGTA-3' and 5'-AAAGGCCTCGCAGTGGTAAT-3' for exon 15 (144bp). The PCR reaction was performed by denaturation at 94 °C for 40s, annealing at 60 °C for 40s and extension at 72 °C for 40s for 30 cycles using 2.5 units of Taq DNA polymerase (BBST Corporation) per 25 μl reaction volume. The PCR products were detected on 2 % agarose gels. SSCP analysis was performed as follows: 15 μl of PCR sample plus 20 μl of formamide loading dye (95 % formamide, 0.05 % bromphenol blue, 10 mmol/L EDTA) were boiled for 10 min, snap-frozen on ice and electrophoresed on a 12 % non-denaturing polyacrylamide gel at 300 V for 5 min, then 120 V for 3-4 h, depending on the size of PCR fragment. Silver Staining for SSCP consisted of fixation in 10 % alcohol for 5 min, sensitizing in 1 % HNO_3 for 5 min, washing twice with distilled water for 2 min, silver reaction (silver nitrate 0.25 g, formaldehyde 50 μl, topped up with distilled water to 100 ml) for 10 min, washing with distilled water for 10s, developing (anhydrous sodium carbonate 6.0 g, formaldehyde 200 μl, 10 % sodium thiosulfate 20 μl, topped up with distilled water to 200 ml) for 10 min, stopping in 10 % glacial acetic acid for 10 min and anhydration in absolute alcohol for 2 min.

Statistical analysis

The statistical differences between different groups were analyzed by Student t-test or One-Way ANOVA. A value of $P<0.05$ was considered significant. All data were disposed by SPSS9.0 statistical software.

RESULTS

Expression of hTcf-4 mRNA in HCC specimens, para-cancerous tissues and normal liver tissues

2 % Agarose gel electrophoresis showed a 406bp hTcf-4 fragment by RT-PCR amplification from normal liver tissues, para-cancerous tissues and HCC tissues. The hTcf-4 mRNA amplification was successful in 29 of 32 HCC tissues (90.6 %), 23 of 32 para-cancerous liver tissues (71.9 %) and 4 of 5 normal liver tissues (80 %). The expression level was 0.71±0.13 in tumor, much higher than that in para-cancerous liver (0.29±0.05, $P<0.001$) and normal liver (0.26±0.05, $P<0.001$) (Figure1). However, there was no significant difference in hTcf-4 expression level between para-cancerous tissues and normal liver tissues ($P>0.05$).

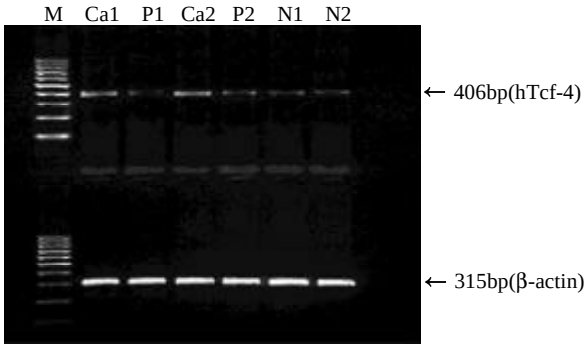

Figure1 Expression of hTcf-4 and ß-actin mRNA in HCC (Ca), para-cancerous tissue (P) and normal liver (N). Semiquantitative RT-PCR analysis revealed that the expression level of hTcf-4 gene in HCC was much higher than that in para-cancerous tissues and normal livers. M: 100bp DNA Ladder

The relationship between hTcf-4 mRNA expression and clinicopathological features of the patients

Statistical analysis showed that the expression level of hTcf-4 mRNA in poorly encapsulated tumors and in tumors with intrahepatic metastasis was much higher than that in well encapsulated tumors and in tumors without metastasis ($P<0.05$). However, no significant difference in hTcf-4 mRNA level was observed with variants of serum AFP levels, liver cirrhosis degree and tumor size (Table 1).

Table1 The relationship between hTcf-4 mRNA expression and clinicopathological features of the patients

	n	hTcf-4/β-actin	*P*
AFP			
≤20μg/L	16	0.71±0.14	>0.05
>20 μg/L	16	0.67±0.12	
Cirrhotic nodules			
<0.3cm	14	0.75±0.11	>0.05
≥0.3cm	18	0.67±0.14	
Tumor size			
≤5cm	15	0.66±0.13	>0.05
>5cm	17	0.76±0.12	
Capsule			
well capsule	14	0.61±0.10	<0.05
poor capsule	18	0.79±0.10	
Metastasis			
Yes	15	0.78±0.12	<0.05
No	17	0.66±0.12	

N: number; Yes: tumors with cancerous thrombi in portal vein or intrahepatic metastasis; No: tumors without cancerous thrombi in portal vein and intrahepatic metastasis.

Detection of mutation by PCR-SSCP-silver staining

Abnormal SSCP migration bands were detected in exon 15 of the hTcf-4 gene from 2 of 32 tumor tissues (6.25 %), compared with the mobility pattern of the para-cancerous liver, demonstrating that there existed mutation in the exon 15. Among these two cases, one was complicated by portal vein thrombosis and the other had tumor without metastasis. The mobility pattern of PCR products from exons 1, 4, and 9 of the hTcf-4 gene was not altered (Figure 2).

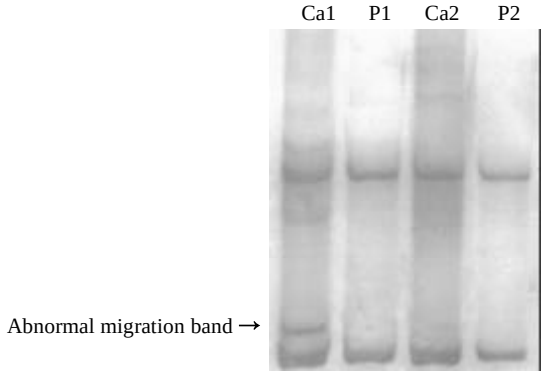

Figure 2 SSCP analysis of hTcf-4 exon 15 in HCC (Ca) and para-cancerous liver (P). Lane 1, abnormal SSCP pattern detected in exon 15 of patient 1, compared with the pattern of exon 15 from the corresponding para-cancerous liver in lane 2. Lane 3, normal SSCP pattern of exon 15 from patient 2, compared with the pattern of exon 15 from the corresponding para-cancerous liver in lane 4.

DISCUSSION

The Tcf-4 gene is a member of the APC/β-Catenin/Tcf pathway that is well known to play a crucial role in many developmental processes and human carcinogenesis[19-27]. Mapping to chromosome band 10q25.3[28], Tcf-4 encodes a transcription factor that interacts functionally with β-catenin to transactivate target genes[29-31]. Morin has recently shown that the nuclei of colon carcinoma cell lines contain constitutively active Tcf-4/β-catenin complexes as a direct consequence of either loss of function of the tumor suppressor protein APC or gain of function by mutations in β-catenin itself[12]. This is believed to result in the uncontrolled transcription of Tcf target genes, leading to transformation of colon epithelial cells and initiation of polyp formation. High level of hTcf-4 expression has been identified in colon cancer, mammary carcinoma and a variety of colorectal cancer cells[21,29]. Tcf factors have also been reported as tumor inducers which aberrantly activate their target genes, now known as the c-myc gene and cyclin D1 gene, in many types of cancer[30-32]. It has been reported that *c-myc* gene and cyclin D1 gene had a high expression level in HCC and were implicated in tumor progression and metastasis with the unclear mechanism[33-39]. Therefore, it is important to elucidate the internal link between APC/β-Catenin/Tcf pathway and liver cancer. Our present studies showed that the level of hTcf-4 expression in cancer tissues was much higher than that in para-cancerous tissues and normal liver tissues ($P<0.001$). Moreover, we found that hTcf-4 gene expression was closely correlated with the integrity of tumor capsule and intrahepatic metastasis of HCC but not with serum AFP levels, liver cirrhosis degree and tumor size, suggesting that hTcf-4 expression was associated with invasion and metastasis of HCC. This may be due to interaction between Tcf and E-cadherin. Huber *et al*. reported that the complex of Tcf andb-catenin in the nucleus binds to the E-cadherin gene promoter and down-regulates E-cadherin gene transcription[40]. On the other hand, loss of E-cadherin expression can contribute to the up-regulation of Tcf-β-Catenin pathway in human cancers[41]. As a result, the role of E-cadherin in cell-cell adhesion is reduced, which may contribute to the metastatic potential of tumor cells.

With regard to the relationship between Tcf-4 mutations and tumor, Duval *et al*[42]. reported that 50 % of human MSI-H (high frequency microsatellite instability) colorectal cell lines and 39 % of MSI-H colorectal primary tumors were found to have a 1-bp deletion in an (A)9 repeat within the coding region of this gene. The (A)9 repeat normally codes for several isoforms that could serve as modulators of Tcf-4 transcriptional activity. The deletion of one nucleotide in this repeat could change Tcf-4 transactivating properties by modifying the respective proportions of the different isoforms. In addition, one frameshift mutation in the β-Catenin binding domain (exon 1), one missense mutation in exon 4 and six nonsense or frameshift mutations localized in the 3' part of the gene were detected in a series of 24 colorectal cancer cell lines[43]. The latter alterations interfered with the Tcf-4 capacity to interact with COOH-terminal binding protein that was implicated in the repression of the Tcf family transcriptional activity. As a result, the Tcf-4 transcriptional activity was enhanced. This indicated that the mutation of Tcf could be an important event during colorectal carcinogenesis by modifying Wnt signaling. In our experiment, we used PCR-SSCP-silver staining analysis to detect mutation of hTcf-4 exons 1, 4, 9 and 15 from human liver cancer tissues. The sensitivity of this method is generally high and greater than 80 % of mutations in most DNA fragments of 300 bp or shorter can be detected since variation of DNA sequence often results in a shift in electrophoretic mobility, which is believed to be caused by sequence-dependent alteration in the tertiary structure of single-stranded DNA. We found SSCP variants in exon 15 of 2 HCC cases (6.25%) only, of which one was complicated by portal vein thrombosis and the other originated from the tumor tissue without metastasis. It seemed that, unlike in colorectal tumor, hTcf-4 mutation may play less important role in HCC and was irrelevant to invasion and metastasis of HCC. Therefore, the enhanced transcriptional activity of hTcf-4 due to aberrant mRNA expression may be the key to HCC occurrence and development. Further study on structure and function of hTcf-4 and its interaction with oncogenes should contribute to clarification of the mechanism of liver carcinogenesis and may provide the theoretical principle for the gene therapy.

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• LIVER CANCER •

Preparation of monoclonal antibody against apoptosis-associated antigens of hepatoma cells by subtractive immunization

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Abstract

AIM: To elucidate the expression of the apoptosis-associated molecules in human primary hepatocellular carcinoma (HCC) cells, and prepare the monoclonal antibodies (mAb) against the apoptosis-associated antigens of HCC cells.

METHODS: Human HCC cell line HCC-9204 cells were induced apoptosis with 60 mL·L^{-1} ethanol for 6 h and their morphological changes were observed by transmission electron microscope. The cell DNA fragmentations were detected by Terminal Deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, and the cell DNA contents by flow cytometry. Ten mice were immunized with ethanol-induced apoptotic HCC-9204 cells with the method of subtractive immunization, while the other 10 mice used as the control were immunized by the routine procedures. The tail blood of all the mice were prepared after the last immunization, and the produced antibodies were determined by the immunocytochemical ABC staining. The splenic cells of the mice whose tail blood sera-HCC-9204 cells serum reactions were most different between the apoptotic and the non-apoptotic were prepared and fused with the mouse myeloma cell line SP2/0 cells. The positive antibodies were selected by ELISA assay. The fusion rates of hybridoma cells and the producing rates of antibodies were calculated. The fused cells that secreted candidate objective antibody were cloned continually with the of limited dilution method, and then selected and analyzed further by the immunocytochemical ABC staining. The chromosomes of the cloned hybridoma cells that secreted objective mAb and the mAb immunoglobulin (Ig) subtype of the prepared mAb were also determined. The molecular mass of the mAb associated antigen was analyzed by Western blot assay.

RESULTS: HCC-9204 cells treated with 60 mL·L^{-1} ethanol for 6 h, manifested obvious apoptotic morphological changes, the majority of the cells were TUNEL-positive, and the sub-G1 apoptotic peak was evident. There were 2 mice in the experimental group whose tail blood serum reacted strongly with the apoptotic HCC-9204 cells, but weakly with their non-apoptotic counterparts.In the fusion rates of hybridoma cells as well as the producing rates of the antibody deseribed above, there did not show significant difference between the experimental and the control group, but weakly with non-apoptotic HCC-9204. However, the total producing rate of antibodies in the experimental group was significantly lower compared with the control ($P<0.01$), and so was the producing rate of the antibodies which reacted strongly with both apoptotic and non-apoptotic HCC-9204 cells($P<0.01$). After cloned continually for several times the cell that produce mAb which reacted strongly with the nuclei of ethanol-induced apoptotic HCC-9204 cells, but very weakly with that of non-apoptotic cells was selected out. Chromosome analysis revealed that the selected cell was with the universal characteristics of the monoclonal hybridoma cells which secreted mAb, and the Ig subtype of the prepared mAb was IgG1. The molecular mass of this mAb associated antigen of was about 75 ku.

CONCLUSION: Subtractive immunization is a useful method to prepare the mAb against the apoptosis-associated antigens of cells. The expression of some molecules increases to some extent in HCC-9204 cells in the process of apoptosis induced by low-concentration ethanol. The mAb that may be against ethanol-induced apoptosis-associated antigens of HCC cells was successfully prepared and primarily identified.

Yang LJ, Wang WL. Prepartion of monoclonal antibody against apoptosis-associated antigens of hepatoma cells by subtractive immunization. *Would J Gastroenterol* 2002;8(5):808-814

INTRODUCTION

Apoptosis is a process of active programmed cell death(PCD), which can be regulated by many kinds of biological factors encoded by a lot of mammalian genes[1-16]. There is not only the increase or decrease of the expression of some already-existed proteins in the process of apoptosis, but also the production and presentation of some new apoptosis-associated molecules that do not express in non-apoptotic cells[17-20]. Currently, the knowledge about apoptosis-associated molecules is still limited. The conventional process to discover new apoptosis-associated molecules is to clone and sequence the apoptosis-associated genes of cells by the methods such as differentiated PCR and phage display first, and then performe the experiments to study the function of the expressed product of the candidate apoptosis-associated genes[21-25]. Preparing the antibodies against the associated antigens of apoptotic cells is also a very hopeful way to investigate apoptosis-associated molecules. There are already some reports about the successful preparation of the polyclonal antibodies against apoptosis-associated molecules while their antigens are unclear and even monoclonal antibodies (mAb) at the condition that their associated antigens are specific[19, 26,27]. However, there has been no report until now about preparing the mAb against apoptosis-associated antigens at the condition that its associated antigens are still unspecific. In the present study, some mice were immunized by the method of subtractive immunization, and the splenic cells of the effectively immunized mice were fused

with mouse myeloma cells to prepare the mAb against the apoptosis-associated antigens of human primary hepatocellular carcinoma (HCC) cells, so as to elucidate the expression of the apoptosis-associated molecules of HCC cells and investigate the methodology and feasibility to prepare mAb against the apoptosis-associated molecules of cells at the condition that the associated antigens are not clear.

MATERIALS AND METHODS

Cells, animals and main reagents

Human HCC cell line HCC-9204 was established previously by our department. Balb/c mouse myeloma cell line SP2/0 was kindly provided by Mrs. Su-Zhen Zhang, Department of Genetic and Developmental biology of our university. Ten-wk old female Balb/c mice were provided by Expereimental Animals Center of our university. Cyclophosphamide (CP), PEG4000, HT (The compound of hypoxanthine and thymidine), HAT (The compound of hypoxanthine, aminopterin and thymidine), 3,3' -diaminobenzine tetrahydrochloride (DAB), 3-aminopropyltriethoxysilane (APES), propidium iodium (PI), colchicine and Giemsa were the products of Sigma Chemical Co., USA. RNase A was the product of Promega Co., USA. HRP-labeled sheep anti-mouse IgG mAb was the product of Dako Co., USA. Terminal Deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit was the product of Boehringer Mannheim Co., Germany. Immunohistochemical ABC kit was the product of Vector Laboratories Inc., USA. Mouse hybridoma immunoglobulin (Ig) subtyping kit was the product of Roche Molecular Biochemicals, USA.

Apoptosis induction and detection

Apoptosis induction HCC-9204 cells at log phage were cultured with RPMI1640 medium containing 60 mL· L^{-1} ethanol for 6 h to induce cellular apoptosis, and non-treated HCC-9204 cells was used as control.

Electron microscope observation The cells were fixed in glutaraldehyde, fixed further in osmium tetroxide, dehydrated and embedded in epikote. The sections were ultra thin, stained doubly with uranyl acetate and lead citrate, and bobserved with transmission electron microscope (JEC Co., Japan).

TUNEL assay[28] The cells cultured on cover slips were fixed in formitrol, and then put into penetrating solution (1 g· L^{-1} sodium citrate containing 1 g· L^{-1} Triton X-100) and reacted at 4 ℃ for 2 min. The cells were stained with TUNEL reaction solution contained FITC-labeled nucleotide and deoxynucleotide terminal transferase at 37 ℃ for 60 min, and then observed with fluorescence microscope.

DNA contents analysis The cells were fixed in 700 mL· L^{-1} cold ethanol. RNase A (1 g· L^{-1}) was added into the suspending solution of cells and reacted at 37 ℃ for 30 min. Then PI (0.1 g· L^{-1}) was added with the four times volume of that of the above solution and reacted at 4 ℃ for 30 min. The DNA contents of different cell cycles were analyzed with flow cytometer (Coulter Co., USA).

Methods of antibody detection

ELISA assay The cells adhered to 96-well culture plate with polylysine were fixed in 0.2 mL· L^{-1} glutaraldehyde. The supernatant of the culture medium of hybridoma cells were used as the primary antibody, and the PBS as its negative control. The HRP-labeled sheep anti-mouse IgG mAb was used as the second antibody. The indirect ELISA assay was performed with the standard procedures. Optical absorbance (*A*) was measured at 490 nm with ELISA detector (Bio-Rad Co., USA).

Immunocytochemical staining[29] The cells cultured on cover slips were fixed in 950 mL· L^{-1} ethanol. The supernatant of the culture medium of hybridoma cells was used as the primary antibody, and the PBS as its negative control. The immunocytochemical ABC staining was performed with routine procedures illuminated by the protocol of Vector Laboratories Inc.

Subtractive immunization for mice

Twenty Balb/c mice were divided randomly into 2 groups. The ten mice in the experimental group were immunized firstly with the non-apoptotic HCC-9204 cells at log phase through cavum abdominis. Then the mice were injected with CP (0.1 g· kg^{-1} body mass) into the cavum abdominis at 10 min, 24 h and 48 h respectively after the first immunization. At the 14th, 21st and 28th d after the first immunization, the mice were further immunized with the ethanol- induced apoptotic HCC-9204 cells through the same route. The ten mice in the control group were immunized with the ethanol-induced apoptotic HCC-9204 cells through cavum abdominis for 3 times at an interval of 2 wk. The tail blood sera of animals in both group were respectively prepared after the last immunization, and the immunocytochemical ABC staining was performed as described above to detect the production of antibodies.

Cell fusion and cloning

The mice whose tail blood sera-HCC-9204 cells reactions were most different between the apoptotic and the non-apoptotic were selected from the experimental group. The splenic cells of the selected mice were prepared at the 3rd day after the last immunization, and fused with SP2/0 cells at log phase promoted by PEG4000. Two 96-well culture plates were used for every mouse. The fused cells were cultured continually with RPMI1640 medium containing HAT and HT. Five d later, the culture wells where the monoclonal hybridoma cells were growing were marked and the producing rate of the clones, i. e. the fusion rate, of hybridoma cells was calculated. When the growing hybridoma cells had covered about 1/2 of the bottom area of a culture well, the antibodies that reacted strongly with ethanol-induced apoptotic HCC-9204 cells, but weakly with the non-apoptotic were selected by the ELISA assay as described earlier and the producing rate of antibodies was calculated. The data was statistically analyzed by χ^2 test. The interested cells were cloned continually by the method of limited dilution, selected and analyzed further by the immunocytochemical ABC staining.

Identification of the characteristic of mAb

Chromosomes of hybridoma cells The cells cultured on cover slips were treated with colchicine and stained with Giemsa by the routine procedures. One hundred hybridoma cells at metaphase were counted and observed.

Ig subtype of mAb The detection and analysis of the Ig subtype of mAb was performed with standard procedures illuminated by the protocol of Roche Molecular Biochemicals.

Molecular mass of mAb-associated antigen Some ethanol-induced apoptotic and non-apoptotic HCC-9204 cells were burst and shattered respectively by ultrasonic and the proteins of cells were prepared by the routine methods. Then SDS-PAGE was performed. The supernatant of the culture medium of hybridoma cells that secreted objective mAb was used as the primary antibody. HRP-labeled sheep anti-mouse IgG mAb was used as the second antibody. The proteins in the gel of SDS-PAGE were transferred to colloxylin filter film by Western blot system (Bio-Rad Co., USA) and stained following the protocol of Bio-Rad Co.. The color was developed with freshly dispensed DAB.

RESULTS

Detection of apoptotic cells

Electron microscope observation The majority of the cells treated with 60 mL· L^{-1} ethanol for 6 h showed cellular pyknosis and were densely stained. The processes and microvilli on the cell surface decreased significantly or even disappeared. Granular, crescent or cricoid nuclei (Figure 1), chromatin margination, as well as cytoplasm bubbling were observed. Endoplasmic reticulums expanded and its vacuolation occurred in some cells.

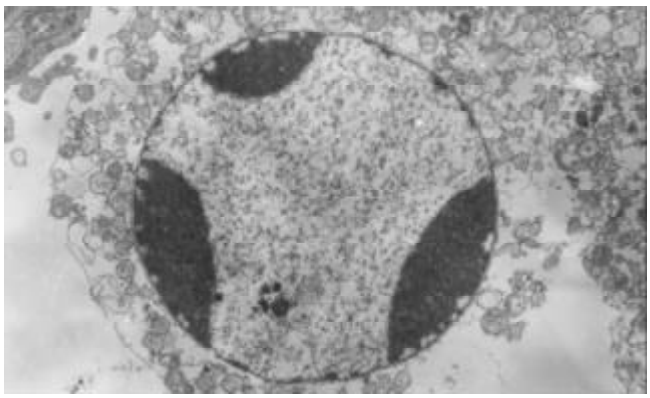

Figure 1 Morpholoical changes of HCC-9204 cells treated with 60 mL·L^{-1} etjanol for 6 h TEM ×6000

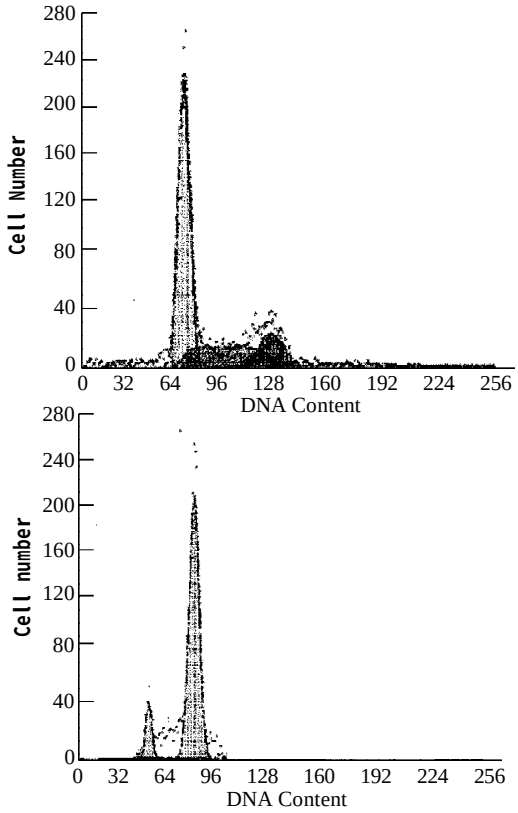

Figure 3 DNA contents of HCC-9204 cells treated with 60 mL·L^{-1} for 6 h. A: Non-treated HCC-9204 cells; B:HCC-9204 cells treated with 60 mL·L^{-1} etjanol for 6 h

TUNEL assay

The positive signal of TUNEL reaction was green or yellow in color within the round, oval, crescent or cricoid, nuclei of cells. The majority of the apoptotic cells in the experimental group were TUNEL-positive (Figure 2).

DNA contents analysis

Compared with that in the control of non-treated HCC-9204 cells, there was obvious sub-G1 apoptotic peak in the experimental group (Figure 3).

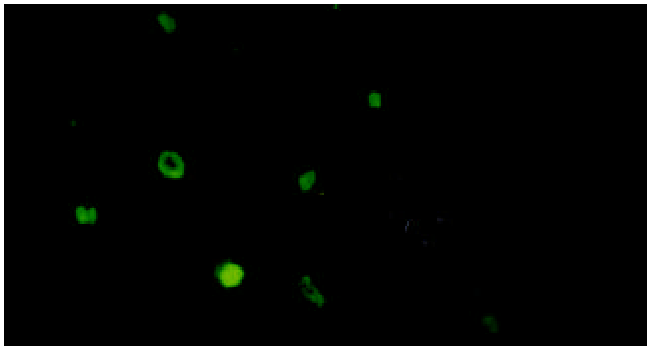

Figure 2 Detection of HCC-9204 cells treated with 60 mL·L^{-1} ethanol for 6 h by TUNEL assay ×200

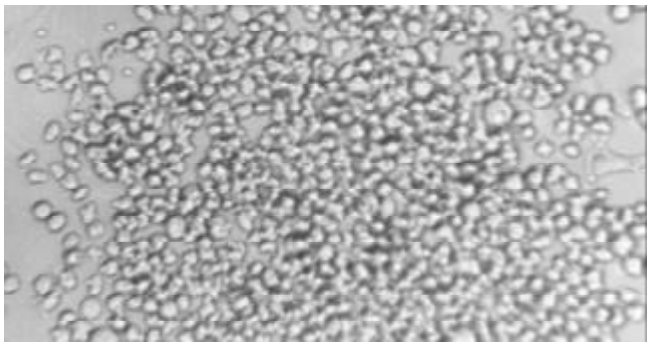

Figure 4 Monoclonal hybridoma cells growing in culture well. ×100

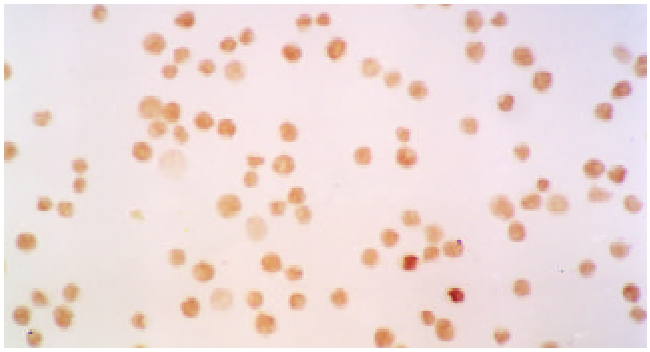

Figure 5 Immunocytochemical localization of the prepared mAb in HCC-9204 cells treated with 60 mL·L^{-1} ethanol for 6 h. ABC ×200

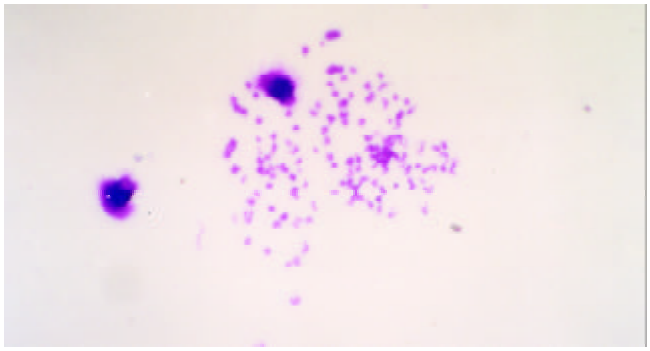

Figure 6 The chromosomes of the hybridoma cells which secrete objective mAb. ×400

Table 1 The fusion rates of hybridoma cells and the producing rates of antibodies

Group	Fusion wells / Incubated wells (Fusion rate)	I(I / Fusion wells)	II(II / Fusion wells)	III(III / Fusion wells)	(I+II+III) ((I+II +III) / Fusion wells)
Experiment	241 / 384(62.8%)	46(19.1%)[a]	5(2.0%)	0(0%)	51(21.2%)[a]
Control	262 / 384(68.2%)	101(38.5%)	4(1.5%)	1(0.4%)	106(40.5%)

[a]$P<0.01$ *vs* Control. I: The wells whose antibodies reacted strongly with both apoptotic and non-apoptotic cells; II: The wells whose antibodies reacted strongly with apoptotic cells, but weakly with non-apoptotic cells(Difference is>8 times); III: The wells whose antibodies reacted weakly with apoptotic cells, but strongly with non-apoptotic cells (Difference is >8 times).

Subtractive immunization for mice

The tail blood sera of 8 mice in the experimental group and 10 mice in the control reacted strongly with both apoptotic and non-apoptotic HCC-9204 cells. However, the tail blood sera of the 2 other mice in the experimental group reacted strongly with apoptotic, but weakly with non-apoptotic HCC-9204 cells.

Cell fusion and cloning

Monoclonal hybridoma cells were growing in many wells of the culture plates at the 5th d after cell fusion (Figure 4).

In the fusion rate of hybridoma cells, as well as the producing rate of the antibodies that reacted strongly with apoptotic, but weakly with non-apoptotic HCC-9204 cells, there did not show significant difference between the experimental and the control group. The total producing rate of antibodies in the experimental group was significant. However, lower compared with that in the control group ($P<0.01$), and so was the producing rate of the antibodies that reacted strongly with both apoptotic and non-apoptotic HCC- 9204 cells ($P<0.01$, Table 1).

An mAb that reacted strongly with the nuclei of ethanol-induced apoptotic, but very weakly with that of non-apoptotic HCC-9204 cells was obtained after the hybridoma cells which secreted candidate objective mAb had been cloned continually for several times (Figure 5).

Characteristic identification of mAb

Chromosomes of hybridoma cells The number of the chromosomes in the hybridoma cells which secreted objective mAb was 83 - 105, their modal number was 94 - 98, and the majority of the chromosomes were the type of terminal centromere, which is consonant with the universal characteristics of the hybridoma cells which secrete mAb (Figure 6).

Ig subtype of mAb The subtype Ig of the prepared mAb was IgG1.

Molecular mass of mAb-associated antigen The molecular mass of the prepared mAb associated antigen was about 75 ku (Figure 7).

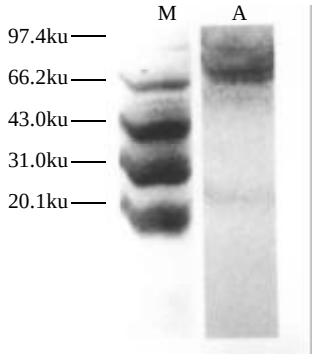

Figure 7 The molecular mass of the associated antigen of the prepared mAb. M: Standard molecular mass; A:HCC-9204 cells treated with 60 mL·L^{-1} ethanol for 6 h

DISCUSSION

To investigate the changes of the apoptosis-associated molecules on cell surface and the production of new antigens in the process of apoptosis is important in many fields of biomedical studies such as the mechanism of signal transduction and molecular regulation in cells and cellular immunology, *etc*., and many mammalian gene-encoded proteins can induce or suppress apoptosis in a variety of cell types[1-16]. It was discovered previously by the method of 2-dimentional gel electrophoresis that there are 17 new proteins, whose molecular mass are 12 - 80 ku, expressed in some cell types undergoing apoptosis induced by the transfection of wild-type *p53*[17]. A mitochondrial membrane protein 7A6 expresses only in some kinds of apoptotic but not in non-apoptotic cells, and its mAb has been used in the detection of apoptosis in some cell types[19, 26, 30-32]. The facts cifed above indicate that in the process of apoptosis, there exists not only the up or down-regulation of some proteins expressed already in non-apoptotic cells, but also the production of new proteins that did not exist in non-apoptotic cells.

Low-concentration ethanol can induce apoptosis in some cell types such as T lymphocytes, natural killer cells, macrophage, neutrophils, nerve cells, reproduction stem cells, mucosa epithelial cells, salivary gland cells, osteoblast, thyroid epithelial cells, leukemia cells, hepatocytes and HCC cells, *etc*.[33-40]. The results of present study showed that the majority of the HCC-9204 cells treated with 60 mL·L^{-1} ethanol for 6 h manifested marked morphological changes of apoptosis,the majority of the cells were TUNEL-positive, and the sub-G1 apoptotic peak detected by flow cytometry was obvious, suggested that low-concentration ethanol could induce apoptosis obviously in HCC-9204 cells and the model of ethanol-induced apoptosis in HCC cells was established successfully. The previous study with SDS-PAGE and folium scan in our laboratory have revealed that at least 7 kinds of new proteins whose molecular mass are more than 67 ku expressed in the HCC-9204 cells treated with 60 mL·L^{-1} ethanol for 6 h but not in non-apoptotic cells. It is well known that HCC is a common malignant tumor in China and some other places in the world, and its oncogenesis and development are related with apoptosis[41-60]. Therefore the ethanol-induced apoptotic HCC-9204 cells were used as antigen to prepare mAb against the apoptosis-associated antigens of HCC cells in this experiment.

Most of the proteins encoded by apoptosis-associated genes express only instantaneously in cells, and the quantity of the expressed products of apoptosis-associated genes is usually very little. The general procedures of the routine of immunization for the preparation of mAb are that the mice were further immunized twice respectively with the same antigen as the first immunization at an interval of 2 wk. In our previous studies, more than ten rabbits have been immunized with ethanol-induced apoptotic HCC-9204 cells by routine immunization procedures, there was no rabbit found whose

ear blood serum reacted strongly with apoptotic, but weakly with non-apoptotic cells. There is also no mouse whose tail blood serum reacted differently with apoptotic and non-apoptotic cells in all the 10 mice used as control in this experiment. The those results suggest that the antibodies against cell apoptosis-associated antigens are difficult to be prepared by the routine immunization procedures because of the interference of the large amounts of antigens not associated with apoptosis in the apoptotic cells.

Subtractive immunization is a special method of differentiated immunization used for the preparation of antibodies when the antigen components are complicated, unclear or cannot be extracted and purified[61-65]. The mechanism of subtractive immunization is that when certain antigens induce the immunoreaction of a animal body, the lymphocytes will proliferate, and the cells in split phase are prone to be selectively killed by the drug containing alkyls such as CP that is in common use in the procedures of subtractive immunization. When the same antigen is injected into the animal body again after the first immunization, the immunoreaction to the antigen is suppressed. CP can effectively inhibit the production of the antibodies against non-objective components of the antigen, but the production of objective antibodies cannot be influenced obviously, and neither are the fusion rates of hybridoma cells. Therefore the selection workload in the process of preparing mAb could reduce remarkably. The standard procedures of subtractive immunization are as the followings. Firstly, 2 groups of mixed antigens A and B whose components were similar were prepared. There is objective antigen in group A, but no objective antigen in group B. Secondly, group B is firstly used to immunize some animals. At the meantime, the immunized animals are treated with CP, or CP is injected 10 min, 24 h and 48 h later. Thirdly, the animals are further immunized with group A for 3 times respectively at an interval of 1 wk. At this time, the immunoreaction of the animal body to the common components of group A and group B is suppressed. Therefore the interference of non-objective antigens to the preparation of objective mAb is wakened or even may be eliminated, the immunoreaction to the objective antigens in group A is enhanced relatively, and the probability to obtain specific mAb increases significantly[48].

There were 2 mice whose tail blood sera reacted strongly with ethanol-induced apoptotic HCC-9204 cells, but weakly with non-apoptotic HCC-9204 cells in 10 mice immunized by subtractive immunization in this experiment, indicating that there are certain antigens which express strongly in apoptotic cells, but weakly or even do not express in non-apoptotic cells in the process of HCC cells apoptosis. The subtractive immunization is therefore a useful method to prepare the antibodies against the apoptosis-associated antigens of cells. The preferable results obtained only in 20 % (2 / 10) of the mice immunized by subtractive immunization, suggesting that there is obvious individual difference in the effect of subtractive immunization with CP. In order to get ideal effect, more mice are needed to be immunized in an experiment. The fusion rate of hybridoma cells in the experimental group did not differ distinctly from that in the control group, but the total producing rate of antibodies was lower significantly than that in the control group, suggesting that CP can suppress remarkably the production of antibodies while the fusion rate of hybridoma cells is not greatly influenced. The producing rate of the antibodies which reacted strongly with both apoptotic and non-apoptotic cells in the experimental group is significantly lower than that in the control, whereas there is no remarkable difference on the producing rate of the antibodies which reacted strongly with apoptotic, but weakly with non-apoptotic cells between the two groups, indicating that CP had partly suppressed the production of the antibodies against non-objective antigens to some extent, but not influenced that of the antibodies against objective antigens. The subtractive immunization can significantly decrease the selection workload in the process of preparing mAb when the antigen of mAb is unknown.

The mAb whose associated antigen located within the nucleus of ethanol-induced apoptotic HCC-9204 cells and reacted weakly with non-apoptotic HCC-9204 cells was prepared successfully and identified primarily in this experiment, which is consistent with some previous reports that injecting apoptotic cells into the cavum abdominis of mice can lead to the production of anti-nucleus antibodies[66]. The results of Western blot analysis revealed that the molecular mass of the associated antigen of this mAb was about 75 ku, which is consistent with the result of our previous study that new proteins whose molecular mass are more than 67 ku are discovered in 60 $mL \cdot L^{-1}$ ethanol-induced apoptotic HCC-9204 cells. The associated antigen of the mAb prepared in this experiment is the certain molecule that expresses in the nucleus of HCC-9204 cells and may be related with the ethanol-induced apoptosis. No antibody that reacted strongly with apoptotic cells, but completely absent with non-apoptotic cells is obtained in this experiment, suggesting that the antibodies against new apoptosis-associated antigens that do not exist in non-apoptotic cells is difficult to be prepared by the method of cell fusion used in this experiment. Low-concentration ethanol can not only induce apoptosis in HCC-9204 cells, but may also suppresses their proliferation and startup the expression of some molecules related with some other effect of ethanol on cells. The relationship between the mAb prepared in this experiment and its associated antigen and apoptosis in HCC cells is still to be determined and should be studied further. This experiment gives us a hopeful prospect to discover some new apoptosis-associated molecules in HCC cells.

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• LIVER CANCER •

Significance of cyclooxygenase-2 expression in human primary hepatocellular carcinoma

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Abstract

AIM: To clarify the significance of cyclooxygenase-2 (COX-2) expression in human primary hepatocellular carcinoma (HCC) and adjacent nontumorous tissues.

METHODS: The COX-2 protein and mRNA were investigated in 27 HCC tissues with adjacent nontumorous tissues, and 5 histologically normal liver tissues,using immunohistochemistry and in situ hybridization.

RESULTS: The well-differentiated HCC expressed COX-2 protein (5.68±1.19) more strongly than moderated HCC (3.43±1.98) and poor differentiated HCC (3.33±1.50) ($P<0.05$ respectively), adjacent nontumorous tissues (4.93 ±1.05) and normal liver tissues (3.20±1.92) ($P<0.01$ respectively); More intensive staining of COX-2 in adjacent nontumorous tissues was observed than that in normal liver tissues ($P<0.05$). There was no significant difference among adjacent nontumorous tissues, moderately differentiated HCC and poorly differentiated HCC ($P>0.05$). The expression of COX-2 mRNA was observed in the cytoplasm of the cells of HCC and of the hepatocytes in adjacent nontumorous tissues in which COX-2 protein was positive.

CONCLUSION: The overexpression of COX-2 in well-differentiated HCC suggests that COX-2 may play a role in the early stages of hepatocarcinogensis.

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INTRODUCTION

Cyclooxygenase (COX) is the rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H_2, the precursor of various compounds including prostaglandins, prostacyclin and thromboxanes. Two COX genes, COX-1 and COX-2, have been identified,which share greater than 60 % identity at the amino acid level. COX-1 is constitutively expressed in a number of cell types, whereas COX-2 is inducible by a variety of factors such as mitogen, cytokines, growth factor, and tumor promoters[1]. The expression of COX-2 is significantly increased in various types of carcinoma. There is also sufficient evidence indicating that selective COX-2 inhibitors produce effective prevention of carcinogenesis and that these compounds act by inducing of apoptosis of various cancer cells. These findings suggest that COX-2 may be involved in carcinogenesis and/or progression of certain types of human malignancies[2]. In this study, we examined the expression of COX-2 in human primary hepatocellular carcinoma (HCC) and adjacent nontumorous tissues.

MATERIALS AND METHODS

Tissue samples

HCC tissue and adjacent nontumorous liver tissues were obtained from 27 patients with hepatic tumor, who received hepatectomy at Renji hospital. Five specimens of grossly normal liver tissues from the area surrounding benign angiomas were used as controls. Specimens were fixed in 10 % neutral formalin and embedded in paraffin.

Histology

Serial 5-um sections were stained with hematoxylin and eosin. Each HCC was histologically graded into one of three categories: well-differentiated, moderately differentiated, or poorly differentiated, according to the criteria proposed by the Liver Cancer Study Group of Japan[3]. The tumor tissues consisted of 11 well-differentiated, 7 moderately differentiated, and 9 poorly differentiated HCCs, and the nontumorous sites consisted of 3 chronic hepatitis and 24 cirrhosis.

Immunohistochemistry

Immunohistochemical staining was performed on serial sections at room temperature, using the peroxidase method with the polyclonal antibody against human COX-2 (Santa Cruz Co.). The sections were deparaffinized in xylene and rehydrated through graded alcohol, immersed the sections in 3 % hydrogen peroxide for 10 minutes to inactivate endogenous peroxidases, then were incubated for 10 minutes with 10 % normal swine serum in Tris-buffered saline to block non-specific binding, subsequently incubated overnight at 4 ℃ with relevant antibody. The following day, the sections were incubated with biotinylated anti-mouse IgG (Maxim Biotech Inc. USA) for 45 minutes, followed by peroxidase-conjugated streptavidin (Maxim Biotech Inc.). The chromogenic reaction was developed with diaminobenzidine for 10 minutes, and all sections were counterstained with hematoxylin. In controls, the primary antibody was omitted.

The intensity of staining for COX-2 in HCC tissues and nontumorous adjacent tissues was scored in each specimen on a scale of 0 to 3, in which 0=negative staining, 1=weakly positive staining, 2=moderately positive staining, and 3=strongly positive staining. The percentage of positive cells in each specimen was estimated and scored on a scale of 0 to 4, in which 0=negative, 1=positive staining in 1 % to 25 % of cells counted, 2, in 26 % to 50 %; 3, in 51% to 75 %; and 76 % to 100 %. Each section was evaluated for the sum of these two parameters.

In Site Hybridization

Deparaffinized sections of liver tissue were treated with proteinase K (70 mg/ml) for 30 minutes at 37 ℃, followed by fixation with paraformaldehyde (0.4 %, 20 minutes). Sections were prehybridized for 1-hour, then hybridized at 55 ℃ overnight with digoxigenin-labeled (labeling-kit: Boehringer Mannheim) fragments of COX-2 (Cayman Chemical). After extensive washing of the tissue section, hybridization was visualized using anti-digoxiganin, alkaline phosphatase-conjugated antibody and NBT/BCIP.

Statistical analysis

Data are presented as the mean ±SD. Statistical significances were assessed using Mann-Whitney' s *U* test. Significance was accepted when $P<0.05$.

RESULTS

In immunohistochemical analysis, cytoplasmic staining for COX-2 was observed in HCC cells and nontumorous hepatocytes. The distribution of positive cells was mostly extensive, and occasionally focal or scattered. Different histological grades of HCC demonstrated different immunoreactivity for COX-2. A significantly high expression level of COX-2 (5.68±1.19) was found in well-differentiated HCC, compared with that of normal tissue (3.20±1.92), adjacent nontumorous tissues (4.93±1.05) ($P<0.01$, respectively), moderately differentiated HCC (3.43±1.98) and poorly differentiated HCC (3.33±1.50) ($P<0.05$, respectively). More intensive staining of COX-2 in adjacent nontumorous tissues was observed than that in normal liver tissues ($P<0.05$). These is no significant difference among adjacent nontumorous tissue, moderately differentiated HCC and poorly differentiated HCC ($P>0.05$, respectively) (Figure 1). The expression of COX-2 mRNA was observed in the cytoplasm of the cells of HCC and of the hepatocytes in adjacent nontumorous tissues in which COX-2 protein was positive.

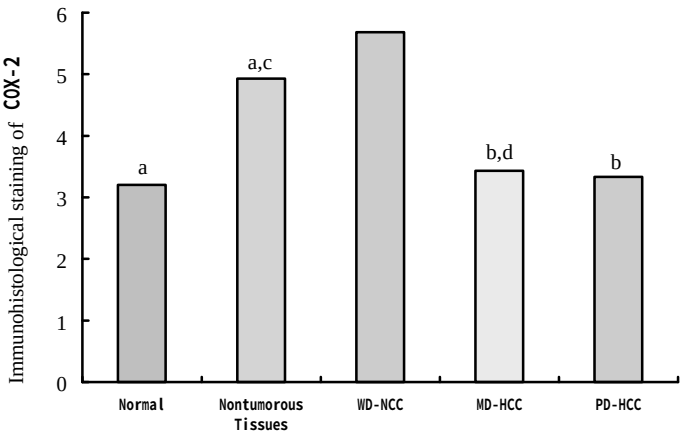

Figure1 Immunohistochemical staining scores of COX-2 in normal, adjacent nontumorous and HCC tissues. ([a]$P<0.01$ *vs* WD-HCC; [b]$P<0.05$ *vs* WD-HCC; [c]$P<0.05$ *vs* normal.)

DISCUSSION

Primary HCC is one of the most common tumors in China. The prognosis of HCC is generally poor, and the 5-year survival rate is limited to 25-39 % after surgery[4]. The present study showed that a significantly increased expression of COX-2 was observed in HCC, suggesting that COX-2 was being involved in hepatocarcinogenesis. Of interest, a profound expression of COX-2 was demonstrated in well-differentiated HCC. It is known that early HCC is usually a well-differentiated carcinoma and then gradually changes into a poorly differentiated phenotype during tumor progression[5]. In this context, it is suggested that COX-2 may be associated with the early process of the progression of HCCs. This result is consistent with those reported by Koga[6].

Recent studies have highlighted the relevance of COX-2 in human carcinogenesis. Epidemiological studies indicate that NSAIDs lead to a regression of colonic polyps in patients with familial adenomatous polyposis. NSAIDs are also known to reduce the risk of colorectal cancers, breast and lung cancers[7]. There is also a sufficient evidence from animal studies indicating that selective COX-2 inhibitors produce effective prevention of carcinogenesis. Introduction of COX-2 cDNA into colon carcinoma cells facilitated growth. COX-2 appears to prevent apoptosis because selective COX-2 inhibitors induced apoptosis in various cell culture systems[8]. Furthermore, it was shown that COX-2 promoted angiogenesis in malignant cells[9]. COX-2 mRNA and protein were recently found to be expressed in human colon carcinoma[10] and gastric carcinoma[11]. However, COX-2 protein was not expressed in human breast carcinoma or in human basal cell carcinoma. These observations have suggested that overexpression of COX-2 in carcinomas is not the universal event in carcinogenesis but may be specific. Recently, Kondo *et al*[12] showed that increased expression of COX-2 in nontumor liver tissue was associated with shorter disease-free survival in patients with hepatocellular carcinoma. Clinicopathological survey indicated a significant correlation between COX-2 expression and differentiated carcinoma. Moreover, high COX-2 expression in nontumorous tissue was significantly correlated with the presence of active inflammation. These findings suggest that COX-2 expression in nontumorous tissue may play a positive role in relapse of HCC after surgery.

Recent chemopreventive strategies for colon carcinogenesis have focused on using COX inhibitors, resulting in interesting data from animal models[13]. Denda et al[14] demonstrated that administration of NSAIDs suppressed cirrhosis and subsequent formation of HCC in the choline-deficient L-amino acid-defined rat model. The present finding of increased COX-2 expression in well-differentiated HCC tissues encourages chemopreventive studies for primary HCC. To clarity whether COX-2 is a principle enzyme involved in liver carcinogenesis, in vivo animal studies should be performed using a specific COX-2 inhibitor.

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Edited by Zhao P

Dear editors:

We found some data errors in our paper entitled "Alternation of AFP-mRNA level detected in blood circulation during liver resection for HCC and its significance", which was published in World J Gastroenterol 2002 October; 8(5):818-821. All authors have made a decision to retract this paper from your journal and all databases as soon as possible.

We are so sorry about it.

Thank you very much.

Best regards,

Xiaoping Chen

2007-11-12

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• LIVER CANCER •

Expression of p53 and C-myc genes and its clinical relevance in the hepatocellular carcinomatous and pericarcinomatous tissues

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Abstract

AIM: To investigate the possible roles of p53 and C-myc genes in the primary hepatocellular carcinogenesis and the relationship between the liver hyperplastic nodule(LHN) and hepatocellular carcinoma(HCC).

METHODS: The expression of p53 and C-myc genes was detected immunohist-ochemically in 73 and 60 cases of HCC and pericarcinomatous tissues, respectively.

RESULTS: The positive expression of p53 in HCC was significantly higher than that in pericarcinomatous tissues ($P<0.05$). In pericarcinomatous tissues, the p53 expression was observed only in LHN, but not in liver cirrhosis (LC) and normal liver tissues. The positive expression rate of C-myc in HCC or LHN was significantly higher than that in LC or normal liver tissues ($P<0.05$ and $P<0.01$), however, no significant difference was found between HCC and LHN ($P>0.05$). The positive expression rate of p53 and C-myc in HCC was correlated with the histological differentiation, that in the poorly differentiated was significantly higher than that in well differentiated samples ($P<0.05$).

CONCLUSION: The overexpression of p53 and C-myc genes might play a role in the carcinogenesis of HCC; And LHN seems a preneoplastic lesion related to hepatocarcinogenesis; No evidence supports that LC contribute directly to the hepatocarcinogenesis.

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INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China[1-11], and the incidence of HCC reported has apparently increased in recent years. Despite a variety of therapeutic strategies, HCC remains a significant cause of cancer death. Therefore, to study the HCC pathogenesis is of the utmost importance to the prevention and treatment of this disease. With the advancement of HCC study, it becomes clear that the biologic behavior of HCC is closely related with the overactivation of the oncogenes and the inactivation of the tumor suppressor genes[12,13].

In the present study, the immunohistochemical LSAB (labelled streptavidin biotin) method was used to detect the expression of p53 and C-myc genes in HCC and pericarcinomatous tissues, in order to investigate the possible roles of these genes played in the HCC carcinogenesis, and to find out the relationship between the liver hyperplastic nodule and HCC. In addition, the relationship between the expression of p53 and C-myc genes and clinicopathological parameters of HCC was preliminarily investigated.

MATERIALS AND METHODS

Materials

HCC specimens of 100 cases obtained from surgical resections or biopsies performed at the Affiliated Hospital of Medical College of Qingdao University, China. Of these patients, 76 were male and 24 female with an average of 50.4 years. None of the patients had received chemo- or radio-therapy before resection. We randomly selected 73 and 60 cases of HCC to detect the expression of p53 and C-myc genes respectively owing to the limitation of antibodies. The specimens for detecting p53 were classified into 4 grades according to Edmondson's grading criteria, 4 specimens were in grade Ⅰ, 24 in grade Ⅱ, 39 in grade Ⅲ, and 6 in grade Ⅳ. All 73 specimens contained pericarcinomatous tissues, in which including 39 liver hyperplastic nodules (LHN), 35 liver cirrhosis(LC) and 10 normal liver tissues. Among the specimens for detecting C-myc gene, 4 were in grade Ⅰ, 17 in grade Ⅱ, 30 in grade Ⅲ, and 9 in grade Ⅳ. All 60 specimens contained pericarcinomatous tissues, in which including 37 LHN, 30 LC and 12 normal liver tissues.

Methods

All specimens were routinely processed, alcohol-fixed and paraffin-embedded. Serial paraffin sections of 4 um thickness were cut and used for hematoxylin and eosin(HE) and immunohistochemical stains. Immunohistochemical LSAB method was used to detect p53 and C-myc genes. Anti-p53 monoclonal antibody DO-7, anti-C-myc monoclonal antibody and LSAB kits were purchased from Dako Co. Before staining, the sections were heated with microwave in 0.05 mol • L^{-1} citric acid solution for antigen retrieval. In each staining , a known p53 or C-myc positive section was added as the positive control, and PBS was used as the substitute of the first antibody for the negative control.

Analysis of immunohistochemical staining

Cells with brown granules under microscope were regarded as positive. The criteria for the evaluation of the p53 expression in the present study were as follows: the positive nuclei number was semiquantitatively evaluated by counting that in 8-10 randomly-chosen medium power (×100 magnification), and the four degrees of the p53 expression were considered as: negative (-), no positive cells; weak positive (+), the positive cells <10 %; moderately positive (+ +), the positive cells between 10-50 %; strong positive (+ + +), the positive cells >50 %. For the evaluation of C-myc expression, the percentage of positively stained cells was employed as an index, which was obtained from counting 500 cells at more than 5 high power fields for each section, and classified into 4 grades: grade Ⅰ, the positive cells between 1-25 %; grade Ⅱ, the positive cells 26-50%; grade Ⅲ, the positive cells 51-75 %; grade Ⅳ, the positive cells 76-100 %.No positive cells were scored negative (-).

Statistical analysis

Results were analysed by χ^2 test. Differences at $P<0.05$ were considered to be statistically significant.

RESULTS

Expression of p53 gene in HCC and its pericarcinomatous tissue

The positive staining for p53 gene expressed as brown granules, which was mainly located in the cell nuclei of tumor cells (Figure 1). The staining intensity and extent varied among tumors, different tumor regions and individual tumor cells. Immunostaining of p53 protein was negative in all tumor stroma, bile duct epithelia, LC and normal liver tissues. A few p53 weakly positive cells were found in LHN (Figure 2). The significant difference existed between HCC and LHN (χ^2 value 10.57, $P<0.01$), and so did between LHN and LC (χ^2 value 6.94, $P<0.01$) (Table 1).

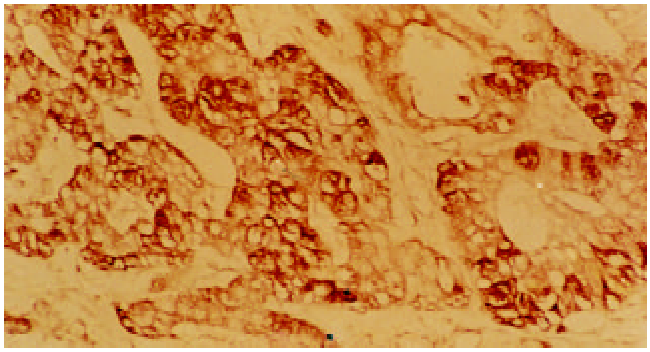

Figure 1 The positive expression of p53 gene in HCC. LSAB×200

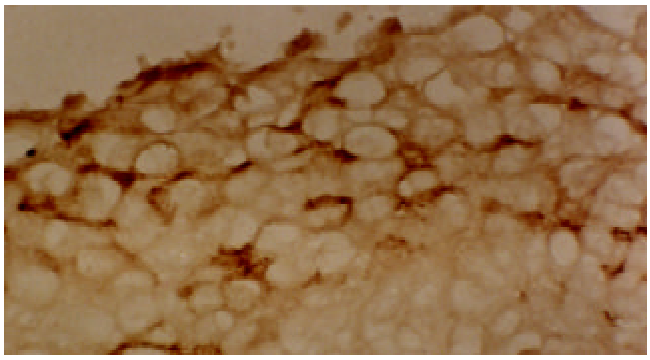

Figure 2 The positive expression of p53 gene in LHN. LSAB×200

Expression of C-myc gene in HCC and its pericarcinomatous tissue

The positive staining for C-myc gene was also expressed as brown granules, which was distributed mainly in cell nuclei, partly in cytoplasms. Although the expression rate of C-myc was higher in LHN than that in HCC, the statistical significance did not reached (χ^2 value 0.05, $P>0.05$). The expression of C-myc gene in HCC and LHN was significantly higher than that in LC (χ^2 values 4.38, 4.51, $P<0.05$). In HCC (Figure 3) and LHN (Figure 4) showing strong expression of C-myc, the positive-staining cells were distributed dominantly in a diffused pattern; whereas in LC (Figure 5) showing weak expression of C-myc, they preferred in a focalized pattern. The expression of C-myc was negative in normal liver tissues (Table 2).

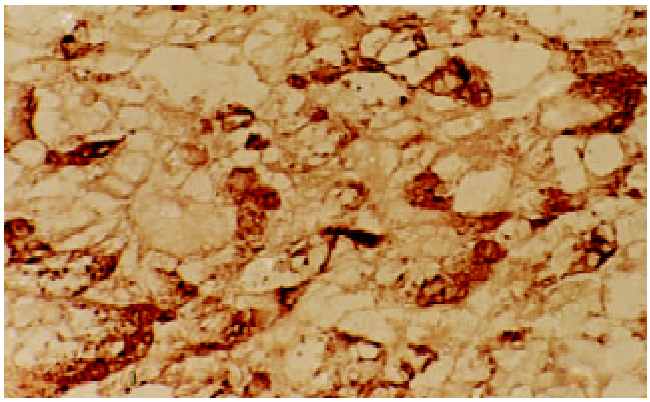

Figure 3 The positive expression of C-myc gene in HCC. LSAB×200

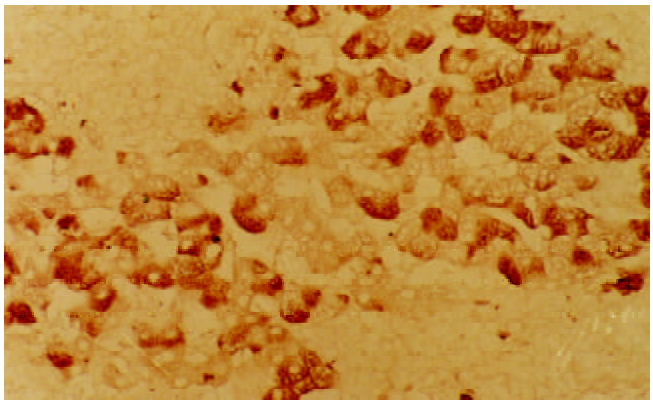

Figure 4 The positive expression of C-myc gene in LHN.LSAB×200

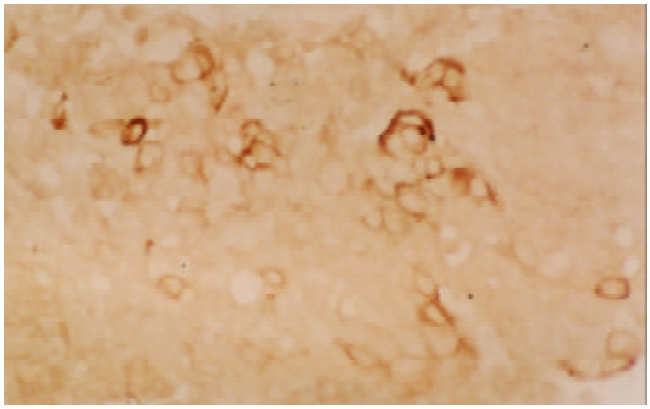

Figure 5 The positive expression of C-myc gene in LC. LSAB×200

Table 1 Expression of p53 gene in HCC and its pericarcinomatous tissue

Histological type	*n*	Expression of p53 gene				Positive rate (%)
		-	+	++	+++	
HCC	73	37	12	19	5	49.3
LHN	39	32	7	0	0	17.9
LC	35	35	0	0	0	0
Normal liver tissues	17	17	0	0	0	0

Table 2 Expression of C-myc gene in HCC and its pericarcinomatous tissue

Histological type	*n*	Expression of C-myc gene					Positive rate (%)
		-	grade Ⅰ	grade Ⅱ	gradeⅢ	gradeⅣ	
HCC	60	37	0	2	9	12	38.3
LHN	37	22	1	3	3	8	40.5
LC	30	25	4	1	0	0	16.7
Normal liver tissues	12	12	0	0	0	0	0

The relationship between the expression of p53 and C-myc genes and histological grade of HCC

There were varieties of positive rates of p53 and C-myc genes in different HCC histological grades of HCC, with a close relationship between the genes expression and the tumor differentiation. The positive rates of p53 expression in Edmondson' s grading Ⅲ and Ⅳ and Edmondson' s grading Ⅰ and Ⅱ were 60 %(27/45), 32.1 %(9/28), respectively, which manifested a significant difference (χ^2 value 5.36, $P<0.05$); The positive rates of C-myc expression in Edmondson' s grading Ⅲ and Ⅳ and Edmondson' s grading Ⅰ and Ⅱ were 48.7 %(19/39), 19 %(4/21), respectively, their differences were also significant(χ^2 value 5.08, $P<0.05$) (Table 3).

Table 3 The relationship between the expression of p53 and C-myc genes and histological grade of HCC

Edmondson's grading	Expression of p53 gene		Expression of C-myc gene	
	Negative	Positive	Negative	Positive
Ⅰ	4	0	3	1
Ⅱ	15	9	14	3
Ⅲ	18	21	18	12
Ⅳ	0	6	2	7

Correlation between p53 and C-myc protein expressions and HCC clinicopathological parameters

No significant relation was found between p53 or C-myc gene expression and patient age, sex and tumor size ($P>0.05$).

DISCUSSION

The p53 gene is one of the most important tumor suppressor genes determined so far[14-19]. In recent years, it has been found that the p53 protein seems not express in the benign and preneoplastic lesions, and that there was no obvious relation between the p53 protein and carcinogenesis[20]. However, in the present study, the positive rate of p53 protein staining in LHN was 17.9 %, which coincided basically with some results in literature[21-25]. The facts that the p53 protein expressed low in LHN and high in HCC, indicate that the high expression of p53 protein is probably associated with the cancerous transformation of hepatocytes, the early event in the carcinogenesis of HCC. It has been proved that wild type p53 protein can induce cell apoptosis whereas the mutant p53 protein can inhibit cell apoptosis and promote cell transformation and proliferation, resulting in carcinogenesis[26-30]; The p53 protein confirmed by immunohistochemical staining was considered as the mutation type[31]; Therefore, we speculate that p53 protein may exert its carcinogenic effect in the early stage of carcinogenesis on hepatocytes by two ways: **(1)** As described above, the mutant p53 protein might be associated with cell rapid proliferation and cell transformation, which ultimately results in hepatocellular carcinogenesis; **(2)** with the increase of the mutant p53 expression, and its inhibiting effect on apoptosis there will be an abnormal in cell numbers, which may eventually initiate the hepatocellular carcinogenesis. In addition, our results indicated that there existed a close relationship between the p53 gene expression and tumor cell differentiation in HCC, which suggests that the expression of p53 gene might serve as an index for the judgement of HCC malignant degree and its clinical prognosis.

It has been reported that the inept expression of C-myc gene correlated with carcinogenesis[23,32,33]. However, some authors held that the overexpression of C-myc gene could not be observed until the hepatocytes had thoroughly transformed into malignancy in the late stage of HCC, which reflected the continuous proliferation of tumor cells[34-36]. On the contrary, most experiments demonstrated the expression of C-myc in HCC and its pericarcinomatous tissue[24,37]. In the present study, the expression of C-myc gene was observed both in LHN and LC with varied degree and LHN was similar to HCC in the expression of C-myc, which were coincident with the results of others and suggests that the overexpression of C-myc gene occurs in the early phase of HCC formation, and correlates with preneoplastic transformation and proliferation. Our results also indicated that the expression of C-myc gene in HCC was related to the cell differentiation, which suggests that C-myc gene expression may exist in the sequential process of hepatocarcinogenesis, and be related to the phase of hepatocarcinogenesis.

In the present study, the expression of p53 gene in LHN was similar to that in HCC to some extent and the overexpression of C-myc gene was seen in both samples. The mutation of p53 gene that may lead to cell malignization, thus may reflect the alterations in different biologic state of LHN, and suggest that LHN is probably in the process of malignant transformation and in relation to hepatocarcinogenesis. In the present study the expression of C-myc gene in LHN had no significant difference from that in HCC, which reveals that parts of LHN were actually in the preneoplastic state or might be cancerous though they seemed normal in histology. Some study indicated that the increased oncogene expression brought cells into a state of active proliferation that resulted in an increased frequency of mutation[38]. This suggests that the overexpression of C-myc gene may make LHN be transformed

malignantly. Another study showed that not all altered hepatocyte foci manifested abnormal expression of C-myc in the early stage of experimental HCC and the high expression of C-myc was only seen in the poorly differentiated foci[39]. It shows that the overexpression of C-myc gene may relate to the tumor' s differentiation. Therefore, the overexpression of C-myc gene may be responsible for the low differentiation of LHN. It has been postulated that C-myc products might serve as a valid index for identifying preneoplastic lesion of HCC, the foci overexpressed C-myc were in danger of carcinogenesis[24,33]. However, few research reports till now have been found on the relationship between LHN and HCC. The present study thus provides a new possible way to diagnose HCC at earliest possible stage, which is of great importance in improving the prognosis because early diagnosis usually means high curability.

For many years, it has been generally considered that LC was closely associated with HCC and hepatocarcinogenesis. According to carcinogenic hypothesis on oncogene, at least two activated oncogenes are required-namely, the ras gene which was representative of transforming gene and the C-myc representative of immortalizing gene. Only when the two sorts of oncogenes function coordinately can stock-cultured cells be transformed malignantly. In the present study, we found that the expression rate of C-myc protein in LC was 26.7 %, but, the expression intensity of C-myc in LC was significantly less than that in LHN. Our previous studies have indicated that there was no mutation of the ras gene in LC, and that the expression of c-erbB-2 oncogene was negative in LC, indicating that there are no mutation and activation of c-erbB-2 in LC, that is, it is impossible in this situation for malignant transformation. In addition, according to the present study, there was no mutation of p53 in LC, suggesting that LC does not necessarily link with hepatocarcinogenesis. Alcohol is the major cause of cirrhosis in European countries and the United States, responsible for 60 to 70 percent of all cases of cirrhosis, but it only infrequently leads to HCC; carbon tetrachloride can lead to LC rather than HCC. However, the reason why the ratio of HCC accompanied by LC in China is obviously higher than that in European countries and the United States perhaps lies in HBV infections, there is not a cause and effect but a accompanying relationship between LC and HCC. The significance of C-myc expression in LC remains to be investigated.

What requires a special explanation is that the LHN used in the present study is totally different from the nodules in LC. We noticed that though the majority of the LHN developed from LC, it is a cell population that differs from LC in properties and proliferative patterns. Although the liver cell cords in LC are in disarray, the hepatocytes are primarily arranged in a single line, most of which are normal in morphology; However, the hepatocytes in LHN grow by expansion, one nodule primarily contains a sort of cells, and in mixed cell nodules there exists a clear margin between the cellgroups, suggesting the nodules are of clone origin.

As to the relationship between the expression of p53 and C-myc genes and clinicopathological parameters of HCC, we found that there was no link of p53 or C-myc gene expression with patient age, sex and tumor size, which was in accordance with the previous reports[40]. Some studies indicated that the overexpression of p53 or C-myc was closely related to the prognosis of HCC[18,33, 41-50], which was not our results have not yet confirmed that p53 or C-myc gene expression is directly associated with the prognosis of HCC. However, in the present study, the low expression of p53 gene and the overexpression of C-myc gene were found in LHN. It can be deduced that although normal in histology, LHN is surely abnormal in gene expression. Whether the phenomenon has a tie with the recurrence of HCC after resection needs to be further studied.

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• LIVER CANCER •

Telomerase inhibition and telomere loss in BEL-7404 human hepatoma cells treated with doxorubicin

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Abstract

AIM: To study the effects of doxorubicin on telomerase activity and telomere length in hepatocellular carcinoma.

METHODS: Telomerase activity was assayed with a non-radioisotopic quantitative telomerase repeat amplification protocal-based method. The effect of doxorubicin (DOX) on the growth of BEL-7404 human hepatoma cells was determined by microculture tetrazolium assay. Mean telomere length (terminal restriction fragment) was detected by Southern blot method. The expression of telomerase subunits genes was investigated by RT-PCR. Cell apoptosis and cell cycle distribution were evaluated by flow cytometry.

RESULTS: Telomerase activity was inhibited in a dose and time-dependent manner in BEL-7404 human hepatoma cells treated with DOX for 24, 48 or 72 h in concentrations from 0.156 to 2.5 μM which was crrelated with the inhibition of cell growth. No changes were found in the mRNA expression of three telomerase subunits (hTERT, hTR and TP1) after drug exposure for 72 h with indicated concentrations. The cells treated with DOX showed shortened mean telomere length and accumulated at the G_2/M phase. However, there was almost no effects on cell apoptosis by DOX.

CONCLUSION: The telomerase inhibition and the telomere shortening by DOX may contribute to its efficiency in the treatment in hepatocellular carcinoma.

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INTRODUCTION

Telomeres form the ends of eukaryotic chromosomes consisting of an array of tandem repeats of hexanucleotide 5'-TTAGGG-3'. Telomeres protect the chromosomes from DNA degradation, end-to-end fushions, rearrangements and maintain nuclear structure[1]. Human telomerase is a ribonucleoprotein complex, composed of a catalytic reverse transcriptase subunit (hTERT), an RNA component (hTR) that serves as a template for the synthesis of telomeric repeats, and an associated protein subunit (TP1)[2-4]. It adds telomeric repeats to the 3' end of telomeric DNA. This telomere stabilization by telomerase can lead to unlimited cell proliferation.

Hepatocellular carcinoma (HCC), one of the most common malignancies in the world especially in Asia and Africa, is an aggressive cancer. It causes approximately 250 000 deaths annually[5]. It was reported that HCC exhibited a high incidence of telomerase activity and that the activity increased in accordance with the HCC degree of histological undifferentiaton which was absent in normal liver tissue[6,7]. Other reports revealed that hTERT expression was the rate-limiting determinant of HCC telomerase activity[8-10].

Doxorubicin (DOX), an antitumor antibiotic, can intercalate into base pairs of DNA and generate toxic oxygen free radicals, which not only causes single-or double-strand DNA breaks but also damages a variety of necessary macromolecules such as proteins, lipids and RNA[11]. DOX is one of the most efficient chemotherapy agents in the treatment of HCC, and its total efficiency rate can be up to 44 %[12]. However, the relationship between the efficiency of DOX and telomerase activity in HCC has not yet been elucidated. In the present study, we investigated the effects of DOX on the telomerase activity and telomere length in BEL-7404 human hepatoma cells.

MATERIALS AND METHODS

Cell and culture condition

BEL-7404 human hepatoma cell line from Cell Bank of Chinese Academy of Sciences[13], was cultured in RPMI-1640 medium (Gibco) supplemented with 10 % heat-inactivated newborn calf serum, at 37 ℃ in a humidified CO_2 incubator containing 5 % CO_2 and 95 % air.

Drug

DOX (Sigma) was dissolved in RPMI-1640 to the final concentration of 5mM and stored at 4 ℃.

Assessment of cell proliferation

An MTT assay was conducted to determine the cell proliferation. Cells were seeded at 1×10^4 cells /well in a 96-well plate and incubated overnight. The drug was added to the cultured cells with the final concentrations from 0.156 μM to 2.5 μM and culturing further for another 24, 48 or 72 h respectively. Following culture, the cells were incubated with 800 mg/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT, Sigma), which was used to assay the activity of mitochondrial dehydrogenases. Four hours later, 10 % sodium dodecyl sulphate - 5 % isobutanol-0.12 % hydrochloric acid solution was added to solubilize the formazan product. The plate was then incubated at 37 ℃ for another 12 h. The absorbance at 570 nm was measured with a model 550 microplate reader(Bio-Rad). The percent of cell growth inhibition was expressed as: (A-B)/A×100 %, where A was the absorbance value from the controls and B was that from the experimental cells.

Telomerase assay

Telomerase activity was assayed with PCR-based telomeric repeat amplification protocol (TRAP) as previously described[14, 15]. Cells were collected and washed with PBS, lysed in 1×3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS, Sigma) buffer, incubated on ice for 30 min, and centrifuged at 12 000 g for 30 min. The protein concentration was determined by Coomassie Protein Assay. Each of TRAP reactions contained 1 μg of total protein. The reaction mixture [20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005 % Tween-20, 1mM EGTA,50 μM of each dNTPs and 0.1 μg TS (5'- AATCCGTCGAGC AGAGTT-3')] was incubated at 30 ℃ for 30 min , heated at 94 ℃ for 5 min. Then 0.1 μg of return primer ACX(5'-GCGCGG[CTTACC]$_3$CTAACC-3'), 0.1 μg of internal control primer NT(5'-ATCGCTTCTCGGCCTTTT-3'), 0.01 aM of internal control template TSNT(5'-AATCCGTCGAGC AGAGTTAAAAGGCCGAGAAGCGAT-3') and 2 units Taq DNA polymerase (Promega) were added. The reaction mixture was then subjected to 28 PCR cycles: 94 ℃ for 30s and 60 ℃for 30 s. PCR products were separated by electrophoresis on 12 % nondenaturing polyacrylamide gels and stained with SYBR Green I (FMC) for 15 min , visualized and analyzed by UVP system. In every experiment, a negative control (1 μl CHAPS lysis buffer) was included. All of experiments were repeated at least twice.

The relative telomerase activity was quantified by the formula: TP=[(A/B)/(A cell control/ B cell control)]×100

Where TP=total product, A=total intensity of telomerase product (50 bp,56 bp,62 bp..), and B=intensity of internal control (36 bp).

RT-PCR

Total cellular RNA was extracted from cells using Trizol (Life Technologies,Inc.) according to the instructions of the manufacturer. In each reaction, 1 μg of total RNA was reverse transcripted into cDNA using M-Mlv reverse transcriptase (Promega). Primer sets used to amplify specific sequences were 5'-CGGAAGAGTGTCTGGAGCAA-3' and 5'-GGATGAAGCGGAGTCGGA-3' for hTERT (146 bp); 5'-TCTAACCCTAACTGAGAAGGGCGTAG-3' and 5'-GTTTGCTCTAGAATGAACGGTGGAAG-3' for hTR (126 bp); 5'-TCAAGCCAAACCTGAATCTGAG-3' for TP1 (264 bp); 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-GTCCTTAATGTCACGCACGATTTC-3' for β-actin (539 bp). The PCR conditions of hTERT and TP1 were 94 ℃, 45s; 60 ℃, 45 s; 72 ℃, 90 s for 31 and 29 cycles, respectively. And the PCR conditions of hTR and β-actin were 94 ℃, 45 s;55℃, 45 s; 72 ℃, 90 s for 28 and 22 cycles, respectively[2].

Telomere length assay

Genomic DNA samples were prepared as described[16]. Cells were lysed and proteins were digested in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA , 0.5 % SDS , 0.1 mg/ml proteinase K at 48 ℃ overnight. Following two extractions with phenol and one with chloroform, DNA was precipitated with ethanol and dissolved in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE) Telomere length was detected using TeloTAGGG telomere length assay (Roche) according to the manufacturer's protocol. For each sample, 1 μg of genomic DNA was digested with Rsa I/Hinf I (Sigma), separated on a 0.8 % agarose gel, transferred to a nylon membrane (Amersham Hybond-N$^+$), and hybridized with a telomere specific digoxigenin(DIG)-labeled probe, incubated with anti-DIG-alkaline phosphatase and detected by chemiluminescence. The blotted signal was divided into 30 equidistant intervals from 1.9 to 21.2 kilobases to calculate mean telomere length (terminal restriction fragment, TRF) using the formula TRF= Σ(ODi)/Σ(ODi/Li), where ODi was the chemiluminescent signal and Li was the length of the TRF fragment at position i[17].

Flow cytometry analysis of cell cycle and apoptosis

The cells were harvested and resuspended in the solution containing 40mM sodium citrate, 250mM sucrose and 5 % DMSO. The suspention was stored at -20℃ for 20 min, then thawed rapidly at room temperature and centrifuged to collect the cells. The cells were resuspended in a solution containing RNase A (5×10^4 unit/g, 50 mg/L) and 20 mg/L propidium iodide (PI). The cell cycle distribution and apoptosis were determined by the fluorescence of individual cells measured with flow cytometry[18].

RESULTS AND DISCUSSION

Inhibition of telomerase activity

Telomerase activity was inhibited in a dose and time-dependent manner in BEL-7404 human hepatoma cells treated with DOX (Figure 1A). To analyze the telomerase inhibition on the gene expression level, cells treated with DOX for 72 h were employed to study the telmerase mRNA expressions of its three major gene components , hTERT, hTR and TP1, using RT-PCR. Results showed that no changes were observed in the mRNA expression pattern of these subunits after the DOX exposure for 72 h with indicated concentrations (Figure 1B).

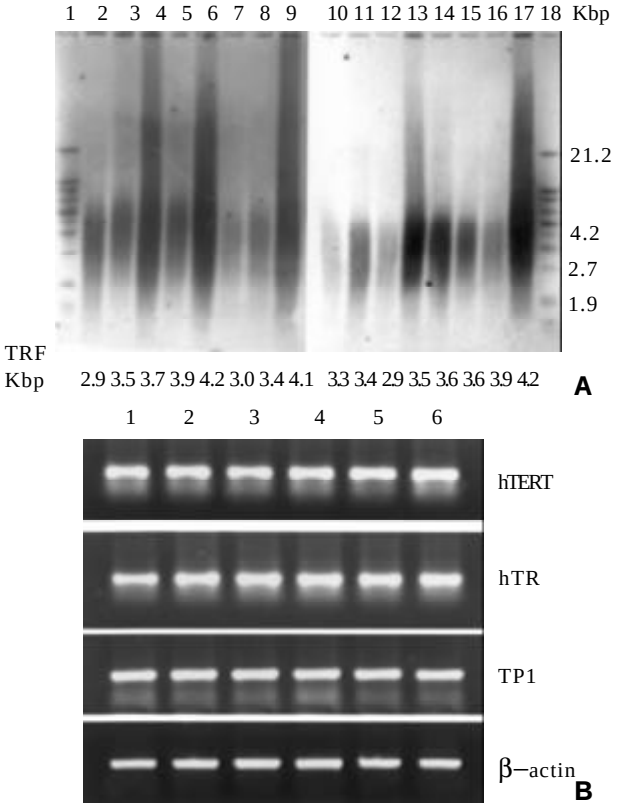


Figure 1 Telomerase inhibition by DOX in BEL-7404 human hepatoma cells. **(A)** Inhibition of telomerase activity by DOX in a dose and time-dependent manner in BEL-7404 human hepatoma cells. Lane 1, negative control; Lane 2, cell control; Lane 3–7, telomerase activity in presence of DOX for 24 h (2.5, 1.25, 0.625, 0.313 and 0.156 μM) ; Lane 8-12, telomerase activity in presence of DOX for 48 h (2.5, 1.25, 0.625, 0.313 and 0.156 μM); Lane 13-17, telomerase activity in presence of DOX for 72 h (2.5, 1.25, 0.625, 0.313 and 0.156 μM). All experiments were repeated at least twice and representative results were shown here. **(B)** RT-PCR analysis of hTERT, hTR and TP1 mRNA expression with DOX treatment for 72 h in a concentration range from 0.156

to 2.5 μM. Lane 1, cell control; Lane 2, 2.5 μM; Lane 3, 1.25 μM; Lane 4, 0.625 μM; Lane 5, 0.313 μM; Lane 6, 0.156 μM. β-actin was used as standard. Amplified sequences for hTERT, hTR, TP1 and β-actin are 146, 126,264 and 539 bp, respectively.

The first report on the telomerase inhibition by DOX was presented by Zhu et al, regarding the SW480 colon carcinoma cells[19]. However, the opposite observations were reported later, in which authors found no effect of DOX on telomerase regulation in nasopharyngeal cancer, testicular cancer and squamous cell carcinoma[20-22]. In this study, we found that the DOX treatment inhibited the telomerase activity in a dose and time-dependent manner in BEL-7404 human hepatoma cells. Considering the different results above, We think that this kind of inhibition might be cell-type specific and may be correlated with the clinical different efficiency of DOX in the treatment of different kinds and stages of tumors.

Abundant evidence indicated that the regulation of telomerase was multifactorial in mammalian cells and involves telomerase gene expression, post-translational protein-to-protein interactions, and protein phosphorylation[23]. However, there was no information regarding three telomerase subunits expression affected by DOX in Zhu's study[18]. In our investigation, there were no changes found in the expression of hTERT, hTR or TP1 mRNA. This indicated that the telomerase inhibition by DOX might be indirect with its antibiotic activity (eg.J damaging of necessary macromolecules).

Shortening of mean telomere length

Mean telomere length of BEL-7404 human hepatoma cells was decreased by the DOX treatment (Figure 2). Here, we believe that this is the first report regarding the shortening of telomere length by DOX treatment in BEL-7404 human hepatoma cells.

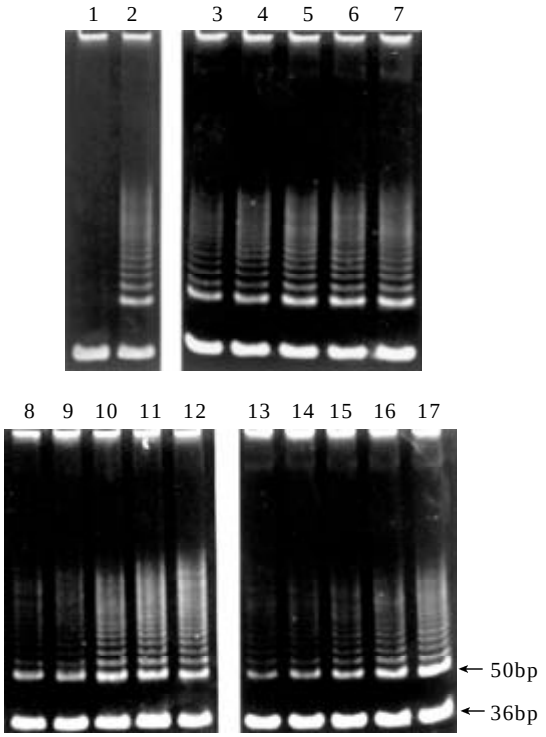


Figure 2 Shortening of mean telomere length (terminal restriction fragments,TRF) in DOX treated BEL-7404 human hepatoma cells. Lane 1 and 18, molecular marker;Lane 17,cell control; Lane 2 – 6, DOX treated for 72 h (2.5, 1.25, 0.625, 0.313 and 0.156 μM); Lane 7–11:DOX treated for 48 h (2.5,1.25,0.625, 0.313 and 0.156 μM); Lane 12-16:DOX treated for 24 h (2.5,1.25, 0.625, 0.313 and 0.156 μM).

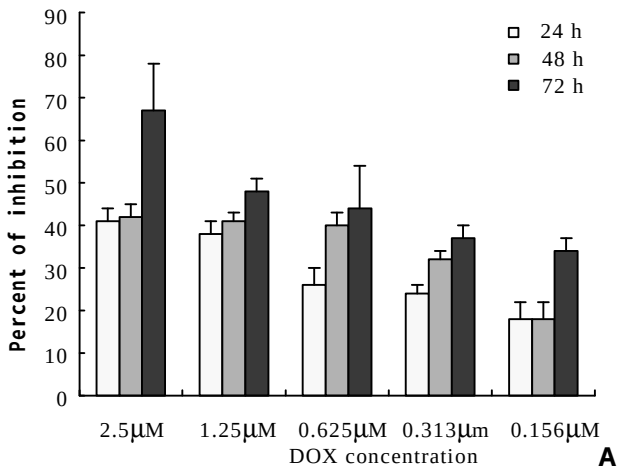
Compared with normal cells and some other carcinoma cells (such as Hela cells), the mean telomere length of BEL-7404 human hepatoma cells is relatively short. As a result, the hepatoma cells are sensitive to telomere shortening, which may account, at least in part, for their chemosensitivity. Results from telomere shortening by cisplatin indicate that the nucleotide-excision repair system and cell division dynamics might be involved in the telomere shortening process, which make the process showing in a non- dose or time dependent manner[24]. We assume that both of the factors may likewise participate in telomere shortening by DOX, because DOX can intercalate into DNA base pairs and result in non-dose or time dependent manner of telomere shortening.

Accumulation of cell cycle in G_2/M phase

The cell growth inhibition and cell cycle progression during DOX treatment were monitored in the present study to correlate these effects with telomerase activity inhibition and telomere loss. The growth of BEL-7404 cells was inhibited by DOX, which was indicated in (Figure 3 A). Following exposure to DOX, the hepatoma cells were accumulated in G_2/M phase, which revealed by flow cytometry (Figure 3 B). There were no marked changes observed in cell apoptosis in the experimental cells exposure to DOX for 24, 48 or 72 h with the concentrations from 0.156 to 2.5 μM, compared with the control cell.

Zhu *et al*[19] attributed the reduction in telomerase activity by DOX to the accumulation of cell cycle in G_2/M phase. But Holt *et al*[25] found that telomerase inhibition did not correlate with the cell cycle arrested at G_2/M phase but with the increasing in cell death. In present study, the cell apoptosis did not increase markedly compared with the control. Our previous research has also showed that antisense oligonucleotide to telomerase RNA component accumulated the cell cycle in G_2/M phase [26]. The present observation confirmed the reports above, which indicated that telomerase inhibition was correlated with the cell cycle arrested at G_2/M phase.

Previously, Ishibashi *et al*[24] have reported that telomere loss in HeLa cells associated with the apoptosis inducted by cisplatin. However, in our investigation the percent of cell apoptosis did not change markedly (Figure 3 B), although the mean telomere length was reduced by DOX. In BEL-7404 human hepatoma cells, we previously found that the cell apoptosis occurred when the mean telomere length reached to about 1.7Kb[27]. In this study, the mean telomere did not reach to this critical length, and the apoptosis had not been induced which was consistent with the previous observation. Therefore, we deduce that telomere shortening may not be correlated with the apoptosis.



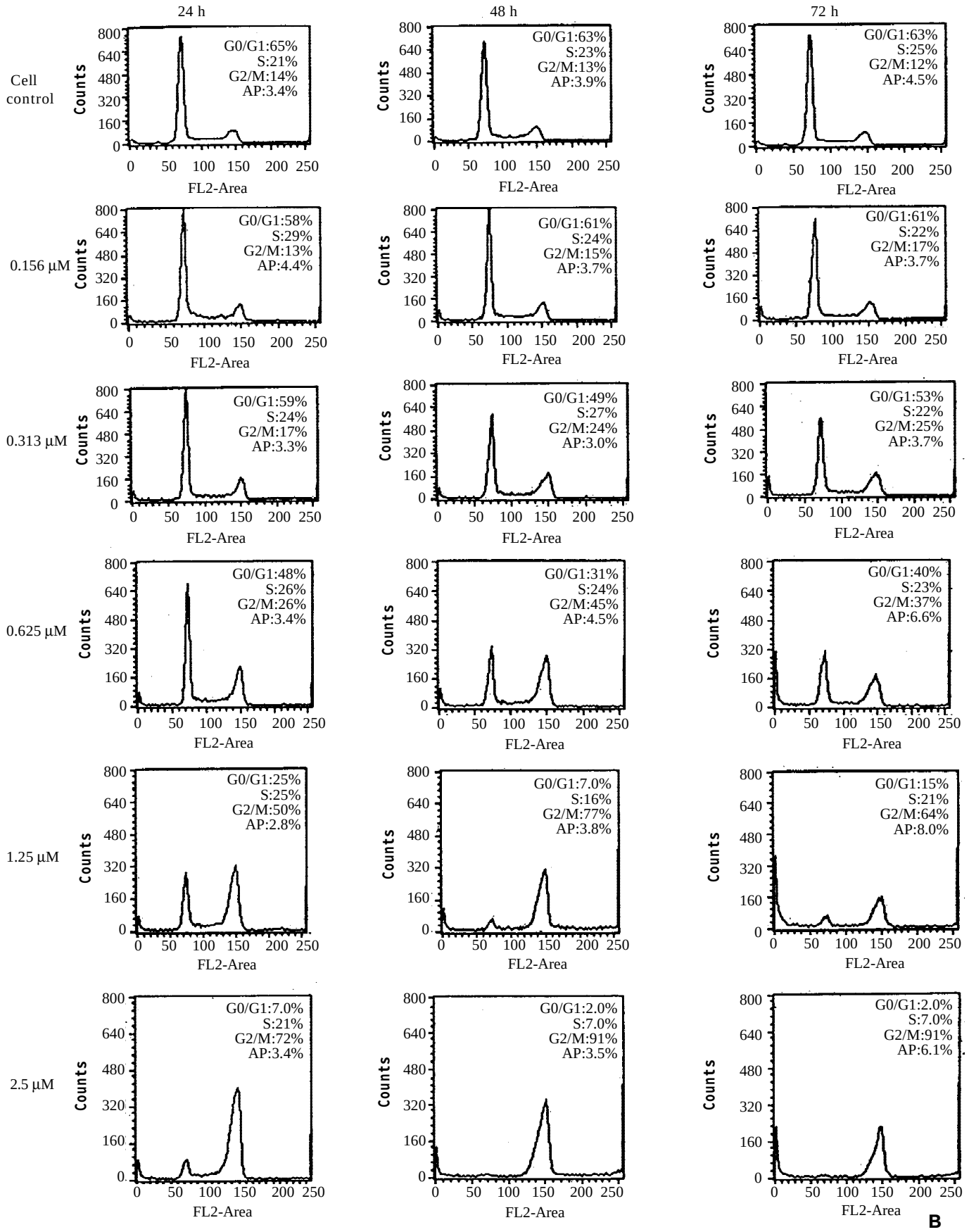


Figure 3 Effects of DOX on cell growth, cell cycle and cell apoptosis in BEL-7404 human hepatoma cells.
(A) Growth inhibition of BEL-7404 human hepatoma cells treated with DOX. Each value represents mean ± SD from triplicate wells.
(B) Cell cycle distribution and apoptosis in DOX treated BEL-7404 human hepatoma cells, analyzed by flow cytometry. Cell cycle was arrested at G_2/M with the treatment of DOX. Histograms of DNA contents of untreated cell control and treated with 0.156, 0.313, 0.625, 1.25 and 2.5 μM were shown. Cells were maintained in the presence of DOX for 24, 48, and 72 h without a change in medium and collected at the times indicated. AP means the percent of apoptosis.

Many studies have shown that the telomerase activity was correlated with the cell growth[28]. Our finding also revealed a good correlation between the inhibition of telomerase activity and the reduction in cell growth. However, in this study, the cell growth inhibition was mainly the result of cell cycle arrest, but not the increasing of cell apoptosis.

In conclusion, we found that telomerase activity was inhibited in a dose and time - dependent manner and mean telomere length was decreased by the treatment of DOX in BEL-7404 human hepatoma cells. This process correlated with the cell growth inhibition and the cell cycle accumulation in G_2/M phase. The present study indicates that the telomerase inhibition and the telomere shortening by DOX may contribute to its efficiency in the treatment of HCC.

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• LIVER CANCER •

Apoptosis of hepatoma cells SMMC-7721 induced by *Ginkgo biloba* seed polysaccharide

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Abstract

AIM: To study the apoptosis of hepatoma cells SMMC-7721 induced by polysaccharide isolated from *Ginkgo biloba* seed.

METHODS: *Ginkgo biloba* seed polysaccharide (GBSP) was isolated by ethanol fractionation of *Ginkgo biloba* seed and purified by Sephadex G-200 chromatography. The purity of GBSP was verified by reaction with iodine-potassium iodide and ninhydrin and confirmed by UV spectrophotometer, cellulose acetate membrane electrophoresis and Sepharose 4B gel filtration chromatography. The Scanning Electron Microscope (SEM) and Flow Cytometry (FCM) were used to examine the SMMC-7721 cells with and without GBSP treatment at 500 mg/ml for 36 h.

RESULTS: GBSP product obtained was of high purity with the average molecular weight of 1.86×10^5. Quantitative analysis of SMMC-7721 cells *in vitro* with FCM showed that the percentages of G_2-M cells without and with GBSP treatment were 17.01±1.28 % and 11.77±1.50% ($P<0.05$), the debris ratio of the cells were 0.46±0.12 % and 0.06±0.06 % ($P<0.01$), and the apoptosis ratio of cells was 3.84±0.55 % and 9.13±1.48 %($P<0.01$) respectively. Following GBSP treatment, microvilli of SMMC-7721 cells appeared thinner and the number of spherical cells increased markedly. Most significantly, the apoptosis bodies were formed on and around the spherical cells treated with GBSP.

CONCLUSION: GBSP could potentially induce the apoptosis of SMMC-7721 cells.

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INTRODUCTION

Ginkgo biloba L., also named after white-seed tree and gongsun tree, is one of the immemorial gymnosperm of the mesozoic era. It is regarded as a living fossil and is also best known for its pharmaceutical value. According to Pen-ts' so Kan-mu (i.e. Compendium of Materia Medica), *Ginkgo biloba* L. can help cure about 20 different diseases. More recently, it has been widely accepted that flavonoid and terpeneand are the effective components of leaves of *Ginkgo biloba*[1-5] for treating cardiovascular and nervous system diseases, scavenging free radicals and antioxidating, etc.[6-11]. Water-soluble polysaccharides of *Ginkgo biloba* leaves, endocarp, seeds and cultured cells were isolated and purified and their structures and some biological activities such as immunoregulation, antineoplastic action, scavenging free radicals and antioxidating were identified[12-16]. Furthermore, *in vivo* and *in vitro* induction of apoptosis of cancer cells by polysaccharides has been reported lately[17-22]. However, there was no report about the effects on apoptosis of tumor cells by polysaccharides isolated from *Ginkgo biloba* L. except from *Ginkgo biloba* endocarp[23]. In this study, high-purity polysaccharide was extracted from *Ginkgo biloba* seeds and the apoptotic effect of *Ginkgo biloba* seed polysaccharides (GBSP) on hepatoma cell line SMMC-7721 was investigated by scanning electron microscope (SEM) and flow cytometry (FCM).

MATERIALS AND METHODS

Materials

High-quality *Ginkgo biloba* seeds (*i.e.* milkwhite color, equal weight, and smooth surface without mildew) were purchased from Jianlian Chinese Traditional Medicine Store in Jinan, Shandong Province. Hepatoma cell line SMMC-7721 was obtained from Shanghai Cell Institute, China Academy of Sciences. Culture medium RPMI1640 was obtained from Gibco Co. (USA). Sephadex G-200, Sepharose 4B, monose as standard, calf serum and propidium iodide (PI) were obtained from Sigma Co. (USA).

Isolation and purification of GBSP

Ginkgo biloba seeds of 200 g were crushed into fine particles and extracted with 3000 ml of distilled water for 8 hours at 75 ℃ for 3 times. The extracts were pooled, concentrated to 30 % of the original volume in a rotary evaporator at 45 ℃ and then centrifuged at 3000 rpm for 15 min. The supernatant was collected and added with 3 volumes of 95 % ethanol to precipitate the polysaccharide. Following centrifugation at 4000 rpm for 15 min, the polysaccharide pellet was dissolved in appropriate volume of distilled water completely, dialyzed with distilled water and decontaminated by means of Sevag to remove protein. The polysaccharide was then freeze-dried, re-dissolved in salt solution and purified further by Sephadex G-200 chromatography. The purity of the resulting GBSP was analyzed by Sepharose 4B gel filtration chromatography and cellulose acetate membrane electrophoresis[24].

Culture of SMMC-7721 cells and treatment with GBSP[25-28]

The SMMC-7721 cells were grown to logarithmic phase of proliferation, washed 3 times with culture medium RPMI1640 and collected at a concentration of 10^6 cells /ml. This cell suspension was then aliquoted into 6 culture bottles and cultured at 37 ℃ and 5 % CO_2 (CO_2 incubator, MCO-17AC, SANYO, Japan) for 24h. For cultures that were prepared for

SCM test, cover slips were placed into bottles in advance. After the cells stuck on the walls of culture bottles, GBSP solution made up with culture medium was added into 3 of 6 culture bottles at the final concentration of 500 mg/ml. The other 3 culture bottles were added with equal volume of culture medium. The cells were cultured for further 36 h under the same conditions.

Flow Cytometry[29-31]

Supernatants of the cultures were discarded and SMMC-7721 cells with and without GBSP treatment were collected by digestion with pancreatin followed by centrifugation. PI was added to the cells for 15 min to label DNA. FCM (FACS/420, Becton Dickinson, USA) was used to analyze cell cycles and apoptosis ratios.

Scanning Electron Microscopy[32-33]

Supernatants of the cultures were discarded and SMMC-7721 cells stuck on the cover slips with and without GBSP treatment were examined by SEM (S-570, Hitachi, Japan).

RESULTS

Characterization of GBSP

One of the objectives of this work was to obtain high-purity GBSP product from the G*inkgo biloba* seeds. The purity of GBSP was first tested by reactions with iodine-potassium iodide and ninhydrin respectively. The results of these two reactions were negative, indicating absence of starch and protein in the GBSP product obtained. The reaction of GBSP with Molish reagent was positive, indicating that the GBSP product was composed of monose. The GBSP solution was then analyzed by UV absorption (UV-1200, The Second Beijing Optical Instrument Manufactory, China). As shown in Figure 1, there were no peaks at wavelengths of 260nm and 280nm on the UV spectrum, indicating that the GBSP product was not contaminated with nucleic acid and protein. The purity of GBSP was further analyzed by Sepharose 4B gel filtration chromatography and cellulose acetate membrane electrophoresis. The elution profile of Sepharose 4B was a single symmetrical peak (Figure 2). The cellulose acetate membrane electrophoresis displayed a single stripe of GBSP on the membrane. These indicated that the present GBSP was of high purity. The average molecular weight of the GBSP was 1.86×10^5 based on the linear calibration curve derived from Sephadex G-200 chromatography, as shown in Figure 3.

G_2-M cell ratio with and without GBSP treatment

The percentages of G_2-M cells without and with GBSP treatment were 17.01%±1.28% and 11.77%±1.50% ($P<0.05$) respectively (Figures 4, 5 and Table). This indicated that GBSP inhibited the proliferation of SMMC-7721 cells.

Table 1 The cycles and apoptosis ratios of SMMC-7721 cell with and without GBSP treatment

GBSP treatment (mg/ml)	Number of culture bottles tested	G_2-M cell ratio (%)	Apoptosis cell ratio (%)	Debris ratio (%)
0	3	17.01±1.28	3.84±0.55	0.46±0.12
500	3	11.77±1.50[a]	9.13±1.48[b]	0.06±0.06[b]

[a]$P<0.05$, [b]$P<0.01$ *vs* control group (*t* test)

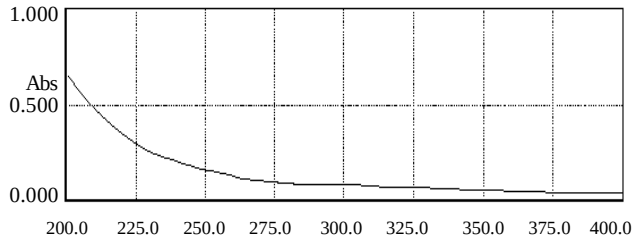

Figure 1 The UV spectrum adsorption curve of GBSP from 200nm to 400nm

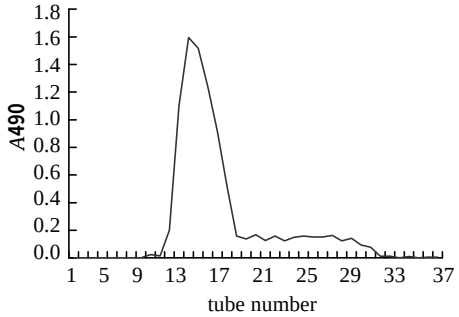

Figure 2 The profile of Sepharose 4B gel filtration chromatography

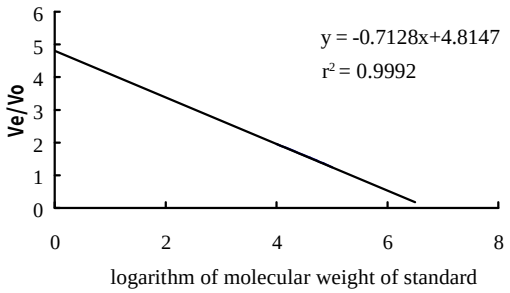

Figure 3 Standard linear calibration curve of Sephadex G-200 chromatography

Apoptosis ratio of the SMMC-7721 cell with and without GBSP treatment

The apoptosis ratio of SMMC-7721 cells without GBSP treatment was 3.84±0.55 % (Figure 4 and Table 1). After GBSP treatment, the apoptosis ratio increased to 9.13±1.48 % ($P<0.01$ *vs* control group) (Figure 5 and Table 1). The debris ratio of the cells without and with GBSP treatment was 0.46± 0.12 % and 0.06±0.06 %, respectively ($P<0.01$) (Figures 4, 5 and Table 1). These results showed that GBSP could induce and promote the apoptosis of SMMC-7721 cells, rather than kill SMMC-7721 cells directly.

Morphology of the SMMC-7721 cell with and without GBSP treatment

The morphology of SMMC-7721 cells with and without GBSP treatment was studied by SEM. The majority of SMMC-7721 cells without GBSP treatment was of shuttle shape (Figure 6) and small proportion of cells was of spherical shape. Close examination revealed dense microvilli on the surface of the cells, and occasionally, 2 to 3 protuberances were also observed on the cell surface. After the treatment of GBSP, these microvilli became thinner, protuberances disappeared (Figure 7) and number of spherical cells increased markedly. These spherical cells shrunk

so that their volume decreased and wrinkles were formed on their surface. Most significantly, apoptosis bodies were formed on and around the spherical cells (Figure 7). These observations suggest that GBSP could induce apoptosis in hepatoma cell line SMMC-7721, consistent with the results of FCM analysis.

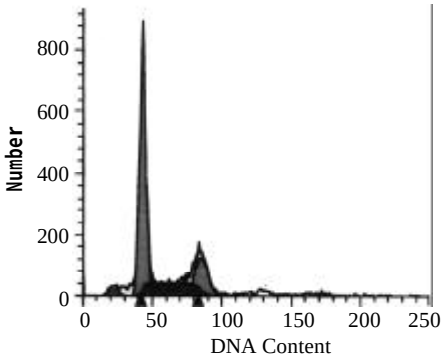

Figure 4 In the absence of GBSP treatment, the apoptosis ratio of SMMC-7721 cells was 3.84±0.55 %, the cells debris was 0.46 ±0.12 % and the percentage of G2-M cells was 17.01±1.28 %.

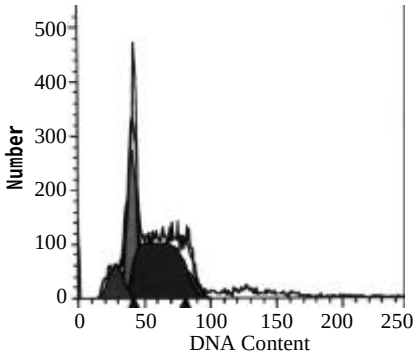

Figure 5 In the presence of GBSP treatment, the apoptosis ratio of SMMC-7721 cells was 9.13±1.48 %, the cells debris was 0.06±0.06 %,and the percentage of G2-M cells was 11.77±1.50%.

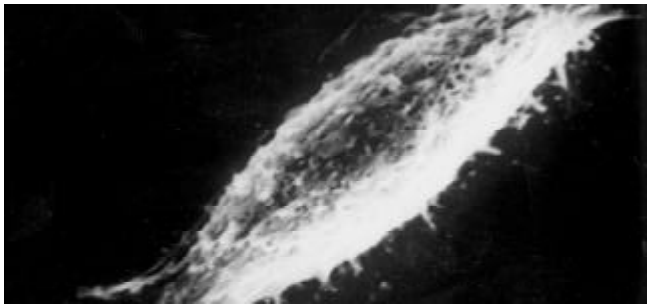

Figure 6 The majority of SMMC-7721 cells were of shuttle shape without GBSP treatment (×2500)

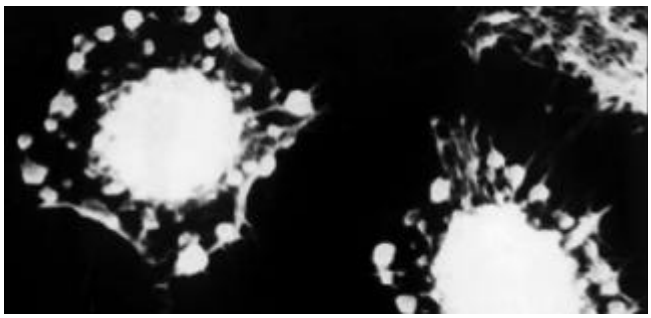

Figure 7 There were more spherical SMMC-7721 cells after GBSP treatment (×2200). These cells shrunk and apoptosis bodies were formed on and around them.

DISCUSSION

The expansion of neoplasma is associated with inhibition of apoptosis of tumor cells. There is evidence that high expression of the inhibition gene bcl-2 led to inactivation of the tumor suppression gene p53[34-45]. When apoptosis was inhibited, tumour cells would not be eliminated in time *in vivo* and, therefore, would proliferate and diffuse more rapidly. At present, drugs such as VP-16, ADR, MTX, and Hydroxyl urea etc. are commonly used in clinical chemotherapy. These agents interfere with growth, metabolism and proliferation processes of the cells so that the apoptosis of tumour cells can be induced [46-48]. However, like most of chemotherapical medicines, they have cytotoxic side-effects which cause damage to normal cells while killing neoplasma cells.

Experiments showed that ingredients of Chinese herbal medicine could induce apoptosis in tumour cells. For example, β-elemi-olefin, arabinose-cytidine and etoposide could induce apoptosis in leukemia cell line[49-55]. It was also reported that polysaccharides isolated from Chinese herbs could inhibit the growth of tumour by activating immune system *in vivo* rather than killing tumour cells directly[56,57]. In this *in vitro* study, we found that GBSP could effectively inhibit division of the SMMC-7721 cells and, meanwhile, induced apoptosis in hepatoma cell line SMMC-7721. Although *Ginkgo biloba* seed has been mainly used for treatment of asthm for over hundred years, it has not been known to have anti-tumour activity. The results of our present study provided valuable data for exploring the clinical application of GBSP for cancer therapy.

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• LARGE INTESTINAL CANCER •

Analysis for phenotype of HNPCC in China

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Abstract

AIM: The aims of this study were to identify the clinicopathological features of Chinese HNPCC families and to evaluate the value of criteria for suspected HNPCC (sHNPCC) in clinical diagnosis.

METHODS: According to the follow-up records, 54 HNPCC families (including 12 ICG-HNPCC families and 42 sHNPCC families) were screened out from patients with colorectal cancers (CRCs), operated upon in 2nd Affiliated Hospital of Zhejiang University from 1984 to 2001. Clinical data of probands and tumor spectrum in these families were listed and analyzed.

RESULTS: (1) Mean age, proportion of colonic cancer, poorly differentiated cancer, multiple CRCs and Dukes' A+B of the probands in ICG-HNPCC and sHNPCC kindred were 39ys and 47.5ys, 75 % and 62 %, 0 and 12.8 %, 16.7 % and 14.3 %, 58.3 % and 81 %,respectively. Compared with sporadic colorectal cancers, probands from ICG-HNPCC and sHNPCC families were obviously different at age of onset(P=0.025 and 0.031), tumor location (P=0.001 and 0.000), differentiation(P=0.002 and 0.011) and development of multiple tumors (P=0.014 and 0.002). (2) A total of 178 malignant neoplasms were found in 54 HNPCC families, including 139 colorectal cancers. Besides of colorectal cancer, extracolonic tumors occurred in stomach, endometrium, hepatobiliary system, and so on (8 gastric cancers, 6 endometrial cancers, 6 hepatobiliary system cancers and 19 others) can also be seen in Chinese ICG-HNPCC and sHNPCC families.

CONCLUSION: (1) Chinese HNPCC families have specific clinicopathological features, such as early onset, predilection for the involvement of colon, tendency of multiple CRCs, development of extracolonic tumors and well differentiation. (2) The criteria for suspected HNPCC is useful in clinical diagnosis and management of HNPCC.

Song YM, Zheng S. Analysis for phenotype of HNPCC in China. *World J Gastroenterol* 2002; 8(5): 837-840

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors. Various factors and mechanisms are involved in the development of CRC[1-5]. In recent years, much progress has been made in the studies of CRC[6-13], but its morbidity is still rising[14,15]. It has been thought that one-third of CRC have a genetic background. Hereditary nonpolyposis colorectal cancer (HNPCC) is a common autosomal dominant colorectal disorder with special clinicopathological features[16]. In clinic, it is diagnosed by Amsterdam criteria[17]: (1) three or more relatives with histologically verified colorectal cancer, one of whom is the first-degree to the other two; (2) colorectal cancer affecting at least two generations; and (3) one or more colorectal cancer cases diagnosed before the age of 50. Families fulfilling Amsterdam criteria were named as ICG-HNPCC in this study. As the criteria are too rigid for small families and it excludes extracolonic cancers associated with HNPCC, the Korean Hereditary Colorectal Cancer Registry designated the term"suspected HNPCC" [18] and designed its criteria for the families who do not fulfill Amsterdam criteria but in whom a genetic basis for colorectal cancer is strongly suspected. The criteria for suspected HNPCC are as following: (1) vertical transmission of colorectal cancer or at least two siblings affected with colorectal cancer in a family and (2) development of multiple colorectal tumors (including adenomas), or at least one colorectal cancer case diagnosed before the age 50, or development of extracolonic cancer in family member. Families fulfilling these criteria were named as sHNPCC in this paper. The criteria were accepted by the International Collaborative Group (ICG) on HNPCC for international collaborative studies. It[19] had been reported that ICG-HNPCC and suspected HNPCC have the similar molecular basis and genetic background. In China, there is still no systematic research on ICG-HNPCC or sHNPCC. Therefore, it is necessary and urgent to find out the characteristics of HNPCC in Chinese populations.

In this study we were to find out clinical phenotype of Chinese HNPCC families and to evaluate whether the criteria for suspected HNPCC is useful in clinical diagnosis and management of HNPCC.

MATERIALS AND METHODS

According to the follow-up records, 54 probands from HNPCC families (including 12 ICG-HNPCC families and 42 s HNPCC families) were screened out from patients with colorectal cancers operated upon in 2nd Affiliated Hospital of Zhejiang University from 1984 to 2001. Their clinicopathological features were compared with those of sporadic CRCs operated upon in the hospital[20]. There were 34 male and 20 female, age ranged from 19-76ys (average 45.7), 35 cases of colonic cancer and 19 cases of rectal cancer.The tumor spectrums in these families were also analyzed. All patients were diagnosed by Amsterdam criteria or criteria for suspected HNPCC.

All data were analyzed by software STATISTICA 5.0. A value of $P<0.05$ is considered to be statistically significant.

RESULTS

Clinicopathological features of Chinese HNPCC families

Data of 54 probands with HNPCC and sporadic CRCs were listed in Table 1. From Table 1, we found that mean age of the probands of 12 ICG-HNPCC families was 39ys, proportion of

colonic cancer, well differentiated cancer, poorly differentiated cancer, mucinous tumor, Dukes' A+B and multiple CRCs were 75 %, 54.5 %, 0 %, 8.3 %, 58.3 % and 16.7 % respectively. For the probands of 42 sHNPCC families were 47.5ys, 62 %, 30.8 %, 12.8 %, 21.4 %, 81 % and 14.3 % respectively. Compared with sporadic colorectal cancers, probands from ICG-HNPCC families were obviously different at mean age (P=0.025), tumor site (P=0.001), tumor differentiation (P=0.002) and multiple tumors (P=0.014). Similar result was obtained when compared probands of sHNPCC families to sporadic cases: there were obviously different at mean age (P=0.031), tumor site (P=0.000), tumor differentiation (P=0.011) and multiple tumors (P=0.002).

Table 1 Clinical data of 54 probands

Parameters		ICG-HNPCC	sHNPCC	Total	Sporadic CRC
Sex	male	6(50%)	14(33.3%)	20(37%)	319(43%)
	female	6(50%)	28(66.7%)	34(63.0%)	423(57%)
Mean age		39y	47.5y	45.7y	56y
tumor site	colon	9(75.0%)	26(62.0%)	35(64.8%)	226(30.5%)
	rectum	3(25.0%)	16(38.0%)	19(35.2%)	516(69.5%)
Dukes' stage	A+B	7(58.3%)	34(81.0%)	41(75.9%)	347(47.8%)
	C+D	5(41.7%)	8(19.0%)	13(24.1%)	380(52.2%)
Differentiation	high	6(54.5%)	12(30.8%)	18(36.0%)	89(15.3%)
	moderate	5(45.5%)	22(56.4%)	27(54.0%)	397(68.1%)
	poorly	0	5(12.8%)	5(10.0%)	97(16.6%)
Pathology	mucinous tumors	1(8.3%)	9(21.4%)	10(18.5%)	159(21.4%)
	Non-mucinous tumors	11(91.7%)	33(78.6%)	44(81.5%)	583(78.6%)
Multiple CRCs		2(16.7%)	6(14.3%)	8(14.8%)	25(3.4%)

Except mean age (P=0.035), there was no obvious statistical difference between probands of ICG-HNPCC and sHNPCC families, in clinicopathological features: tumor site (P=0.402), Dukes' stage (P=0.106), tumor differentiation (P=0.147), Pathology (P=0.303) and multiple tumors (P=0.838).

Tumor spectrum in HNPCC families

A total of 178 malignant neoplasms were found in 54 HNPCC families including 139 colorectal cancers, 8 gastric cancers, 6 endometrial cancers, 6 hepatobiliary cancers and 19 others. In 12 ICG-HNPCC families, there were 53 tumors, including 47 CRCs, and each family had 3.92 CRCs patients. In 42 sHNPCC families, there were 125 tumors, including 92 CRCs, and each family had 2.19 CRCs patients. Except CRCs, carcinomas of stomach, endometrium, hepatobiliary system and urologic system were the most common extracolonic malignancies in HNPCC families. Extracolonic malignancies were less in ICG-HNPCC than those in sHNPCC.

Table 2 Tumor spectrum in HNPCC families

Organs	ICG-HNPCC	sHNPCC	Total
CRCs	47	92	139
Stomach	1	7	8
Endometrium	0	6	6
Hepatobiliary system	2	4	6
Lymphatic/hemato-poieticsystem	1	1	2
Esophage	0	2	2
Breast	0	1	1
Urologic system	0	5	5
Pancreas	1	0	1
Others	1	7	8
Total	53	125	178

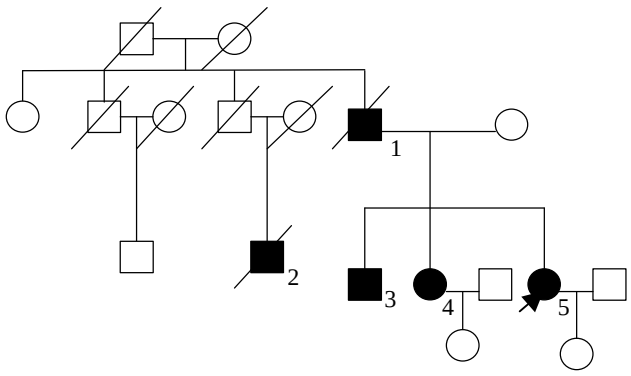

Figure 1 Pedigree of a ICG-HNPCC family.
1: father of proband, with CRC in 64ys, dead; 2: son of proband's uncle, with liver cancer, dead; 3: little brother of proband, with colonic polyps; 4: little sister of proband, with colonic cancer in 40ys, with gastric cancer 1 year later, alive; 5: arrow indicates the proband, 48ys, with colonic cancer, alive.

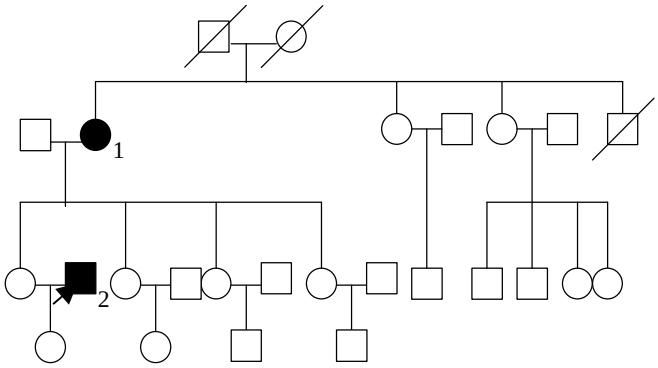

Figure 2 Pedigree of a sHNPCC family. 1: mother of proband, with CRC in 55ys, alive(80ys); 2: arrow indicates the proband, 38ys, with rectal cancer,alive.

DISCUSSION

In 1913, Warthin first described some families with an excess of colorectal, uterine and gastric cancers. It took more than half a century before Lynch undertook the task of collecting data that led to accurate description of these cancer-prone families[21]. According to the absence or presence of extracolonic malignancies, these families were divided into Lynch Ⅰ syndrome and Lynch Ⅱ syndrome[22]. Later, the syndrome was specifically called HNPCC. So HNPCC is also called Lynch syndrome.

HNPCC is an autosomal dominant disorder with special clinicopathological features[23-27], early onset (average <45ys), high frequency of cancers in proximal colon (60-70 %), excess of synchronous (18.1 %) and metachronous (24.2 %) tumors, development of extracolonic malignancies, excess of mucinous (30-40 %) and poorly differentiated tumors (23-39 %). It is often diagnosed by Amsterdam criteria, but the criteria is too strict for little families with strong genetic basis for CRCs, then sHNPCC criteria will be useful.

Mo[28] had described 10 Chinese HNPCC kindred. Among 10 kindred, patients had a mean age of 44.8ys and showed an excess of multiple CRCs (20.2%), a high frequency of cancers in colon (82.3 %). Zhao[29] got the similar results. In our study, compared with sporadic CRCs, 12 probands of ICG-HNPCC kindred showed special features, such as early onset (P<0.05),

predilection for involvement of colon (P<0.05) and tendency of multiple CRCs (P<0.05). They also had well but not poorly differentiated appearance (proportion of well differentiated cancer, poorly differentiated cancer, mucinous tumors and Dukes' A+B were 54.5 %, 0, 8.3 % and 58.3 % respectively). Perhaps it was due to the small sample of our study or the racial difference.

For the families who do not fulfill Amsterdam criteria but in whom a genetic basis for colorectal cancer is strongly suspected, sHNPCC criteria were used. It was proved that sHNPCC had the similar genetic background with ICG-HNPCC[19]. In our study, 42 probands of sHNPCC kindred had the similar clinicopathological features with ICG-HNPCC, early onset (mean age 47.5ys), predilection for involvement of colon (62 %), tendency of multiple CRCs (14.3 %) and well differentiation appearance.

No obvious difference was found between ICG-HNPCC and sHNPCC in their clinicopathological features (P>0.05), except the mean age (P<0.05). This difference might be due to the limited cases studied. According to phenotype of Chinese ICG-HNPCC and sHNPCC, it indicated that they might have similar genetic background. The criteria for sHNPCC will be helpful for clinical diagnosis and treatment of HNPCC.

Except CRCs, extracolonic tumors were often seen in HNPCC kindred[30-41], such as cancers of ovary, brain, small bowel, urologic system, breast, larynx, stomach, pancreas and biliary system as well as leukemia, lymphoma, soft tissue sarcoma, desoid tumor and cutaneous tumors. Gastric cancer, endometrial cancer, carcinomas of hepatobiliary system were the most common extracolonic malignancies. In our study, gastric cancer, endometrial cancer, carcinomas of hepatobiliary system and urologic system were the four common extracolonic malignancies. Cancers of esophagus, breast, pancreas, leukemia and lymphoma were also seen in Chinese HNPCC kindreds. We also found that extracolonic malignancies were less seen in ICG-HNPCC than those in sHNPCC, perhaps it was due to the limited cases studied.

Our results suggest that both ICG-HNPCC and sHNPCC families in China had the clinicopathological features, early onset, predilection for involvement of colon, tendency of multiple CRCs, extracolonic tumors and well differentiated appearance. They might have the similar genetic background. In recent years, Chinese families are becoming smaller and smaller due to the practising of family planning[42]. So criteria for sHNPCC will become more and more useful in clinical diagnosis of HNPCC. Now in China, establishment of HNPCC registry, genetic counseling and surveillance of high-risk asymptomatic family members are important and necessary. Endoscopic surveillance is recommended if any tumor found in some cases, and prophylactic operation is suggested[43-49].

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• LARGE INTESTINAL CANCER •

Less cytotoxicity to combination therapy of 5-fluorouracil and cisplatin than 5-fluorouracil alone in human colon cancer cell lines

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Abstract

AIM: Our previous studies showed increased sensitivity to 5-FU in colon cancer cell lines with microsatellite instability, and considered that mutations of TGFβ-RⅡ, IGFⅡR, RIZ gene might enhance the potentials of cell growth and proliferation, which increased the sensitivity to 5-FU. Here we compared the distribution of cell cycle and P53 status between two human colon cancer cell lines with different sensitivity to 5-FU. Because mechanistic differences exist between 5-FU and CDDP, we also analyzed the efficacy of CDDP and combination therapy on two human colon cancer cell lines.

METHODS: We compared the sensitivity to CDDP of these two cell lines by MTT assay. Distribution of cell cycle under treatment of 5-FU, CDDP alone or both was analyzed by Flow Cytometry, and expression of P53 was detected by immunocytochemical staining.

RESULTS: SW480 cells were more sensitive to CDDP than LoVo cells at the concentrations above 16 μmol/l (Ratio of absorption is 0.64 and 0.79 at 16 μmol/l, respectively; $P<0.01$). Efficacy of combination therapy was conversely lower than that of single-therapy of 5-FU (Ratio of absorption in LoVo+5-FU, SW480+5-FU, LoVo+5-FU+CDDP and SW480+5-FU+CDDP is 0.53, 0.54, 0.72, 0.78, respectively; $P<0.01$). LoVo cells were negative whereas SW480 cells positive in P53 expression. 5-FU induced G1-phase arrest in both cell lines, but LoVo cells peaked 24 hours earlier than SW480 cells, and 48 hours earlier for an apparent hypodiploid DNA. However, CDDP showed the contrary, inducing S-phase arrest, and SW480 cells peaking 36 hours earlier. Both cell lines showed hypodipliod nuclei 48 hours after CDDP treatment. Percentage of cells in G1-phase and S-phase dominated alternatively under combination therapy in both cell lines.

CONCLUSION: These results suggest that colon cancer cells with microsatellite instability are more sensitive to 5-FU, whereas more resistant to CDDP. Combination therapy of 5-FU and CDDP shows fewer efficacies than 5-FU single-therapy, although it can render a cell cycle arrest. P53 may be involved in the shift of G1-phase to S-phase, but inessentially.

Chen XX, Lai MD, Zhang YL, Huang Q. Less cytotoxicity to combination therapy of 5-fluorouracil and cisplatin than 5-fluorouracil alone in human colon cancer cell lines. *World J Gastroenterol* 2002; 8(5): 841-846

INTRODUCTION

5-FU is currently the first-line agent for colorectal cancer after surgical cytoreduction with an overall response rate of less than 15 %[1,2], this has stimulated intensive effort in the development of novel compounds with improved pharmacological properties and new regimens for colorectal cancer patients. Efforts have been made in combination of 5-FU with several second-line agents, such as paclitaxel, mitomycin, calcium folinate, INF-α, irinotecan, leucovorin, suramin and tegafur, and so on, unfortunately, improvement is far from satisfaction. Our studies previously demonstrated that colorectal cancer cell lines with microsatellite instability showed increased sensitivity to 5-FU, and that mutations were found in 8 loci from different genes, among which 3 loci harbored in the exon of TGF-RⅡ, IGFⅡR, and RIZ, respectively. All these three genes are closely associated with cell growth and proliferation. On basis of these results, we proposed that these mutations may enhance the proliferative potentials of cancer cells and increase chemosensitivity to 5-FU.

In this study, We explored the differences of cell cycle arrest and apoptosis between two cell lines under 5-FU treatment, and found that in G1-phase arrest and presence of hypodiploid DNA in LoVo cells happened 48 hours earlier than that in SW480 cells. Because the options available to colorectal cancer patients for second-line therapy were limited, and mechanistic differences existed between 5-FU and CDDP, we analyzed the efficacies of CDDP and combination therapy on these two cell lines. Results indicated that SW480 cells were more sensitive to CDDP than LoVo cells, and that combination therapy of 5-FU and CDDP showed less efficacy than single-therapy of 5-FU in both cell lines, although it can render a cell cycle arrest. P53 may be involved in the entry of G1-phase to S-phase, but inessentially.

MATERIALS AND METHODS

Cell lines

LoVo, a human colon adenocarcinoma cell line, was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China; SW480, a human colon adenocarcinoma cell lines, was donated by the Cancer Institute, Zhe Jiang University. They were maintained in RPMI1640 supplemented with 10 % fetal bovine serum in a humidified 5 % CO_2 atmosphere at 37 ℃.

Drugs and agents

5-fluorouracil (5-FU), [cis-diamminedichloroplatinum (Ⅱ)] (cisplatin/CDDP), 3-[4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO) and propidium iodide (PI), were all purchased from the Sigma Chemical Co. The primary mouse antibody of P53 (DO-7), biotinylated anti-mouse immunoglobulin, horseradish peroxidase-conjugated streptavidin, and the chromogenic substrate solution 3,3-diaminobenzidine were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

MTT assay

MTT assay was performed as described by Lu *et al*[3]. Briefly, logarithmically growing cells were seeded in the 96-well plate at a concentration of 1×10^4 per well and incubated for 12 hours. Then medium with various concentrations of drugs was added in quadruplicate and exposed for 72 hours. The culture medium was then removed and about 300 μl fresh medium containing 0.5 mg/ml MTT was added to each well. 4 hours later, the medium was replaced with 100 μl DMSO and vortexed for 10 minutes. Absorbance (A) was then recorded at 570nm using an Enzyme-linked Immunosorbent Assay device DG3022A. Cell viability was assessed as follows: Viability [%]=$A_{treat}/A_{control}\times100$ %.

Immunocytochemical staining

Immunocytochemical staining was performed using DO-7 anti-P53 on logarithmically growing cell lines, LoVo and SW480, on coverslip. Firstly, cells were plated onto coverslips, adhered overnight. Then, rinsed three times with PBS, cells were fixed in cold acetone for 8-10 min. Endogenous peroxidase was blocked with 1 % hydrogen peroxide in absolute methanol for 30 min. The primary antibodies were applied for 2 hours at 37 ℃ at 1:300 dilution in a humidified chamber. Then the typical SP strategy followed.

Flow Cytometric analysis

Cells were incubated in medium containing 4 μmol/L 5-FU, 10 μmol/L CDDP alone or both (4 μmol/L 5-FU+10 μmol/L CDDP) continuously, and then were fixed in ice-cold 70 % ethanol at 0, 12, 24, 48, 72 and 96 h after initial treatment. Then approximately 10 000 cells each specimen stained by 10 μg/ml PI were analyzed by Flow Cytometry (FACS®), as described by Bunz *et al*[4].

Statistical analysis

Data of MTT assay were mean values of at least three different experiments and expressed as mean ±SD, analyzed by two-tailed Student's *t*-test and General Linear Model, *P* value of less than 0.05 was considered as statistically significant.

RESULTS

Response to CDDP in LoVo cells and SW480 cells

We compared the sensitivity and responsiveness of LoVo cells and SW480 cells to CDDP and the combination of CDDP+5-FU in cytotoxicity assays. As shown by the dose-effect curve, both cell lines are sensitive to CDDP, but more for SW480 cells (Figure 1A). Neither of the cell lines examined showed significantly synergetic response when the two drugs were combined simultaneously, when compared with single-therapy of 5-FU, indicating that CDDP, to some degree, may block the effects of 5-FU (Figure 1B).

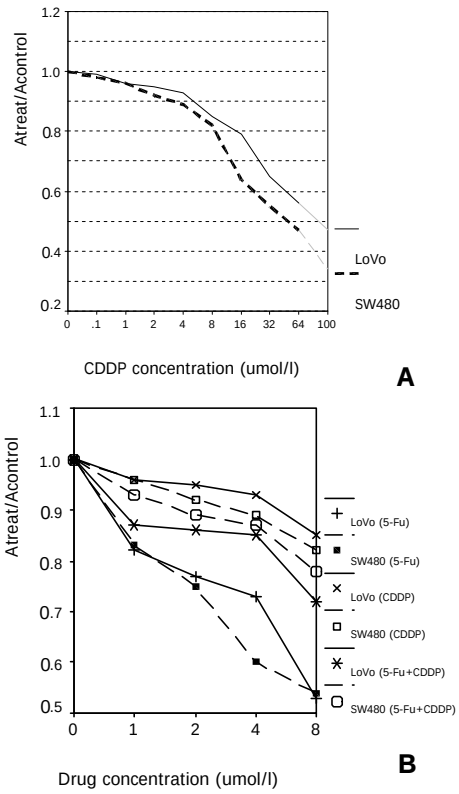

Figure 1 Comparison of sensitivity to 5-FU, CDDP or combination therapy in LoVo cells and SW480 cells. **(A)** SW480 cells show increased sensitivity to CDDP than LoVo cells at the concentrations above 16 μmol/l (Ratio of absorption is 0.64 and 0.79 at 16 μmol/l, respectively;*P*<0.01). **(B)** The dose-effect curve tells that the cytotoxicity diminishes in combination therapy of 5-FU and CDDP in both cell lines when compared with 5-FU single-therapy (Ratio of absorption in LoVo+5-FU, SW480+5-FU, LoVo+5-FU+CDDP and SW480+5-FU+CDDP is 0.53, 0.54, 0.72, 0.78, respectively; *P*<0.01).

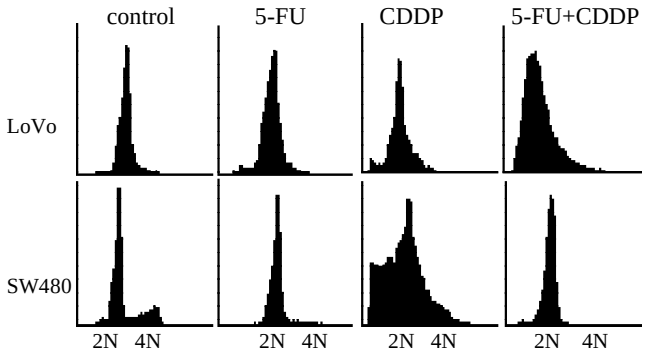

Figure 2 DNA content distribution of LoVo and SW480 cells after 48 hours exposure to 5-FU and/or CDDP. 5-FU causes a more apparent increase of the proportion of hypo diploid DNA cells in LoVo than in SW480. CDDP renders an apparent increase of the hypodiploid DNA cells in both cell lines, but SW480 has more than LoVo cells. Few hypodiploid DNA cells are observed in combination of 5-FU and CDDP treatment.

Distribution of cell cycle by FCM analysis

LoVo cells demonstrated an apparent peak of cells with hypodiploid DNA 48 hours after 5-FU exposure, 48 hours earlier than SW480 cells, which indicated that LoVo cells are more sensitive to 5-FU (Figure 2, 3A). When treated with CDDP for 48 hours, SW480 cells showed more cells with hypodiploid DNA than LoVo cells, about 2-fold increase at 96 hours (55.1 % and 28.5 %, respectively) (Figure 2, 3B).

However, when the two lines were exposed to 5-FU in combination with CDDP, this cytotoxicity significantly diminished. SW480 cells showed an 18 % cells with hypodiploid DNA, but not in LoVo cells in 96 hours (Figure 2, 3C). Both cell lines exposed to 5-FU showed an accumulation in G1-phase and a significantly decreased proportion of S-phase. On the contrary, CDDP arrests both lines mainly in S-phase instead of G1-phase. Percentage of cells in G1-phase and S-phase dominates alternatively in both lines treated with combination of 5-FU and CDDP (Figure 4).

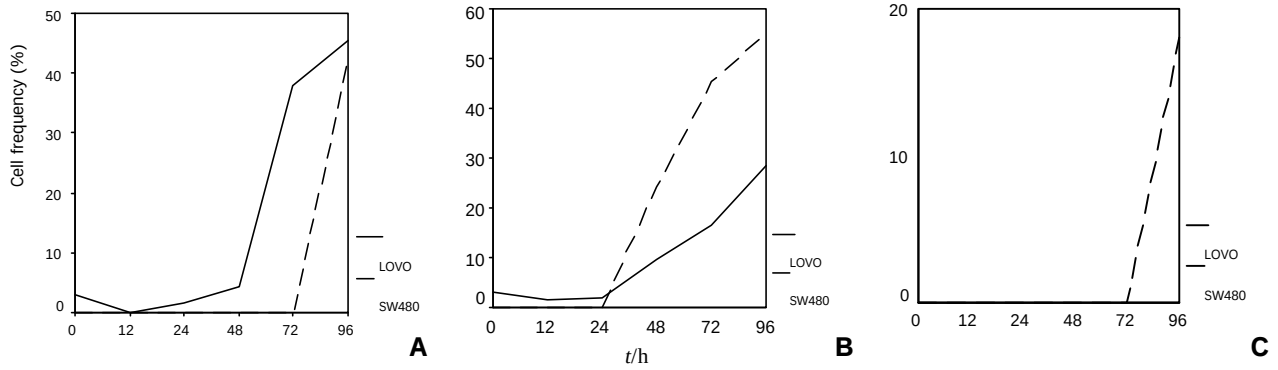

Figure 3 Hypodiploid DNA induced by 5-FU, CDDP or a combination therapy in LoVo cells and SW480 cells. LoVo cells treated with 5-FU show an earlier presence and higher percentage of cells with hypodiploid DNA. **B** When exposed to CDDP, SW480 cells showed a more dramatic increase in presence of hypodiploid DNA than LoVo cells after 48 hours. **C** When the two cell lines were exposed to 5-FU in combination with CDDP, SW480 cells have an 18% hypodiploid DNA, but not in LoVo cells in 96 hours after treatment.

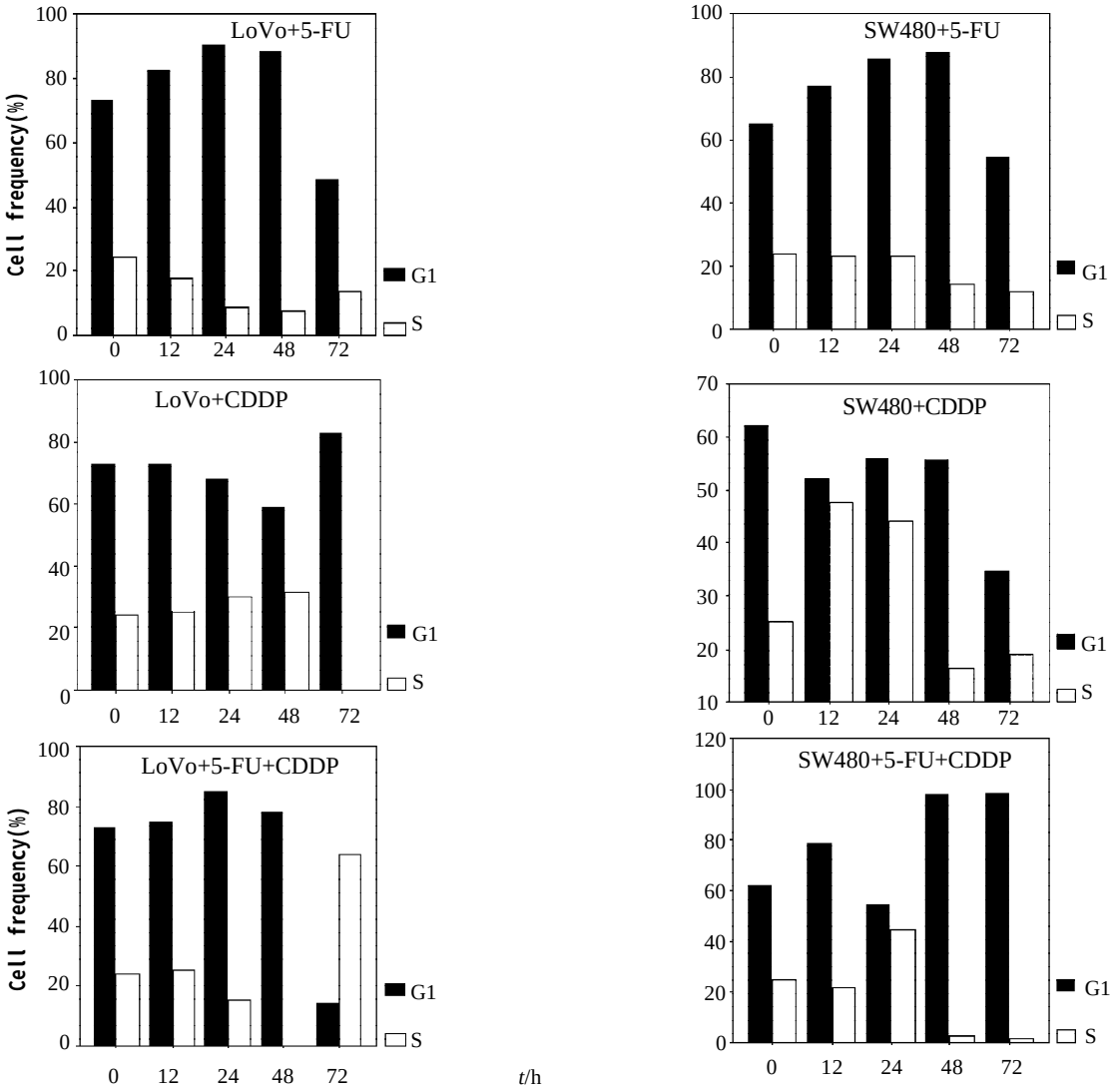

Figure 4 Effects on cell cycle distribution of LoVo cells & SW480 cells treated with5-FU, CDDP or combination therapy. Both cell lines show an accumulation of G1-phase exposed to5-FU. On the contrary, CDDP mainly renders an accumulation of S-phase. Percentage of G1-and S-phase dominates alternatively in both lines treated with combination of 5-FU and CDDP.

P53 expression by immunocytochemical staining

P53 staining scattered nestedly in LoVo cells, predominantly in nuclei, and the proportion of positive cells only accounts for less than 1 %, which was thus considered as wtP53. However, SW480 cells showed extensively and strongly P53 expression, and more than 98 % cells were labeled in nuclei, so it suggested a mutated P53 in this cell line (Figure 5).

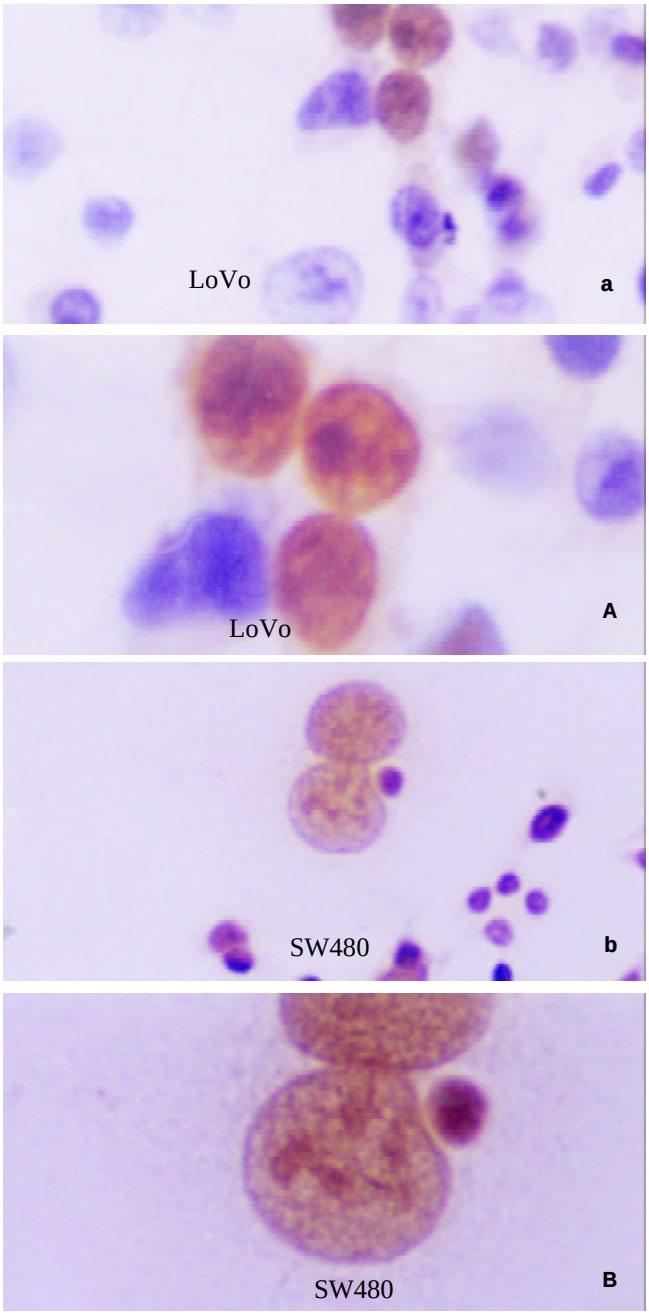

Figure 5 P53 staining with the anti-P53 mAb Do-7 in LoVo and SW480 cells.(**a,b**×100; **A,B**×200)

DISCUSSION

Our previous studies showed increased sensitivity to 5-FU in colon cancer cell lines with microsatellite instability, and considered that mutations of TGF-RⅡ, IGFⅡR and RIZ gene maybe enhance the potentials of cell growth and proliferation, which increases the sensitivity to 5-FU. This assumption is supported greatly by findings that the generation time of LoVo cells is significantly shorter than that of SW480 cells[4], and by our results here, we found that both cell lines demonstrated an accumulation of G1-phase after 12 hours 5-FU exposure, but apoptosis occurred earlier in LoVo cells.

Many researches have been done to reveal biochemical factors associated with 5-FU response, meanwhile, efforts are made to improve the efficacy of chemotherapy by combining 5-FU with other second-line drugs, such as paclitaxel, oxaliplatin, mitomycin, calcium folinate, INF-α, irinotecan, leucovorin, suramin, tegafur, and so on[5-10]. Considering mechanistic differences exist between 5-FU and CDDP, we supposed the possibility of their synergism and analyzed the efficacy of combination therapy of these two drugs.

However, our data didn' t agree with this supposition. We know that after administration 5-FU is rapidly taken up by cells and metabolized by enzymes by several pathways to produce two active metabolites, i.e. 5-FUTP, which may be incorporated directly into RNA, and 5-FdUMP. 5-FdUMP in the presence of reduced folates inhibits thymidylate synthase (TS) activity and depletes dTTP, a necessary precursor of DNA synthesis. Alternatively, it may be phosphorylated to the triphosphate and 5-FdUTP incorporated directly into DNA, inhibiting chain elongation and altering DNA stability, resulting in the production of single-strand breaks and DNA fragmentation[11-13]. Thus, 5-FU belongs to the phase-specific anticancer drug that means improved cytotoxicity to cells in S-phase. However, CDDP acts differently, it binds to DNA base pairs, creating adducts, crosslinks, and strand breaks that inhibit DNA replication.

As pointed above, the two cell lines both arrest in G1-phase, and LoVo cells precede SW480 cells in presence of hypodiploid nuclei with treatment of 5-FU. But CDDP rendered an apparent peak of cells with hypodiploid DNA after 48 hour, and LoVo cells showed less percentage of hypodiploid DNA cells, which suggested that, from view of population, SW480 cells are more sensitive to CDDP than LoVo cells. This result is consistent with most reports, i.e. colorectal cancer cells with microsatellite instability are more resistant to CDDP, and several assumptions have been made to explain this phenomenon: firstly, an assumption so-called *"Recognition, Excision, Futility of repairing"*[14,15]. The DNA-CDDP adducts are recognized and then excised by the mismatch repair system (MMR), the underlying molecular mechanism responsible for correction of mismatch base pairs or some sorts of DNA damages, but incapable to be repaired because of certain reasons. This failure may then lead to permanent single- or double-strand breaks which are now considered to be the initiation of cell death or apoptosis. Secondly, *"Protection mechanism"* [16]. DNA-CDDP adducts are recognized by MMR system or other nuclear factors, then the following binding functions a shelter which protects the damage from repairing by other mechanisms independent of MMR system, which renders cells to death or apoptosis. Thirdly, *"Cell cycle pathway"*. It is supposed that response of cancer cells to CDDP depends on the ability of G2/M arresting. Some workers considered that P53 is responsible for the shift of G1/S phases, whereas MMR system can inactivate CDK1-CylinB complex by phosphorylation of two amino acid residues, Thr14 and Thr15, of CDK1, and blocks cells in G2/M phase for repair, unrepairable DNA damage often results in activation of the apoptotic pathway (Hawn *et al*, 1995). This assumption is supported by many data[17-19]. Here, the first explanation disclaims itself because of the homogenous loss of hMSH2, one of most important members responsible for DNA-CDDP adducts recognition in MMR system. We also failed to detect

the G2/M phase arrest which is emphasized in the third assumption. As for the second supposition, further evidences are required to confirm it.

Combination therapy of 5-FU and CDDP showed less efficacy than single-therapy of 5-FU. Here we found that there was less cells with hypodiploid DNA in both cell lines treated with a combination of 5-FU and CDDP, which suggested that the 5-FU-induced cytotoxicity may, at least partially, diminish by the concomitant presence of CDDP. There are at least two mechanisms may explain the observed dominance of CDDP over 5-FU. One may simply involve a CDDP-induced cell cycle blockade, we called it *"cell cycle disturbance"*, analogous to that recently described by Judson et al in paclitaxel[20]. By arresting cells in S-phase of cell cycle, CDDP inhibited both cell lines undergoing apoptosis after exposure to 5-FU. Both cell lines firstly showed a G1-phase increase, a "5-FU-like response", and then followed by S-phase increase, a "CDDP-like response". In SW480 cells, particularly in SW480 cells, the percentage of G1-phase and S-phase dominates alternatively. These results demonstrate clearly that disturbance of cell cycle arrest and apoptosis occurred in the combination therapy. An alternative mechanism by which CDDP may exert dominance over 5-FU centers on the ability of each drug to modulate level of many biochemical molecules, we called it *"molecular antagonism"*. CDDP intercalates into DNA, forming adducts, and has been shown to both activate and block a variety of biochemical molecules, including transcription factors, such as *c-myc*, AP-1/AP-2, Oct-1, E2F1, P53 and P73; or molecules involved in cell signal transduction, such as Ras, PKA, EGF4, PKC-α/-ε/-θ; or factors associated with proliferation, DNA replication and cell cycle regulation, such as PCNA, TS,DNA pol-α/β,Topo Ⅰ, Cyclin E/D, P16, P21, P27; or Bax and Bcl-2, and so on. We have recently shown that there is a direct correlation between cytotoxicity and 5-FU induced transcriptional activation, i.e. some of these factors are also downstream elements induced by 5-FU and, in turn, affects sensitivity to 5-FU[21-24], leading us to postulate that at least part of the mechanism involves the antagonism of factors induced by each drug. In fact, these two possibilities are compatible with each other, the latter might just be the underlying biochemical explanation of the former.

Many evidences have shown that sensitivity of cancer cells to 5-FU is associated with a variety of mechanisms, including the key enzyme required for its activation and catabolism, folate substrate and the TS activity, and so on. The concept that P53 is involved in chemotherapy-induced cell cycle arrest and apoptosis is accepted by most scientists[5,12,25-32]. Yoshikawa *et al*[1] found there was no relationship between the sensitivity to 5-FU and P53 in colorectal cancer chemotherapy according to evidences from clinical trials, combining with these findings, they proposed that that 5-FU might act via two different pathways, depending on dose: (a) G1/S-phase cell cycle arrest and apoptosis at 1 000 ng/ml, and (b) G2/M-phase cell cycle arrest and mitotic catastrophe at 100 ng/ml in SW480. Our results accord with the higher concentration group they reported, i.e. cells undergo G1-phase or S-phase arrest and apoptosis. Controversies exist in the role of P53 in CDDP chemosensitivity, evidences from malignancies of lung, esophagus, cervix and bladder showed that wtP53 is a favorable prognostic predictor in chemotherapy, and that mutation of P53 will lead cells to chemoresistance[33-36]. However, in agreement with the findings of Pestell *et al*[37] in ovarian cancer, colon cancer cell line with mutant P53 exhibited more sensitivity to CDDP. We think this discrepancy may result from the different type of tissue. Up-regulation of P53 in response to 5-FU/CDDP-induced DNA damage may activates P21 and wee1/mik1, which inhibits the CDK activity, and consequentially, E2F1 failed to release itself from E2F1:RB complex due to down-regulation of RB phosphorylation, as a result, cells arrest in G1/S-phase. Alternatively, it is recently reported that P53-induced increase of P21 activity may also be mediated by the PI3K-AKT1/AKT2 signal transduction pathway[38]. Lin *et al*[39] found that activation of ATM induced by DNA damage can directly phosphorylate specific residues at the NH_2-terminal of E2F1 and can increase P53 expression. Nagashima *et al* described that P53 can also be acetylated and activated by DNA damage-induced $P33^{ING2}$ in CDDP and paclitaxel exposure[40]. All these evidences proved that P53 plays an important role in chemotherapy-induced cell cycle arrest and apoptosis. We' ve known that P21 and P53 are both mutant in SW480 cells, So blockage of cell cycle in G1/S-phase in this cell line may be P53-independent, compared with LoVo cells, apoptosis of SW480 cells treated with 5-FU or CDDP delayed, which may imply that apoptosis induced by P53 pathway is more effective than others. Huang once reported that a few or even one double-strand break of DNA would be enough to increase expression of P53, and led cells to cycle arrest for repairing, if failed, undergoing apoptosis[21,41,42]. So P53, cell cycle status, damage repair system and apoptotic pathway together determine cells to survive or not.

In conclusion, we have demonstrated that colon cancer cell lines with microsatellite instability are more sensitive to 5-FU, but CDDP goes conversely. Combination therapy of 5-FU and CDDP can lead cells to cycle arrest, but it shows less cytotoxicity than single-therapy of 5-FU. P53 may be involved in cell cycle shift of G1-phase to S-phase, but inessentially.

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• LARGE INTESTINAL CANCER •

Variability of cell proliferation in the proximal and distal colon of normal rats and rats with dimethylhydrazine induced carcinogenesis

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Abstract

AIM: To investigate the patterns of cell proliferation in proximal and distal colons in normal rats and rats with 1,2-dimethylhydrazine (DMH) induced carcinogenesis using the thymidine analogue bromodeoxyuridine.

METHODS: Colonic crypt cell proliferation was immunohistochemically detected using the anti-bromodeoxyuridine Bu20a monoclonal antibody.

RESULTS: Marked regional differences were found in both groups. Total labelling index (LI) and proliferative zone size in both normal (8.65±0.34 *vs* 7.2±0.45, 27.74±1.07 *vs* 16.75±1.45) and DMH groups (13.13±0.46 *vs* 11.55±0.45, 39.60±1.32 *vs* 35.52±1.58) were significantly higher in distal than in proximal colon ($P<0.05$), although the number of cells per proximal crypt was greater (31.45±0.20 *vs* 34.45 ±0.39, 42.68±0.53 *vs* 49.09±0.65, $P<0.0001$). Crypt length, total LI and proliferative zone size all increased in both proximal and distal regions of DMH rats compared to normal controls ($P<0.0001$). In DMH-treated rat colon a shift of labelled cells to higher crypt cell positions was demonstrated distally whilst a bi-directional shift was evident proximally ($P<0.05$).

CONCLUSION: Our results show that changes in cell proliferation patterns, as assessed by bromodeoxyuridine uptake, can act as a reliable intermediate marker of colonic cancer formation. Observed differences between proliferation patterns in distal and proximal colon may be associated with the higher incidence of tumors in the distal colon.

Ma QY, Williamson KE, Rowlands BJ. Variability of cell proliferation in the proximal and distal colon of normal rats and rats with dimethylhydrazine induced carcinogenesis. *World J Gastroenterol* 2002; 8(5):847-852

INTRODUCTION

Abnormality in epithelial proliferation is considered to be a characteristic of both human diseases associated with higher risk of colonic cancer and animal colonic cancer models[1-10]. Hyperproliferation of colonic epithelial cells was observed in tritiated thymidine-labelled colorectal specimens from patients with familial polyposis *Coli*, adenomas and hereditary non-polyposis colon cancer[11-16]. Assessment of colonic crypt cell proliferation, including detection of increased proliferative state and expansion of the proliferative zone is suggested as a putative intermediate marker of colon cancer risk[17-19]. Evidence from animal studies showed that experimental colonic tumors induced by procarcinogen 1,2-dimethylhydrazine (DMH) are of epithelial origin with a similar histology, morphology and anatomy to human colonic neoplasms[20,21]. Furthermore, prior to the development of colonic cancer, DMH injections result in increased colonic crypt cellularity, colonic crypt cell proliferation and colonic crypt proliferative zone[22,23]. This procarcinogen thus provides an adequate model for kinetic and therapeutic studies of the colorectal cancer[24-33]. In the normal rat colon, the location of the stem cells and the direction of colonocyte migration differ between the distal colon and the proximal colon[34]. Interestingly, differences in the incidence, morphology and clinical behaviour of colonic carcinoma have been identified in the proximal and distal colon[35,36]. However, there are no studies which use bromodeoxyuridine (BrdUrd) to compare cell proliferation patterns in the distal and proximal colonic locations of both normal and colonic cancer animals.

BrdUrd is a thymidine analogue which, after incorporation into normal and malignant cells during the S-phase of the cell cycle, can be detected using a monoclonal antibody. BrdUrd immunohistochemistry offers several advantages over thymidine autoradiography. Firstly, because a radioactive precursor is not required, there is less background interference in the tissue sections. Secondly, a clear distinction between the labelled cells and unlabelled cells is provided. Thirdly, the BrdUrd technique is less time-consuming, thus being more suitable for incorporation into routine diagnostic services. Finally, because BrdUrd is also a therapeutic agent, BrdUrd may be safely injected into the human body to study the cell proliferation in the patients without introducing additional procedures[37]. Several studies have demonstrated that the LI estimated by BrdUrd immunohistochemistry is equivalent to that obtained by thymidine autoradiography when an *in vitro* labelling technique is used[38]. This suggests that BrdUrd LI may be used to replace thymidine LI in both *in vitro* and *in vivo* studies.

In the present study, BrdUrd *in vivo* cell labelling was employed to determine the crypt cell proliferation patterns in the proximal and distal rat colons from normal and DMH-induced colon cancer animals.

MATERIALS AND METHODS

Twelve male Wistar rats with initial weight between 180 g and 220 g were housed, 3 in a cage, and maintained on standard laboratory diet (41 B (M)) with free access to water. Six of

these animals received weekly subcutaneous injections of colonic procarcinogen 1,2-dimethylhydrazine (DMH, Aldrich Chemical Company Inc) at a dosage of 20 mg/kg body weight for 20 weeks. The DMH was prepared as a 0.5 % solution in 1 mM ethylenediaminetetra-acetic acid (EDTA, BDH Limited Poole, England) adjusted to pH 7.0 with 10 % sodium bicarbonate immediately before injection. Animals were injected at the same time on the same day each week. Animals were sacrificed 2 weeks after the last DMH injection. Fifteen minutes before removal of the colon, the anaesthetized rats received a peritoneal injection of 5 mg 2 % BrdUrd (Sigma B-5002). This was always done between 9 a.m. and 11 a.m. to avoid diurnal variation. The colon was removed and rinsed with tap water. Following excision of the caecum and rectum, the remaining colon was divided into proximal and distal halves. A 1-2 cm segment of each end of the proximal and distal colon was discarded. After fixation in 70 % ethanol for 4 hours,the segments were rolled prior to processing and embedding in paraffin wax.

BrdUrd immunohistochemistry

Several 3 μm sections were cut and placed on poly-L-lysine coated slides. The slides were dewaxed before DNA was denatured in 1M HCl at 37 ℃ for 12 minutes. After rinsing in phosphate buffered saline (PBS, pH 7.1) the sections were incubated with 30 μl mouse anti-BrdUrd monoclonal antibody (M 744 Dako, Bucks, England) diluted 1:50 in PBS with 0.05 % Tween 20 (PBST) with added normal rat serum diluted 1:25 for 60 minutes at room temperature. After a further rinsing in PBS, the sections were incubated with biotinylated rabbit anti-mouse $F(ab')_2$ antibody (E 413 Dako, Bucks, England) at a dilution of 1:200 in PBST with added rat serum for 30 minutes at room temperature. Slides were again rinsed in PBS and then incubated with Streptavidin-Biotin Peroxidase complex (K 377 Dako, Bucks, England) for 30 minutes at room temperature. Finally, the reaction product was visualized using diaminobenzidine hydrochloride (DAB) (Sigma, Dorset, England) primed with 100 μl of 30 % H_2O_2 (diluted 1:20 with distilled water) for approximately 5 minutes. After DAB was washed off with distilled water, the sections were lightly counterstained in Harris haematoxylin before dehydration and mounting in DPX.

Counting and scoring

Only complete well-orientated longitudinally sectioned crypts which displayed lumen at the top and muscularis mucosae at the base was used for analysis. Comparisons between the following 4 groups were undertaken: distal colon from normal rats, proximal colon from normal rats, distal colon from DMH rats and proximal colon from DMH rats. To facilitate scoring, each crypt was divided at the base into 2 crypt columns (hemicrypts). Starting at the base of the hemicrypt, cells were numbered up to the luminal surface of the colon to determine the number of cells per hemicrypt (CPC) and then divided into 5 equal compartments each containing the same number of cells. The number and the position of BrdUrd-labelled cells in the hemicrypt were recorded. The proliferative zone, which was expressed as a percentage, was obtained by calculating the difference between the highest and lowest labelled cells in each hemicrypt and dividing this Figureure by the total number of cells in the hemicrypt. Labelling index (LI) was determined for the whole hemicrypt, for each compartment and for the proliferative zone, by dividing the number of labelled cells by the total cells and multiplying by 100. Each hemicrypt was then normalised to a notional 100 cell positions. The frequency of BrdUrd positive cells in each of the 100 positions was recorded.

Statistical analysis

All results are presented as the mean ±SEM. Two design variables were generated: (1) DMH, where 1 represents a case where DMH has been administered, 0 represents otherwise; and (2) POSITION, where 1 represents a sample from the proximal colonic crypt, while 0 represents one from distal colonic crypt. Multivariate linear regression analyses were performed using the two design variables as covariates against each of the measured parameters (Table 1). Whenever appropriate a two sided Student' s *t* test was used to identify the differences between individual variables. Kolmogorov-Smirnov 2 sample test (3) was used to compare the BrdUrd cumulative labelling frequency curves in the four subsets in the sample identified with reference to (1) the presence of DMH treatment, and (2) the site of origin of the sample from the colon. Results were considered to be significant when $P<0.05$ in all cases.

Table 1 Multivariate linear regression analyses with 2 design variables against 7 measured parameters

Response variable	Covariate	Coefficient	*P*-value
CPC	DMH	12.386	<0.05
	POSITION	5.004	<0.05
TOTLI	DMH	4.433	<0.05
	POSITION	-1.528	<0.05
INDLI 1	DMH	3.915	<0.05
	POSITION	-9.006	<0.05
INDLI 2	DMH	4.504	<0.05
	POSITION	-2.159	0.14
INDLI 3	DMH	10.425	<0.05
	POSITION	3.343	<0.05
PZONE	DMH	18.169	<0.05
	POSITION	-4.454	<0.05
PZONELI	DMH	-18.312	<0.05
	POSITION	1.725	0.43

CPC, cell per hemicrypt; TOTLI, total hemicrypt labelling index; INDLI 1, individual LI in compartment 1; INDLI 2 individual LI in compartment 2; INDL 3, individual LI in compartment 3; PZONE, proliferative zone; PZONELI, labelling index of proliferative zone.

RESULTS

Proximal compared to distal colon

CPC was significantly greater in the proximal compared to the distal colon in both normal (t=7.42, P<0.0001) and DMH-treated groups (t=7.76, P<0.0001, Table 1). The total hemicrypt LI was significantly higher in distal than proximal colon in both normal (t=2.43 , P=0.016) and DMH treated animals (t=2.47, P=0.014). In the normal controls, BrdUrd labelled cells in the proximal colon were located predominantly in compartment 2 and 3 (88.4 %), whereas the labelled cells in the distal colon were found mostly in compartment 1 and 2 (85.3 %). In compartment 1, LI was significantly higher in distal than in proximal colon (t=8.05, P<0.0001), while in compartment 3 LI was significantly lower in distal than in proximal colon (t=-4.46, P=0.0001, Figure 1). Labelled cells

never appeared in compartment 5. The size of the proliferative zone was higher distally (t=2.57, P=0.011). When the cumulative labelling distribution curves of the proximal and distal colons of normal rats were compared the distal colon showed a significant shift to the left (K-S=2.192, P<0.0001, Figure 2).

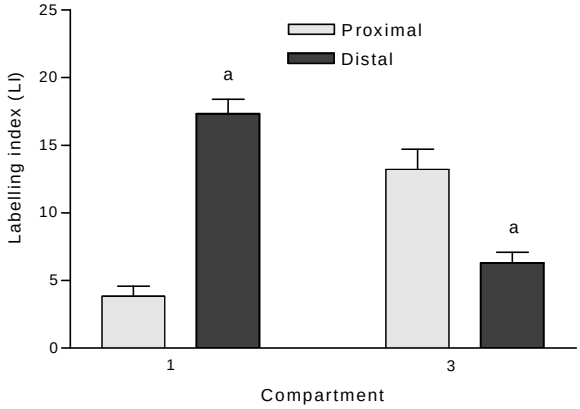

Figure 1 Labelling indices of compartment 1 and compartment 3 in proximal and distal colon of normal rats. [a]P<0.0001 when distal is compared to proximal colon in the same compartment. Values are mean±SEM.

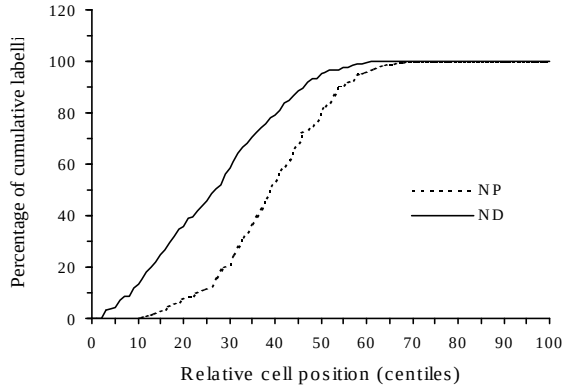

Figure 2 The different patterns of cumulative labelling distributions in proximal (NP) and distal (ND) rat colon of normal controls. The curve is significantly shifted towards the right when the proximal colon is compared to distal colon.

DMH compared with control rats

DMH treatment significantly increased CPC in both proximal (t=15.9, P<0.0001) and distal colon (t=20.6, P<0.0001) when compared with normal controls. Total LI was also increased significantly by DMH injections in both proximal (t=6.23, P <0.0001) and distal colon (t=7.94, P<0.0001, Table 2). The effects of DMH on the individual LIs for the 5 compartments in the proximal and distal colon are shown in Figures 3 and 4. In proximal colon, the increase in LI was in compartments 1 and 3, whereas in distal colon, the increase in LI in DMH rats was most marked in compartments 2 and 3. The extent of the increase in LI in compartment 3 of the distal colon was significantly greater than the corresponding increase in compartment 3 LI of the proximal colon (t=3.44, P=0.001). Additionally, DMH increased the size of the proliferative zone in both proximal (t=7.9, P<0.0001) and distal colonic crypts (t=10.6, P<0.0001). However the LI of proliferative zone was reduced because of the increased size. Further analysis of the cumulative labelling distributions showed a shift of the DMH distal curve to the 81st centile which was to the right of the plateau of the normal distal colon located at 61st centile (K-S=1.89, P=0.001, Figure 5). In contrast the cumulative labelling distribution curve in proximal colon demonstrated a shift to the left in the lower crypt cell positions and then shifted to the right high up the crypt (K-S=3.625, P<0.0001, Figure 6).

Table 2 Comparison of cell proliferation in proximal and distal hemicrypts of normal and DMH rat colon

	Normal control		DMH	
Parameters	Proximal	Distal	Proximal	Distal
Cells/hemi-crypt	34.45±0.39	31.45±0.2[a]	49.09±0.65[b]	42.68±0.53[a,c]
Labelled cells	2.49±0.16	2.74±0.11	5.7±0.24[b]	5.67±0.23[c]
Total LI	7.2±0.45	8.65±0.34[a]	11.55±0.45[b]	13.13±0.46[a,c]
Proliferative zone	16.75±1.45	21.74±1.07[a]	35.52±1.58[b]	39.6±1.32[a,c]
LI of proliferative zone	59.43±3.39	56.55±2.13	39.84±1.77[b]	38.89±1.5[c]

Values expressed as mean ±SEM. [a]P<0.05 when distal is compared to proximal in the same group, [b]P<0.05 when DMH proximal is compared to normal proximal, [c]P<0.05 when DMH distal compared to normal distal.

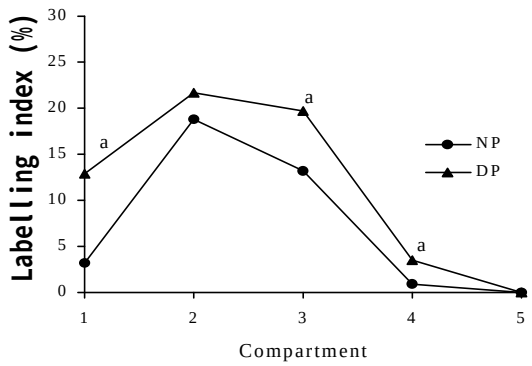

Figure 3 Labelling indices of normal proximal colon (NP) and DMH proximal colon (DP) for the 5 compartments. [a]P <0.0001 when labelling indices in the compartments of DMH proximal colons were compared to those found in normal proximal colons in the same compartments.

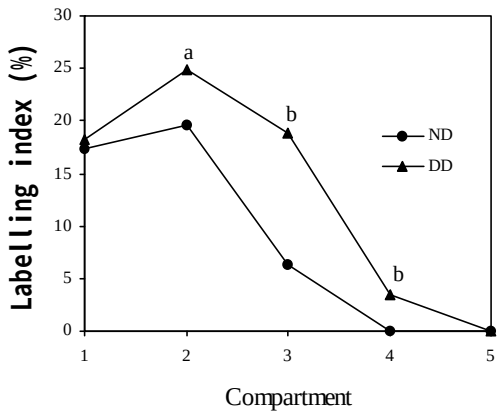

Figure 4 Labelling indices of normal distal colon (ND) and DMH distal colon (DD) for the 5 compartments. [a]P<0.05, [b]P<0.0001 when labelling indices in the compartments of DMH distal colons were compared to those found in normal distal colons in the same compartments.

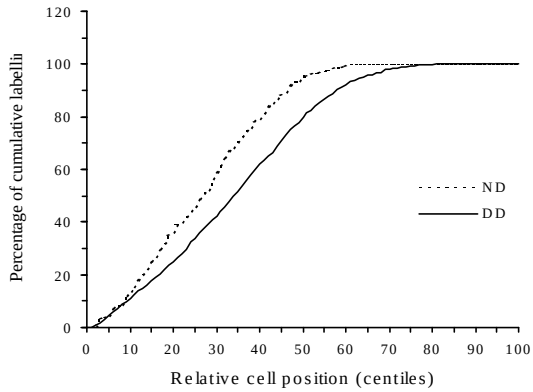

Figure 5 Cumulative labelling distribution in normal (ND) and DMH distal (DD) rat colon. The curve is significantly shifted towards the right in DMH distal colon compared to normal distal colon.

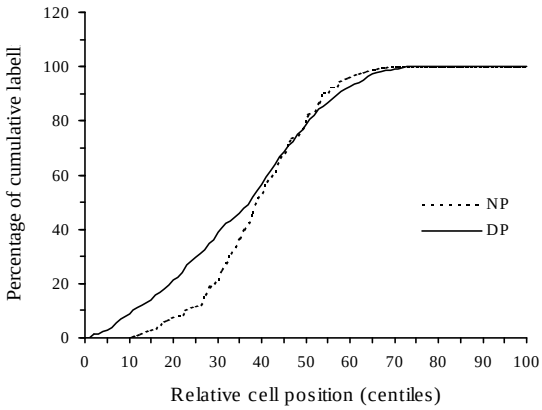

Figure 6 Cumulative labelling distribution in normal (NP) and DMH proximal (DP) rat colon. The curve of DMH treated proximal rat colon is initially significantly shifted towards the left and then in the higher centiles shifted to the right.

DISCUSSION

Significant regional differences in the distribution of BrdUrd-labelled cells located in proximal and distal rat colon have been demonstrated in this study. The total LI and the proliferative zone size are all significantly larger in the distal than in the proximal colonic crypt both in normal and DMH-induced carcinogenesis animals. These regional differences in proliferative cell distribution between proximal and distal colon were previously shown using other markers, e.g. ^{3}H-thymidine autoradiography and proliferating cell nuclear antigen (PCNA) immunohistochemistry. ^{3}H-thymidine LI and proliferative zone size have been reported to be significantly larger distally than proximally[39]. In the distal colon, PCNA expression was strictly confined in the lower third of the crypt, whereas in the proximal colon it was located in the mid-crypt[23].

Our results show that in the distal colon the proliferative cells are located predominantly in the compartments 1 and 2, whereas in the proximal colon, the proliferative cells are located in the 2nd and 3rd compartments. In earlier studies, using tritiated thymidine, Sunter *et al*[40] found the differences in the distribution of proliferative activity within the crypt from site to site along the length of the rat colon. In the proximal colon, Sunter noted that the peak LI was located in the middle third of the crypt while in distal colon the peak was located in the lower third near the base of the crypt.

These findings together with ours, tend to support the explanation of crypt cell origin and colonocyte migration described by Sato and Ahnen[34]. After double labelling with ^{3}H-thymidine and BrdUrd, they investigated the location of stem cells and the direction of colonocyte migration in normal rat colonic crypt. They reported that distal stem cells are located in the crypt base while proximal stem cells are located in the mid-crypt. They postulated that colonocytes migrate up toward the luminal surface in distal colon in contrast to the bidirectional migration, i.e. up toward the luminal surface and down toward the crypt base in proximal colon.

Our results show that after DMH injection colonic crypt cell proliferation is significantly increased regardless of the position (proximal or distal) and irrespective of the proliferative parameter assessed except the LI of proliferative zone (Table 1). The LI of the proliferative zone in the DMH animals may not increase because of the concomitant increase of the zone size. DMH treatment not only increases the colonic crypt cellularity, total BrdUrd LI but also increases the size of the proliferative zone in both proximal and distal colon. The LI of each compartment from 1 to 3 is also increased, especially in the distal colon. This can be confirmed by the cumulative labelling distribution curves (Figures 5 and 6). Although DMH treatment increases LI in both proximal and distal colon the cumulative labelling distribution is markedly shifted to the right in distal colon whereas the proximal curve shifts to the left (i. e. downwards in the crypt). These results are interesting because of the differences in colonic cancer distribution between proximal and distal colon. The distribution of DMH-induced colorectal cancer resembles human large bowel carcinoma, in which the majority of tumors are recorded distally[41,42]. In our previous work when total colon was exposed to the procarcinogen DMH, 73 % tumors occurred distally and only 12 % occurred proximally[33].

Further investigation is required to resolve the questions concerning the differences of tumor distribution and their relationship to the different crypt cell proliferation patterns observed in proximal and distal colon. It has been shown that in the proximal colon the lower one-third of the crypt contains predominantly mucous cells whereas the upper one-third mainly has columnar cells[43]. In contrast, crypts of the distal colon contain only a small number of mucous cells in basal positions. The undifferentiated cells or the cells with the lowest level of differentiation (presumptive stem cells) are the vacuolated cells located near or at the crypt base[44]. When the vacuolated cells migrate upward, they transformed into columnar cells and when they migrate downward, they give rise to mucous cells[45]. The major role which stem cells play in carcinogenesis is presumably to transform the malignancy [46]. If this hypothesis is true, there should be more mucinous tumors expected in proximal colon and the tumors in distal colon should originate from columnar cells. In DMH-induced rat colonic carcinogenesis the tumors tended to be more frequently sessile, often mucinous and invasive adenocarcinomas in proximal regions and polyps in distal areas[42].

In this study we observed a greater baseline value of crypt length in proximal than in distal colon, which is contrary to some other published literature[39,40]. This disparity may be due to the differences in defining the criteria for handling the overlapping nuclei, selecting crypts or ascertaining the top of the crypt. We have noticed that the nuclei in the proximal colon are not as typical as those in distal colon. In this study longitudinally well-oriented crypts was selected and all visible nuclei were counted.

Our study demonstrated regional differences in crypt cellularity and cell proliferation patterns. Additionally, we can

conclude that the procarcinogen DMH increased crypt cell proliferation and shifted the cumulative BrdUrd labelling distribution in both distal and proximal rat colon. The histochemical similarity of the distal rat colon to the human colon[43] permits the distal rat colon to be used as a model of colonic cancer[47-54]. Therefore, BrdUrd is an appropriate intermediate marker for the early detection of colorectal cancer in patients at high risk and the correct assessment of dietary interference.

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• LARGE INTESTINAL CANCER •

Angiogenesis inhibitor TNP-470 suppresses growth of peritoneal disseminating foci of human colon cancer line Lovo

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Abstract

AIM: To study the effect of angiogenesis inhibitor TNP-470 on peritoneal dissemination of colon cancer in nude mice.

METHODS: The MTT assay was used to evaluate the inhibitory effect of TNP-470 on human colon cancer cell line Lovo. Lovo cells were injected into the peritoneal cavity of BABL/C nu/nu mice and the models of peritoneal dissemination were developed. Thirty nude mice were randomly divided into control and TNP-470-treated group. In TNP-470-treated group, TNP-470 was injected subcutaneously every other day from day 1 until sacrifice or death (30 $mg \cdot kg^{-1}$). The control group received a sham injection of the same volume saline solution.

RESULTS: *In vitro*, TNP-470 inhibited the growth of Lovo cells, with its IC50 at 2.14×10^2 $\mu g \cdot L^{-1}$. *In vivo*, TNP-470 demonstrated growth inhibition of tumors. Mice body weight and abdominal circumferences were significantly different between TNP-470-treated group (24.5±3.2 g, 7.0±1.1 cm) and control group (29.5±2.1 g, 10.3±1.5 cm), P=0.005 and P=0.001. The number of disseminated foci was significantly different between the control group (92.1±20.6) and the TNP-470-treated group (40.3±12.3), P<0.001. The maximal size of foci was significantly smaller in TNP-470-treated group (3.3±0.7 mm) than that of control (7.3±2.3 mm), P=0.004. Mean survival time was significantly longer in TNP-470-treated group(98.00±12.06 d) than that in control group (41.86±9.51 d), P<0.001.

CONCLUSION: Angiogenesis inhibitor TNP-470 might be effective in treating peritoneal dissemination of colon cancer and improve the survival rate of nude mice.

Fan YF, Huang ZH. Angiogenesis inhibitor TNP-470 suppresses growth of peritoneal disseminating foci of human colon cancer line Lovo. *World J Gastroenterol* 2002; 8(5):853-856

INTRODUCTION

Colorectal cancer still remains the most frequent malignancy in Japan, Unite States of American and China. Although combined therapies, including chemotherapy, radiation therapy and immunotherapy are performed in addition to surgical radical resection, nearly 50 % of patients still die of recurrence and a major form of recurrence was peritoneal dissemination[1]. Therefore, new therapeutic programs are needed to raise the survival rate of colorectal cancer patients. The importance of tumor angiogenesis is widely accepted in cases of blood-born metastases[2,3]. Although the form of blood supply is markedly different between metastases in solid organs and those at the peritoneum, it has been generally accepted that any foci larger than 0.2 mm require new tumor vessels for their growth[1-4]. Thus, inhibition of angiogenesis would prevent the tumor growth and their peritoneal dissemination.

TNP-470 is a semisynthetic analogue of fumagillin isolated from *Aspergillus fumigatus*. TNP-470 has been reported to inhibit neovascularization by preventing endothelial cells growth and proliferation[5-9]. Recently, the therapeutic effects of TNP-470 on various human and rodent tumors have been reported and this agent shows a marked inhibitory effect on tumor growth and metastasis *in vivo*[10-11]. However, the importance of angiogenesis in the establishment and growth of peritoneal dissemination remains unknown and there has been no report that evaluates the effect of TNP-470 on establishment and growth of peritoneal dissemination and ascites production of human colon cancer.

In this study, we investigated the inhibitory effects of TNP-470 on an establishment and growth of intraperitoneally inoculated human colon cancer cell line, Lovo, and survival of nude mice with this tumor *in vivo*. We also examined the inhibitory effect of TNP-470 on cell growth *in vitro*.

MATERIALS AND METHODS

Drug and reagents

TNP-470 was a generous gift from Takeda Chemical Industries (Osaka, Japan). 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), gum arabic and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO); RPMI 1640 and heat-inactivated fetal calf serum (FCS) were purchased from Gibco (Grand Island, NY).

TNP-470 was stored dry at -20 ℃. *in vitro* experiments, TNP-470 was dissolved in DMSO and RPMI 1640 medium supplemented with 10 % FCS. The final concentration of DMSO was 0.1 %, while *in vivo* experiments, TNP-470 was suspended in a vehicle of 3 % ethanol and 5 % gum arabic in saline.

Cell line

Human colon adenocarcinoma cell line, Lovo was kindly provided by the Department of Pathology, Cancer Center, First Military Medical University(FIMMU). Cells were cultured in RPMI 1640 supplemented with 10 % FCS, and were maintained at 37 ℃ in 5 % CO.All experiments were performed using cells harvested at the 80-90 % subconfluent stage.

Animals

Female BALB/c nude mice were obtained from the Experimental Animal Center, FIMMU, and reared under specific pathogene-free condition. Four-week-old mice weighing 17-22 g were used in the experiments.

In vitro experiments

The MTT assays were made to evaluate the sensitivity of TNP-470 to Lovo cells[8]. Lovo cells were plated in 96-well mocrotiter plates at a concentration of 5×10^4 cells in 50 μL of RPMI 1640 medium. After 24 h incubation, the medium was changed to RPMI 1640 medium with various concentrations of TNP-470(5×10^{-4} μg· L^{-1}-5×10^5 μg· L^{-1}), and the medium was incubated for 48 h, 20 μL MTT (5 g· L^{-1}) solution was then added to each well. After the plates were incubated for 3 h, 150 μL DMSO was added. The absorbance at 570 nm was determined using a microplate reader (Bio-Rad Model 3550, Hercules, CA). Dose-response curves were plotted, and the 50 % inhibitory concentration (IC50) was extrapolated as the drug dose causing a 50 % reduction in absorbance as compared with control values. The experiments were repeated in three independent experiments.

In vivo experiments

Lovo cells were harvested after being cultured for 48 h and the model of peritoneal dissemination in nude mice was developed as follows. Approximately 5×10^7 cells in 0.2 mL saline solution were injected into the peritoneal cavity of an nude mouse (day 0). Thirty nude mice were randomly divided into a control group (n=16) and a TNP-470-treated group (n=14). In the TNP-470-treated group,TNP-470 of 30 mg· kg^{-1} was injected subcutaneously every other day from day 1 until sacrifice. The control group received a sham injection of the same volume of saline. On day 10, two mice in control group were sacrificed and disseminated nodules on the peritoneum were evaluated. Seven mice each were sacrificed on day 30. Body weight and abdominal circumferences (substitute abdominal circumferences for ascites) of each mouse in two groups were measured. The number and maximum size of disseminated nodules on the peritoneum and mesentery were evaluated. The remaining 14 mice, 7 in each group, were followed for the survival experiment.

Statistical analysis

Data were expressed as mean ±standard deviation. Comparison between two groups was made by the independent samples t test. The survival curve was calculated by the Kaplan-meier method and compared by the Log-rank test. $P<0.05$ was considered statistically significant. All statistics were carried out using SPSS10.0 statistics software.

RESULTS

Effects of TNP-470 on cell growth in vitro

In the colorimetric MTT assay, significant growth inhibition was observed in a dose-dependent manner. The IC50 value was 2.14×10^2 μg· L^{-1} extrapolated from the dose-response curve following 48 h exposure (Figure 1).

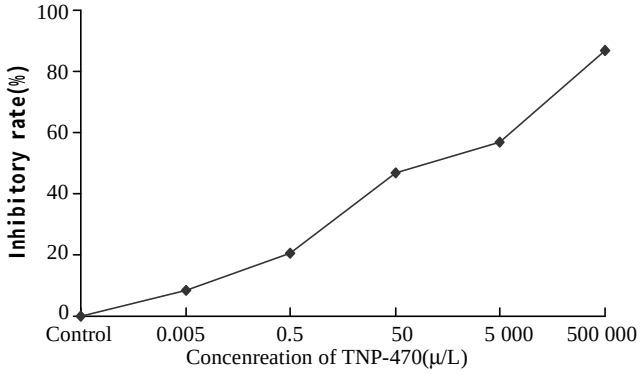

Figure 1 Inhibition curve of Lovo cells after TNP-470 treated 48 h

Effects of TNP-470 on establishment and growth of peritoneal dissemination in vivo

Two mice in control group sacrificed on day 10 developed disseminated nodules, suggesting that small nodules at the peritoneum developed within 10 days after inoculation in this model. Body weight and abdominal circumferences were gained in two groups when mice sacrificed on day 30. Body weight and abdominal circumferences in the control group and the TNP-470-treated group are summarized in Table 1. The difference of body weight and abdominal circumferences were statistically significant (P=0.005 and P=0.001). The number and maximum size of disseminated foci of the control group and the TNP-470-treated group are shown in Table 2. The difference of foci number and maximal size of disseminated foci were also statistically significant (P<0.001 and P=0.004).

Table 1 Body weight and abdominal circumference of two groups ($\bar{x}\pm s$)

Groups	Mice	Body weight (g)	Abdominal circumference (cm)
Control	7	29.5±2.1	10.3±1.5
TNP-470	7	24.5±3.2[a]	7.0±1.1[b]

[a]t=3.394, P=0.005, [b]t=4.624, P=0.001, *vs* control

Table 2 Numbers and Maximum size of disseminated foci of two groups ($\bar{x}\pm s$)

Groups	Mice	Number of foci	Maximum size of foci (mm)
Control	7	92.1±20.6	7.3±2.3
TNP-470	7	40.3±12.3[a]	3.3±0.7[b]

[a]t=5.715, P<0.001, [b]t=4.319, P=0.004, *vs* control

In the control group, mice died from day 31 to day 57. In a TNP-470-treated group, 5 mice were died from day 74 to day 110. Two mice survived more than 120 days and TNP-470 treatment was continued. These 2 mice did not have any disseminated foci and were sacrificed on day 120 and day 130, respectively. The median survival time in the control group and the TNP-470-treated group were 40 and 92 days, the mean survival time being 41.86±9.51 days and 98.00±12.06 days, respectively (P<0.001). The survival rate was significantly smaller in those in the control group than those of TNP-470-treated group (P<0.001) (Figure 2).

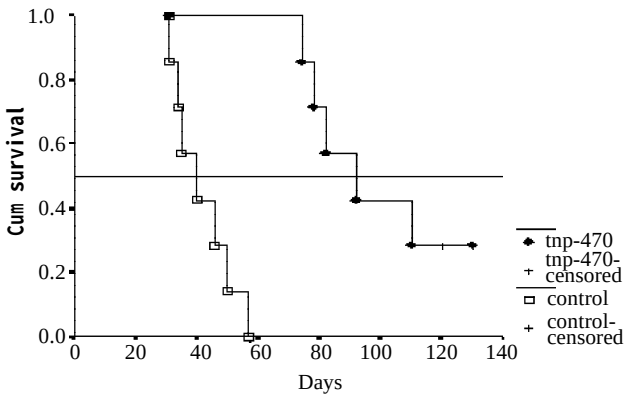

Figure 2 Survival curves in the control and TNP-470-treated groups

DISCUSSION

About 33 % patients with colorectal cancer have recurrence after operation and 50 % patients died of tumor metastasis[13,14], and the peritoneal dissemination or liver metastasis represents the most common form of recurrence. When the tumor has extended through the serosa or been resected , tumor cells are carried to distant points of the peritoneal cavity and "seeding" on peritoneum. Supported by peritoneal permeability and growth of neovascularization, these tumor cells would develop into micro-metastatic nodules, eventually producing generalized peritoneal dissemination[15,16]. With tumor recurrence as peritoneal dissemination, patient prognoses are extremely poor. Although combined therapies, including chemotherapy, radiation therapy and immunotherapy are performed in addition to surgical radical resection, no effective treatment can prevent the recurrence. New therapeutic strategies have to be invented to overcome the poor prognosis.

Angiogenesis, has been shown to be essential for tumor growth not only at the primary but also at the site of metastases, and the peritoneum would not be an exception[17-20]. Inhibition of angiogenesis is emerging as a promising strategy for cancer treatment[21-24]. Anti-angiogenic agents have demonstrated a remarkable inhibition effect on tumor growth and metastasis, and anti-angiogenic therapy may prevent the tumor recurrence[25].

Among the most potent inhibitors of angiogenesis is the fumagillin family of natural products. An analog of fumagillin, known as TNP-470 or AGM-1470, has the anti-angiogenic activity by inhibiting endothelial cell growth with high potency both *in vitro* and *in vivo*[26,27]. Studies have shown that the molecular mechanism of TNP-470 inhibiting endothelial cell proliferation is associated with the two type methionine aminopeptidase (MetAp-2). TNP-470 was found to bind MetAp-2 covalently, leading to specific inhibition of its activity, and strong correlation has been found between inhibition of MetAp-2 enzymatic activity and inhibition of endothelial cell proliferation[28-33]. Investigators for TNP-470 have demonstrated suppression of neovascularization, tumor growth, and distant metastases *in vivo*[34-44], and the proliferation of various cancer cell lines *in vitro*[45-49]. There are very few studies on peritoneal dissemination[50-52], and no data are available about preventing peritoneal dissemination or survival benefit of colon cancer treated by TNP-470.

Tsujimoto *et al*[53] reported that angiogenesis occurred in peritoneal foci 1 week after intraperitoneal inoculation of tumor cells in a mouse model. Our study indicated that TNP-470 inhibied the proliferation of Lovo cells *in vitro*, with its IC50 at 2.14×10^2 ug/L, and markedly suppressed the growth of peritoneal dissemination in vivo. The mice survival time was siginificantly longer in TNP-470 treated group than that of control. Our results suggest that these effects are exerted not only by inhibiting neovascularization necessary for tumor growth, but also by directly inhibiting the proliferantion of Lovo cells.

Body weight loss was known to be the major side effect of TNP-470. There was a slight body weight gain when mice were sacrificed on day 30, and the increase was associated with the production of malignant ascites. In the survival experiments, body weight loss was observed in the control group and 3 tumor-bearing mice became cachectic with the progression of tumor. No body weight loss was observed in the treatment group, suggesting that suppression of the growth of peritoneal dissemination foci by TNP-470 resulted in a preservation of body weight and prevention of cachexia in this model.

In conclusion, angiogenesis inhibitor TNP-470 might be effective in treating peritoneal dissemination of colon cancer by inhibiting the growth of the seeded tumor cells on the peritoneum.

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• VIRAL HEPATITIS •

Investigation of HGV and TTV infection in sera and saliva from non-hepatitis patients with oral diseases

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Abstract

AIM: To determine the frequencies of HGV and TTV infections in serum and saliva samples of non-hepatitis patients with oral diseases in Hangzhou area, and to understand the correlation between detected results of HGV RNA and/or TTV DNA in sera and in saliva from the same patients.

METHODS: RT-nested PCR for HGV RNA detection and semi-nested PCR for TTV DNA detection were performed in the serum and saliva samples from 226 non-hepatitis patients with oral diseases, and nucleotide sequence analysis.

RESULTS: Twenty-seven (11.9 %) and 21 (9.3 %) of the 226 serum samples were only positive for HGV RNA and TTV DNA, respectively. 10 (4.4 %) and 9 (3.9 %) of the 226 saliva samples were only positive for HGV RNA and TTV DNA, respectively. And 7 (3.1 %) of the serum samples and 2 (0.9 %) of the saliva samples showed the positive amplification results for both HGV RNA and TTV DNA. 12 saliva samples from the 34 patients (35.3 %) with HGV or HGV/TTV viremia and 11 saliva samples from the 28 patients (39.3 %) with TTV or HGV/TTV viremia were HGV RNA detectable, respectively, including two patients positive for both HGV RNA and TTV DNA in serum and saliva samples. No saliva samples from the 226 patients were found to be HGV RNA or TTV DNA detectable while their serum samples were negative for HGV or TTV. Homologies of the nucleotide sequences of HGV and TTV amplification products from the serum and saliva samples of the two patients compared with the reported sequences were 88.65-91.49 % and 65.32-66.67 %, respectively. In comparison with the nucleotide sequences of amplification products between serum and from saliva sample from any one of the two patients, the homologies were 98.58 % and 99.29 % for HGV, and were 98.65 % and 98.20 % for TTV, respectively.

CONCLUSION: Relatively high carrying rates of HGV and/or TTV in the sera of non-hepatitis patients with oral diseases in Hangzhou area are demonstrated. Parts of the carriers are HGV and/or TTV positive in their saliva. The results of this study indicate that dentists may be one of the populations with high risk for HGV and/or TTV infection, and by way of saliva HGV and TTV may be transmitted among individuals.

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INTRODUCTION

Viral hepatitis is relatively common in China[1-13]. Except for human A-E hepatitis viruses, hepatitis G virus (HGV) and GB virus C (GBV-C) were recently identified as the causative agents of human non-A-E hepatitis[14,15]. Transfusion transmitted virus (TTV), another novel virus isolated from a patient suffering from non-A-G hepatitis, was considered to be responsible for human non-A-G hepatitis[16]. Since the nucleotide sequences and putative amino acid sequences of HGV and GBV-C genomes show high homologies of 85 % and 100 %, respectively, the two viral agents are considered to be the different isolates of the same virus belonging to *Flaviviridae* family[14,15]. TTV was proposed to be a member of a new virus family temporarily named *Circinoviridae*[17]. Although HGV is a positive-stranded RNA virus and TTV is a negative-stranded DNA virus, the two viruses are mainly transmitted through a hematogenous pathway[18-21], especially through blood transfusion[22-25]. Many investigation data demonstrated that HGV or TTV was able to cause a long-term asymptomatic viremia in non A-E hepatitis patients and only a few patients could suffer from hepatitis with mild liver injury[26-32]. On the contrary, a few references reported that HGV infection might accelerate the progression of chronic liver diseases and cause fulminant hepatic failure[33-37]. TTV was frequently found in non-A-G hepatitis patients with a single elevation of alanine aminotransferase (ALT)[38-40]. Recent published literatures revealed that HGV or TTV infection frequency in peripheral bloods of normal populations including healthy blood donors was found to be relatively high[41-50] and part of the infected individuals were HGV RNA or TTV DNA detectable not only in the serum samples but also in their saliva samples[51-55]. Therefore, dentists may be one of the populations at high risk of HGV and TTV infection, and may play an important role in HGV and TTV transmission among individuals. On the other hand, the possibility of HGV and TTV transmission through saliva is also an interesting subject for investigation.

To determine the frequency of HGV or TTV infection in non-hepatitis patients with oral diseases and to understand the correlation of HGV and TTV detection between in sera and in saliva from the same one patients, RT-nested PCR and semi-nested PCR were applied to detect HGV and TTV respectively in the serum and saliva samples from 226 non-hepatitis patients for oral treatment in Hangzhou area. The HGV RNA or TTV DNA positive amplification products from representatives of the serum and saliva samples were cloned and then sequenced. The results of this study may contribute to understand

transmission mechanism of the two viruses and populations with high risk of HGV and TTV infection, and may be helpful for the control of the prevalence of human hepatitis associated with HGV or TTV.

MATERIALS AND METHODS

Materials

Total 226 patients with tooth, pulp or periodontal diseases came from the oral departments of three hospitals in Hangzhou, Zhejiang Province of China, respectively. Each of the patients was told the aim and content of this study and all of them agreed to be the volunteers. A serum sample as well as a saliva sample from each of the patients was collected. These samples were immediately performed for HGV and TTV detection or temporarily stored at -20 ℃. The patients were confirmed to have no any clinical signals of hepatitis and no elevation of ALT by conventional hepatic examinations, and the serum samples were negative for hepatitis A-C viruses by PCR and EIA. The reagents used in reverse transcription (RT) and PCR were purchased from Boehringer and BMI, and the other reagents used in this study came from Sigma.

Methods

Isolation of total RNA and DNA in sera Total RNA in 200 μl of each serum samples was prepared by Trizol method according to the manufacturer' s instruction and then dissolved in 50 μl of DEPC treated super-purified water. 200 μl of each serum samples was mixed with 200 μl DNA extraction solution containing 20 mmol· L^{-1} EDTA, 0.5 % (W/W) SDS, 10 mmol· L^{-1} Tris-HCl and 40 mg proteinase K (pH8.0) and was treated at 55 ℃ for 30 min. Total DNA in the mixture was extracted by phenol-chloroform method[56] and then dissolved in 50 μl of TE buffer (pH 8.0).

Isolation of total RNA and DNA in saliva 200 μl of each saliva samples was mixed with 400 ml RNA extraction solution containing 4 mol· L^{-1} guanidine thiocyanate,0.1 mol· L^{-1} 2-mercaptoethanol, 0.5 % (W/W) sodium N-lauroylsarcosine and 25 mmol· L^{-1} sodium citrate (pH7.0). Vortex the tube gently for 15 min and lay the tube at room temperature for 5 min. 100 mL 2 mol· L^{-1} sodium acetate (pH4.0), 600 mL TE buffer (pH8.0) and 200 ml phenol (pH4.5)-chloroform (5:1, V:V) were added into the tube and then mixed. Supernatant of the mixture after centrifugation was added with 400 ml 2-propanol (-20 ℃) and then mixed. Lay the tube at -20 ℃ for 2 h to precipitate RNA. RNA pellet obtained by using centrifugation at 4 ℃ was washed with 75 % ethanol and then dissolved in 50 μl of DEPC treated super-purified water[51, 52]. 200 μl of each saliva samples was mixed with 200 ml DNA extraction solution containing 20 mmol· L^{-1} EDTA, 100 mmol· L^{-1} NaCl, 0.5 % (W/W) SDS, 10 mmol· L^{-1} Tris-HCl (pH8.0)and 80 μg proteinase K. Incubate the tube at 55 ℃ for 60 min. Total DNA was obtained by phenol-chloroform method[56] and then dissolved in 50 μl of TE buffer (pH 8.0)[53-55].

RT-nested PCR for HGV RNA detection Ten microliters of total RNA preparation was mixed with 10 μl RT master mixture containing 5 μmol· L^{-1} hexanucleotide as random primers, 12.5 mol· L^{-1} each of dNTP, 20U M-MuLV-reverse transcriptase, 20U RNase inhibitor and 4 μl of 5×RT buffer (pH8.3). The steps of RT were described as the following: at 70 ℃×5 min for denaturation, at 42℃×60 min for cDNA synthesis, and at 70 ℃×10 min to stop the reaction.

Primers derived from HGV 5' -NCR were used in the RT-nested PCR for HGV detection[13, 50]. The external primers: 5' -ATGACAGGGTTGGTAGGTCGTAAATC -3' (sense), 5' -CCCCACTGGTCCTTGTCAACTCGCCG-3' (antisense). The internal primers:5' -TGGTAGCCACTATAGGTGGGTCTTAA -3' (sense), 5' -ACATTGAAGGGCGACGTGGACCGTAC-3' (antisense). For the first round PCR, 10 μl of RT product was mixed with 90 μl PCR master mixture containing 250 nmol· L^{-1} each of the external primers, 2.5 mol· L^{-1} each of dNTP, 25 mol· L^{-1} $MgCl_2$, 2.5U of *Taq* DNA polymerase and 10 μl 10×PCR buffer (pH8.3). For the second round PCR, 5 μl product from the first round PCR was used as template, and the other reaction reagents were the same as that in the first round PCR except of the primers. DNA Thermal cycler 480 (Perkin Elmer) was used for amplification. The parameters for each of the PCR rounds were described as the following: 94 ℃5 min (×1); 94℃1 min, 56 ℃ 1 min, 72℃1.5 min (×35); 72 ℃7 min (×1). The expected size of target fragments amplified from HGV 5' -NCR RNA was 193bp.

Semi-nested PCR for TTV DNA detection The primers used in the semi-nested PCR for TTV DNA detection were the same described by Okamoto *et al*[57]. The external primers: 5' -ACAGACAGAGGAGAAGGCAACATG-3' (sense), 5' -CTGGC ATTTTACCATTTCCAAAGTT-3' (antisense). The internal primers: 5' -GGCAACAT GTTATGATAGACTGG-3' (sense), 5' -CTGGCATTTTACCATTTCCAAAGTT-3' (antisense). Except the primers, $MgCl_2$ concentration (15 mol· L^{-1}), total reaction volume (50 μl) and annealing temperature (60 ℃), the compositions and concentrations of other reaction agents and the parameters for the semi-nested PCR was the same as that of the RT-nested PCR for HGV detection. The expected size of target fragments amplified from TTV DNA was 271bp.

Examination of amplification products The results of amplification reactions were observed on UV light after electrophoresis of 20 g· L^{-1} agarose stained with ethidium bromide. 100bp DNA ladder was used as a size marker to estimate the length of products.

Cloning and sequencing of PCR amplification products The target DNA fragments of HGV or TTV amplification products by PCR were cloned into plasmid pGEM-T vector by using T-A cloning kit according to the manufacturer' s instruction. The recombinant plasmid was amplified in *E.coli* strain DH 5α and then recovered by Sambrook' s method. A professional company (Sangon) was responsible for the analysis of nucleotide sequences of the inserted fragments. Homology of the nucleotide sequences was compared with those reported respectively[14, 56].

RESULTS

Positive rates of HGV RNA and TTV DNA detection in the serum and saliva samples

Twenty-seven (11.9 %) and 21 (9.3 %) of the 226 serum samples were only positive for HGV RNA and TTV DNA, respectively. 10 (4.4 %) and 9 (3.9 %) of the 226 saliva samples were only detectable for HGV RNA and TTV DNA, respectively. And 7 (3.1 %) of the serum samples and 2 (0.9 %) of the saliva samples showed the positive results for both HGV RNA and TTV DNA. 171of the 226 serum samples (75.7 %) and 205 of the 226 saliva samples (90.7 %) were undetectable for both HGV RNA and TTV DNA. Representatives of the target fragments respectively amplified from HGV and TTV genomes in the serum and saliva samples were shown in Figure 1.

Distribution of HGV and TTV detection results in serum and saliva samples

Twelve saliva samples from the 34 patients (35.3 %) positive

for HGV RNA and 11 saliva samples from the 28 patients (39.3 %) positive for TTV DNA in their sera were HGV RNA and TTV DNA detectable respectively, including 2 patients with both HGV RNA and TTV in their serum and saliva samples. No saliva samples from the 226 patients were found to be positive for HGV RNA or TTV DNA but their serum samples were negative for HGV or TTV. Distribution of HGV and TTV detection results in serum and saliva samples of the 226 patients was shown in Table 1.

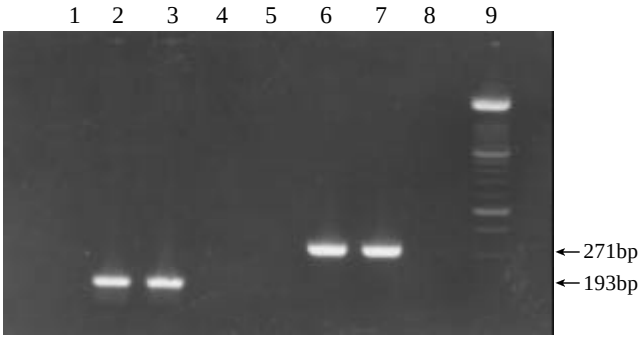

Figure 1 Target fragments respectively amplified from HGV RNA and TTV DNA in serum and saliva samples. (1: HGV negative serum sample; 2 and 3: HGV positive serum sample and saliva sample (No.: 010906); 4: blank control for HGV detection; 5: TTV negative serum sample; 6 and 7: TTV positive serum sample and saliva sample (No.: 011022); 8: blank control for TTV detection; 9: marker)

Table 1 Distribution of HGV and TTV detection results in serum and saliva samples of the 226 patients

serum samples		saliva samples		cases (N)	percentage (%)
HGV	TTV	HGV	TTV		
+[a]	-[b]	-	-	18	8.0
-	+	-	-	13	5.8
+	+	-	-	3	1.3
+	-	+	-	9	4.0
-	+	-	+	8	3.5
+	+	+	+	2	0.9
+	+	+	-	1	0.4
+	+	-	+	1	0.4
-	-	-	-	171	75.7
Total				226	100.0

[a]+: positive detection results for HGV or TTV; [b]-: negative detection results for HGV or TTV

Nucleotide sequence analysis and homology comparison

In comparison with the reported HGV[14] and TTV genotype-1a[57] nucleotide sequences, the homologies of HGV and TTV amplification products from 2 serum and 2 saliva samples from the two same patients (No.: 010906 and 011022) were 88.65-91.49 % and 65.32-66.67 %, respectively. When to compare the nucleotide sequences of HGV and TTV amplification products between the 2 serum samples and between the 2 sliva samples, the homologies were as high as 98.65 % and 98.20 %, 93.24 % and 94.59 %, respectively. The homology comparison mentioned above did not contain the primer sequences (Figure 2 and 3).

```
1:1   TGGTAGCCACTATAGGTGGGTCTTAAGAGAAGGTTAAGATTCCTCTTGTGCCTGCGGCGA
2:1   ..........................G.TT..C....G.C........C..T..T.....
3:1   ..........................G.TT..C....G.C........C..T..T.....
4:1   ..........................G.....C....G.C.....G.C...........
5:1   ..........................G.....C....G.C.....G.C...........

1:61  GACCGCGCACGGTCCACAGGTGTTGGCCCTACCGGTGGGAATAAGGGCCCGACGTCAGGC
2:61  A.AA.................................T......................
3:61  A.AA.................................T......................
4:61  ...AA.......................T.........T......................
5:61  ...AA................................T........A...........

1:121 TCGTCGTTAAACCGAGCCCGTTACCCACCTGGGCAAACGACGCCCACGTACGGTCCACGT
2:121 .G................A...................A...................
3:121 ...................A...................A...................
4:121 ...................A.A.................................
5:121 ...................A.A.................................

1:181 CGCCCTTCAATGT
1:181 .............
3:181 .............
4:181 .............
5:181 .............
```

Figure 2 Homology of the nucleotide sequences from HGV RT-nested PCR products of the 2 serum and 2 saliva samples from 2 patients compared with the reported sequence. (1: The reported HGV sequence[14]; 2 and 3: the sequences of HGV RT-nested PCR products from the serum sample and saliva sample from the patient (No.: 010906); 4 and 5: the sequences of HGV RT-nested PCR products from the serum sample and saliva sample from the patient (No.: 011022). Underlined areas indicate the primer positions.

```
1:1   GGCAACATGTTATGGATAGACTGGCTAAGCAAAAAAAACATGAACTATGACAAAGTACAA
2:1   . . . . . . . . . . . . . . . . . . . . . . . . C T . . . G . T G . . T C A G T A . . . . . . . . G C A G . . .
3:1   . . . . . . . . . . . . . . . . . . . . . . . . C T . . . G . T G . . T C A G T A . . . . . . . . G C A G . . .
4:1   . . . . . . . . . . . . . . . . . . . . . . . . C T . . . . . . G G . . T C A G T A . . . . . . . . G C A G . . .
5:1   . . . . . . . . . . . . . . . . . . . . . . . . C T . . . . . . G G . . T C A G T A . . . . . . . . G C A G . . .

1:61  AGTAAATGCTTAATATCAGACCTACCTCTATGGGCAGCAGCATATGGATATGTAGAATTT
2:61  . . . . . . . C . . . . . . . . C A . . . . . A . . . C T . . . . . . . C T . . . T . . . . . . . . . C A C . . . . A C
3:61  . . . . . . . . . . . . . . . . C A . . . . . A . . . C T . . . . . . . C T . . . . . . . . . . . . . C A C . . . . A C
4:61  . . . . . T . . T C . . . . . C A . . . . . . . . . . C T . G . . . . C T . . . . . . . . . . . . . C A C . . . . A C
5:61  . . . . . . . . . T C . . . . . C A . . . . . . . . . . C T . G . . . . C T . . . T . . . . . . . . . C A C . . . . A C

1:121 TGTGCAAAAAGTACAGGAGACCAAAACATACACATGAATGCCAGGCTACTAATAAGAAGT
2:121 . . C A G C . . . G T A . . . . . . . . . A C . . . . . . . G . A C A C . . . T G T . . A T G T G . . . . T . . . . C
3:121 . . C A G C . . . G T A . . . . . . . . . A C . . . . . . . G . A C A C . . . T G T . . A T G T G . . . . T . . . . C
4:121 . . C A G C . . . G T A . . . C . . . A C . T . . . . . . . . . C A . . . . T G T . . A T G T G . . . . T . . . . .
5:121 . . C A G C . . . G T A . . . C . . . A C . T . . . . . . . . . C A . . . . T G T . . A T G T G . . . . T . . . . .

1:181 CCCTTTACAGACCCACAACTACTAGTACACACAGACCCCACAAAAGGCTTTGTTCCTTAC
2:181 . . . . A . . . . . T A . . T . . G . . . . . . . A C . . . . A C A . . A G . . T T . G G . . A . A C . . . . . G . . .
3:181 . . . . A . . . . . T A . . T . . G . . . . . . . A C . . . . A C A . . A G . . T T . G G . . A . A C . . . . . C . . .
4:181 . G . . . . . T A . . T . . G . . . . . . . A C . . . . A C A . . A G . . T T . G G . . A . A C . . . . . G . . .
5:181 . . . . . T A . . T . . G . . . . . . . A C . . . . A C A . . A G . . T T . G G . . A . A C . . . . . C . . .

1:241 TCTTTAAACTTTGGAAATGGTAAAATGCCAG
2:241 A G C A . . . . . .
3:241 A G C A . . . . . .
4:241 A G C A . . . . . .
5:241 A G C A . . . . . .
```

Figure 3 Homology of the nucleotide sequences from TTV semi-nested PCR products of the 2 serum and 2 saliva samples from 2 patients compared with the reported sequence. (1: The reported TTV sequence [57]; 2 and 3: the sequences of TTV semi-nested PCR products from the serum sample and saliva sample from the patient (No.: 010906); 4 and 5: the sequences of TTV semi-nested PCR products from the serum sample and saliva sample from the patient (No.: 011022). Underlined areas indicate the primer positions.

DISCUSSION

Viral hepatitis is a common infectious disease of human beings, which is prevalent in all countries of the world and causes a serious healthy problem. Although hepatitis virus A-E have been demonstrated to be the causative agents of human hepatitis A-E respectively, approximately 20 % of acute and 15 % of chronic hepatitis were associated with unknown etiology[23, 24]. HGV and TTV were recently identified as transfusion-transmitted viruses and were considered to be responsible for human non-A-E hepatitis[14-17]. Many published literatures reported that among the patients infected with HGV or TTV, a few of them showed mild liver injury, which resulting in a conclusion of a low clinical importance of HGV or TTV infection[26-32]. However, a wide variety of questions about the potential pathogenicity of HGV and TTV infection still remain unanswered[13, 23]. So far the assays for HGV and TTV detection are not the routing laboratory examination items in many countries and areas. Not few of investigation data indicated that high frequencies of HGV or TTV infection were frequently found in different normal populations such as in healthy blood donors[41-50]. Some investigation results revealed that the genomic nuclear of HGV or TTV in saliva of partial infected individuals was also detectable[51-55], suggesting the possibility by way of saliva for transmission of the two viruses. Although HGV or TTV infection might be not clinically significant, the existence of extensive and potential infectious sources of the two viruses will still generate a great problem for human health.

In the present study, HGV and TTV infection rates in serum samples of the 226 non-hepatitis patients with oral diseases were 15.0 % (34/226) and 12.4 % (28/226) respectively, which indicated that HGV infection rate was obviously higher than that of the reported in other areas of China and abroad (1.3-10.6%)[7,8], and TTV infection rate was higher than that of the reported abroad (<5 %) [41, 43-47] but similar to the reported in China (9.3-16.8 %)[42, 48-50]. Such high HGV and TTV infection frequencies in the non-hepatitis population in Hangzhou are probably due to geographic difference. In the previous study, we found 2.5 % of healthy blood donors in Hangzhou coinfected with both HGV and TTV[50]. In this study, the serum samples from 7 of the 226 patients (3.1 %) were positive for both HGV RNA and TTV DNA, which offered further evidence for the existence of coinfection of the two viruses. Therefore, it is valuable to make further studies to determine the pathological and clinical significance of the coinfection.

Since the patients with oral diseases in this study did not show any clinical symptoms and laboratory markers for hepatitis, which suggested that most of the patients infected with HGV and/or TTV are usually asymptomatic. However, these asymptomatic patients carrying HGV and/or TTV may be more risky and important for the source of infection. It was worthy to notice that 5.3 % and 4.9 % of the non-hepatitis patients with oral diseases were HGV RNA and/or TTV DNA detectable in the saliva, indicating dentists may be one of the populations at high risk of HGV and/or TTV infection except of blood precipitants and hemodialysed patients[18, 25, 31, 32, 57, 58].

It was more important that HGV and TTV in saliva might be transmitted among the patients for oral treatment through dentists' hands and therapeutic instruments. So it is necessary to take effective measures to block the possible transmission of HGV and TTV by way of saliva.

In this study, 2 serum samples and 2 saliva samples from the two patients showed positive results for both HGV RNA and TTV DNA. The nucleotide sequences of HGV amplification products from these samples are highly homologous (88.65-91.49 %) to the sequence reported by Linnen *et al*[14]. This result of sequence analysis indicates that the RT-nested PCR used in this study is reliable for HGV RNA detection. To analyze the details of the nucleotide sequencing data, it seems to show a HGV genotype different from the reported[59-62]. Lower homology (65.32-66.67 %) of the nucleotide sequences of TTV Semi-nested PCR products from these samples of the 2 patients compared with the reported TTV genotype 1a sequence[56] reveals the genomic heterogeneity of TTV isolates from different areas, and this finding accords with the conclusions of previously published literatures[31, 56]. In this study, no saliva samples from the 226 patients were found to be positive for HGV RNA or TTV DNA while their serum samples were negative for HGV or TTV. Furthermore, the nucleotide sequence homology of HGV amplification products between the serum and saliva samples from any one of the 2 patients is 98.65 % and 98.20 %, respectively, and so is the nucleotide sequence homology of TTV amplification products (93.24 % and 94.59 %), which are much higher than that compared with the reported HGV and TTV sequences[14, 56]. These data indicate a closely and directly correlation between HGV and TTV genomes detected from serum and saliva from the same one patients.

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• VIRAL HEPATITIS •

Preparation of human single chain Fv antibody against hepatitis C virus E2 protein and its identification in immunohistochemistry

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Abstract

AIM: To screen human single chain Fv antibody (scFv) against hepatitis C virus E2 antigen and identify its application in immunohistochemistry.

METHODS: The phage antibody library was panned by HCV E2 antigen, which was coated in microtiter plate. After five rounds of biopanning,56 phage clones were identified specific to HCV E2 antigen. The selected scFv clones were digested by SfiI/NotI and DNA was sequenced. Then it was subcloned into the vector pCANTAB5E for expression as E-tagged soluble scFv. The liver tissue sections from normal person and patients with chronic hepatitis B and chronic hepatitis C were immunostained with HCV E2 scFv antibody.

RESULTS: The data of scFv-E2 DNA digestion and DNA sequencing showed that the scFv gene is composed of 750 bp. ELISA and immunohistochemistry demonstrated that the human single chain Fv antibody against hepatitis C E2 antigen has a specific binding character with hepatitis virus E2 antigen and paraffin-embedded tissue, but did not react with liver tissues from healthy persons or patients with chronic hepatitis B.

CONCLUSION: We have successfully screened and identified HCV E2 scFv and the scFv could be used in the immunostaining of liver tissue sections from patients with chronic hepatitis C.

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INTRODUCTION

Hepatitis C virus (HCV) has been identified as the major etiological agent of post-transfusion non-A non-B hepatitis[1-10], responsible for most cases of non-A non-B hepatitis. Hepatitis C is a disease of clinical importance because of its high infection rate in blood donors and its persistence as chronic infections which may lead to cirrhosis and hepatocellular carcinoma in the long term[11-26]. The variability of the HCV genome has difficulties in serological detection and vaccine design. Recent advance in phage technology offers a means of cloning human anti-HCV antibodies of a defined specificity that may have potential therapeutic use[27]. We now report the generation of phage display antibody using phage antibody library. From this library we obtained specific single-chain Fv antibody that recognizes the viral envelope protein E2, using HCV E2 protein as the immobilized antigen and proceeding immunohistochemistry.

MATERIALS AND METHODS

Materials

Humanized scFv antibody phage library in which the variable region coding gene of VL and VH were amplified by polymerase chain reaction (PCR) with degenerate primers and connected with a glycine linker [(Gly4Ser)3] was widely used in the screen and identification of humanized scFv to various antigens[28-34]. The recombinant HCV E2 protein was purchased from Virostat Co, USA. Phage M13K07 was purchased from Pharmacia Co., Sweden. Other reagents used in this experiment are all domestic products of analytical grade.

Biopanning

The phage library was amplified in 37 ℃. The host *E. coli* TG1was infected with phage M13K07 and incubated at 37 ℃ for 12 hours, the phage in the supernatant was harvested and concentrated by PEG. Culture plate (Nunc) was coated with recombinant HCV E2 protein at the concentration of 80 mg/L. The coating buffer was 0.05 mol/L $NaHCO_3$, pH 9.6. The plate was blocked with BSA at the concentration of 20 g/L for 2 hours and the concentrated phages were added to the well of the plate, incubated at the room temperature for 90 min. The plates were washed 20 times with PBST and PBS buffer respectively. The bound phage was eluted by the 0.1M of triethylamine, and neutralized with 1M Tris buffer (pH 7.4). Recovered phages were used to infect the host *E. coli* TG1 at the log phase growth and HCV E2 protein-binding phages were amplified. The procedure of absorption-elution-amplification was repeated 5 times.

Identification of phage clones

After 5 rounds of biopanning, 56 phage clones were selected randomly. The clones grew in 400 μl 2×TY-AMP-Glu at 37 ℃ overnight. The culture was transformed to another Eppendorf tube when its A600 nm reached 0.5. The culture was continued at 30 ℃ overnight after adding helper phage. ELISA for determining the supernatants was repeated at least two times. The cross-reaction of the supernatants to the BSA antigen was conducted. According to the ELISA results to the HCV E2 and BSA, one clone with high reaction to HCV E2 and low reaction to BSA was selected.

Sequencing analysis

The plasmid DNA was prepared using Wizard plus minipreps DNA Purification System (Promega Co., USA) and sequenced using ABI automated DNA sequencing machine.

Expression of human HCV E2-scFv in E.coli

The selected HCV E2 scFv clone was subcloned as SfiI/NotI fragments into the vector pCANTAB5E for expression as E-tagged soluble scFv. DNA digestion and electrophoresis confirmed the recombinant vector pCANTAB5E-E2-scFv. Competent *E. coli* XL1-Blue was transformed with pCANTAB5E-E2-scFv and transformed XL1-Blue was induced by IPTG for 20 h. The *E. coli* was harvested by centrifugation at 10 000rpm. The culture supernatant was rendered for ELISA test according to the standard procedure. In ELISA detection, Nunc plate was coated with 1μg/well of recombinant HCV E2 antigen and blocked with 2 % bovine serum albumin (BSA) at 37 ℃ for 2 h. The supernatants from induced and non-induced transformed *E. coli* were added and incubated at 37 ℃ for 2 h. The plate was washed with PBS buffer, and 100 μl of HRP/anti-E Tag 1:4000 ratio diluted in PBS buffer containing 1 % BSA was added, and incubated at 37 ℃ for 1 h. The substrate solution was added and A450nm value was measured.

Immunohistochemical identification of scFv in liver tissue

Paraffin-embedded liver tissue slices were from patients with positive anti-HCV antibodies and HCV-RNA. After deactivating endogenous hyperoxidase, these slices were submersed in the methanol solution with 0.5 % H_2O_2 in the room temperature. Fifty min later, they were washed with PBS buffer for 3 times,5 % BSA was added and slices were stored overnight at 4 ℃. Self-made scFv primary anti- HCV E2 single-chain antibodies were diluted at 1:100 ratio and added on to the slice. They were kept in the 37 ℃ incubator for 1 h, then 4 ℃ refrigerator overnight. HRP-sheep anti-M13 antibodies (diluted to 1:200) solutions were dropped on to the tissue sections, incubated at 37℃ for 40 min. After washing 3 times with PBS buffer, DAB solutions (9 mg DAB, 13.5 ml Tris.cl, 1.5 ml $CoCl_2$, 15 μl 30 % H_2O_2) were dropped on to the tissue sections at room temperature. After ten minutes, the slices were washed with PBS buffer for 3 times again, and 1 % heamatin solution was used to stain the cell nucleus. Gradient ethanol was utilized to dehydrate and dimethylbenzene to clear the sections, then neutral resin to envelope them. The resultant slices were observed under microscope. The controls were set as follows: 1) PBS buffer instead of anti- HCV E2 scFv; 2) HBsAg, HBcAg double-positive liver tissue sections; and 3) Normal liver tissue sections.

RESULTS

Screening and identification phage clones

Using HCV E2 protein as immobilized antigen, the humanized scFv phage library was biopanned. After 5 rounds of biopanning, 56 phage clones were selected randomly. ELISA and cross-reaction of these clones to BSA confirmed their specificity to HCV E2. Among the 56 phage clones, 16 showed good reactivity to the recombinant HCV E2 protein with high A value in the ELISA. In the cross-reaction screen, 6 among the 16 showed low cross-reaction with BSA. The combined results indicated that 1 of the 6 showed the highest reaction to HCV E2 protein and lowest reaction to BSA. One clone has been selected for further DNA digestion and sequence analysis. The DNA sequence digestion was made by SfiI/NotI in Figure 1. Its nucleic acid sequence and deduced amino acid sequence about HCV-E2-scFv fragment are shown in Figure 2.

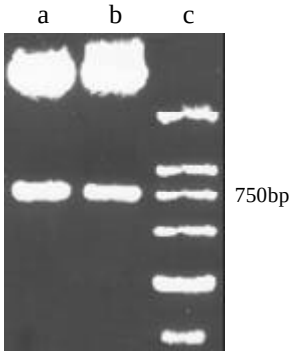

Figure 1 Restriction map of HCV E2-scFv by SfiI/NotI digestion. A,B: HCV E2-scFv; C: DNA Marker

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ATG GCC CAG GTG CAG CTG GTG CAG TCT GGG GCT GAG GTG AAG AAG CCT GGG GCC
 M   A   Q   V   Q   L   V   Q   S   G   A   E   V   K   K   P   G   A
TCA GTG AAG GTT TCC TGC AAG GCT TCT GGA TAC ACC TTC ACT AGC TAT GCT ATG
 S   V   K   V   S   C   K   A   S   G   Y   T   F   T   S   Y   A   M
CAT TGG GTG CGC CAG GCC CCC GGA CAA AGG CTT GAG TGG ATG GGA TGG ATC AAC
 H   W   V   R   Q   A   P   G   Q   R   L   E   W   M   G   W   I   N
GCT GGC AAT GGT AAC ACA AAA TAT TCA CAG AAG TTC CAG GGC AGA GTC ACC ATT
 A   G   N   G   N   T   K   Y   S   Q   K   F   Q   G   R   V   T   I
ACC AGG GAC ACA TCC GCG AGC ACA GCC TAC ATG GAG CTG AGC AGC CTG AGA TCT
 T   R   D   T   S   A   S   T   A   Y   M   E   L   S   S   L   R   S
GAA GAC ACG GCC GTG TAT TAC TGT GCA AGA TCG AGT GGG CCG ATG CAT CGT GAG
 E   D   T   A   V   Y   Y   C   A   R   S   S   G   P   M   H   R   E
TGG GGC CAA GGT ACC CTG GTC ACC GTG TCG AGA GGT GGA GGC GGT TCA GGC GGA
 W   G   Q   G   T   L   V   T   V   S   R   G   G   G   G   S   G   G
GGT GGC TCT GGC GGT GGC GGA TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT
 G   G   S   G   G   G   G   S   S   E   L   T   Q   D   P   A   V   S
GTG GCC TTG GGA CAG ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC
 V   A   L   G   Q   T   V   R   I   T   C   Q   G   D   S   L   R   S
TAT TAT GCA AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC
 Y   Y   A   S   W   Y   Q   Q   K   P   G   Q   A   P   V   L   V   I
TAT GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC AGC
 Y   G   K   N   N   R   P   S   G   I   P   D   R   F   S   G   S   S
TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA GAT GAG GCT
 S   G   N   T   A   S   L   T   I   T   G   A   Q   A   E   D   E   A
GAC TAT TAC TGT AAC TCC CGG GAC AGC AGT GGT AAC CAT GTG GTA TTC GGC GGA
 D   Y   Y   C   N   S   R   D   S   S   G   N   H   V   V   F   G   G
GGG ACC AAG CTG ACC GTC CTA GGT GCG GCC GCA GAA CAA AAA CTC ATC TCA GAA
 G   T   K   L   T   V   L   G   A   A   A   E   Q   K   L   I   S   E
GAG GAT CTG AAT GGG GCC GCA TAG
 E   D   L   N   G   A   A   *
```

Figure 2 Nucleic acid and deduced amino acid sequences of scFv for HCV E2 protein GenBank accession number for this sequence is AF317001

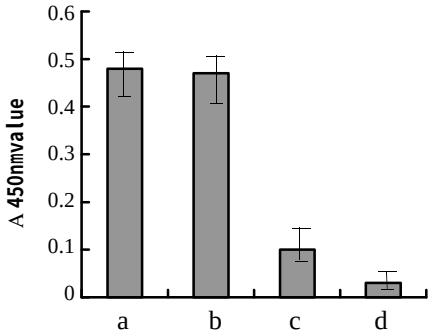

Figure 3 Absorbances of HCV-E2-scFv binding to E2 antigen by ELISA. (a). supernant from induced XL1-blue transformed with pCANTAB5E-E2-scFv; (b). posive control; (c). supernant from non-induced XL1-blue transformed with pCANTAB5E-E2-scFv, d. negtive control

Expression of human HCV-E2-scFv in E. coli.

The expressed HCV-E2-scFv antibody from *E. coli* XL1-blue transformed by pCANTAB5E and induced by IPTG was confirmed by ELISA as shown in Figure3. The recombinant HCV E2 antigen was taken as the positive control. The protein from induced and non-induced *E. coli* XL1-blue transformed by expression vector was positive. But the protein derived from the *E. coli* non-transformed by pCANTAB5E- HCV E2- scFv was negative. These results indicated that the soluble form of human HCV E2- scFv antibody has been successfully expressed in this procedure.

Immunostaining of HCV E2 antigen of liver tissue sections

The different sections from liver tissues of healthy persons and patients with chronic hepatitis B or C were immunostained. The positive immunostaining was seen only in the liver tissue section of patients with chronic hepatitis C, but not in the liver tissue of normal person and patients with chronic hepatitis B as seen in (Figure 4A). The HCV E2 antigen was mainly located in the cytoplasm of the hepatocytes infected by HCV virus (Figure 4B).

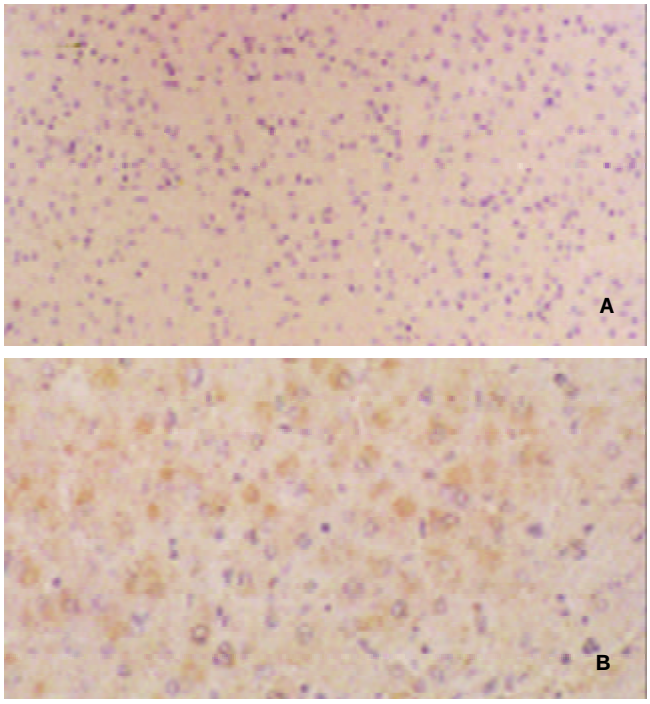

Figure 4 A. Immunohistochemistry of liver tissue from health person. B. Immunohistochemistry of liver tissue from patients with chronic hepatitis C E2 antigen was detected in the cytoplasm of some liver cells

DISCUSSION

HCV is the etiological agent responsible for most cases of non-A non-B hepatitis. It is a blood-related disease of clinical importance since a large number of cases developed chronic infections which may lead to cirrhosis and hepatocellular carcinoma. The variability of the HCV genome has posed serious problems in serological detection of HCV and vaccine design[27-38]. The possibility of using neutralizing antibodies in passive immunization is also hampered by the existence of genotypes and quasispecies[39-50]. To circumvent this, it is possible to envisage the use of cross reactive antibodies with a broad reactivity directed against functionally conserved surface domains common to various genotypes. There has been *in vitro* and *in vivo* evidence that HCV infection elicits a neutralizing antibody response in humans. The virus envelope protein is the primary target for the host immune system. Despite the high variability of the HCV envelope sequence, certain domains of biological importance, e.g. ligands required in viral attachment to host cell receptors, have to be preserved. Antibody specific for the envelope E2 protein has been shown to be cross-reactive HCV E2 and to be able to block viral attachment to cultured human fibroblast cells. However, attept to obtain human monoclonal anti -HCV antibodies is an urgency.

Phage library is a powerful tool for the selection of important and useful antibody specificities espeacially for getting humanized scFv[51-53]. It has many advantages. First, it is the only method to get specific antibody by passing the immunization step. It can mimic the maturation procedure of human antibody in vivo, so it is possible to get high affinity antibody from this selection. Second, the scFv with a low molecular weight, can make it potentially applicable in the clinical diagnosis and treatment of both infectious disease and cancer. Finally, it has no Fc fragment, so the backgroud is very low.

The phage technology offers a means of cloning human anti-HCV antibodies of a defined specificity that may be potential for therapeutic and diagnostic use. In this report, we have obtained anti-HCV E2 scFv by affinity selection and purified recombinant hepatitis C virus E2 as coating antigen from synthetic phage display antibody library. After 5 rounds of selection, 56 clones were evaluated by enzyme-linked immunosorbent assay (ELISA). One phage clone was selected from 56 clones according to the highest A450 nm value in the ELISA identification and lowest cross-reaction to the bovine serum albumin (BSA). The coding fragments of scFv were sequenced. The affinity and specificity of scFv were evaluated by ELISA and immunohistochemistry. HCV E2-scFv DNA digestion and sequence data showed that the scFv gene is composed of 750 bp. ELISA and immunohistochemistry demonstrated that the human single chain Fv antibody against HCV E2 antigen has a specific binding activity with hepatitis C virus E2 antigen. This study illustrated the feasibility of using antibody-engineering technology with the phage display library that may be useful for future therapeutic and detection purpose.

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• VIRAL HEPATITIS •

Lamivudine does not increase the efficacy of interferon in the treatment of mutant type chronic viral heaptitis B

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Abstract

AIM: To study the role of lamivudine in improving the efficiency of interferon for the treatment of mutant type chronic hepatitis B.

METHODS: Fifteen patients with mutant type chronic hepatitis B were prospectively studied. All patients had liver histology and serology to prove the diagnosis of chronic hepatitis B. Each patient received 4.5 millionunits of interferon alpha-2a thrice weekly and 100 mg of oral lamivudine daily for 24 weeks. Patients were observed and tested for blood chemistry every week for the initial 4 weeks and every 2 weeks thereafter during the treatment until 24 weeks. After the end of treatment, patients were followed up at 4-week intervals for an additional 6 months. Serum HBV DNA levels were tested using the liquid phase molecular hybridization assay. Those with non-detectable HBV DNA were also tested using the real-time polymerase chain reaction. One patient, who did not finish treatment due to depression, was excluded.

RESULTS: At the end of treatment, 7 (50 %) patients had serum ALT levels within normal limits; 12 (86 %) patients had serum HBV DNA levels <5 pg/mL using the liquid phase molecular hybridization assay, but only 8 (67%) were <20 copies/dL using the real-time polymerase chain reaction. Six months after treatment, only two (14 %) patients had a sustained complete response to the combination therapy with serum ALT level <35 iu/L and undetectable serum HBV DNA levels.

CONCLUSION: These pilot data showed that lamivudine did not increase the efficacy of interferon in the treatment of mutant type chronic hepatitis B. The liquid phase molecular hybridization assay was not sensitive enough to detect the low HBV DNA levels during combined interferon and lamivudine therapy.

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INTRODUCTION

Hepatitis B virus (HBV) is one of the major causes of liver disease worldwide[1,2], and chronic hepatitis B can progress to cirrhosis and hepatocellular carcinoma[1,2]. It is thus important to conduct anti-viral therapy against chronic hepatitis B to minimize the amount of liver damage[4]. Recent studies suggest that around half of all patients with chronic HBV infection responded to a 6 to 12 month course of interferon therapy at the end of treatment with loss of serum hepatitis B viral deoxyribonucleic acid (HBV DNA) and hepatitis B e antigen (HBeAg), as well as normalization of serum alanine aminotransferase (ALT) activity[5,6]. However, a mutant type of HBV DNA has been identified in serum from patients with chronic hepatitis B, who presented with negative HBeAg and abnormal serum ALT levels[7]. The mutant type of HBV infection showed mutations at the precore region. Different from those patients with wild type HBV DNA, the respond rate of interferon mono-therapy against mutant type chronic hepatitis B was low[8,9]. Lamivudine has become a recent interest in the treatment of chronic viral hepatitis B[10,11]. However, the relapse rate after the end of lamivudine therapy is high[12-14]. Many different regimens, including a longer course of treatment or higher dosages of interferon, have been claimed to improve the interferon therapy, but the sustained response rate is still disappointing[15,16]. Recent studies from patients with chronic hepatitis C show that the combination of interferon alpha with ribavirin results in a higher sustained response rate than interferon alpha alone[17,18]. However, the role of combination of interferon and lamivudine against chronic hepatitis B remains uncertain[19]. We therefore conducted the present pilot study to investigate the possible role of combined interferon and lamivudine to improve the efficiency of interferon in patients with mutant type chronic hepatitis B.

MATERIALS AND METHODS

We prospectively studied 15 documented patients with mutant type chronic hepatitis B at Cathay General Hospital, Taipei, Taiwan between June 1999 and June 2001. All patients were males aged between 20 and 65 years old (mean ± SD: 44±8 years), and were naive without prior interferon or other anti-viral therapy. Chronic hepatitis B was defined as positive hepatitis B surface antigen (HBsAg, Auszyme, Abbott Laboratory, Abbott Park, IL 60064) and abnormal serum ALT levels (normal <35 IU/L) for more than 6 months. Mutant type chronic hepatitis B was defined as detectable HBV DNA, positive HBsAg, negative HBeAg [HBeAg; HBe (rDNA) EIA, Abbott Labratory], and abnormal serum ALT levels in patients having chronic hepatitis B. All patients had at least three documented occasions of abnormal serum ALT levels higher than twice the upper limit of normal with one month apart, within 6 months prior to enrollment. All patients underwent liver biopsy within one month before the start of treatment to confirm the chronic hepatitis without cirrhosis.

None of our patients were alcoholic, intravenous drug users

or homosexual. None had received hepatotoxic drugs, herbal medicine or immuno-suppressive therapy within the past 6 months. Further, none had decompensated liver function (prolonged prothrombin time >3 seconds *vs.* INR >1.50, serum total bilirubin >3.0 mg/dl, or serum albumin < 3.0 g/dL), cirrhosis, chronic renal failure, clotting abnormalities, hemophiliacs, serious neurological disorders, obesity, chronic viral hepatitis C (Murex anti-HCV, Version III, Murex Diagnostics Ltd., Dartford, England) or delta (Wellcozyme, Wellcome Diagnostics, Dartford, UK), autoimmune disease (anti-nuclear antibody titer >1:40, Fluoro HEPANA, Medical & Biological Lab., Nagoya, Japan), and/or inheritable disorders such as hemochromatosis, alpha-1-antitrypsin deficiency or Wilson' s disease. All patients with peripheral white cells <4 000 per mm^3 and platelet < 100 000 per mm^3 were excluded. Serum HBV DNA was measured using a commercially available liquid phase molecular hybridization assay (Digene Hybrid Capture™ System, Beltsville, MD) according to the manufacturer' s instruction. The lowest detectable HBV DNA level was 5 pg/ml. Serum samples with non-detectable HBV DNA were retrospectively tested using the real-time polymerase chain reaction (RT-PCR) methods after the end of clinical follow-up. The lowest detectable HBV DNA level was 20 copies/mL.

All patients received 4.5 millionunits (MU) of interferon alpha-2a (Roferon, F. Hoffmann-La Roche Ltd., Basel, Switzerland) thrice weekly and 100 mg of oral lamivudine daily for 24 weeks. Patients were observed and tested for blood chemistry every week for the initial 4 weeks and every 2 weeks thereafter during the treatment until 24 weeks. After the end of treatment, patients were followed up at 4-week intervals for an additional 6 months.

Normalization of serum ALT levels and absence of serum HBV DNA were assessed for the efficacy of treatment. A complete response was defined as the normalization of serum ALT levels together with the absence of serum HBV DNA by the end of treatment. A sustained complete response was defined as the continuation of the remission for at least 6 months after the end of treatment.

Informed consent was obtained from the patients before treatment. The study protocol was reviewed and approved by the Institutional Review Board of the hospital under the guidelines of the 1975 Declaration of Helsinki.

Statistical analysis

Was performed using two-tailed Student' s *t*-test and two-tailed Fisher' s exact test where appropriate.

RESULTS

The demographic data, biochemical data and serum HBV DNA levels are shown in Table 1. The total bilirubin were 0.9±0.2 mg/dL (data are presented as mean ±S.D. and so forth; range: 0.4-1.8 mg/dL), and prothrombin time (INR) was 1.01±0.08 seconds (range: 081-1.22) before treatment. The mean serum ALT levels were 132±71 iu/L (range: 42-290 IU/L), and mean serum HBV DNA were 224±255 pg/mL (range: 6-1924 pg/ml) before treatment.

The major side effects of treatment were: flu-like symptoms: 8 (53 %), fatigue: 12 (80 %), insomnia: 4 (29 %), hair loss: 5 (33 %), and depression: 1 (7 %). None developed anemia (hemoglobin <11 g/dL), leukopenia (leukocyte <3500/ μl), thrombocytopenia (thrombocytes $<100\times10^3/\mu l$), hyperbilirubinemia (>2.0 mg/dL), or pancreatitis during therapy.

Fourteen patients finished a 24-week-course of treatment. One patient (HSF) discontinued interferon after 2 months of therapy due to interferon-related depression. His serum ALT remained abnormal from 210 IU/L before treatment to 38 IU/L at the end of treatment, and serum HBV DNA levels dropped from 90 pg/mL before treatment to 29 pg/mL at the end of therapy. None of the remaining 14 patients experienced depression, aggression, hostility or hallucination.

The serum ALT levels dropped from 132±71 IU/L before treatment to 46±17 IU/L at the end of treatment (Table 2, P= 0.0002), and the serum HBV DNA levels decreased from 224±255 pg/ml before treatment to 8±12 pg/mL at the end of treatment (P=0.058). At the end of treatment, 7 (50 %) of the 14 patients had serum ALT levels within normal limits. Although 12 (86 %) patients had undetectable serum HBV DNA levels (<5 pg/mL) using the liquid phase molecular hybridization assay, only 8 of them had undetectable HBV DNA (<20 copies/mL) using the RT-PCR. Both of these patients had complete response with normal serum ALT levels and undetectable at the end of treatment. The remaining 12 patients had abnormal serum ALT levels and/or detectable HBV DNA.

Six months after the end of treatment, the mean serum ALT (90±58 IU/L, P=0.03) and HBV DNA (63±70 pg/ml, P=0.05) levels increased compared to those at the end of treatment. Three patients continued to have undetectable serum HBV DNA using the liquid phase molecular hybridization assay method. However, only two (14 %) of them had both complete response and sustained complete response to the combination therapy with serum ALT level <35 IU/L and undetectable serum HBV DNA levels using the RT-PCR method. One patient with undetectable HBV DNA using the liquid phase molecular hybridization assay had abnormal serum ALT levels after the end of treatment; his serum HBV DNA level was 93 copy/dL using the RT-PCR. All of the remaining 11 patients had abnormal serum ALT levels as well as detectable serum HBV DNA.

Table 1 Demographic data, biochemical data and serum HBV DNA levels of patients

Patient No	Age/Gender (y)	ALT (IU/L)	Total Bilirubin (mg/dL)	Prothrombin time (INR)	HBV DNA (pg/mL)
1*	52/M	210	0.9	1.02	90
2	65/M	42	0.9	0.93	24
3	61/M	162	1.4	1.18	1924
4	43/M	38	0.8	0.91	107
5	45/M	212	0.8	1.00	303
6	34/M	49	0.4	1.02	66
7	38/M	47	0.6	1.05	262
8	36/M	119	0.9	0.81	6
9	35/M	56	0.8	0.90	7
10	48/M	154	0.5	1.12	265
11	46/M	223	1.0	1.14	56
12	38/M	290	1.8	1.16	8
13	41/M	44	1.0	0.93	8
14	39/M	195	0.7	1.03	11
15	60/M	147	0.7	1.07	17

*Patient 1 received only 8 weeks of interferon therapy

Both of the two patients with sustained complete response were less than 40 years old (36 and 38 years old) with higher initial serum ALT levels >100 IU/dL (119 and 290 IU/dL), lower initial serum HBV DNA levels <10 pg/ml (6 and 8 pg/mL), and a histology with moderate intralobular degeneration and focal necrosis (range: 1/3-2/3 of lobules). Using the two-tailed Fisher' s exact test, age<40 year-old (P=0.11), serum ALT level >100 IU/dL (P=0.37), serum HBV DNA < 10 pg/ml (P=0.07), and moderate to severe intralobular degeneration and focal necrosis (P=0.07) were not significant factors in predicting sustained complete response.

Table 2 Serum ALT and HBV DNA levels of patients before, at the end, and at 6 months after treatment

	ALT (iu/L)			HBV DNA				
Patients	start	end	6M	start	end		6M	
					LPMH	PCR	LPMH	PCR
1[a]	210	38	51	90	29	-	-	-
2	42	97	166	24	18	-	30	-
3	162	57	84	1924	ND	24.4	13	-
4	38	34	37	107	ND	78.8	7	-
5	212	26	66	303	ND	5.1	27	-
6	49	46	150	66	63	-	98	-
7	47	35	43	262	ND	94.7	27	-
8	119	24	20	6	ND	ND	ND	ND
9	56	80	41	7	ND	43.8	ND	93.0
10	154	54	254	265	ND	1.3	128	-
11	223	48	215	56	ND	1.2	445	-
12	290	30	24	8	ND	ND	ND	ND
13	44	22	71	8	ND	9.7	10	-
14	195	56	41	11	ND	2.2	39	-
15	147	22	47	17	ND	12.6	10	-
Mean	132	46[b]	90[c]	224	8[d]		63[e]	
S.D.	71	17	58	255	12		70	

ND: HBV DNA <5 pg/ml using the liquid phase molecular hybridization assay (LPMH); HBV DNA < 20 copies/dL using the real-time polymerase chain reaction (PCR). [a]Patient 1 received only 8 weeks of interferon therapy; [b]P=0.0002, [d]P=0.05 compared with those before treatment; [c]P=0.058, [e]P=0.05 compared with those at the end of treatment

DISCUSSION

At the end of treatment, the mean serum ALT level was decreased, and half of the patients had a serum ALT level within normal limits. Although most patients had undetectable HBV DNA levels using the liquid phase molecular hybridization assay, the change of serum HBV DNA levels was not statistically significant, a finding consistent with studies from patients of chronic viral hepatitis B with wild type HBV DNA[11,20].

Six months after the end of treatment, the mean serum ALT level was significantly increased compared with those at the end of treatment. Only 14 % of patients had sustained response with normal serum ALT levels as well as undetectable HBV DNA levels. The low sustained response rate to combined interferon alpha-2a and lamivudine was consistent with those of interferon mono-therapy for patients with mutant type chronic hepatitis B[8,9]. Our pilot data showed that lamivudine did not increase the efficacy of interferon in the treatment of mutant type chronic hepatitis B, a finding consistent with studies stating those having positive HBeAg that IFN and lamivudine did not offer additional benefit compared with lamivudine monotherapy[20].

Although 12 of the 14 patients (86 %) had an undetectable HBV DNA level at the end of treatment using the liquid phase molecular hybridization assay, half of them had abnormal serum ALT levels. Only 14 % and 21 % of the 12 patients with undetectable HBV DNA levels using the liquid phase molecular hybridization assay had normal serum ALT levels and undetectable serum HBV DNA using the RT-PCR method. RT-PCR is much more sensitive than liquid phase molecular hybridization assay in the detection of samples with low HBV DNA levels.

For the 15 serum samples with undetectable HBV DNA using the liquid phase molecular hybridization assay, only 10 of them had an undetectable HBV DNA level using the RT-PCR. These four samples were from eight and two patients at the end and 6 months after treatment. Both of these patients had sustained normal serum ALT levels and undetectable serum HBV DNA at 6 months after treatment. For the remaining 5 patients, low titer HBV DNA was identified using the RT-PCR, and all patients developed abnormal serum levels after the end of treatment. Our data showed that the liquid phase molecular hybridization assay was not sensitive enough to detect the low HBV DNA levels during combined interferon alpha-2a and lamivudine therapy. RT-PCR method is better than liquid phase molecular hybridization assay in the detection of low titer HBV DNA. The presentation of low levels of HBV DNA may result in subsequent abnormal serum ALT levels and increasing HBV DNA levels in 6 months.

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• VIRAL HEPATITIS •

Mutational characteristics in consecutive passage of rapidly replicating variants of hepatitis A virus strain H2 during cell culture adaptation

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Abstract

AIM: To investigate the molecular mechanism of cell adaptation and rapid replication of hepatitis A virus strain H2 in KBM17 cells.

METHODS: Virus of strain H2 at passage 7 was consecutively passaged in KBM17 cells for 22 passages, every passage was incubated for 14 days. Antigenic and infectious titers of every passage and one-step growth dynamics of passage 22 were determined with ELISA. Genomes of passage 6, passage 12, passage 18 and passage 22 were sequenced and compared with H2K7.

RESULTS: During continuous passage of vaccine strain H2 at passage K7 in KMB17 cells, infectious and antigenic titers increased with the increase of passages, infectious titers at day 14 reached 6.77LgCCID$_{50}$ml^{-1} for passage 6 (P6), 7.0 LgCCID$_{50}$ml^{-1} for passage 12 (P12), 7.33 LgCCID$_{50}$ml^{-1} for passage 18 (P18) and 7.83 LgCCID$_{50}$ml^{-1} for passage 22 (P22), respectively. The one-step growth dynamics showed that replicating peak of P22 appeared at day 14 with infectious titers of 7.83 LgCCID$_{50}$ml^{-1} and antigenic titer of 1:1024. After passage 22 a new cell-adapted variant (P22) of H2K7 with rapid and shortened replication cycle from 28 days to 14 days was obtained. Sequencing and comparisons of genomes of P6, P12, P18 and P22 showed that mutational numbers in genomes of different passages increased with adaptive passages, and mutations scattered over the genome. In comparison with that of K7, P6 had only 6 nucleotides (nt) mutations, P12 had 7 mutational changes, in addition to 6 same mutations with P6, there appeared a new mutation in 5'NTR at nucleotide position 591 resulting in a nucleotide exchange from A to G. P18 had 10 nt mutations, among the 10 mutations, 7 mutational changes were same as with P12, three new mutational changes appeared in the genome, one in 5'NTR, one in 3C coding region, one in 3D coding region, at P22 there appeared 18 nucleotide changes in the genome, on the basis of P18, there occured additional 8 nucleotide mutations, two in 5'NTR, three in 2C, one in 3A, one in 3C and one in 3D. The results suggested that although H2K7 was already an attenuated strain, the mutations of genome is not sufficient to completely adapt the KMB17, further mutations caused rapid replication adaptation.

CONCLUSION: 18-nt changes scattering over the genome are cooperatively responsible for further adaptation characterized by rapid and shortened replication cycle from 28 days to 14 days in KMB17 cells. The mutations in 2C coding region play more important role in increase of infectious titer than other mutations, the mutations in 2B coding region show less important role than it usually does in cell adaptation, nucleotide changes in 5' NTR seem to be not relevant to cell adaptation during initial stages (before P6), but do in late stages.

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INTRODUCTION

Hepatitis A virus (HAV), one of the two members of genus *Hepatovirus*, is enterically transmitted primarily through the fecal/oral route causing sporadic and epidemic acute hepatitis in humans[1,2]. Human HAV is a nonenveloped icosahedral particle of 27-32 nm in diameter with a single-stranded, 7.5-kb positive-sense RNA genome with a long 5'-terminal nontranslated RNA segment (5' NTR) and a short 3' NTR with a poly(A) tail[1]. Similar to other picornaviruses, the HAV genome contains one large open reading frame encoding a polyprotein of about 250 kD, which is co-and posttranslationally cleaved into smaller structural proteins (2A, 2B and 2C) and nonstructural proteins (3A, 3B, 3C and 3D) by virus-encoding proteinases[3].

HAV has been adapted to grow in a variety of primate cell lines[4], but in contrast to that of other picornaviruses, the replication cycle of wild-type HAV isolated from infected human in cell culture is extremely low and inefficient. After prolonged incubation and several passages in cell culture, a persistent infection with low virus yields and little apparent impact on cellular growth and metabolism is normally established[5]. Only a few HAV strains have been reported to cause cytophethesis in vitro[6,7].

Unlike other picornaviruses, although HAV can be adapted to grow in a variety of primate cell lines, cell-adapted variants still grow poorly in cell culture. The molecular mechanisms of adaptation and slow replication cycle are still not completely understood.

That the genomes of a number of wild types and cell-adapted HAV strains have been completely or almost completely sequenced made it possible to determine the

nucleotide changes responsible for cell adaptation. By sequence comparison of different cell-adapted variants[8-19], and studies with chimeric HAV composed of sequence from wild-type and cell culture-adapted variants[20-22], mutations increasing virus replication in cell culture were identified. The adaptation of HAV to grow in cell culture is associated with a number of cooperative mutations over the whole genome, but the mutations within 5' NTR containing regulatory internal ribosome entry segment (IRES) for viral translation[23], and within 2B and 2C coding regions encoding enzymes for viral RNA replication[24], play more important role in cell adaptation. Moreover, the mutations in 2B and 2C coding regions were proven to be necessary for adaptation of growth in cell culture of HAV variants[10,11,21], the mutations in 5' NTR had no independent effect , but cooperatively acted with 2B and 2C coding regions to enhance replication in cell culture[21,24].To study further the molecular mechanisms of cell adaptation of HAV in cell culture, a cell-adapted variant of live HAV attenuated vaccine strain H2 was used for consecutive passage for 22 passages in human embryonic lung diploid fibroblast KMB17, during passages the replicating peak cycle was shortened from 28 days to 14 days, the genomes of different passages, 6th passage (HAVH2K7P6), 12th passage (HAVH2K7P12), 18th passage (HAVH2K7P 18) and 22nd passage (HAVH2K7P22), were sequenced and compared to identify the adaptive mutations.

MATERIALS AND METHODS

Cell

Human embryo lung diploid fibroblast strain (KMB17)[25] was used at passage 24-35 in this research.

Virus Strain

Hepatitis A virus strain H2, an attenuated strain at passage K7, was derived from the fecal specimen of a patient with hepatitis A in Hangzhou, China. After isolation and passage in a culture of newborn monkey (*Macaca Mulatta*) kidney cells, adaptation to grow in human embryo lung diploid fibroblast (KMB17), and serial passage at a low temperature (32 ℃) in KMB17 cells, the strain became attenuated[25] and licensed for production of live attenuated HAV vaccine.

H2 strain passage and rapidly replicating adaptation in KMB17 cells

KMB17 cells were grown in 25 cm^2 flask (Nunc, Weisbaden, germany) using Eagle' Minimal Essential Medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 % heat-inactivated newborn calf serum, and passaged every four days. HAV H2M20K7 with 28-day replication cycle were seeded onto KMB17 confluent monolayer cells,after 37 ℃ absorption for 2 hours, maintenance medium with 2 % newborn calf serum were added, and seeded cells were cultured at 35 ℃ for 14 days. The virus was harvested at the 14th day, and then seeded onto the monolayer cells again, the virus was serially passaged for 22 passages with 14-day cycle. The antigen titers and infectious titers were detected with ELISA[26, 27]. The virus at 6th, 12th, 18th, 22nd passage were designated as HAVH2K7P6(P6), HAVH2K7P12(P12), HAVH2K7P18(P18) and HAVH2K7P22(P22), respectively. The respective genomes were cloned and sequenced.

One-step growth dynamics

KMB17 cells were infected with a multiplicity of infection (MOI) of 5. The titer was assayed by inoculating the cells grown in25 cm^2 flask (Nunc, Weisbaden, germany), and infection was checked 4 weeks after inoculation by ELISA[26, 27].

Primers and sequencing strategy

Primers were designed according to the sequence of H2K7[29] with Goldkey software (Table 1), The sequencing strategy was showed as Figure 1.

Table 1 The primers used for amplification of HAV genomic RNA

Cloned fragments	Primers	Sequences
A (0.8kb)	A1	5'-CGCCGGCGTTCAAGAGGGGTGTCCGGAG-3'
	A2	5'-GAATCTCAATGCCAAATCTTGC-3'
B (0.5kb)	B1	5'-TCTGAGGTACTCAGGGGC-3'
	B2	5'-CAGTCAATGATGCTATAGAACC-3'
C (1.1kb)	C1	5'-CCAACAGGGGGGATTGATC-3'
	C2	5'-CGTTAGAAGGAGAGGTCAATC-3'
D (1.0kb)	D1	5'-CCCTGGATTTCTGACACTCC-3'
	D2	5'-CAGTGGATAACATGGCATTTG-3'
E (1.1kb)	E1	5'-GTCTGTCACAGAACAATCAGAG-3'
	E2	5'-GATCCCAGAACAGATATCTCTTAA-3'
F (1.2kb)	F1	5'-GTTAAGAGATATCTGTTCTGGATC-3'
	F2	5'-CCATCCTCCAACGAGCACTCC-3'
G (1.2kb)	G1	5'-CAGTTCTTTAGTCATGACAGTTG-3'
	G2	5'-GCCATTGGATCAATTTCAGC-3'
H (1.1kb)	H1	5'-GAGTCCCATTTATCATCACA-3'
	H2	5'-GTCCAATCAGATCAAGATTATC-3'
I (o.5kb)	I1	5'-GATTCTCTGTTATGGAGATG-3'
	I2	5'-TTTTTTTTTTTTTTTTTTTTTATTT-3'

(1):A-I represent 9 amplified fragments of HAV; (2) 1 and 2 represent positively and negatively oriented primers

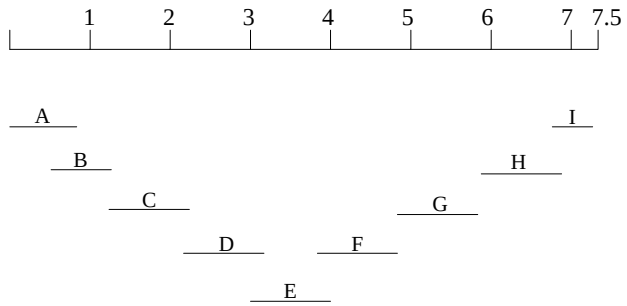

Figure 1 Sequencing strategy of HAV genome. A-I represent 9 amplified fragments of HAV

Antigen-capture PCR amplification of the genomes

cDNA synthesis and cloning Antigen-capture PCR were used to prepare cDNA of genome of different passages[29] with some modifications. Sterile 0.5-ml conical tube (Ependorf) was coated 100 μl of human anti-HAV IgG diluted 1:1 000 in 50 mM sodium carbonate buffer (pH 9.6). After 4 h of incubation at 37 ℃, the unbound IgG was removed , and 150 μl of 1 % bovine serum albumin (Sigma) diluted in the buffer was added. After 1 h at 37 ℃, the tube was washed three times with 300 μl of PBS (pH 7.4) containing 0.05 % Tween 80. purified HAV (100 μl) was then added, and the preparation was incubated overnight at 4 ℃. The tube was washed six times with 500 μl of a 40 mM Tris (pH 8.4)-40 mM KCl-7 mM $MgCl_2$ solution. Then 100 μl of water was added and tube was heated to 95 ℃ for 5 min to disrupt captured viruses and melt any second structures within the viral RNA. The first strand cDNA was synthesized using SuperScriptTM Preamplification System kit (Gibco, Life Technologies), following the instruction by

manufacturer. The clones of different fragments were performed by PCR in a mixture (50 μl) including 5 μl 10×LA PCR buffer, 8 μl 2.5mM dNTPs, 2 μl template of RT-PCR products, 300nM positive-sense primer, 300nM negative-sense primer and 2.5U LA Taq DNA polymerase (TaKaRa), additing water to 50 μl. The reaction mixture was subjected to 95 ℃ for 5 min, then 30 automated cycles of denaturation at 95 ℃ for 30 sec, annealing at 50 ℃ for 30 sec, and extention at 72 ℃ for 1 min or 1 min 30 sec., The final reaction was incubated at 72 ℃ for 10 min. After the PCR products were recovered and purified, the fragments were ligated into pGEM-T Vector (Promega). The resulting products were transformed into competent *E.coli*. DH5α cells. Three ampicillin-resistant clones were picked out for each fragment. The size of inserts in positive clones was estimated with restriction enzyme site at either side of the inserted fragment. Rapid plasmid preparations were made with the Wizard plasmid purification kit (Promega).

DNA sequencing and analysis

Sequencing strategy was shown in Figure 1. Oligonucleotide primers specific for HAV and primers corresponding to the T7/SP6 promoter region of pGEM®-T Vector were used to sequence the Inserted and identified HAV fragment. a Taq DyeDeoxy Terminator Cycle sequencing kit and a 377 DNA sequencer (Perkin Elmer) were used to determine nucleotide sequences. To eliminate the possibility of errors in the sequence due to Taq polymerase for PCR, at least three clones of each amplified fragment derived from two individual PCR products were sequenced. Also to correctly determine the sequence of extreme 5 terminus of HAV genome, a 5' RACE reaction was used to obtain the a cDNA fragment from 5' NTR of genome with 5' -Full RACE Core Set (TaKaRa). Analysis, alignment and translation in the amino acids of the obtained nucleotide sequences were done using the sequence analysis program OMEGA (Oxford Molecular).

RESULTS

Dynamical characteristics of adaptive enhancement of H2K7 replication in KMB17 cells

Although HAV has been adapted to grow in cell culture, even cell culture adapted variants grow considerably slowly with low virus yields and characterized by an asynchronous onset of replication[30,31]. HAV usually established ersistent infection in cell culture with protracted replication cycle, but replication cycle of maximal accumulation of HAV in cell culture was dually shortened with the increase of passages, even cytopathic effect (CPE) appeared[32-35]. Attenuated HAV strain H2K7 didot cause CPE in KMB17 cells, its replicating peak of was days, the infectious titer reached. 0LgCCID$_{50}$ml^{-1}. In this study, attenuated strain H2 at passage K7 were passaged in KMB17 cells every fourteen days. At last the change of incubation cycle, from 28 days to 14 days, resulted in the increase of both antigenic and infectious titers. At first passage of adaptation (P1) the antigenic and infectious titers were only 1:16 ELISA units (ELI.U) and 5.83LgCCID$_{50}$ml^{-1}, respectively, at P6 the antigenic and infectious titers gradually increased o 1:64 ELI. U and 6.77LgCCID$_{50}$ml^{-1}, respectively, at P12 obvious enhancement of antigenic and infectious titers (1:256 ELI.U and 7.0 LgCCID$_{50}$ml^{-1}, respectively) occured, More enhancement appeared at P18 with 1:512 ELI.U and 7.33 LgCCID$_{50}$ml^{-1}, respectively, at P22 antigenic and infectious titers reached the highest levels of 1:1024 ELI.U and 7.83 LgCCID$_{50}$ml^{-1}, respectively (Table 2). During the whole passages no CPE appeared. Furthermore, one-step growth dynamics showed that at P22 at days 2, 4, 6, 8, 10, 12 14 of culture antigenic and infectious titers gradually increased, at day 14 antigenic and infectious titers reached the highest levels of 1:1024 ELI.U and 7.8 LgCCID$_{50}$ml^{-1}, respectively, afterwards, the infectious titer gradually decreased, but antigenic titer remained unchanged until day 26 (Table 3). The increases of antigenic and infectious titers with consecutive passage showed that strain H2K7 had been further adapted successfully in KMB17 cells with shortened replication cycle of 28 days to 14days.

Table 2 Titers of different passages of HAVH2K7 on KMB17 for 14d

Passages	Antigen titers	Infectious titers(LgCCID$_{50}$/ml)
1	1:16	5.83
2	1:8	5.5
3	1:16	5.67
4	1:32	6.0
5	1:32	6.5
6	1:64	6.77
7	1:64	6.67
8	1:64	6.5
9	1:64	6.67
10	1:128	6.83
11	1:128	7.0
12	1:256	7.0
13	1:256	7.17
14	1:256	7.0
15	1:256	7.17
16	1:256	7.0
17	1:512	7.17
18	1:512	7.33
19	1:512	7.5
20	1:512	7.67
21	1:1024	7.67
22	1:1024	7.83

Table 3 One-step growth dynamics of HAVH2K7P22 on KMB17 cells

Post inoculation days	Antigen titers	Infectious titers(LgCCID$_{50}$/ml)
2	1:8	ND*
4	1:16	6.0
6	1:32	6.67
8	1:128	7.17
10	1:512	7.5
12	1:1024	7.8
14	1:1024	7.17
16	1:1024	7.0
18	1:1024	7.0
20	1:1024	6.83
22	1:1024	7.0
24	1:1024	6.8
26	1:2048	6.5

*Not determined

Mutational Characteristics in Consecutive Passage of Rapid Replication of Strain H2 during Cell Culture Adaptation

The process of adaptation of HAV in cell culture was also a process of serials of mutations in genome and results in attenuation[11]. The rapidly adaptive passages of H2K7 in KMB17 cells caused the change of replication cycle, from 28 days to 14 days. The maximal virus yields appeared at 14 days at P22 (Table 3). In order to investigate whether the shortened replication cycle of H2K7P1-22 in cell adaptation was reflected by mutational changes in the genome, which was thought to be responsible for adaptation of HAV, and whether related mutations were responsible for the adjustment of shortened replication cycle, we sequenced and compared the entire genome of four passages (P6, P12, P18, P22), the mutations correlating with adaptation at different passages were identified. The results revealed that mutational numbers in genomes of different passages increased with adaptive passages, and mutations scattered throughout the whole genome. There were only 6 nucleotides mutations appeared in the genome of P6, and nucleotide identity was up to 99.93 % in comparison with that of H2K7, as shown in (Table 4),

Table 4 Differences in the genome sequence and amino acids of the HAV H2 K7 with HAV H2K7 P6, HAV H2K7 P12, HAV H2K7 P18 and HAV H2 K7 P22

Nucleotide position	Location	Nucleotide					Amino acids				
		K7	K7P6	K7P12	K7P18	K7P22	K7	K7P6	K7P12	K7P18	K7P22
33	5'NTR	C	C	C	U	U					
263	5'NTR	U	U	U	U	C					
378	5'NTR	U	U	U	U	C					
591	5'NTR	A	A	G	G	G					
646	5'NTR	A	A	A	U	U					
858	VP2	G	U	U	U	U	A	S	S	S	S
1178	VP2	C	U	U	U	U	I	I	I	I	I
4022	2B	T	C	C	C	C	I	I	I	I	I
4558	2C	A	A	A	A	C	Q	Q	Q	Q	P
4802	2C	U	U	U	U	C	A	A	A	A	A
4949	2C	A	A	A	A	U	T	T	T	T	T
4968	2C	A	C	C	C	C	N	H	H	H	H
5193	3A	C	U	U	U	U	R	C	C	C	C
5217	3A	G	G	G	G	U	A	A	A	A	S
5336	3C	G	G	G	G	A	L	L	L	L	L
5715	3C	A	A	A	G	G	T	T	T	A	A
6427	3D	U	U	U	G	G	V	V	G	G	G
7256	3D	A	U	U	U	U	A	A	A	A	A

two mutations occurred in VP2 coding region (at nucleotide position 858 with exchange from G to U, resulting in amino acid exchange from A to S, at nucleotide position 1178 with silent exchange from C to U, resulting in no amino acid exchange), one mutation in 2B coding region (at nucleotide position 4022 with exchange from U to C, resulting in amino acid exchange from C to I), one mutation in 2C coding region (at nucleotide position 4968 with exchange from A to C, resulting in amino acid exchange from N to H), one in 3A coding region (at nucleotide position 5193 with exchange from C to T, resulting in amino acid exchange from R to C), one in 3D coding region (at nucleotide position 7256 with silent exchange from A to U). When H2K7 was passaged up to P12, 7 mutations appeared, in addition to 6 same mutations with P6, there was a new mutation appeared in 5' NTR at nucleotide position 591 resulting in a nucleotide exchange from A to G. P18 had 10 nucleotide mutations occurred in the entire genome, among 10 mutations 7 mutational changes were the same with P12, three new mutational changes appeared in genome, one in 5' NTR (one at nucleotide position 33 with nucleotide exchange from C to U), one in 3C coding region (at nucleotide position 5715 with exchange from A to G, resulting in amino acid exchange from U to A), one in 3D coding region (at nucleotide position 6427 with exchange from U to G, resulting in amino acid exchange from V to G). At P22 there were 18 nucleotide changes appeared in the genome, on the basis of P18 additional 8 nucleotide mutations appeared, two in 5' NTR (at nucleotide position263 with exchange from U to C, and 378 with exchange from U to C), three in 2C coding region (at nucleotide position 4 558 with exchange from A to C, resulting in amino acid exchange from Q to R, at nucleotide position 4802 with silent exchange from U to C, resulting in no amino acid exchange, at nucleotide position 4 949 with silent exchange from A to U), one in 3A coding region (at nucleotide position 5 217 with exchange from G to U, resulting in amino acid exchange from A to S), one in 3C coding region (at nucleotide position 5 336 with silent exchange from G to A), one in 3D coding region (at nucleotide position 7 256 with silent exchange from A to U).

Identity comparisons of genomes of different passages

Complete nucleotide sequence analysis of four-passage adaptive variants revealed an identity between H2K7 and P6 of 99.93 %, between H2K7 and P12 of 99.9 %, between H2K7 and P18 of 99.87 %, and between H2K7 and P22 of 99.76 %. The identity comparisons showed that mutations increased gradually with the increase of passages, but at high passage obvious mutations were concentrated in 5' NTR region, for example, P22 had 18 nucleotide mutations, 5 of 18 mutations appeared in 5' NTR region, the other mutations scattered throughout the coding region, during adaptive process no mutations appeared in VP4 coding region, VP3 coding region, VP1 coding region, 2A coding region, 3' NTR, the identities were 100 % (Table 5).

Table 5 Identitiy comparisons of nucleotide sequence of HAVH2K7 with HAVH2K7P6 and HAVH2K7P22

Genomic region	HAVH2K7P6 (%)	HAVH2K7P12 (%)	HAVH2K7P18 (%)	HAVH2K7P22 (%)
GENOME	99.93	99.9	99.87	99.76
5'UTR	100	99.86	99.59	99.32
VP4 ENCODING	100	100	100	100
VP2 ENCODING	99.71	99.71	99.71	99.71
VP3 ENCODING	100	100	100	100
VP1 ENCODING	100	100	100	100
2A ENCODING	100	100	100	100
2B ENCODING	99.74	99.74	99.74	99.74
2C ENCODING	99.9	99.9	99.9	99.6
3A ENCODING	99.42	99.42	98.42	98.84
3B ENCODING	100	100	100	98.53
3C ENCODING	100	100	99.85	99.85
3D ENCODING	99.94	99.86	99.86	99.86
3'UTR	100	100	100	100

DISCUSSION

Although the wild-type HAV grew poorly in cell culture, it usually did not cause CPE, and tended to establish persistent infection, after consecutive passages cell-adaptive variants were characterized with higher replication yield, rapid replication cycle and attenuation[19], and nucleotide mutations increased with adaptive passages[17]. On this basis, the development of a live attenuated hepatitis A vaccine (H2 strain) showed good immumogenicity and protective efficacy[36]. a variant of vaccine strain H2 was isolated in the consecutive passages in KMB17 cells without CPE and characterized with rapid replication cycle of 14 days, higher infectious titer of 7.8 $LgCCID_{50}ml^{-1}$.

From the viewpoint of evolution, cell adaptation of HAV depends on the interaction of HAV and cell, the internal cell environments form a selective pressure on the virus, in order to survive the viral genome gradually mutates to form new phenotype to adapt cell environments, so the accumulations of mutation with increasing passages result in more adaptive phenotype, more rapid replication cycle and higher virus yield. In accordance with this, in a continued passage process of vaccine strain H2 at passage K7 in KMB17 cells, with the increase of infectious titers the mutational numbers of genomes of different passages increased, P6 had 6 mutations with infectious titer of 6.77 $LgCCID_{50}ml^{-1}$, P12 had 7 mutations with infectious titer of 7.0$LgCCID_{50}ml^{-1}$, P18 had 10 mutations with infectious titer of 7.33 $LgCCID_{50}ml^{-1}$, P22 had 18 mutations with infectious titer of 7.8 $LgCCID_{50}ml^{-1}$. One-step growth dynamics of P22 also showed that 18 mutations of P22 resulted in shortening the replication cycle from 28 days to 14 days. The results suggested that although H2K7 was already an attenuated strain, the mutations of genome was not sufficient to completely adapt the KMB17, only further mutations increased its adaptive ability in cell KMB17. The sequencing and analysis of genomes of different passages of several cell culture-adapted variants of the HM175 strain of HAV also showed that with the increase of infectious titers the mutational numbers of genomes of different passages increased[9, 19, 24].

In contrast to the other viruses of picornaviruses, mutations of cell adaptation of HAV scattered throughout the whole genome and cooperatively acted to enhance the growth of virus in cells. sequencing of cell culture-adapted variants of the HM175 strain of HAV showed that P16 had 19 mutations[24], P35 had 25 mutations[19] and P59 had 42 mutations[9], P6 of cell culture-adapted variants of the H2 strain in KMB17 had 6 mutations, P12 had 7 mutations, P18 had 10, P22 had 18, although different strains in different cells had different mutations, these variants shared common mutations in 5' NTR and in 2B, 2C, 3A, 3B, 3C, 3D coding region of the genomes. Experiments with chimeric infectious cDNA clones indicated that the mutations in both 5' NTR and in 2B, 2C, 3A, 3B, 3C, 3D coding region contributed to the ability of the virus to grow in cells of African green monkey kidney lineage[11,12]. These results revealed that mutations in 5' NTR and in 2B, 2C, 3A, 3B, 3C, 3D coding region of the genomes played more important role than others in cell adaptation.

The 5' NTR of HAV forms a highly ordered secondary and presumably tertiary structure, and contains elements necessary for both viral translation and RNA replication[37], HAV translation is initiated in a cap-independent fashion by a mechanism that involves the binding of the 40s ribosomal subunit at a site located hundreds of bases downstream of the 5' end of the RNA which has been termed a ribosomal landing pad or an internal ribosome entry segment (IRES)[23,37,38,]. Mutations analysis of 5' NTR showed that the IRES of 450 nucleotides was located downstream of nucleotide (nt)161 and extended to within 40 nt of the first initiator AUG[38-40]. The efficient translation activity by IRES necessitates the interaction of secondary and presumably tertiary RNA structure with a set of specific eukaryotic translation initiation factors and also noncanonical host factors[39-41]. Therefore, it is likely that these 5' NTR mutations enhance translation by altering the affinity of the RNA for cellular proteins that either positively or negatively influence the activity of the IRES.

In our study in the continuous cell adaptation of H2K7, the sequencing showed that increased infectious titers with the increased passages of H2K7 in KMB17 cells correlated well with the increased mutations in 5' NTR. P6 had no mutations in 5' NTR with infectious titer of 6.77 $LgCCID_{50}ml^{-1}$, P12 had 1 mutation in 5' NTR (nt 591) with infectious titer of 7.0 $LgCCID_{50}ml^{-1}$, P18 had 3 mutations in 5' NTR (nt 33, 591, 646) with infectious titer of 7.33 $LgCCID_{50}ml^{-1}$, P22 had 5 mutations in 5' NTR (nt 33, 263, 378, 591, 646) with infectious titer of 7.8 $LgCCID_{50}ml^{-1}$, in addition to 1 mutation (nt 33 of P18 and P22), all mutations appeared in IRES. According to the viewpoint mentioned above, one mutation (nt 591) in IRES of P12 may change the secondary and presumably tertiary structure of 5' NTR of K7, this changed IRES structure enhance the affinity of K7 RNA with cellular proteins, resulting in the enhancement of translation, among 3 mutations in 5' NTR of P18, 2 mutations in IRES further enhance the affinity of K7 RNA with cellular proteins, resulting in further enhancement of translation, among all mutations in 5' NTR of different passages, 4 mutations in IRES of P22 produce the most efficient affinity of RNA with cellular proteins, yielding the maximal progeny virus. A comparison with the modelof 5' NTR of RNA secondary structure of the HAV genome, proposed by Ali *et al*[39], suggest that the G to U mutation (nt 646) of 5' NTR of P22, located in the terminal part of the 5' NTR (loop V), could lead to an RNA duplex folding stabilized by an additional basepairing, resulting in further increase of infectious titer. Consistent with our result, Graff *et al* also showed that increased mutations in IRES of different-passage GBM/Hp8/24 played a role in enhancement of infectious titer and a common G to U mutation (nt 646) of 5' NTR of strain GBM appeared in GBM/Hp8/6, but mutations of different positions of 5' NTR of different HAV strains in different cells may reflect the different interactions of 5' NTR with different cellular proteins. It is worth noting that P6 had no mutation in 5' NTR, but the infectious titer only reached 6.77$LgCCID_{50}ml^{-1}$, 0.9 $LgCCID_{50}ml^{-1}$ more than that at P1, indicating less important role of 5' NTR in early cell adaptation. Consistent with our result, Frings *et al*[42] found that after 5-passage adaptation of primate cell-adapted hepatitis A virus strain HM175 to its growth in guinea pig cells, the infectious titer reached 6.3 $LgCCID_{50}ml^{-1}$, but no mutation in 5' NTR. Taken together with our result, it suggest that during early adaptation there were different ways to improve HAV fit with a specific complement of host cell factors and that growth restrictions in defined host cells had not been determined solely by regulatory interactions of cellular factors with 5' NTR sequence, which concerned mainly cap-independent translation initiation.

Previous studies showed that 2B and 2C coding region mutations are essential for cell adaptation of HAV, mutations in other regions have no independent effect, but act cooperatively with mutations in 2B and 2C coding region to enhance replication[43]. 2B and 2C coding region are found to have 251 and 335 amino acids, respectively. Protein 2B is involved in the rearrangement of cellular membrane[3]. Protein 2C is considered to have helicase and NTPase activities[3]. The

functions of 2B and 2C suggest that the mutations in these two regions play important roles in cell adaptation of HAV. During continuous passages from P6-P22 of H2K7, 4 mutations appeared in 2C coding region, two were silent mutations, two resulted in two- amino acid substitutions, one was common in P6, P12, P18 and P22, one mutation at nt 4558 from A to C only appeared in P22, causing amino acid substitution from Q to P, this substitution may enhance the function of 2C, and further increase the infectious titer at P22. In contrast to the previous results[15], in sequence of entire genome of 4 passages (P6,P12,P18,P22), only one mutation appeared in 2B coding region at nt 4022 from U to C, but not resulting in amino acid mutation, this might suggest that 2B protein play less important role than 2C in KMB17 cell adaptation of H2K7.

Mutations in 3A coding region of the isolate in Italy correlated with CPE[35], but our result showed that although mutations in 3A coding region appeared, even a unique amino acid mutation from A to S occurred at P22, no CPE appeared at all passages of H2K7 in KMB17 cell culture. The mechanism remains unknown. The other mutations in 3A, 3C, 3D coding region of P22 may cooperatively act to further enhance the virus replication.

Comparisons of entire genome of P22 with those of continuous passages of GBM and HM175 in different cells showed that although the mutations existed in the same 5' NTR, 2B, 2C, 3A, 3B, 3C, 3D coding regions, most of the mutational sites were different, these differences revealed different interaction of HAV with different cellular proteins under different selective pressures.

From our investigations of sequence analysis of consecutive H2K7 passages and a comparison of their growth characteristics, we can conclude that 18-nt changes scattering over the genome are cooperatively responsible for further adaptation characterized by rapid and shortened replication cycle from 28 days to 14 days in KMB17 cells, the mutations in 2C coding region play more important roles in increase of infectious titer than other mutations, the mutations in 2B coding region show less important role than it usually does in cell adaptation, nucleotide changes in 5' NTR seem not to be relevant during initial stages (before P6) of cell adaptation because of the significant enhancement in infectious titer not correlating with the mutations in this region, but in late passage (P22) mutations in 5' NTR which seem to affect obviously the replication cycle of H2K7 in cell adaptation.

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• *H.pylori* •

Azithromycin in a triple therapy for *H.pylori* eradication in active duodenal ulcer

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Abstract

AIM: To assess and compare the efficacy and safety of two triple regimes: A) metronidazole, amoxicillin and omeprazole, which is still widely used in Russia, and B) azithromycin, amoxicillin and omeprazole in healing active duodenal ulcer and *H.pylori* eradication.

METHODS: 100 patients with active duodenal ulcer were included in the open, multicentre, randomized study with comparative groups. Patients were randomly assigned to one of the following one-week triple regimes: A) metronidazole 500 mg bid, amoxicillin 1 g bid and omeprazole 20 mg bid (OAM, *n*=50) and B) azithromycin 1 g od for the first 3 days (total dose 3 g), amoxicillin 1 g bid and omeprazole 20 mg bid (OAA, *n*=50). Omeprazole 20 mg od was given after the eradication course as a monotherapy for three weeks. The control endoscopy was performed 8 weeks after the entry. *H.pylori* infection was determined in the entry of the study and four weeks after the cessation of treatment by means of histology and CLO-test.

RESULTS: 97 patients completed the study according to the protocol (1 patient of the OAM group did not come to the control endoscopy, 2 patients of the OAA group stopped the treatment because of mild allergic urticaria). Duodenal ulcers were healed in 48 patients of the OAM group (96 %; CI 90.5-100 %) and in 46 patients of the OAA group (92 %; CI 89.5-94.5 %) (*p*=ns). *H.pylori* infection was eradicated in 15 out of 50 patients with OAM (30 %; CI 17-43 %) and in 36 out of 50 patients treated with OAA (72 %; CI 59-85 %) ($P<0.001$)- ITT analysis.

CONCLUSION: The triple therapy with omeprazole, amoxicillin and metronidazole failed to eradicate *H.pylori* in the majority of patients, which is an essential argument to withdraw this regimen out of the national recommendations. Macrolide with amoxicillin are preferable to achieve higher eradication rates. Azithromycin (1 g od for the first 3 days) can be considered as a successful component of the triple PPI-based regimen.

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INTRODUCTION

A number of antimicrobial agents have been used in various regimens to eradicate *Helicobacter pylori*. The properties of different medications may have some impact on the therapy result. Clinical trails are undertaken to search for simpler but equally effective (or more effective) regimen. The modern macrolides are in the focus of attention from that point of view. Azithromycin, a new generation macrolide, has some special attributes, that makes it a promising compound in the regimens for *H.pylori* eradication. It is acid-stable, has a long half-life and achieves remarkably high concentration in the gastric tissue. Thus after a single oral dose of 500 mg the concentration of azithromycin persisted in the gastric mucosa above the MIC_{90} for *H.pylori* over a five-day period[1]. There were several clinical trails with azithromycin in the therapy of *H.pylori* infection. As pharmacological properties of azithromycin make possible to use shorter courses, the problem was to define an optimal dose and duration of azithromycin in the triple therapy. The triple regimens with the total course dose of azithromycin of 1.5 g gave high eradication rates[2, 3], but the result was not stable[4], the total course dose of 3 g appeared to be more reliable[5, 6].

There were just a few studies of azithromycin in the treatment of *H.pylori* infection in peptic ulcer and chronic gastritis in Russia[7, 8]. The results were satisfactory, and it was clear that further studies to reach the level of evidence-based medicine are needed.

The clinical trial of azithromycin (Sumamed®, PLIVA) in the triple regimen for the eradication of *H.pylori* in active peptic ulcer, that was planned according to the GCP criteria, was of priority significance for Russia. The aim of the study was to assess and compare the efficacy of two triple regimes: (A) omeprazole, amoxicillin and metronidazole, and (B) omeprazole, amoxicillin and azithromycin in healing active duodenal ulcer and *H.pylori* eradication. The safety and tolerability of the two drug combinations were also evaluated and compared.

MATERIALS AND METHODS

Study design

It was an open, randomized study with comparative groups conducted in three Moscow gastroenterological centers: V.

Vasilenko Clinic of internal deseases, gastroenterology and hepatology of the Moscow Sechenov Medical Academy, Gastroenterological Center of the Health Ministry of Russia and Central Institution of Gastroenterology. The study protocol was worked out by PLIVA pharmaceutical company (Zagreb, Croatia).

The study was conducted according to GCP guidelines and the Helsinki Declaration. All patients gave written informed consent and the protocol was approved by the local Ethic Committees of the above-mentioned centers.

Out-patients and in-patients of both sexes aged between 18 and 70 years with endoscopically proven one or more duodenal ulcers were eligible for entry into the study. *H.pylori* presence before the treatment was detected by a rapid urea test and histology. In the CLO-test (Delta-West Ltd, Australia) two biopsy specimens (one from the antrum and one from the corpus on the greater curvature) were examined. The positive result of the CLO-test was needed to involve the patient into the study. Four biopsy specimens (two from the antrum and two from the mid-corpus on the greater and lesser curvature) underwent histopathological assessment. Sections of paraffin-embedded specimens were routinely stained with haematoxylin-eosin for morphologic examination and with Giemsa for *H.pylori* detection.

The information about the study was accessible to all patients before the entry. The written consent was necessary for participation in the clinical trial. The criteria for exclusion were: intake of proton pump inhibitors, antibiotic or bismuth salts within 4 weeks prior to the study, ulcer complications, concomitant gastric ulcer or reflux oesophagitis of grade II or more according to the classification of Savari et Miller, stomach surgery (except for a simple closure of perforation), known hypersensitivity to one of the study medications, severe concomitant diseases with metabolic changes, suspected poor compliance. Patients were required to be a male or nonpregnant, nonlactating females; females were postmenopausal or using a contraceptive.

At the entry the patients had a full physical examination. Routine haemotological and biochemical (serum creatinine, urea, transaminases, alkaline phosphatase, total and direct bilirubine) screening was carried out.

The patients,that satisfied the inclusion criteria,were randomly assigned to one of the following one-week triple regimes: (A) metronidazole 500 mg bid, amoxicillin 1 g bid and omeprazole (Losec®, AstraZeneca) 20 mg bid (OAM) and (B) azithromycin (Sumamed®, PLIVA) 1 g od for the first 3 days (total dose 3 g), amoxicillin 1 g bid and omeprazole (Losec®, AstraZeneca) 20 mg bid (OAA).

Omeprazole (Losec®, AstraZeneca) 20 mg od was given after the eradication course as a monotherapy for three weeks.

Control examination was performed 4 weeks after the cessation of omeprazole monotherapy (8 weeks after entry). Physical status, adverse events, haemotological and biochemical analysis, endoscopy (ulcer healing) were assessed. *H.pylori* infection was determined by histology and CLO-test: cure of the infection was established if two tests of all biopsy specimens gave negative results (two from the antrum, two from the corpus on the greater and lesser curvature for histology and one from the antrum, one from the corpus on the greater curvature for CLO-test).

Statistical analysis

The duodenal ulcer healing rates and *H.pylori* eradication rates were compared between the two treatment groups using a χ^2-test. A two-sided 95 % confidence interval (95 % CI) was calculated using the normal approximation to the binominal distribution.

Patient population

100 patients entered the trial: 50 patients were randomized to group A, and 50-to group B. The two treatment groups had similar demographic characteristics.

The patients at the entry usually had the dyspeptic symptoms typical for active duodenal ulcer. The ulcers localized in the duodenum bulb were between 0.3 cm and 1.5 cm in size (the two thirds of patents had ulcers between 0.5 cm and 1.0 cm in size). Three patients with two duodenal ulcers were randomized to group B.

RESULTS

49 patients (out of 50) of group A (metronidazole, amoxicillin and omeprazole) completed the study without contravention to the protocol: one patient was lost for the follow-up. Two patients of group B (azithromycin, amoxicillin and omeprazole) had mild allergic symptoms (urticaria) in the beginning of the eradication course and stopped treatment. A short use of antihistamine medications led to relief of allergy. Thus, 48 patients of group B (out of 50) completed the study according to the protocol.

The efficacy of treatment regimens was assessed by duodenal ulcer healing. Endoscopy performed 4 weeks after the cessation of omeprazole monoterapy revealed that duodenal ulcers healed in 48 patients of the OAM group and in 46 patients of the OAA group. The causes of ulcer persistence were failed *H.pylori* eradication and drop-out from the protocol. A patient with missing data from group A and two patients of group B (drop-outs due to adverse events) were included in the analysis for ulcer healing as “not healed”. Ulcer healing rate in group A was 96 % (CI 90.5-100 %) and in group B 92 % (CI 89.5-94.5 %). Statistical difference was not significant.

The main indicator of the triple regimen effectiveness was the rate of *H.pylori* eradication. Eradication rates were estimated for the population that completed the study according to the protocol (per protocol analysis) and for the population that was involved in the study (intention-to-treat analysis). One patient of group A, that did not come to the control examination, and two patients of group B, withdrawn from the study because of the adverse events, were estimated as a negative eradication result. *H.pylori* infection was eradicated in 15 out of 50 patients with OAM: eradication rate was 30.6 % (95 % CI: 17.6 %- 43.6 %) PP and 30 % (95 % CI: 17-43 %) ITT. *H.pylori* infection was eradicated in 36 out of 50 patients treated with OAA: eradication rate was 75 % (95 % CI: 63-87 %) PP and 72 % (95 % CI: 59-85 %) - ITT analysis (Figure 1).

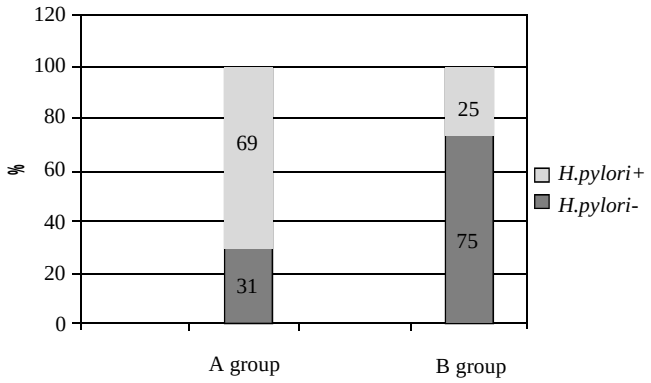

Figure 1 The results (per protocol) of *H.pylori* eradication in the treatment groups.

The difference between the treatment groups was statistically significant: $P<0.001$.

The treatment safety was assessed by adverse events recording, the laboratory tests deviation of clinical significance were also taken into consideration. The two cases of withdrawal because of an allergic urticaria were registered in group B. Quick relief of the allergic symptoms due to the antihistamine preparations allowed us to consider this adverse events as mild. The laboratory parameters of haemotological and biochemical tests (serum creatinine, urea, transaminases, alkaline phosphatase, total and direct bilirubine) were usually normal. There was no clinically significant changes from the baseline in the laboratory results.

DISCUSSION

The new triple regime for *H.pylori* eradication with new macrolide azithromycin was compared with the combination of metronidazole, amoxicillin and omeprazole, which is still widely used in Russia.

Both combinations were highly effective in ulcer healing. The analysis of cases with persistent duodenal ulceration revealed that most of them were connected with protocol nonfulfilment and failed eradication. High healing rates are expected results of triple therapies based on proton pump inhibitors. Both proton pump inhibitors action and the effect of *H.pylori* eradication are of importance in ulcer healing. Persistence of *H.pylori* infection in gastric mucosa is considered now as one of the factors preventing ulcer healing, or to be more precise, as a factor of distortion of the normal regeneration process[9]. This molecular events are not so evident in every-day clinical practice as potent effect of omeprazole. Proton pump inhibitors-based triple therapies even without "posteradicational" antisecretory monotherapy are advantageous in active duodenal ulcer. Rapid symptoms relief and ulcer healing in 96 % patients of group A and in 96 % patients of group B (per protocol) once more proved the effectiveness of eradication therapy based on omeprazole. Rapid ulcer healing did not depend on antibiotic composition.

There are no therapies that eradicate *H.pylori* infection in every case. That is why the search for better anti-*H.pylori* regimes is of present interest. Clinical experience of the Russian trials that are organized according to the principals of evidence-based medicine is of great importance. They give information about the possibility of application of foreign data to the Russian patient population. The therapy used in group A "metronidazole 500 mg bid, amoxicillin 1 g bid and omeprazole 20 mg bid" is widely used in the Russian practice. This regime is well-known to general practitioners and is included in "The guidelines for the management of *Helicobacter pylori* infection" of the Russian *Helicobacter pylori* Study Group and Russian Gastroenterological Association, adopted in 1997[10]. Since that time there were some reports that informed about very low eradication rates with proton pump inhibitor, metronidazole and amoxicillin[11, 12]. The present clinical trial demonstrated (by the example of the patients from three Moscow gastroenterological centers) discouraging eradication rate in the OAM triple therapy. This is a real argument in favour of withdrawing regime "proton pump inhibitor, metronidazole and amoxicillin" from the national guidelines as it was done in the Maastrcht 2-2000 European Consensus report[13].

Antimicrobial resistance of *H.pylori* strains is one of the main causes of treatment failure. *H.pylori* resistance to nitroimidazoles (metronidazole and tinidazole) is quite an often event in the Russian populations. Thus, in Moscow *H.pylori* strains with primary metronidazole resistance were found in more than 50 % of isolates[14]. Unsuccessful anti-*H.pylori* course of metronidazole-containing regimen usually leads to secondary resistance. One-third of the patients in the present study had a long duration of peptic ulcer disease (>5 years), almost all of them had used metronidazole. *H.pylori* susceptibility testing was not considered in the present trial, but we could suspect, with high probability, nitroimidazole resistance as a cause of eradication failure in two-thirds of the group A patients.

The proton pump inhibitor-based triple therapy with amoxicillin and clarithromycin gives steady high eradication rates. The European clinical trial MACH1 demonstrated the best result using omeprazole 40 mg with amoxicillin 1 g bid and clarithromycin 500 mg bid among five omeprazole-based combinations with different antimicribials for seven days[15]. The proton pump inhibitor (or ranitidine bismuth citrate) in combination with clarithromycin 500 mg bid and amoxicillin 1 g bid were named the preferable regimen for first-line eradication therapy in the Maastrcht-2 Consensus report[13]. The real chance to enhance the eradication rates in the countries with high levels of metronidazole resistance is to avoid metronidazole in the anti-*H.pylori* treatment. Provided the levels of clarithromycin resistance are low the macrolide and amoxicillin regimens would be the most beneficial.

The treatment regimen of group B "azithromycin 1 g od for the first 3 days, amoxicillin 1 g bid and omeprazole 20 mg bid for 7 days" eradicated *H.pylori* infection in 72 % of patients, the result is good for the Russian populations. This rate of *H. pylori* eradication seems to be lower that is acceptable. But according to some publications it is quite common with accepted PPI-based triple therapies. Thus J.P.Gisbert *et al*[16] . gave mean eradication rate (weighted mean) as 71 % for ITT analysis for 7-day to 14-day omeprazole-based therapies.

H.pylori has cross resistance to macrolides: the strain resitant e.g. to clarithromycin is resistant to every other macrolide. The level of clarithromycin resistance in Moscow is 8-14 %, unfortunately with the tendency for an increase[14]. The effect of drug synergism is of great value in combination treatment to heal *H.pylori* infection. P.M.Lepper *et al*[17].demonstrated in vitro synergistical effect of azithromycin and proton pump inhibitor lansoprazole. They speculate that this effect may enhance eradication rates even with macrolide-resistant *H.pylori* strains because of the unique pharmacological properties of the combination. Azithromycin could provide a potent anti-*H.pylori* effect and could simplify the bulky triple therapy. Of macrolides azithromycin develops the highest concentration in gastric tissue and mucus, its pharmacokinetic properties makes it possible to take azithromycin only once a day and only during three days in a week course. Clarithromycin for standard eradication is administered for 7 days twice a day (usually 4 tablets of 250 mg). Azithromycin is really an advantageous medication to reach simpler therapy, improving both tolerability and compliance. The correctness of the azithromycin dose chosen in our trial -1 g daily for three days - was confirmed by recent results[18].

In conclusion, we have shown that azithromycin has clinical (*in vivo*) activity against *H.pylori* infection. Azithromycin (1 g od for the first 3 days in a week course) can be considered as a successful component of the triple proton pump inhibitor-based regimen. It is necessary to eradicate *H.pylori* in peptic ulcer patients, using effective and simple regimens.

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• BASIC RESEARCH •

Estrogen reduces CCL_4- induced liver fibrosis in rats

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Abstract

AIM: Chronic liver diseases, such as fibrosis or cirrhosis, are more common in men than in women. This gender difference may be related to the effects of sex hormones on the liver. The aim of the present work was to investigate the effects of estrogen on CCL_4-induced fibrosis of the liver in rats.

METHODS: Liver fibrosis was induced in male, female and ovariectomized rats by CCL_4 administration . All the groups were treated with estradiol(1 mg/kg) twice weekly. And tamoxifen was given to male fibrosis model. At the end of 8 weeks, all the rats were killed to study serum indicators and the livers.

RESULTS: Estradiol treatment reduced aspartate aminotransferase(AST) , alanine aminotransferase (ALT), hyaluronic acid(HA) and type IV collagen(CIV) in sera, suppressed hepatic collagen content, decreased the areas of hepatic stellate cells (HSC) positive for α-smooth muscle actin (α-SMA), and lowered the synthesis of hepatic type I collagen significantly in both sexes and ovariectomy fibrotic rats induced by CCL_4 administration. Whereas, tamoxifen had the opposite effect. The fibrotic response of the female liver to CCL_4 treatment was significantly weaker than that of male liver.

CONCLUSION: Estradiol reduces CCL_4-induced hepatic fibrosis in rats. The antifibrogenic role of estrogen in the liver may be one reason for the sex associated differences in the progression from hepatic fibrosis to cirrhosis.

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INTRODUCTION

Estrogen is frequently used for anticonception and treatments of menopausal disorders. Its clinical use has increased steadily during the last years due to reports of decreased morbidity and mortality during postmenopausal estrogen treatment[1]. There have been reports of decreased morbidity in cardiovascular disease, suggesting estrogenic effects on tissues other than on the classic reproductive organs.

Population data have long suggested that chronic liver disease progresses at unequal rates in both sexes for viral hepatitis and other forms of injury with a similar incidence in males and females. In chronic viral hepatitis the major sequelae, such as fibrosis or cirrhosis, are more common in men than in women[2,3]. Although establishing the actual rate of fibrosis in a patient would require serial liver biopsy, which is seldom done, a reasonable approximation can be inferred from the incidence of fibrosis-related complications[4-6]. The development of cirrhosis is more common in men than in women(2.3 to 2.6:1). Although the liver is not a classic sex hormone target, livers in both men and women have been shown to contain estrogen receptors and respond to estrogens by regulating liver function. Therefore, sex hormones may play a role in the progression from hepatic fibrosis to cirrhosis. It showed that estradiol treatment resulted in reducing hepatic fibrosis in rats induced by dimethylnitrosamine(DMN). However, much current evidence suggests that women develop alcoholic liver disease at lower levels of alcohol intake and over a shorter period of time as compared to men. In other words, females are more susceptible to alcohol-induced liver injury than males[7]. The specific mechanisms concerning a gender-related difference in susceptibility are largely unknown.

CCL_4-induced fibrosis shares several characteristics with human fibrosis of different etiologies; thus, it is an adequate model of human fibrosis[8-10] . The aim of the present work was to study the effects of estrogen on CCL_4-induced fibrosis of the liver in rats, and to investigate the possible mechanisms.

MATERIALS AND METHODS

Animals

Male and female Spratgue-Dawley rats (Experimental Animal Holding Unit of Shaanxi Province, China) were housed in a temperature-humidity-controlled environment with 12-h light-dark cycles (lights on from 07:00 to 19:00) and had unrestricted access to food and water. Forty male rats, weighing 220±21 g, corresponding to an age of approximately 10 weeks, were divided into four groups of ten each. For CCL_4 group, 400 mL/L CCL_4 in peanut oil were injected subcutaneously at a dose of 2 ml/kg twice weekly, and the first dosage was doubled. The estrogen group, apart from the used of CCL_4, was treated subcutaneously with estradiol 1 mg/kg twice weekly (The Ninth Pharmaceutical Plant of Shanghai,China). The anti-estrogen group, along with the CCL_4 treatment described above, was given Tamoxifen 6 mg/kg every day orally (The First Pharmaceutical Plant of Suzhou, China). The rats were fed a modified high fat diet containing 5 g/kg cholesteral and 200 g/kg pig oil. The control group was given normal food and water, and received injection of peanut oil vehicle twice weekly.

Fifty female rats 10 weeks old, weighing 208±17 g, were divided into five groups with ten each. The ovariectomy (Ovx) group was initiated with a bilateral ovariectomy and the sham operation group was initiated with just a sham operation. The

two estrogen groups, with bilateral ovariectomy and with sham operation, were treated subcutaneously with estradiol (1 mg/kg twice weekly). All of the above four groups received 400 ml/L of CCL_4 in peanut oil at a dose of 2 ml/kg twice weekly, and were fed with a high fat diet containing 5 g/kg cholesteral and 200 g/kg pig oil. The CCL_4 and estradiol were used after 2 weeks of operation. The control group was given normal food and water, and received injection of peanut oil vehicle twice weekly.

At the end of the 8-week experimental period, all the rats were fasted overnight and put to death by cervical dislocation after anaesthetised by intramuscular injection of sodium pentobarbital (40 mg/kg). Blood was collected from the animals and the serum obtained was analysed. The liver was removed rapidly.

Estimation of serum indicators

In serum, activities of aspartate aminotransferase(AST) and alanine aminotransferase (ALT) were assayed by a 917-Hitachi Automatic Analyzer. Serum hyaluronic acid(HA) and type IV collagen(CIV) concentrations were measured radioimmunologically using commercial kit(Shanghai Navy Medical Institute, China).

Parameters of hepatic antioxidation

Parameters of antioxidation in the liver was determined by measuring the levels of hepatic malondialdehyde (MDA) and superoxide dismutase (SOD) (kit: Jiancheng Medical Institute, Nanjing, China).

Histopathological study

Excised liver tissues from each rat were fixed in 100 ml/L neutral formalin, embedded in paraffin, and stained with hematoxylin-eosin(HE) and masson's trichrome. The evaluation of hepatic fibrosis was determined by a semi-quantitative method to assess the degree of histologic injury in chronic hepatic fibrosis[11,12].

Immunohistochemical examination

Liver tissue sections were mounted on slides, deparaffinized in xylene, and rehydrated in alcohol. The level of α-smooth muscle actin (α-SMA) (Neomarkers,USA), type Ⅰ collagens (Boster, Wuhan, China), transforming growth factor β_1 (TGF β_1) and platelet -derived growth factor(PDGF) (Dako,USA) were determined by immunohistochemical methods in female groups. Based on the extent of histological staging, the α-SMA, type Ⅰ collagens, TGFβ_1 and PDGF positive cells were expressed as a percentage of the total area of the specimen.

Statistical analysis

Data are presented as $\bar{x}\pm s$ unless otherwise indicated. The Mann-Whitney *u* test for nonparametric and unpaired values, student's *t*-test or Fisher's exact test was used as appropriate. Results were considered significant when $P<0.05$.

RESULTS

Changes of serum indicators and hepatic antioxidation date

At the end of 8-week experimental period, 8 rats were dead because of infection at the region of injection and hepatic crack by unsuitable handling. Table 1 gives the values for the activities of the serum indicator enzymes, the markers of hepatic fibrosis, and the hepatic antioxidation data.

It is evident that CCL_4 produced a marked increase in the activities of serum ALT and AST in both male and female rats. Although the extent of that was lower in female group than in male group, it was not statistically significant ($P>0.05$). The CCL_4 plus estradiol group showed a significant decrease in the enzyme levels, but the levels were still higher than those of control groups. In ovariectomy rats, when CCL_4 were given, the enzyme levels were higher than those of the sham operation rats in both estradiol used or none-used groups, but the differences were not statistically significant($P>0.05$). The levels of serum ALT and AST in Tamoxifen group were significantly higher than those of the CCL_4 group and estrogen used groups.

As for the changing trend of fibrotic markers in serums, HA and CIV were similar with those of the enzyme levels in all groups. The results showed that the levels of HA and CIV in CCL_4 used groups were significantly higher than those of control groups, especially in male group. Tamoxifen could increase the extent of that and estrogen could decrease it significantly. In ovariectomy groups, the HA and CIV were significantly higher than those of shame operation groups.

Tissue antioxidation date indicated that MDA was increased and SOD was decreased in CCL_4 treated rats significantly, whereas estradiol had the opposite effect. The MDA was higher and SOD was lower in males than those of females treated with CCL_4.

Table 1 Liver enzymes, serum fibrosis indicators and hepatic antioxidation ($\bar{x}\pm s$)

Group	*n*	ALT (nkat/L)	AST (nkat/L)	HA (μg/L)	CIV (μg/L)	MDA (nmol/g)	SOD (kNU/g)
Males							
Control	10	31±6	66±18	115±31	18±5	3.1±0.9	8.0±1.6
CCL_4	10	576±262[a]	699±241[a]	530±122[a]	54±14[a]	10.5±3.4[a]	2.2±1.1[a]
CCL_4+E	8	355±125[c]	314±179[c]	232±78[c]	30±10[c]	6.6±2.8[c]	5.0±1.6[c]
CCL_4+Tam	8	884±294[c]	1073±453[c]	703±187[c]	69±15[c]		
Females							
Control	10	35.8±7.9	64.5±20.8	121±26	17±4	3.0±0.8	8.7±2.8
CCL_4	9	540±252[a]	631±268[a]	388±81[a]	41±11[a]	7.1±2.1[a]	4.0±1.5[a]
CCL_4+Ovx	10	658±220	697±240	586±145[c]	53±14[c]	9.1±2.9[c]	2.8±1.0[c]
CCL_4+E	8	314±163[c]	302±153[c]	267±83[c]	29±7[c]	4.7±2.2[c]	5.9±2.0[c]
CCL_4+Ovx+E	9	311±146[c]	321±121[c]	236±119[c]	31±7[c]	4.8±2.3[c]	6.2±2.5[c]

[a]$P<0.05$, *vs* control; [c]$P<0.05$, *vs* CCL_4

Histopathological and immunohistochemical changes

The control livers showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A). Prolonged administration of CCL_4 causes severe pathological damages: inflammation, necrosis, and collagen deposition (Figure 1B,male). The semiquantitative hepatic collagen staging value was 3.3±0.7 in males, and 2.4±1.1 in females. It was showed that the staging value was significantly decreased in female rats. After administration of estradiol, the extent of hepatic fibrosis was significantly weaker than that of CCL_4 groups (Figure 1C:male): the semiquantitative staging value was 2.0±1.1 in males and 1.6±0.9 in females respectively. Ovariectomy significantly increased the staging value (3.1± 0.7). Moreover, the staging value was highest when given Tamoxifen to the experimented rats (3.7±0.5 Figure 1D).

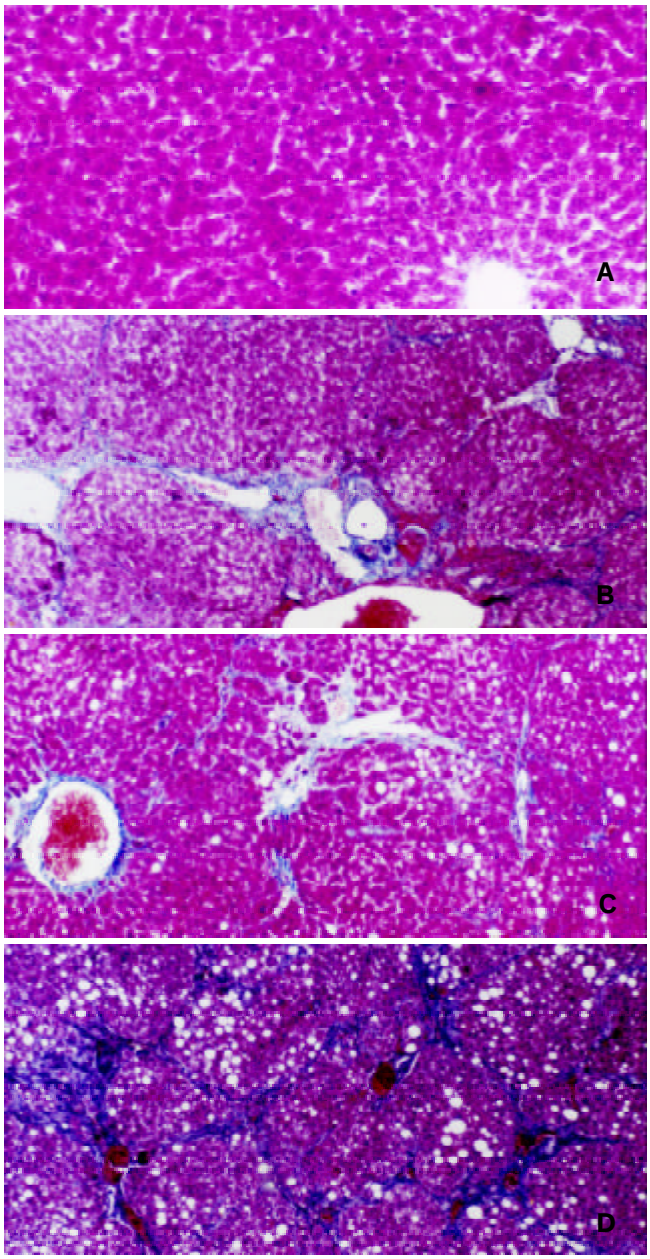

Figure 1 Effects of estradiol and tamoxifen on the histology of CCL_4 induced fibrotic rat liver. Masson trichome stain, scale bar=40μm, original magnification,×100
1A: Normal rat liver; 1B: CCL_4 group shows fibrosis; 1C:Estrogen group with less fibrosis than in group B; 1D: Tamoxifen group shows marked fibrosis than in group B.

Analysis of α-SMA, an activation marker of rat hepatic stellate cells (HSC) by immunohistochemistry showed staining in vascular smooth muscle cell of control rat livers, but not in sinusoids. In the CCL_4 model, the positive cells of α-SMA, type I collagen, $TGF\beta_1$ and PDGF within centrilobular and periportal fibrotic bands. The percentage areas of these staining in the liver of female rats were showed in Figure 2. The results suggest that administration of CCL_4 significantly increased the percentage areas of all of the four marker staining. Ovariectomy group has a marked higher percentage area than that of CCL_4 group and it could be significantly suppresed by estradiol used.

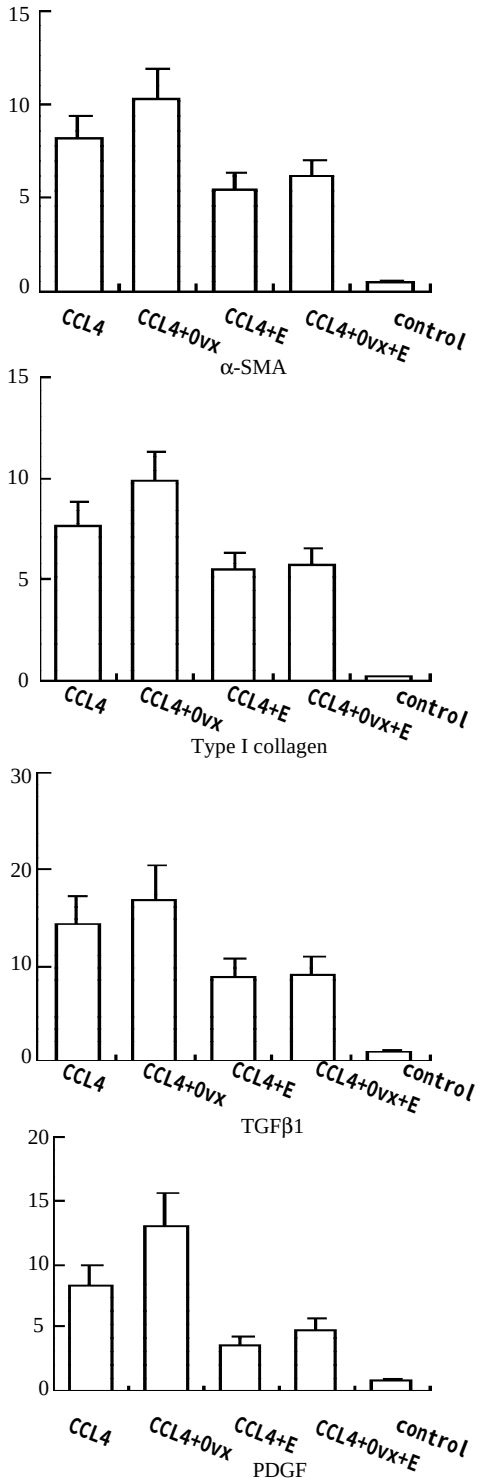

Figure 2 Percentage area (%) of α-SMA, type I collagen, $TGF\beta_1$, and PDGF in female rats

DISCUSSION

Hepatic fibrosis is usually initiated by hepatocyte damage, leading to recruitment of inflammatory cells and platelets, activation of kupffer cells and subsequent release of cytokines and growth factors (e.g. TGFβ_1 and PDGF)[13,14]. These factors probably link the inflammatory and reparative phase of liver cirrhosis, by activating HSC[15-18]. Upon activation, HSC proliferate and transform into myofibroblast-like cells that deposit large amounts of connective tissue components[19-27].

The present study showed that estradiol reduces CCL_4-induced hepatic fibrosis in rats. Estradiol administration reduces HA and CIV in serums, suppresses hepatic collagen content, reduces the areas of HSC positive for α-SMA, and lowers the synthesis of hepatic type I collagen in both sexes. The fibrotic response of the female liver to CCL_4 treatment was significantly weaker than that of male liver. It suggested that physiological levels of estrogen have an antifibrogenic effect. These effects of estrogen were also confirmed by ovariectomy in female rats at the time of CCL_4 administration. These findings suggest that the antifibrogenic role of estrogen in the liver may be one reason for the sex associated differences in the progression from hepatic fibrosis to cirrhosis.

Hepatic fibrogenesis is often associated with hepatocellular necrosis and inflammation accompanied by the repair processes[28-29]. Chronic administration of CCL_4 caused fibrosis as indicated by an increase in serum marker enzymes. Raised serum enzyme levels in CCL_4-injected rats can be attributed to the damaged hepatocellulal structural integrity[30-32]. The administration of estradiol in this study seems to decrease the serum enzymes (ALT, AST), and then preserve the structural integrity of the hepatocellular membrane. Decreased hepatocyte damage suppressing the stimulant effect to the Kupffer cells and subsequent by lower the HSC activation[33].

Peroxidation of lipids can dramatically change the properties of biological membranes, resulting in severe cell damage and could play a significant role in the pathogenesis of disease. It has showed that lipid peroxidation, free-radical-mediated process, and certain lipid peroxidation products induce genetic overexpression of fibrogenic cytokines and increase the synthesis of collagen. Free radicals and MDA can stimulate the synthesis of collagen and initiate the activation of HSC[34,35]. Relevant to the latter findings, estradiol and its derivatives are strong endogenous anti-oxidants that reduce lipid peroxide levels in liver and serum. Free radicals, generated mainly by Kupffer cells, are thought to cause tissue injury by initiating lipid peroxidation and irreversibly modifying membrane structure. The present study shows that CCL_4 administration leads to parallel increase in MDA and collagen, and subsequent decrease in SOD, a radical scavenging agent, and that estradiol had the opposite effect. These findings suggest that the antifibrotic effect of estradiol may be caused, at least in part, by its radical scavenging action or antioxidant activity.

Cell proliferation, α-SMA expression, retinoid disappearance, and the formation of collagens and other ECM materials are characteristics of the activated phenotype of HSC[36]. So HSC are regarded as the primary target cells of hepatic fibrogenesis. Studies in vitro have shown that HSC and their activated counterparts may be induced to proliferate by polypeptide growth factors and cytokines, such as TGFβ_1 and PDGF[37,38]. As these growth factors are produced by infiltrating inflammatory cells, Kupffer cells and sinusoidal endothelial cells, they might act as paracrine mediators which trigger the transformation of HSC *in vivo*. The present data show that estradiol suppresses HSC proliferation and parallel with inhibit TGFβ_1 and PDGF expression in CCL_4-induced fibrosis in male and female rats. These findings suggest that estrogen may exert its suppressive effect on hepatic fibrosis by indirectly modulating the synthesis and releasing of cytokines and other growth factors which in turn altering HSC activation and proliferation.

Chronic fibrotic diseases can differ from each other in etiology. But, in terms of pathogenesis, they share some basic common features[1]. For instance, three serious chronic disease-atherosclerosis, glomerulosclerosis, and liver fibrosis-may appear very different in their development. In all three, though, a central, and indeed an essential, role is playd by macrophages and by ECM-producing cells: smooth muscle cells (SMC) in atherosclerosis, mesangial cell in glomerulosclerosis, and HSC in liver fibrosis. The three types of cells have many properties in common both structurally and functionally, including a changes in cell biology with deposition of matrix proteins. Therefore, factors which affect the development of atherosclerosis or glomerulosclerosis may affect liver fibrossis by similar mechanisms. Studies show that estradiol suppresses atherosclerosis and glomerulosclerosis in rats by directly affecting the estrogen receptor on SMC and mesangial cell. Livers in both male and Female rats have shown to contain high affinity, low capacity estrogen receptors and respond to estrogen by regulating liver function. In the present study, Tamoxifen, an antiestrogen act by occupying the estrogen-binding site of the receptor protein, increases fibrogenesis in CCL_4-induced fibrosis of the liver. It suggests that estradiol may suppress hepatic fibrosis also by a direct receptor mechanism. This remains to be confirmed.

In conclusion, estrogen may play an important role as an endogenous fibrosuppressant, accounting for sex-associated differences in the progression from hepatic fibrosis to cirrhosis. The following mechanisms have been hypothesized to explain the antifibrogenic effect of estrogens: (A) a hepatocellular membrane protection and radical scavenging action. (B) a modulation of HSC proliferation and collagen synthesis, (C) a modulation in the expression of pro-and anti-fibrogenic cytokines and may be (D) a estrogen receptor mechanism. However, the real importance of these mechanisms is still to be elucidated.

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• BASIC RESEARCH •

Establishment of a transgenic cell line stably expressing human cytochrome P450 2C18 and identification of a *CYP2C18* clone with exon 5 missing

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Abstract

AIM: The human cytochrome P-450 2C18(CYP2C18) has been characterized. However, the protein has not been purified from liver and very little is known regarding the specific substrate of CYP2C18. In order to study its enzymatic activity for drug metabolism, the *CYP2C18* cDNA was cloned and a stable CHL cell line expressing recombinant CYP 2C18 was established.

METHODS: The human *CYP2C18* cDNA was amplified with reverse transcription-polymerase chain reaction (RT-PCR) from total RNAs extracted from human liver and cloned into pGEM-T vector. The cDNA segment was identified by DNA sequencing and subcloned into a mammalian expression vector pREP9. A transgenic cell line was established by transfecting the recombinant plasmid of pREP9-*CYP2C18* to Chinese hamster lung (CHL) cell. The enzyme activity of *CYP2C18* catalyzing oxidation of tolbutamide to hydroxytolbutamide in postmitochondrial supernant(S9) fraction of the cell was determined by high performance liquid chromatography(HPLC).

RESULTS: The amino acid sequence predicted from the cloned cDNA segment was identical to that of reported by Romkes *et al* (GenBank accession number: M61856, J05326). The S9 fraction of the established cell line metabolizes tolbutamide to hydroxytolbutamide. Tolbutamide hydroxylase activity was found to be 0.509 ± 0.052 $\mu mol \cdot min^{-1} \cdot g^{-1}$ S9 protein or 8.82 ± 0.90 $mol \cdot min^{-1} \cdot mol^{-1}$ CYP, but was undetectable in parental CHL cell. In addition, we have identified a *CYP2C18* cDNA clone with exon 5 missing.

CONCLUSION: The cDNA of human *CYP2C18* was successfully cloned and a cell line, CHL-CYP2C18, efficiently expressing the protein of *CYP2C18*, was established. A spliced variant of CYP2C18 with exon 5 missing was identified in the cloning process.

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INTRODUCTION

Cytochrome P450 (CYP) is a heme-containing enzyme widely distributed from bacteria to mammals, and catalyzes the oxidative and reductive metabolism of a wide variety of compounds including endogenous as well as exogenous compounds. Mammalian CYP present in liver microsomes is characteristic of its nature in metabolizing exogenous compounds including drugs, pesticides, environmental pollutants, and carcinogens[1]. Mammals contain at least 17 distinct *CYP* gene families that together code for an estimated 50-60 individual *CYP* genes in any given species[2]. The human CYP2C subfamily comprises four members, CYP2C8, CYP2C9, CYP2C18 and CYP2C19[3], accounting for 20 % of the total CYP in human liver. CYP2C18 mRNA was found in liver, albeit at mean levels 7-8-fold lower than those of mRNAs encoding CYP2C8 and CYP2C9[4,5]. The cDNA encoding human *CYP2C18* has been characterized, but the protein has not been purified from liver, and very little is known regarding the specific substrate of CYP2C18[4].

The combination of gene technology and cell culture technology has provided new opportunities for studying proteins because any gene from any species encoding a protein may be cloned and expressed in bacterial, yeast, or mammalian cell in a defined way[6-11]. This approach in drug metabolism is of particular importance because some of the enzymes are difficult to purify and to prepare in sufficient quantities, for its low expression levels, organ-specificity of its expression, or low abundance of native organ material. These restrictions apply especially for human enzymes. The heterologous expression of the cDNA bypasses these restrictions[12]. The human *CYP2C18* cDNA had been expressed in yeast[13,14], COS-1 cells[3], lymphoblast cells[15], and human liver epithelial cells THLE[16]. Several cell lines stably expressing human CYP1A1[17],CYP2B6[17],CYP2A6[18], CYP3A4[19],CYP2C9[20] and a phase II metabolism enzyme UDP-glucuronosyltransferase, UGT1A9[21] have been established in our laboratory. In this study human *CYP2C18* cDNA was amplified with reverse transcription-polymerase chain reaction (RT-PCR), and a transgenic cell line stably expressing CYP2C18 was established. In the cloning process, we have identified a spliced variant of CYP2C18 with exon 5 missing.

MATERIALS AND METHODS

Materials

Restriction endonucleases, moloney murine leukemia virus (M-MuLV) reverse transcriptase were supplied by MBI Fermentas AB, Lithuania. PCR primers, DNA sequence primers, random hexamer primer, Taq plus I, and dNTPs were synthesized or supplied by Shanghai Sangon Biotechnology Corp. DNA sequencing kit was purchased from Perkin-Elmer Corp. The TRIzol Reagent, G418, Minimum Essential Media(MEM) and newborn bovine calf sera were from Gibco. NADPH was from Roche molecular biochemicals. Diethyl pyrocarbonate (DEPC), tolbutamide and hydroxytolbutamide were purchased from Sigma Chemical Company. T4 DNA ligase and pGEM-T vector system were from Promega. Other chemical reagents used are all of analytical purity from the commercial sources.

Methods

Cloning of human CYP2C18 cDNA from a Chinese human liver The total RNA was extracted from a surgical specimen of human liver with TRIzol reagent according to the manufacture' s instructions. The RT-PCR amplifications were described before[20]. Two specific 28 mer oligonucleotide PCR primers were designed according to the mRNA sequence of CYP2C18 reported by Romkes *et al*[3] (GenBank accession no. M61856, J05326). The sense primer corresponding to base position 38 to 65 was 5' -TTATCTTCTTCAGCTAGCCAATGTTCAT-3' , with a restriction site of *Nhe* I, and the anti-sense primer, corresponding to the base position from 1681 to 1708, was 5' -TGACAGCACTCGAGCAGCCAAACTATCT-3' , with a restriction site of *Xho* I. The PCR was performed at 94 ℃ 2 min, then 35 cycles of 94 ℃ 60 s, 55 ℃ 60 s, 72 ℃ 2 min, and lastly 72 ℃ 10 min. An aliquot (10 ml) from the PCR was subjected to electrophoresis in a 1 % agarose gel stained with ethidum bromide.

Construction of recombinant pGEM-CYP2C18 and sequencing of CYP2C18 cDNA[22] The PCR products were ligated with pGEM-T vector, and transformed to the *E.coli* DH5a. Several cDNAs of *CYP2C18* cloned in pGEM-T were sequenced on both strands by dideoxy chain-termination method marked with BigDye with primers of T7 and SP6 promoters and two specific primers of 5' -GGACATGAGCAAATCCTTA-3' (343-361), and 5' -TGGGGATGAGGTAGTTTTTG-3' (1327-1346). The termination products were resolved and detected using an automated DNA sequencer (Perkin-Elmer-ABI Prism 310).

Construction of the pREP9 based expression plasmid for CYP2C18[22] The *Nhe* I/ *Xho* I fragment having the total span of human *CYP2C18* cDNA and the correctly deduced amino acids sequence in pGEM-*CYP2C18* was subcloned to a mammalian expression vector pREP9 (Invitrogen). The recombinant was transformed to *E.coli* Top 10, and screened by ampicillin resistant. The recombinant was identified by restriction mapping.

Transfection and selection[20,22] Chinese hamster lung (CHL) cells were transfected with the resultant recombinant plasmid, pREP9-*CYP2C18*, using a modified calcium phosphate method. A tansgenic cell line named CHL-*CYP2C18* was established.

Preparation of postmitochondrial supernant (S9) of CHL-CYP2C18 The procedure of preparation of the S9 fraction was described before[20]. The protein in S9 was determined by the Lowry' s method, with bovine serum albumin as standard. CYP was measured spectrally using the method of Johannesen *et al*[23].

Tolbutamide hydroxylase assays[20,24,25] The CYP2C18 tolbutamide hydroxylase activity of S9 was determined by high performance liquid chromatography (HPLC) as described before[20] .

RESULTS

Construction of recombinants and detection of an exon 5 skipping in transcripts of the human CYP2C18 gene

The recombinant of pGEM-*CYP2C18* (Figure 1) was constructed with the human *CYP2C18* cDNA inserted into the cloning site of pGEM-T vector. Selection and identification of the recombinant was carried out by *Nhe* I/ *Xho* I endonuclease digestion and agarose gel electorphoresis (Figure 2). Two cloned cDNA segments were sequenced completely. Comparing with the cDNA sequence reported by Romkes *et al*[3] (GenBank accession no. M61856, J05326), one has two base differences, 222T>C, 828C>T, while the encoding amino acid sequence was the same, I74 and H276. However, another cDNA clone was found to have only 8 exons (Figure 3). There is no base difference between this cDNA and the wild type one except the missing exon 5.

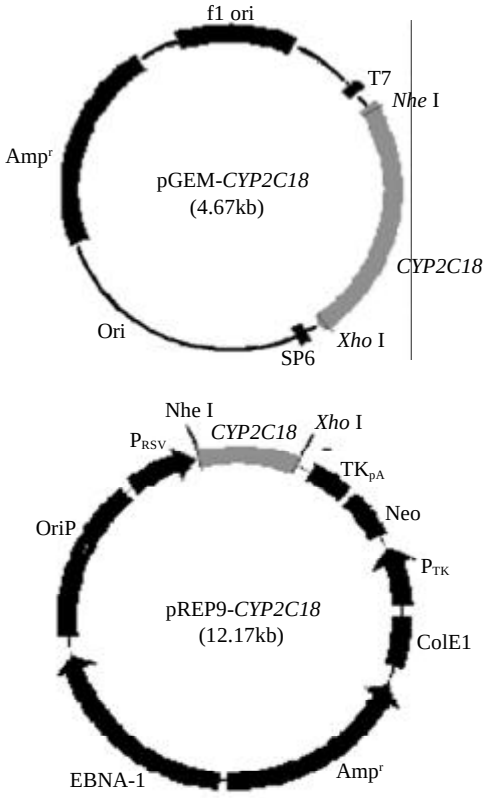

Figure 1 Scheme of recombinants of pGEM-*CYP2C18* and pREP9-*CYP2C18*

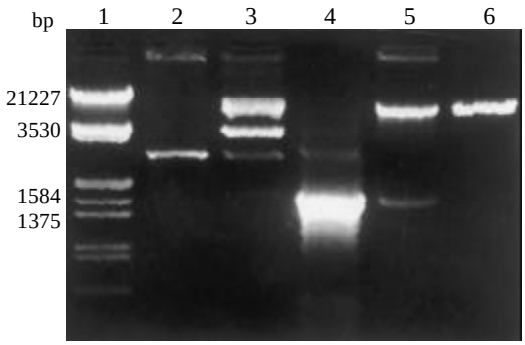

Figure 2 Electrophoresis identification of recombinants of pGEM-*CYP2C18* and pREP9-*CYP2C18*. Lane 1: Marker (λ/*Eco*R I and *Hin*d III); 2: pGEM-T vector; 3: Recombinant of pGEM-*CYP2C18* digested by *Nhe* I and *Xho* I(incompleted digestion); 4: PCR products of *CYP2C18*(1.67 kb); 5: Recombinant of pREP9-*CYP2C18* digested by *Nhe* I and *Xho* I; 6: pREP9 vector.

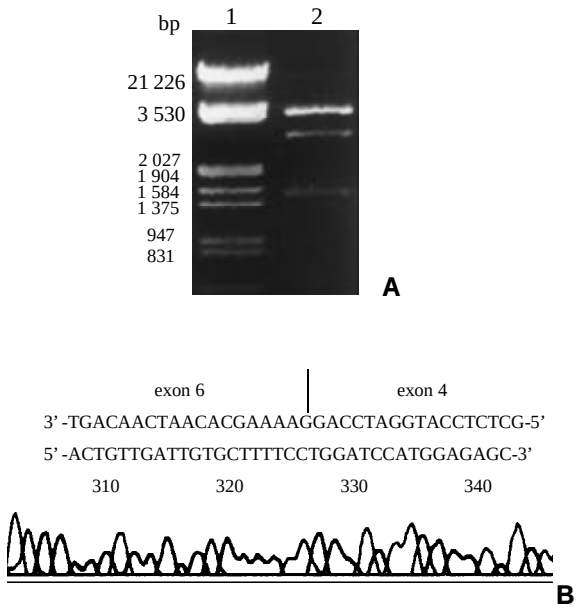

Figure 3 Electrophoresis and sequencing identification of a *CYP2C18* clone with exon 5 missing. A: Electrophoresis identification of a *CYP2C18* clone with an exon 5 being skipped; Lane 1: Marker (λ/*Eco*R I and *Hin*d III); 2: Recombinant of pGEM-*CYP2C18* digested by *Nhe* I and *Xho* I (incompleted digestion); B: Partial sequencing of an *CYP2C18* cDNA clone with an exon 5 being skipped. The upper sequence represent the sense strand and the underside sequence represent the antisense strand being sequenced.

The *Nhe* I/*Xho* I fragment (1.65 kb) containing the complete *CYP2C18* cDNA was subcloned into the *Nhe* I/ *Xho* I site of mammalian expression vector pREP9 (Figure 1). Selection and identification of the recombinant was carried out by *Nhe* I/ *Xho* I endonuclease digestion and agarose gel electrophoresis (Figure 2). The resulting plasmid was designated as pREP9-*CYP2C18* and contains the entire coding region, along with 162 bp of the 5' and 36 bp of the 3' untranslation region of the *CYP2C18* cDNA, respectively.

Establishment of transgenic cell lines with CYP2C18 enzyme activity

CHL cells were transfected with pREP9-*CYP2C18*, and selected with G418. The surviving clones were propagated and the cell line termed CHL-CYP2C18 was established. The tolbutamide hydroxylase activity of CYP2C18 in S9 of CHL-CYP2C18 cells was assayed by HPLC. A typical elution profile of hydroxytolbutamide in extracts was shown (Figure 4).

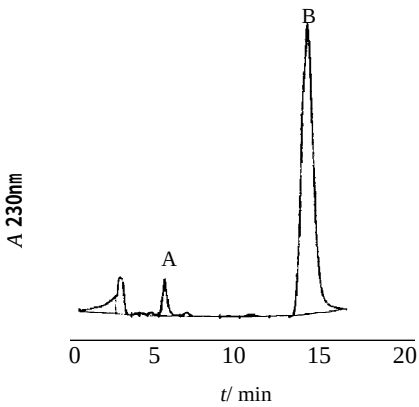

Figure 4 Representative chromatogram of extracts. A Shim-pack CLC-ODS column (15×0.6 cm i.d.) was used. The mobile phase was constituted with 0.05 % phosphoric acid (pH 2.6), acetonitrile (6:4/V:V) with the flow rate of 1 mL·min^{-1}. Hydroxytolbutamide was monitored at 230 nm. A: hydroxytolbutamide; B:tolbutamide

The CYP2C18 enzyme activity towards tolbutamide was found to be 0.509±0.052 mmol·min^{-1}·g^{-1} S9 protein or 8.82 ±0.90 mol·min^{-1}·mol^{-1} CYP (n=3), but was not detectable in parental CHL cells. The CYP content was 53.9 pmol·mg^{-1} S9 protein from CHL-CYP2C18 and no detectable CYP was present in CHL cells.

DISCUSSION

The human *CYP2C18* gene is located on chromosome 10q24. Four *CYP2C18* alleles have been reported. T385 and M385 alleles exhibited the same regio-and stereoselectivities for warfarin[25]. There was no substantial difference in the ability of the T385 and M385 of *CYP2C18* to metabolize mephenytoin[26]. $CYP2C18_{m1}$, a substitution of 204T>A in exon 2, which creates a stop codon that yields a truncated protein lacking the heme-binding site[27]. $CYP2C18_{mFR}$, consisting of -460A>T substitution in the 5'-flanking region, the functional property of this mutant allele remains unclear[28]. One of the *CYP2C18* cDNA cloned by us belongs to the T385 allele in amino acids sequence. Although there are two base substitutions, 222T>C, 828C>T, the encoding amino acids have not been changed.

To express the functional activity of a CYP, a cell must have adequate heme biosynthetic capabilities and ample intracellular membrane[29]. CYPs also require other enzymatic components for full activity, including the flavoprotein NADPH-P450 oxidoreductase (OR) and, in some cases, cytochrome b_5. The OR must interact directly with the CYP to transfer the required two electrons from NADPH. Cytochrome b5 is necessary for increasing electron transfer for certain CYP forms and specific substrates. The CHL is a cell line originally derived from the lung of a newborn female Chinese hamster and has no or very limited activities of CYP enzymes, but has adequate OR and cytochrome b5 levels to support CYP activities.

To achieve high expression levels of CYP2C18, the *CYP2C18* cDNA was cloned into the eukaryotic expression vector pREP9, which had previously been used in this laboratory for the expression of human CYP1A1[17], CYP2B6[17], CYP3A4[18], CYP2A6[19], CYP2C9[20] and UGT1A9[21] in CHL cells. The salient feature of this vector has an EBV origin of replication and nuclear antigen (EBNA-1) to allow high-copy episomal replication in mammal cell lines. The Rous sarcoma virus long terminal repeat (RSV LTR) early promoter controls the expression of the *CYP2C18* cDNA.

CYP2C18 was expressed at a very low level in human liver[4]. Its protein had not been detected and its mRNA can not be induced by rifampicin or phenobarbital in human livers and cultured primary hepatocytes[5]. But it seems to be a major CYP2C in the skin and the lung judged by its mRNA levels[30]. Its mRNA was found in the brain, uterus, mammary gland, kidney, and duodenum[31]. It has been reported that CYP2C18 can metabolize (S)-mephenytoin[26], tolbutamide[26], warfarin[25], tienilic acid[20], diclofenac[13], aminopyrine[32], and bisphenol A[14] by yeast expression and metabolize all-trans-retinoic acid[15] by lymphoblast expression. Cyclophosphamide(CPA) and ifosfamide can be metabolized by M385 CYP2C18. CYP2C18-M385 plus CYP OR appear to be excellent gene combinations for use with CPA in P450/prodrug activation-based cancer gene therapy[33]. A 2-aroylthiophenes derived from tienilic acid by replacement of its OCH_2COOH substituent with $O(CH_2)_3OH$ function, appears to be a particularly good

substrate of CYP2C18[34].

Tolbutamide (1-butyl-3-p-tolylsulfonylurea) is an oral hypoglycemic agent which is being used in the treatment of diabetes. In humans it undergoes CYP-catalyzed hydroxylation of the tolyl methyl group which is the initial and rate-limiting reaction followed by further oxidation by cytosolic dehydrogenases yielding carboxytolbutamide. Overall this pathway accounts for up to 85 % of tolbutamide clearance in humans[35]. We used tolbutamide as a substrate for evaluating the expression of human CYP2C18 activity in CHL-CYP2C18 cells. The tolbutamide hydroxylase activity was 0.509 ± 0.052 mmol· min^{-1}· g^{-1} S9 protein or 8.82 ± 0.90 mol· min^{-1}· mol^{-1} CYP, which is slightly higher than that of our cloned human CYP2C9: 0.465 ± 0.109 μmol· min^{-1}· g^{-1} S9 protein or 8.62 ± 2.02 mol· min^{-1}· mol^{-1} CYP[20]. The tolbutamide hydroxylase activity was reported to be 0.273 ± 0.066[36] or 0.189 ± 0.008[37] mmol· min^{-1}· g^{-1} of human liver microsomes. Considering the low expression of CYP2C18 in liver, the tolbutamide hydroxylase activity in human liver might be contributed by CYP2C9. CHL-CYP2C18 cells efficiently expressing the CYP2C18 may be a useful tool for further studies of its enzymatic function and mechanism.

Interestingly, we find one of our cloned *CYP2C18* cDNAs has only 8 exons by DNA sequencing. This cDNA clone is identical to the wild-type one except the missing exon 5. This is consistent with Zaphiropoulos's report that an exon 5 skipping of human *CYP2C18* gene in epidermis[30]. The exon is spliced at the predicted sites indicating that it pre-exists in the mRNA samples and is not a PCR artifact. Transcripts that have skiped *CYP2C18* exon 4, exon 4,5 and 6, or exon 4,5,6, and 7 were also identified in epidermis[30]. The splicing process in higher eukaryotes is chracterized by the precise excision of introns that can be longer than 50 000 bases and the joining of exons that are rarely over 300 bases. Yet the mechanisms that regulate the splicing process and the generation of alternatively spliced mRNA products are still poorly understood[38]. In addition to alternative splicing in intragenic RNA molecule, chimeric RNA production had been detected in *CYP2Cs*[39-41] and *CYP3As*[42] genes. This finding suggests that intergenic mRNA formation may represent a generalized splicing pathway that deepens the complexity of splicing patterns in gene families[42] and the concept of gene transcription may not suffice to include all variations in expressed genomic sequences. Finta and Zaphiropoulos[43] proposed a more general term, "genome transcription".

The difference in enzyme activity between the full length clone and the spliced variant will be studied in the future.

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• BASIC RESEARCH •

Effects of electroporation on primary rat hepatocytes *in vitro*

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Abstract

AIM: To investigate the effects of electroporation on primary rat hepatocyte and to optimize the electroporation conditions introducing foreign genes into primary hepatocytes.

METHODS: A single-pulse procedure was performed at low voltage (220-400 V) but with high capacitance (500-950 μF). Hepatocytes were divided into 4 groups according to the electroporation conditions: group Ⅰ, 220 V and 500 μF; group Ⅱ, 220 V and 950 μF; group Ⅲ, 400 V and 950 μF, and group IV. The control group was freshly isolated hepatocytes and directly cultured under the same conditions as those of electroporation groups. The effects of electroporation on primary rat hepatocytes were detected by trypan blue exclusion (TBE) and MTT analysis. Besides, albumin (Alb), alanine transaminase (ALT) and lactate dehydrogenase (LDH) in the supernatants of cultured hepatocytes were measured by biochemical assay.

RESULTS: Between day 1 and day 15 after incubation, primary rat hepatocytes of each electroporation group appeared normal, being the same with those of control group. TBE staining showed that slight hepatocyte damage and high survival rate were found in the electroporation groups and the control group. Cultured for 3, 7, 11 and 15 days, hepatocyte viability was approximatly 92.6±2.5 %, 89.5±3.3 %, 82.0±3.5 % and 74.3±1.2 %, respectively. MTT analysis indicated that the viabilities of hepatocytes had no significant difference between each electroporation group, and those were similar to that of control group. At the 36th hour after electroporation, Alb, ALT and LDH in the supernatants of control group were 5.3±0.1 g·L^{-1}, 183.7±8.4 nkat·L^{-1} and 896.8±58.5 nkat·L^{-1}; those of group Ⅱ were 5.7±0.1 g·L^{-1}, 215.4±16.7 nkat·L^{-1} and 1063.8±51.8 nkat·L^{-1}; and those of group Ⅲ were 5.8±0.2 g·L^{-1}, 217.1±8.4 nkat·L^{-1} and 1063.8±10.0 nkat·L^{-1} . Statistically, the proteins of group Ⅱ and group Ⅲ were significantly higher than those of control group (P<0.05), whereas the protein production of group Ⅰ, Alb, ALT and LDH were 5.3±0.2 g·L^{-1}, 205.4±3.3 nkat·L^{-1} and 1035.4±116.9 nkat·L^{-1}, were similar to those of control group. At the same time, TBE and MTT analysis indicated that there was no significant cell viability difference between electroporation groups and control group.

CONCLUSION: This single-pulse electroporation procedure performed at low voltage (220-400 V) but with high capacitance (950 μF) is one of the optimal choices to introduce foreign genes into primary rat hepatocyte.

Yao YQ, Zhang DF, Huang AL, Luo Y, Zhang DZ, Wang B, Zhou WP, Ren H, Guo SH. Effects of electroporation on primary rat hepatocytes *in vitro. World J Gastroenterol* 2002; 8(5):893-896

INTRODUCTION

Electroporation technique has been widely used in the experiments of foreign gene transfection into cultural cells. Little is known about the effect of electroporation on the primary culture cells, especially on the primary hepatocytes, for most of the target cells are immortal carcinoma cells. At present, in the *in vitro* study of liver diseases, primary cultured hepatocytes have been used as one of the most important target cells, which are increasingly used in the research of basic and clinical medicine[1-17], and electroporation has also been used as preferred means introducing foreign genes into primary hepatocytes. It is known that primary hepatocyte does not proliferate in common culture, so the biological activities of electroporated hepatocytes must be the key points affecting the efficiency of transfection and expression of foreign genes in the target cells. In order to optimize the electroporation conditions introducing foreign genes into hepatocytes, we studied the effect of electroporation on the biological activities of primary cultured rat hepatocyte.

MATERIALS AND METHODS

Isolation and culture of hepatocytes

Wistar rats, male, about 200 g of body mass, were provided by the Center for Laboratory Animal at Chongqing University of Medical Sciences. Hepatocytes were harvested from adult Wistar rats using the *in situ* collagenase perfusion technique[18]. Injected into the peritoneal cavity with pentobarbital sodium (100 mg·kg^{-1} body weight) and heparin (200 IU·kg^{-1} body weight), the abdomen of the animals were opened and the portal vein was exposed and cannulated. Then the liver was perfused at 37 ℃ *in situ* first with a calcium-free Hanks' Balanced Salt Solution (HBSS) for 10 min, next with 0.2 g·L^{-1} collagenase (Type Ⅳ, 390 kU·g^{-1}) in calcium-presented HBSS for 15 min. The liver was removed and the cells were combed gently in tissue culture medium. Hepatocytes were pelleted, washed, and separated from nonparenchymal cells by differential centrifugations at 50×g. Viability of hepatocytes exceeded 98 % as determined by trypan blue exclusion (TBE).

Electroporation

Freshly isolated hepatocytes were diluted into 1×10^{10} cells·L^{-1} suspension with serum-free RPMI 1640 medium for

electroporation (electroporation apparatus is Bio Rad Gene Pulser Ⅱ), and were divided into three groups. In group Ⅰ, voltage was 220 V, and capacitance was 500 μF, in group Ⅱ, 220 V and 950 μF, and in group Ⅲ, 400 V and 950 μF. All of three groups were electroporated by single-pulse procedure, and electroporation time was about 19 ms. Before and after electroporation hepatocytes were put onto ice for 10 min. Electroporated hepatocytes diluted into 3×10^8.cells.L^{-1} suspension with hepatocyte culture medium was inoculated into 96-well plastic cell-culture plates (3×10^4 cells in each well). Medium contained RPMI 1640 with insulin (100 $U\cdot L^{-1}$), penicillin, streptomycin and 100 $ml\cdot L^{-1}$ fetal bovine serum. Control group, group Ⅳ, was freshly isolated hepatocytes and was directly cultured under the same conditions as those of electroporation groups. The medium was changed after 36 h incubation and later changed every two days.

Hepatocyte viability and function

To detect the viabilities of hepatocytes of electroporatin groups and control group, TBE staining was used from day 1 to day 15 after incubation. Besides, MTT analysis was also used to measure the cell viability. At 36 h after incubation, the medium was changed with phenol red-free RPMI 1640 medium (0.1 mL each well), 10 μL MTT (5 $g\cdot L^{-1}$) was added into each well, and incubated for another 4 h. Then discard the supernatants, add 0.1 ml dimethyl sulfoxide into each well and oscillate the culture plate for 5 min. Next, the 96-well culture plate was put into Elx800 type automatic reader and absorbance values in each well was detected at 405 nm wave length. Each group had more than 6 wells each time. Three more of these tests were repeated. To measure albumin (Alb), alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) in the supernatants of cultured hepatocytes of electroporation groups and control group, biochemical assays were used.

RESULTS

Effect of electroporation on the viability of hepatocytes

From day 1 to day 15 after incubation, primary rat hepatocytes of each electroporation group appeared normal, the same as those of control group. TBE staining showed that the viabilities of hepatocytes had no significant difference between each electroporation group, which were similar to that of control group (Table 1). Culture for 7 d showed that hepatocyte viability was still around 90 %, and about 74 % hepatocytes survived 15 d after electroporation. MTT analysis also showed the same results, after 36 h of culture, primary rat hepatocytes of electroporation groups had the same viabilities as that of control group ($P>0.05$, Student' s *t* test), the absorbance values (405 nm wave length) of group Ⅰ to Ⅳ were 0.317±0.069, 0.369±0.059, 0.279±0.085 and 0.332±0.072, respectively.

Table 1 Viabilities of electroporated hepatocytes measured by TBE

Culture time (day)	Survival rate (%)
1	95.4±1.8
3	92.6±2.5
5	91.3±2.4
7	89.5±3.3
9	87.2±4.6
11	82.0±3.5
13	77.0±0.9
15	74.3±1.2

Effect of electroporation on hepatocyte function

To detect the effect of electroporation on Alb, ALT and LDH production of primary rat hepatocytes, biochemical assays were used to measure these proteins in the supernatants of cultured hepatocytes. Amounts of Alb, ALT and LDH in the supernatants were assayed 36 h after incubation. The results (Table 2) showed that Alb, ALT and LDH production of electroporation groups Ⅱ and Ⅲ were significantly higher than those of control group, whereas the protein production of group Ⅰ was similar to those of control group.

Table 2 Alb, ALT and LDH production in the supernatants of primary cultured rat hepatocytes

Groups	Alb/($g\cdot L^{-1}$)	ALT/($nkat\cdot L^{-1}$)	LDH/($nkat\cdot L^{-1}$)
Ⅰ	5.3±0.2	205.4±3.3	1035.4±116.9
Ⅱ	5.7±0.1[a]	215.4±16.7	1063.8±51.8[a]
Ⅲ	5.8±0.2[a]	217.1±8.4[a]	1063.8±10.0[a]
Ⅳ	5.3±0.1	183.7±8.4	896.8±58.5

[a]$P<0.05$, *vs* control.

DISCUSSION

Primary hepatocytes have been increasingly used in basic and clinical medicine researches, such as the study of hepatocyte function, the mechanism of hepatocyte injuries and the protective approaches of hepatocyte biological activities[19-25], and the use of hepatocytes in bioartificial liver support system (BALSS)[26,27] and gene therapy[28]. Besides, more and more researchers utilize primarily cultured hepatocyte model to study foreign genes' activities and effects on host cells, for example, hepatitis B and C viruses replication and expression in hepatocytes[29-31]. In order to keep the normal biological activities and functions *in vitro* of hepatocytes, more methods have been used to optimize primary hepatocyte model, especially the introduction techniques of foreign genes into hepatocytes.

The introduction and stable expression of foreign genes in mammalian hepatocytes have been demonstrated by several techniques[32-37], including the use of physical approaches such as direct injection of a DNA calcium phosphate precipitate, electroporation of plasmid DNA and the exposure to liposome-erythrocyte ghost complexes as well as the biological approach of infection of primary hepatocyte cultured with retrovirus vectors. Among these techniques, electroporation has been considered as one of the most useful methods introducing foreign into target cells[38-41]. However, it is not defined that which electroporation condition is the best one under which foreign genes can be efficiently transduced into primarily cultured hepatocytes, and this condition could cause little damage to target cells.

It is known that primarily cultured hepatocytes are well-differentiated cells, most of which are resting cells, arrested in G0, and do not proliferate in common culture conditions. To be an ideal model, in which foreign gene can get highly efficient transfection and expression, primarily cultured hepatocytes after electroporation could not only keep a high survival rate, but also maintain excellent cellular functions. Therefore, optimizing electroporation conditions has become the key point affecting the biological activities of primary hepatocytes.

Our results showed that a single-pulse procedure performed at low voltage (220-400 V) but with a high capacitance (950

μF) caused minimal cell damage and kept hepatocyte with a high survival rate, cultured for 7 d and 15 d, greater than 90 % and 74 % respectively, and hepatocyte viabilities of electroporation groups were similar to that of control group and much higher than those reported by other savants. Our current data further indicated that this electroporation procedure could not only maintain hepatocyte function, but also facilitate Alb, ALT and LDH expression in hepatocytes and production in the culture medium. Its mechanism is not clear, possibly related to two reasons: first, Alb, ALT and LDH might leak into culture medium through electroporated hepatocyte membrane, while minimal cell damage had been found by TBE and MTT assay. Second, this electroporation condition may have a potentiation to stimulate wave of DNA synthesis in electroporated rat hepatocytes. In conclusion, this single-pulse procedure performed at low voltage (220-400 V) but with a high capacitance (950 μF) could not only cause minimal cell damage, but also potentiate DNA synthesis in rat hepatocytes. For instance, up to date, we have used this electroporation procedure in transduceing hepatitis B virus gene into primary rat hepatocytes and primary duck hepatocytes and successfully observed that hepatocytes are competent for transfection with HBV gene, which can stably replicate and express in primary hepatocytes[42,43]. Therefore, this single-pulse procedure can be used as one of the optimized electroporation procedures introducing foreign genes into primarily cultured hepatocytes.

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• BASIC RESEARCH •

Effect of N-desulfated heparin on hepatic/renal ischemia reperfusion injury in rats

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Abstract

AIM: To investigate the effect of N-desulfated heparin on hepatic/renal ischemia and reperfusion injury in rats.

METHODS: Using rat models of 60 minutes hepatic or renal ischemia followed by 1 h,3 h,6 h and 24 h reperfusion,animals were randomly divided into following groups,the sham operated controls,ischemic group receiving only normal saline,and treated group receiving N-desulfated heparin at a dose of 12 mg/kg at 5 minutes before reperfusion. P-selectin expression was detected in hepatic/renal tissues with immunohistochemistry method.

RESULTS: P-selectin expression, serum ALT, AST, BUN and Cr levels were significantly increased during 60 minute ischemia and 1 h, 3 h, 6 h and 24 h reperfusion,while the increment was significantly inhibited,and hepatic/renal pathology observed by light microscopy was remarkably improved by treatment with the N-desulfated heparin. Furthermore,the heparin was found no effects on PT and KPTT.

CONCLUSION: P-selectin might mediate neutrophil infiltration and contribute to hepatic/renal ischemia and reperfusion. The N-desulfated heparin might prevent hepatic/renal damage induced by ischemia and reperfusion injury without significant anticoagulant activity.

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INTRODUCTION

Hepatic/renal ischemia-reperfusion injury is very common clinically. So far, no effective treatment for this pathological injury is available. It has been found that cell adhesion molecule P-selectin plays an important role in hepatic/renal ischemia-reperfusion injury by mediating the interactions of polymophonuclear neutrophils with endothelium. P-seletin monoclonal antibody has been demonstrated to prevent effectively reperfusion-induced hepatic/renal tissue damage[1-22]. Heparin, a highly sulfated proteoglycan, has anti-inflammatory activity besides its anticoagulant function. Recently reports suggested that heparin could prevent leukocyte adhesion through inhibiting the role of P-selectin and might be used to treat inflammatory diseases[23]. However, the clinical use of heparin for treatment of inflammation is impeded by its strong anticoagulant activity[24]. Chemically modified heparins which have been developed have relatively low anticoagulant activity remain their anti-inflammatory activities[25,26]. In this study, we investigated the effect of N-desulfated heparin (NNH) on hepatic/renal ischemia-reperfusion injury in rats.

MATERIALS AND METHODS

Animal model

Ninety male Wistar rats (Shanghai Experimental Animal Centers of Chinese Academy of Sciences), weighing 200±10 g, were given free access to food and water for three days before the experiments. The rats were anesthetized with 2.5 % sodium pentobarbital intraperitoneally, and randomly divided into 2 groups. In one group of rats, the ligament linking liver, diaphragm and abdominal wall were separated, portal vein and liver artery that drain blood to left hepatic lobe were freed by blunt dissection and then blocked with a microvascular clamp for 60 minutes, after that clamp was removed, and reperfusion was started; while in another group, the left renal artery was freed, blocked with a microvascular clamp for 60 minutes, then the clamp was removed and reperfusion was started, simutaneously, the right kidney was cut off. The two groups of rats were randomly divided into NNH-treated group (n=20) and non-treated group (n=20). They were divided into subgroups according to the indicated time: 1, 3, 6, 24 hours after reperfusion. NNH (12 mg/kg, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences), or saline were injeted by veins five minutes before reperfusion. A sham-operated group (n=5, anesthesia and opening celiac cavity, no blocking of hepatic or renal blood flow) served as control.

Collection and measurement methods of specimens

Blood, hepatic and renal tissues were harvested at the indicated time. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and blood urea nitrogen (BUN) and creatinine (Cr) were measured with a 747 automatic analyzer (Hitachi boehringer Mannhein, Mannhein, Germany). Hepatic and renal tissue samples were fixed in 10 % formalin and embedded in paraffin. Sections were cut 5 μm thick and stained with hematoxylin and eosin for light microscope examination. Expression of P-selectin in hepatic/renal tissue

was detected by an immunohistochemistry method with a labelled streptavidin biotin (LSAB) kit (Fujian Maixin Biotechnology Co., products of Biotechnology co. CA, USA). Activated partial thromboplastin time (APTT) and protothrombinogen time (PT) were also measured.

Statistical analysis

Data was presented with $\bar{x}\pm s$, and Student' s *t* test was used to determine changed between different groups. $P<0.05$ was considered significant.

RESULTS

Histopathologic evaluation

One hour after reperfusion, visual observation revealed that the left hepatic lobe was more swollen than the right lobe, and was dark in color. Under the light microscope, interstitial congestion and infiltration of inflammatory cells were observed. 1 hour after reperfusion, with the observation of naked eyes, the renal cortex was pale, the renal medulla displayed blood stagnation and was dark in color. Under the light microscope, edema, denaturation with different extent and necrosis of renal tubular epithelial cells were observed. Simultaneously, interstitial congestion, edema and infiltration of inflammatory cells were also observed. However, in the N-desulfated heparin-treated group. The outward apperance of the liver and kidney was similar to that of normal control. Hepatic cells and tubular cells showed less swelling and no denaturation or necrosis, and interstitial changes were not obvious.

Hepatic and renal function evaluation

Twenty four hours after hepatic reperfusion, the serum levels of ALT (628±91 μ/L) and AST (1608±199 μ/L) in the saline-treated group were much higher than the sham-operated group (52±11 μ/L and 80±17 μ/L respectively, $P<0.01$). The NNH-treated group revealed significantly lower levels of ALT (161±24 μ/L) and AST (360±49 μ/L) compared with saline-treated group ($P<0.01$).

Twenty four hours after renal reperfusion, the serum levels of BUN (14.54±0.67 mmol/L) and Cr (102.2±4.67 μmol/L) were much higher than the sham-operated group (7.88±0.57 mmol/L and 39.00±4.47 μmol/L, respectively, $P<0.01$). The NNH-treated group presented with significantly lower levels of BUN (10.60±0.80 mmol/L) and Cr (67.78±5.01 μmol/L) compared with saline-treated group ($P<0.01$).

P-selectin expression in hepatic and renal tissues

P-selectin was expressed widely within hepatic and renal tissues 1 hour after reperfusion, which was mainly distributed on small vessels of left hepatic lobe and tubular epithelium. In addition, it was also expressed on part of hepatic cellular membrane, glomerulomesangium, capillary loops, and interstitium. After treatment with the NNH, there were no obvious yellow-brown positive granules in the hepatic and renal tissue, suggesting that the P-selectin expression was not displayed.

PT and KPTT assays

Twenty four hours after hepatic reperfusion, PT (15.0±1.6 s) and KPTT (21.9±4.1 s) in the saline-treated group were similar to that of sham-operated group (13.4±1.7 s and 17.9±2.9 s respectively, $P>0.05$). PT(14.6±1.9 s) and KPTT (18.7±3.7 s) in NNH-treated group did not differ from those in saline-treated group ($P>0.05$). 24 hours after renal reperfusion, PT and KPTT were 13.7±1.4 s and 17.6±3.1 s respectively, while in NNH-treated group they were 13.3±1.6 s and 17.1±3.5 s respectively ($P>0.05$).

DISCUSSION

Recently, the role of cell adhesion molecules and neutrophil in orgen ischemia and reperfusion injury has attracted attention[27-48]. As a potential member of the selectin family, P-selectin is found in both weibel-plade body of epithelial cell of middle and small blood vessels and a-granule of platelet. It is expressed rapidly on the surface of these cells after their activation. P-selectin plays an important role in inflammation by initiating neutrophil rolling, adhesion and recruitment to injured tissue[27]. Blockade of P-selectin expression or interaction with its ligands can attenuate leukocyte adherence and infiltration during ischemia and reperfusion injury. And P-selectin monoclonal antibody was found to have protective effects on the injury[21,22].

Heparin and its analogue have been shown to bind to cell adhesion molecule P-selectin. The binding can inhibit adhesion of leukocytes mediated specifically by the adhesion molecules and are of anti-inflammatory activity[25,26]. Although heparin can be used to treat inflammatory diseases, their clinical potentials are limited due to their potent anticoagulant activity. Several chemical modifications of heparin that have reduced anticoagulant activities while preserving their anti-inflammatory activities have been reported. For example, the O-desulfated heparin derivatives have been shown to be of treatment for inflammation, while they still retained 5-30 % anticoagulant activities as compared with heparin[49]. Further, N-acetylheparin is known to have lower anticoagulant activity while preserving the anti-inflammatory activity[50]. N-desulfated heparin has been reported to have significantly reduced anticoagulant activity[51,52]. However, the anti-inflammatory roles of the heparin have not been extensively studied before.

The effect of N-desulfated heparin on ischemia and reperfusion injury was observed in this study based on the established rat model of hepatic/renal ischemia-reperfusion.

Hepatic and renal tissue displayed significant histopathologic damage after hepatic/renal ischemia-reperfusion while the serum levels of ALT and AST as well as BUN and Cr were increased. We showed that ischemia-reperfusion induced hepatic/renal injury was remarkably attenuated when NNH was given 5 min before reperfusion as shown by improved hepatic/renal function and less pathologic damage. The results suggest that the NNH has a protective effect on hepatic/renal reperfusion injury by inhibiting the interaction of neutrophils and endothelium.

After ischemia and reperfusion, P-selectin expression was up-regulated in hepatic and renal tissue, suggesting that P-selectin is related to hepatic/renal reperfusion injury. It has been found that leukocyte rolling and recuitment was delayed when deficient mice were infected,suggesting that P-selectin is involved in the early events of inflammation mediated by leukocytes[53]. Results from this study showed that P-selectin expression in hepatic and renal tissue was inhibited in NNH-treated group. This is consistent with down-regulated expression of sialyl lewis X, a ligand for P-selectin located mainly in neutrophils, as with anti-P-selectin therapy (unpublished data). These suggest that P-selectin might mediate neutrophil infiltration within the liver and kidney in the early stage of hepatic/renal reperfusion injury. Furthermore, blockade of P-selectin can attenuate inflammatory cell infiltration and pathological damage. N-desulfated heparin can be of protective role in the hepatic and renal injury caused by ischemia-reperfusion by inhibiting the adhesion and activation of neutrophils mediated by P-selectin. In addition, we found the heparin had no significant effects on PT and KPTT. It is

consistent with Bjornsson's study[51]. Therefore, the N-desulfated heparin might be an efficient approach for the treatment of reperfusion injury with no obvious anticoagulant activity.

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• BASIC RESEARCH •

Expression of exogenous rat collagenase *in vitro* and in a rat model of liver fibrosis

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Abstract

AIM: The present study was conducted to test the hypothesis that the introduction of the collagenase gene into tissue culture cells and into a rat model of liver fibrosis would result in the expression of enzymatically active product.

METHODS: FLAG-tagged full-length rat collagenase cDNA was PCR amplified and cloned into a mammalian expression vector. NIH3T3 cells were then transiently transfected with this construct. Expression of exogenous collagenase mRNA was assessed by RT-PCR, and the exogenous collagenase detected by Western blotting using anti-FLAG monoclonal antibody. Enzymatic activity was detected by gelatin zymography. To determine the effects of exogenous collagenase production *in vivo*, the construct was bound to glycosyl-poly-L-lysine and then transduced into rats that had developed liver fibrosis as a result of CCl_4 plus ethanol treatment. The hepatic expression of the construct and its effect on the formation of liver fibrosis were demonstrated using RT-PCR and immunohistochemistry.

RESULTS: It was found that exogenously expressed rat collagenase mRNA could be detected in NIH3T3 cells following transfection. Enzymatically active collagenase could also be detected in the culture medium. The recombinant plasmid was also expressed in rat liver after *in vivo* gene transfer. Expression of exogenous rat collagenase correlated with decreased deposition of collagen types I and III in the livers of rats with experimentally induced liver fibrosis.

CONCLUSION: The expression of active exogenous rat collagenase could be achieved *in vitro* and *in vivo*. It was suggested that *in vivo* expression of active exogenous collagenase may have therapeutic effects on the formation of liver fibrosis.

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INTRODUCTION

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes that require zinc ions for their activities, they take part in a variety of normal and disease-associated matrix lysis and remodeling events[1-3]. For example, interstitial collagenase, one member of MMPs, also called collagenase, mainly initiates the degradation of collagen types I, II, III, V, VII, and X, which are fibrillar collagens[4-6]. Interstitial collagenase probably contributes importantly to tissue repair, embryogenesis, bone renewal, and to some pathogenic processes such as rheumatoid arthritis, tumor metastasis, and also organ fibrosis[7-10]. Changing the expression or the activities of interstitial collagenase by regulatory factors or by gene transfer may have therapeutic effects on those disease processes.

Liver cirrhosis often shortens life expectancy and decreases the quality of life. In liver cirrhosis, a major pathological feature is the accumulation of extracellular matrix (ECM). Within the major components of ECM, collagen types I and III constitute more than 95 % of the total content of increased collagen in fibrotic liver. The abnormal accumulation of ECM may reflect alterations in the synthesis of matrix proteins, their degradation, or both[11,12]. The reversibility of liver fibrosis/ cirrhosis has been found in patients with various chronic hepatopathy[13, 14] and in experimental cirrhotic animals[15], indicating a therapeutic approach of this disease condition through improving ECM degradation. It has been reported that the mRNA transcription of interstitial collagenase is not up-regulated in experimental hepatic fibrosis[16,17] and the interstitial collagenase proteins in cirrhotic human livers and in serum of chronic hepatitis patients decrease[18,19]. Previous study also shows that the activity of collagenase decreases in advanced liver fibrosis[20-22] and in serum from patients with chronic liver disease[23,24]. Therefore, we proposed that exogenous gene expression of active collagenase might have therapeutic effects on the chronic liver diseases processes. The present experiments were performed to test the hypothesis that the introduction of a collagenase expression vector into tissue culture cells resulted in the enzyme expression, and that similar introduction of exogenous collagenase into a rat model of chemically induced liver fibrosis, altered the development of fibrosis. The *in vitro* results suggest that rat collagenase could be transiently expressed in NIH3T3 cells, and that rat liver fibrosis could be delayed by transducing the collagenase gene *in vivo*.

MATERIALS AND METHODS

Vector construction

A 6 amino acids deleted rat collagenase cDNA containing plasmid (UMR5.4) was kindly provided by Dr John J. Jeffrey[25] and used as starting material. Full-length rat collagenase cDNA was PCR amplified using the primers RIC-UP1 and RIC-DP1 with UMR5.4 as the DNA template (Table 1). The sense primer RIC-UP1 contained the sequences of the deleted 6 amino acids. The PCR fragments were then inserted into the PCR2.1 vector (Invitrogen, Carlsbad, CA, USA) to form PCR2.1-RIC. Using PCR2.1-RIC as template, the cDNA was further PCR amplified with RIC-UP2 and RIC-DP2 as primers (Table 1). The FLAG epitope was added to the C-terminus of the rat collagenase

gene in this construct by using RIC-DP2 that contained the FLAG sequence at the 3' end adjacent to the translation stop codon TAA (Table 1). The PCR reaction was performed as follows: the total reaction volume was 100 μl which contained 1ng DNA template (PCR2.1-RIC), 10 pmol· L^{-1} of each primer of RIC-UP2 and RIC-DP2, 2.5 μmol· L^{-1} dNTP, 10×PCR buffer and 5U Taq polymerase; 35 PCR cycles at 95 ℃ for 1.5 min, 55 ℃ for 1.5 min, and 72 ℃ for 2 min. The 1400 kb PCR product was purified and inserted into mammalian expression vector pTarge™ by the method of TA cloning following the protocol suggested by manufacture (Promega, Madison, Wis, USA) and named as pCMV-RC-F (Figure 1, Map of the plasmid pCMV-RC-F). The pCMV-RC-F was DNA sequenced using T7 primer. The large-scale of plasmid DNA was prepared using Qiagen tip100 (Qiagen, Hilden, Germany). The vector pTarge™ that was self-ligated without any inserted DNA was also isolated and used as control in the transfection study.

Table 1 Primer pairs for PCR and RT-RCR reactions

RIC-UP1: 5'-CATGCATTCAGCTATCCTGGCCACCTTCTTCTTGTTG-3'
RIC-DP1: 5'-CTCCATAGATGAACTCCCC-3'
RIC-UP2: 5'-CCACCATGCATTCAGCTATCCTGG-3'
RIC-DP2: 5'-GTTACTTATCATCGTCGTCCTTGTAGTCACACCAC AATAAGGAATTC-3'
RIC-UP3: 5'-GAATCCAGTCTCTCTATGGTCCAG-3' (785-808)
RIC-DP3: 5'-TCATCGTCGTCCTTGTAGTCACAC-3' (1413-1436)
IC-UP4: 5'-TGGAGCCCTGATGTTTCCCATCTA--3' (693-717)
IC-DP4: 5'-ACCCAAATACTCTTTGGGAGATAA--3' (1423-1446)
β-UP5: 5'-AACCGCGAGAAGATGAACCAGATCATGTTT-3' (2135-2164)
β-DP5: 5'-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3' (2456-2485)

( ): binding location of primer in relative cDNA sequence

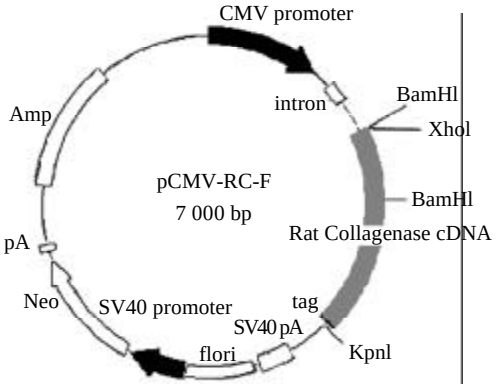

Figure 1 Map of plasmid pCMV-RC-F. The FLAG tagged full length rat collagenase cDNA was inserted into a mammalian expression pasmid vector Ptarge™ which carries the human cytomegalovirus (CMV) immediate-early enhancer / promoter region.

In vitro transfection

Exponentially growing NIH 3T3 cells were seeded into 6-well tissue culture dishes at 5×10^5 cells/well and grown overnight to 60 % confluency in culture medium supplemented with 10 % FCS. Each well was then transfected with 5 μg DNA constructs using lipofectamine according to the instruction provided by the manufacturer (Gibico BRL, Eggenstein, Germany). Encapsulated DNA was incubated for 5 h on cells in serum free medium, then in medium containing 10 % bovine serum. After further incubation of the cells for 24 h, the culture medium was discarded and replaced by normal culture medium. Fifty-six h after transfection, the NIH 3T3 cells and their media were harvested for further analysis.

RT-PCR analysis for mRNA expression

The collected cells were washed twice with ice-cold phosphate-buffered saline (PBS), and the mRNA extracted with mRNA Capture Kit (Boehringer Mannheim). The samples were further incubated with biotin-labeled oligo $(dT)_{20}$ working solution for 5 min to hybridize the mRNA with oligo $(dA)_n$. The mixture was added to a streptavidin-coated PCR tube, and incubated for 3 min at 37 ℃ to immobilize the poly $(A)_n$ RNA. Tubes were then washed three times and amplified by RT-PCR. The RT-PCR reaction system contained 0.3 μmol· L^{-1} each of the primer pairs RIC-UP3 and RIC-DP3 (corresponded to nucleotides 785-808 of the collagenase encoding region and the FLAG domain coding region, amplificated a PCR product of 652 bp), 5 mmol· L^{-1} DTT, 6 U RNAsin, 1.5 mmol· L^{-1} $MgCl_2$, 5×RT-PCR buffer and 1 μl Titan™ Enzyme mix provided with the Titan™ RT-PCR System (Boehringer Mannheim). Samples were placed in a thermocycler equilibrated at 50 ℃for 30 min, at 94 ℃ for 5 min, then for 34 cycles at 94 ℃ for 1 min, 55 ℃ for 2 min, and 72 ℃ for 3 min, with a final extension at 72 ℃ for 10 min. The amplification products were then analyzed on 10 g· L^{-1} agarose gels containing 0.5 mg· L^{-1} ethidium bromide.

Western blot analysis of FLAG-tagged rat collagenase expression

The amount of protein in the collected culture medium of transfected cells was determined with Dye-binding method (Bio-Rad, Hercules, CA, USA). For each samples 30 μg total protein was electrophoresed on sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membrane (Gelman Inc, Ann Arbor, MI, USA) by electroblotting. Pre-stained rainbrow recombinant protein molecular weight markers (Amersham International plc, Little Chalfont, Buckinghamshire, England) were used for molecular weight determinations. Membranes were blocked with a blocking buffer containing 50 g· L^{-1} non-fat milk powder, 10 mmol· L^{-1} Tris/HCl (pH 7.5), 100 mmol· L^{-1} NaCl and 1 g· L^{-1} Tween 20 and incubated with 1:100 diluted solution of anti-FLAG M_2 McAb[26] (Kodak, New Haven, CT, USA) at 37 ℃for 1 h. After washing for 30 min the membranes were treated with HRP-conjugated secondary antibody (1:5000) (Bio-Rad) for 1 h at room temperature followed by another 30 min of washings. The ECL Western blotting system (Amersham Life Sciences) was used in accordance to the manufacturer' s instructions for chemiluminescence of proteins, and the blots were then exposed to photographic films (Fuji Photo Film Co., Tokyo, Japan).

Gelatin zymography assay for the enzyme activity

The activity of collagenase was detected by gelatin zymography as previously described[27, 28] with minor modifications. Culture supernatants that contained 60 μg /lane total protein were prepared and mixed with 5× zymogram sample buffer consisting of 0.4 mol· L^{-1} Tris (pH 6.8), 50 g· L^{-1} SDS, 200 g· L^{-1} glycerol, and 0.3 g· L^{-1} bromphenol blue, then electrophoresed with protein molecular weight markers as described before on 100 g· L^{-1} sodium dodecyl sulfate-polyacrylamide gels containing 10 g· L^{-1} gelatin under an ice water cycle. After electrophoresis, the position of molecular weight markers were marked and the gels were rinsed in washing buffer consisting of 50 mmol· L^{-1} Tris, pH 7.6, 1 μmol· L^{-1} $ZnCl_2$, 5 mmol· L^{-1} $CaCl_2$, 250 g· L^{-1} TritonX-100 at 4 ℃ on a rotary shaker. The Triton X-100 solution was decanted and replaced with rinsing

buffer consisting of 50 mmol· L^{-1} Tris (pH 7.6), 1 μmol· L^{-1} $ZnCl_2$, 5 mmol· L^{-1} $CaCl_2$ for further washing. The gel was then incubated with enzyme buffer containing 50 mmol· L^{-1} Tris (pH 7.6), 1 μmol· L^{-1} $ZnCl_2$, 5 mmol· L^{-1} $CaCl_2$, 0.2 g· L^{-1} Brij-35 at 37 ℃ for 42 h. Each gel was stained with 0.5 g· L^{-1} Coomassie blue G-250 in an aqueous solution of 300 g· L^{-1} methanol and 100 g· L^{-1} acetic acid and destained with three changes of 300 g· L^{-1} methanol, 100 g· L^{-1} acetic acid. Areas of digestion were visualized as non-staining regions of the gel and photographed.

Animal model of liver fibrosis

Thirty-six male Wistar rats (Shanghai Experimental Animal Center, Chinese Academy of Sciences) weighing 130±10 g were randomly distributed into four groups, 6 in a normal control group (A), 10 in an experimental liver fibrosis model without plasmid treatment as disease control group (B), and 10 in each of two pCMV-RC-F plasmid transfection as treatment groups (C1 and C2). Animals in the normal control group were treated with olive oil and received food and water *ad libitum*. Rats in the other three groups received a subcutaneous injection of CCl_4 solution (500 g· L^{-1} in olive oil) twice a week for eight weeks at a dose of 0.3 ml per 100 g of body weight after an initial dose of 0.6 ml per 100 g. The only source of fluid for the rats was 100 g· L^{-1} ethanol in water during the entire period of experiment. In addition, animals in the C1 group were intravenous injected with of 100 μg of galactosyl poly-L-lysine (G-PLL, obtained from Dr. Wen Shouming, The Air Force General Hospital of Chinese Peoples Liberation Army, Beijing, China) encapsulated recombinant plasmid through tails vein every two weeks, beginning two weeks after the start of treatment with CCl_4. G-PLL was included to accelerate the targeting effect of the transduced plasmid to the livers[29-31]. Animals in the C2 group received the same treatment as those in group C1 but the plasmid was administrated four weeks after starting the treatment with CCl_4. Animals in the B group received G-PLL encapsulated pTarge™ empty plasmid vector at the same dosage and the same time as those in the C1 group. The molecular ratio of galactose and poly-L-lysine is 15:28 and the average molecular weight of the G-PLL is M_r 8 500.

After weeks 8 treatment, the surviving 6 or 7 rats in each of the C1, C2, B groups and all the 6 rats in the A group were narcotized with 20 g· L^{-1} pentobarbital sodium. The middle lobes of the livers were removed and specimens were fixed in Carnoy' s fixative (glacial acetic acid, chloroform, and ethanol in volume ratio of 1:3:6) and then embedded in paraffin for histological analysis. The remaining tissue was quickly partitioned and immediately frozen and stored under liquid nitrogen for RNA extraction and RT-PCR analysis. Then the animals were humanly killed.

Collagenase mRNA expression in rat liver

Total RNA was isolated from the liver tissues using Trizol (Gibco BRL) according to the manufacturer' s directions. Integrity of RNA was confirmed by visual examination on an ethidium bromide-stained 10 g· L^{-1} Tris-acetate and EDTA agarose gel. Total RNA of 1 mg from each sample was used for reverse transcription and amplification using a RT-PCR kit (Sino-American Biotechnology Co. Shanghai. China). The RT-PCR reaction contained 10 pmol each of the primer pairs RIC-UP3 and RIC-DP3 for detecting the recombinant collagenase mRNA (a product of 652 bp), or each primer pairs of IC-UP4 and IC-DP4 (designed within nucleotides 711-734 of the coding region and nucleotides 1440-1463 of 3' untranslated region of collagenase, Table 1) for amplifying the endogenous collagenase mRNA (a product of 753 bp)[25]. The later primer pairs could not amplify the expression of recombinant collagenase that was lack of the nature 3' untranslated region of collagenase gene. Additionally primer pairs β-UP5 and β-DP5 were included for amplifying the expression of b-actin 335 bp DNA fragment) as internal control[32]. Samples were placed in a thermocycler with the incubation programme of 37 ℃ for 40 min, 94 ℃ for 7 min, then 36 cycles of 94 ℃ for 1 min, 56 ℃ for 1 min, and 72 ℃ for 1 min, with a final extension of 72 ℃ for 10 min. Products of RT-PCR were electrophoresed on a 20 g· L^{-1} agarose gel to show the amplified bands.

Histology and Immunohistochemistry

For immunohistochemical staining, 5 mm sections of each group were treated with anti-FLAG M_2 McAb to assay the expression of recombinant collagenase. After dewaxed with xylene and rehydrated through a graded alcohol series, sections were digested with 4 g· L^{-1} trypsin. Nonspecific binding was blocked with calf serum 1:5 in PBS. The sections were incubated with mouse anti-FLAG M_2 McAb (1:100) overnight at 4 ℃ and then with biotinylated horse anti-mouse IgG (Vector) for 30 min, avidin-peroxidase complex for 30 min, and the substrate solution (3,3-diaminobenzidine tetrahydrochloride in H_2O_2 in Tris buffer, pH 7.4) for 10 min, followed by counterstaining with hematoxylin. The sections were washed with 0.01 mol· L^{-1} PBS (pH 7.4) three times after each step. Finally, ten high power fields of each section were observed under microscope and the positive signals were counted.

Some sections of each group were treated with the monoclonal antibodies against collagen types I and III (1:100), then with secondary antibody and the procedure of the immunostaining were same to the above-mentioned except without counterstaining with hematoxylin. The slides were then analyzed with an Image Analyzing system (TJTY 300 System) to obtain the integral light density (ILD) of stained collagen types I and III.

Morphological examination was performed with Hematoxylin and Eosin staining (H&E) for histological changes of liver fibrosis.

Statistical analysis

The ILD values of collagen types I and III were analyzed by one-way ANOVA. The statistics were calculated by software SPSS 7.0 for Windows. The data are expressed as mean ±S. E. M. Values of $P<0.05$ were considered statistically significant.

RESULTS

In vitro expression and secretion of active FLAG-tagged rat collagenase

After *in vitro* transfection of plasmid in NIH 3T3 cells, the transcription of recombinant collagenase gene was confirmed by RT-PCR amplification of 652 bp cDNA fragment from cells transfected with pCMV-RC-F, but not from cells transfected with control plasmid pTarge™ (Figure 2). This product was of the size expected from mRNA of the tagged collagenase. Further demonstration of transfected gene expression was performed by Western blot analysis using anti-FLAG McAb. The immunoreactive band at about M_r 55 000 was observed in the culture supernatant of cells transfected with pCMV-RC-F but not in that with pTarge™ transfection or in culture medium alone (Figure 3). Collagenase activities were also found around the M_r 55 000 in gelatin zymogram analysis of the culture supernatant of pCMV-RC-F transfected cells and still could be observed after 1:64 dilution. A weak activity of collagenase was also observed in the culture supernatant of pTarge™

transfected cells but was almost undetectable after 1:2 dilution. No enzyme activity of M_r 55 000 collagenase could be detected in culture medium alone (Figure 4).

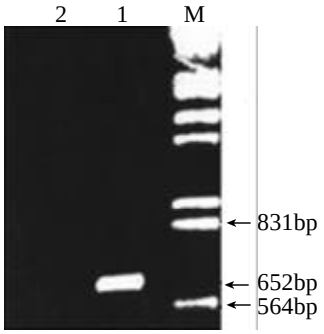

Figure 2 RT-PCR assay of the mRNA expression of recombinant collagenase in NIH 3T3 cells. The PCR-amplified 652 bp fragment of recombinant collagenase cDNA derived from their respective mRNA was found in NIH 3T3 cells which were transfected with pCMV-RC-F (lane 1), while no signal was detected in cells which transfected with control plasmid pTarge™ (lane 2). Lane M is the molecular weight marker (DNA / *Hind* III, *BamH* 1).

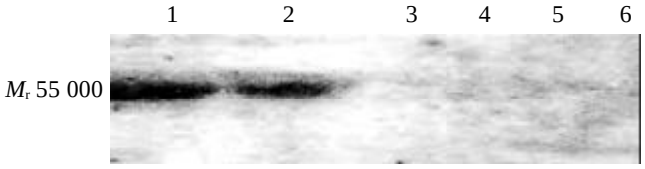

Figure 3 Westernblot analysis of the FLAG-tagged rat collagenase expression using mouse anti-FLAG M_2 McAb. The immunoreactive band with anti-FLAG M_2 McAb at about M_r 55 000 was observed in the culture supernatant of cells transfected with PCMV-RC-F (lane 1) and the supernatant of 1:2 dilution (lane 2). No immunoreactive was found in the supernatant of cells transfected with pTarge™ (lane 3, 4) or in culture medium alone (lane 5, 6).

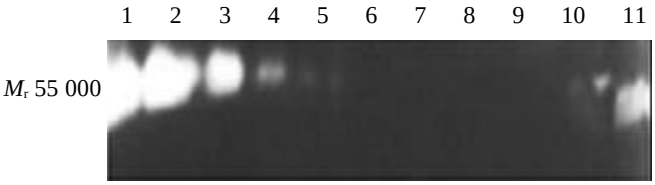

Figure 4 Gelatin zymography analysis for the activity of collagenase. The enhanced gelatin degradation activity was found in the culture supernatant of pCMV-RC-F transfected cells (lane 1-7 represent the zymograph of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 diluted solutions of the supernatant) when compared with pTarge™ transfected cells (lane 10,11 represent the zymograph of 1:2 and 1:1 diluted solutions of the surpernatant). No activity was found at about M_r 55 000 in the culture medium (lane 8, 9).

In vivo expresson of FLAG-tagged rat collagenase in rat livers and its effects on liver fibrosis

The 650bp amplified product was detected in the RT-PCR samples from pCMV-RIC-F plasmid transduced rat groups (C1 and C2 group) but not in the control animal groups (A and B groups) (Figure 5). The 753 bp amplified product by primer IC-UP4 and IC-DP4 which represent the endogenous collagenase gene expression was detected in all samples (Figure 6). In addition to RT-PCR, the expression of exogenous collagenase was observed in C1 and C2 groups by immunostaining with anti-FLAG antibody (Figure 7). Nearly about 30 percent of total cells presented positive signals in C1 and C2 groups, and the distributions of positive signals were found in both the hepatocytes and the perisinusoidal cells.

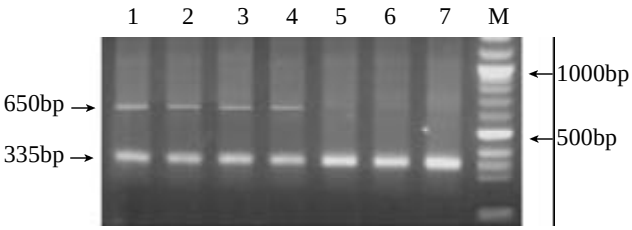

Figure 5 RT-PCR assay for the expression of recombinant interstitial collagenase in rat livers. The 652bp PCR amplified fragment of recombinant collagenase cDNA which was derived from their respective mRNA could be detected in liver samples from pCMV-RIC-F plasmid transduced rat groups C1 (lane 1, 2) and C2 (lane 3, 4) but not in the normal control group A (lane 5, 6) and disease control group B (lane 7). 335 bp fragment was PCR amplified b-actin cDNA. Lane M was 100 bp-1200 bp DNA marker ladders.

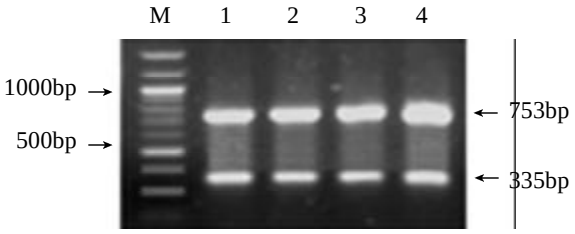

Figure 6 RT-PCR assay for the endogenous expression of collagenase in rat livers. The 753bp PCR-amplified fragment of endogenous collagenase cDNA which was derived from their respective mRNA could be detected in all liver samples from the normal control groups A (lane 1) and disease control group B (lane 4), as well as pCMV-RIC-F plasmid transduced rat groups C1 (lane 3) and C2 (lane 4). 335 bp fragment was PCR amplified b-actin cDNA. Lane M was 100bp-1200bp DNA marker ladders.

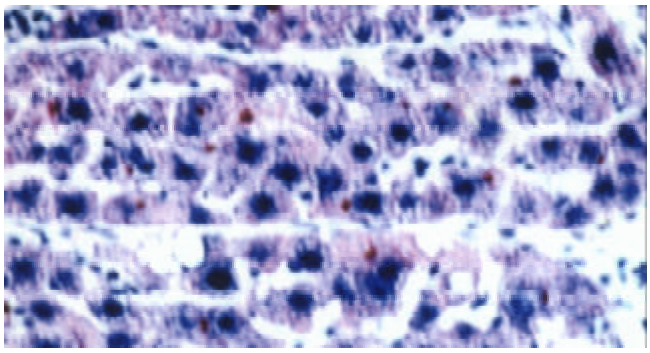

Figure 7 Immunohistochemical analysis of the expression of recombinant plasmid with anti-FLAG antibody in pCMV-RIC-F plasmid transduced group C1 (×400). Nearly about 30 percent of total cells presented positive signals. The distributions of positive signals were found in both the hepatocytes and the perisinusoidal cells.

The accumulation of collagen types I and III in the livers of animals in group B was more severe then that in group C1 and C2 according to the quantitative analysis of the immunohistochemical findings ($P<0.05$) (Table 2,Figure 8A-D).

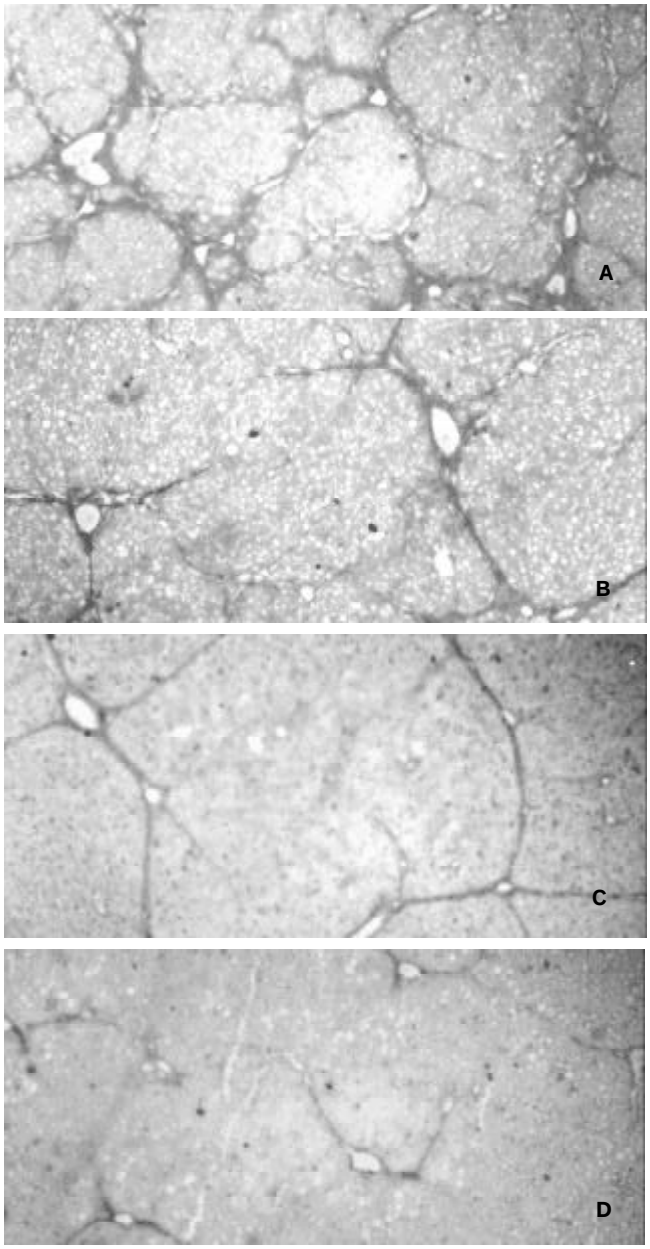

Figure 8 Immunohistochemical analysis of collagen types I (A, B) and III (C, D) in livers (×100). The accumulations of collagen in the disease control group (A, C) were more severe than that in the pCMV-RIC-F plasmid transduced C1 group (B, D).

Table 2 Effects of in vivo transfection of FLAG-tagged rat collagenase on the accumulation of collagen type I and III in experimental liver fibrosis

Group	*n*	ILD of collagen I	ILD of collagen III
A	6	17.35±2.62	18.94±3.78
B	6	86.83±11.93[a]	75.21±10.07[a]
C1	7	69.25±12.31[ab]	62.10±9.72[ab]
C2	7	72.18±14.18[ab]	64.80±11.69[ab]

[a]$P<0.05$ when compared with normal control group (group A); [b]$P<0.05$ when compared with disease control group (group B). ILD: Integral Light Density; N: number of rats in each group

DISCUSSION

In the present study the expression of FLAG-fusion rat collagenase was detected in pCMV-RC-F transfected NIH 3T3 cells and the secreted FLAG-fusion collagenase was found in the culture supernatant. This recombinant collagenase also exhibited enhanced gelatin degradation activity. These results suggest that the construction of recombinant collagenase plasmid is successfully function *in vitro* and could be used in further study *in vivo*. NIH 3T3 cell is a cell line of mouse embryonic fibroblasts. The mouse and the rat interstitial collagenase share highly homology when comparison was made between the amino acid sequences of their active forms[33]. Constitutive expression of collagenase gene had been found in NIH 3T3 cells before[34]. In our study, the weak activity of collagenase found in the culture supernatant of pTarge™ transfected cells in our gelatin zymography study might reflect this constitutive expression of mouse collagenase. Similar situation may also exist in liver tissues *in vivo*. Using anti-FLAG McAb we can easily distinct the recombinant rat collagenase that is tagged with FLAG domain from the natural mouse collagenase or the natural rat collagenase.

Collagen types I and III constituted the main components found in the increase of ECM[35, 36]. It has been proposed that the degradation of collagen types I and III is very important in the reversion of liver fibrosis. Theoretically the degradation of collagen could be enhanced if the expression of active collagenase increases. In this study, it was found that the recombinant plasmid could be delivered to liver by G-PLL and could be expressed in the tissue of liver. It was observed immunohistochemically that there were significant decrease of collagen types I and III deposition after transducing the recombinant collagenase plasmid into fibrostic livers. This suggests that the exogenous collagenase gene was able to degrade collagen.

Experimental gene therapies of liver cirrhosis have been tried by other research groups with different gene transfer methods and targeted genes[37-42]. In our study glycosyl-poly-L-lysine was used in the recombinant collagenase gene transfer into the livers since hepatocytes possess receptors that recognize galactose-terminal (asialo-) glycoproteins and thus they are particularly well suited for receptor-mediated methods of gene transfer. In our previous study it was proved that the recombinant plasmid could be delivered to liver and hepatocytes more specifically by G-PLL than lipofectamine[33]. In the present study it was also found that the positive signals of anti-FLAG McAb binding protein were in the hepatocytes of the rats of C1 and C2 groups. The less amounts of perisinusoidal cells which had positive signals as well might be the activated kupffer cells that had the function of phagocytosis. In addition, the previous study also observed the obvious expression of recombinant FLAG-tagged rat collagenase in liver at 24 h after plasmid transfection and persisting for longer than three weeks[31]. No detrimental effects of the transfection and expression of plasmid on important organs of normal rats such as liver, lung, heart and kidney were found by monitoring the serum levels of alanine transaminase, aspartic transaminase and creatinine and by observing the histological manifestation of these organs before and after transfection. These results indicated that the *in vivo* transfection of recombinant collagenase in liver that was mediated by glycosyl-poly-L-lysine was functionally expressed in liver and safe for other tissues.

The regulation of collagenase activity was affected by multiple factors[43, 44]. These include regulation of gene-transcription and protein-biosynthesis by cytokines or other factorsm, transformation of proenzymes into active forms, the

influence of specific or non-specific inhibitors on the activity of activated enzyme. Some studies have shown that the level of tissue inhibitor of metalloproteinase-1 (TIMP-1), which is a specific inhibitor of metalloproteinase, became very high during progressive liver fibrosis[45-49]. However the changes of regulation mechanisms such as the expression of TIMP-1 after collagenase gene transduction are still unknown. Whether the treatment effect on liver fibrosis by transferring an antisense gene of TIMP-1 is better than directly transferring collagenase gene as it was performed in this study worth further investigation.

In summary, the exogenous FLAG-tagged rat collagenase can be transferred by a recombinant plasmid and is expressed functionally in NIH3T3 cells and in a rat liver fibrosis model. As the result demonstrated that *in vivo* transduction of exogenous FLAG-tagged rat collagenase reduced liver fibrosis, this may stimulate the effort for which, the new therapeutic strategies in the management of liver fibrosis through regulation of collagenase activity and/or its inhibitors.

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• BASIC RESEARCH •

The immunotherapeutic effect of dendritic cells vaccine modified with interleukin-18 gene and tumor cell lysate on mice with pancreatic carcinoma

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Abstract

AIM: To estimate the effect of a therapeutic vaccine against pancreatic carcinoma based on dendritic cell (DC) vaccine modified with tumor lysate and Interleukin-18 gene.

METHODS: The BALB/C mice model of pancreatic carcinoma was induced with DMBA. DC vaccine was constructed through pulsed with tumor lysate and transfected by the recombinant adenoviral vector encoding IL-18 gene.The immnotherapeutic effects of DC vaccine on mice with pancreatic carcinoma were assessed (divided into DC-IL18-Lysate group, DC-Lysate group, DC-IL18 group, DC group, PBS group).

RESULTS: After vaccination of the DC vaccine, the concentration of IL-18 and IFN-γ were 2161±439 ng • L^{-1} and 435±72 ng • L^{-1} in DC-IL18-Lysate group and there was significant difference compared with other groups (P<0.01). After vaccination of the DC vaccine, the transplanted tumors were observed on 30 days in DC-Lysate groups, on 16 days in DC-IL18 groups, on 3 days in control group, but mice remained tumor-free for at least 50 days in DC-IL18-Lysate group and there was significant difference between DC-IL18-Lysate group and other groups (P<0.01). The median survival exceeds 62 days in DC-IL18-Lysate group. But the median survival was 48.6 days in DC-Lysate group, 33 days in DC-IL18 group, 17 days in PBS group. The survival period was obviously prolonged in DC-IL18-Lysate group than in other groups (P<0.05, P<0.01). The weight of pancreatic tumor was 0.22±0.083 g in DC-IL18-Lysate group, 1.45±0.74 g in DC-Lysate group, 1.89±1.34 g in DC-IL18 group, 3.0±1.6 g in DC group, 2.9±2.0 g in PBS group and the weight of tumor obviously reduced in DC-IL18-Lysate group than in other groups (P<0.05, P<0.01).

CONCLUSION: DC vaccine modified with tumor lysate and Interleukin-18 gene can induce a specific and effective immune response against pancreatic carcinoma cell.

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INTRODUCTION

Because of the lack of methods for early diagnosis and the limited effects of surgical treatment, chemotherapy and radiation therapy, about 98 % of the patients with pancreatic carcinoma don' t live more than 5 years[1-4]. So we clearly need the new therapies to improve the prognosis of the patients with pancreatic carcinoma. Immunotherapy is moving close to become a promising approach of anticancer therapy as it has fewer side effects and, more importantly, the opportunity to generate long-term immunity[5-7]. Dendritic cell (DC) is highly effective antigen presenting cell (APC) with the unique capability of inducing primary immune response against tumor-associated antigens. Animal studies have shown that the DC vaccine pulsed with tumor antigen could elicit specific T-cell response against tumor[8-12]. Recently, *Geiger et al*[13] reported that they have completed the first phase trial of tumor lysate-pulsed DC vaccine in the therapy of pediatric solid tumor, including osteosarcoma and fibrosarcoma, which were partial or complete regression.

Interleukin-18 (IL-18), originally termed IFN-inducing factor, induces IFN-γ production in both T cells and NK cells[14-15]. In addition, IL-18 induces T cells to produce GM-CSF, and enhances the cytolytic activity of T cells and NK cells[16]. In some animal model systems, IL-18 gene transfected into tumor cells should enhance both specific and nonspecific antitumor immune responses, which indicate if IL-18 gene were transfered into DC, it should induce highly effective antitumor immune responses[17-20].

In this study, an *in vivo* model to estimate the effect of a therapeutic vaccine against pancreatic carcinoma based on DC vaccine modified with tumor lysate and Interleukin-18 gene was designed. We hope those results should provided a scientific basis for our next step, clinical trials, in the future.

MATERIALS AND METHODS

Materials

BALB/c mice (6-8 week old, male) were purchased from the experimental animal center of the TongJi Medical Collegy. ^{3}H-TdR,^{51}Gr was from Beijing Institute of Atomic Energy, 7,12-Dimethylbenzanthracene(DMBA) from Aldrich Co. Germany. IL-18, IFN-γ ELISA Kits from Zhongke Biotech Co. Wuhan. A recombinant adenoviral vector encoding IL-18 gene termed pCR3.1-IL-18 was kind gift from Dr. Chenwen Ye (Department of Immunology, Institut Pasteur de Lille, Paris, France). The mouse dendritic cell line MTSC4 derived from

the thymic of BALB/c mice (4 week old) were obtained from the department of immunology of Beijing medical university, which were maintained in CM.

Methods

The mice model of pancreatic carcinoma The membrance and partial parenchyma of BALB/c mice' s pancreas were opened about 1 mm depth and the DMBA (7 mg) was put into there, as previously described [21]. After 3-4 months, the mice developed pancreatic ductal adenocarcinomas with glandular duct-like distribution of cancer cells. The tumors from mice' s pancrease were removed, carefully detached with a cell scraper, washed twice in PBS, and resuspended at a density of 2×10^7/ml in serum-free medium. 0.5 ml (2×10^7 /ml) viable tumor cells were injected into the left flank of BALB/c to develop the mice model of pancreatic carcinoma.

DC pulsed with tumor lysate Pancreatic carcinoma cells from the fresh solid tumor of mice were incubated with 0.01 % EDTA-solution for 10 min, washed twice in PBS, and resuspended at a density of 5×10^6/ml in serum-free medium. The cell suspensions were frozen at -80 ℃ (2 min) and thawed in 37 ℃ water (4 min), which were disrupted by four freeze-thaw cycles. For the removal of crude debris, the lysate was centrifuged for 10 min at 300×g. The supernatant was collected and passed through a 0.2-um filter for later use. Dendritic cells (DCs) were incubated with tumor lysates at a ratio of three tumor cells equivalents to one DC (i.e., 3:1) in CM. After 18 hr of incubation, DCs were harvested, washed twice in HBSS, and resuspended in HBSS for further study.

Cell transfection A recombinant adenoviral vector encoding IL-18 gene termed pCR3.1-IL-18 was kind gift from Dr. Chemen Ye (Immunology, Paris, France). For the transfection, DC and DC pulsed with tumor lysates were washed twice in HBSS, and incubated at 37 ℃ with the adenoviral vectors, respectively. Virus was used at a dose of 100 multiplicity of infection (MOI). Under these conditions, more than 80 % of DCs were infected.

Induction of tumor-specific CTL *in vivo* 30 BALB/c mice were at random divided into five groups. Every group included 6 mice, which were immunized s.c. in the right flank,(1)0.2 ml DC-IL18-Lysate (2×10^4 DCs modified with tumor lysate and IL-18, DC-IL18-Lysate group);(2)0.2 ml DC-Lysate (2×10^4 DCs modified with tumor lysate, DC-lysate group);(3)0.2 ml DC-IL18 (2×10^4 DCs modified with IL-18, DC-IL18 group);(4) 0.2 ml DC (2×10^4 DCs, DC group);(5)0.2 ml PBS (control group), respectively, twice at 7 days. After 7 days, spleen-derived T cells were isolated from mice by Nylon wool-separated and were cocultured *in vitro* with tumor cells for 5 days. After 5 days, T cells were tested for cytolytic activity in a standard 4-hr ^{51}Cr-release assay. Effector-to target (E/T) ratio were from 20:1 to 100:1. Each assay was performed in triplicate and triplicate wells were averaged and percentage of special CTL was calculated by the formula [(sample-spontaneous release)/(maximum release-spontaneous release)×100 %][22].

Immunological protection of DCs vaccine 30 BALB/c mice were at random divided into five groups and were immunized s.c. in the right flank with DC-IL18-Lysate, DC-Lysate,DC-IL18,DC,PBS, respectively, twice at 7 days (described above). These mice were challenged 7 days after the last immunization with 0.5 ml(1×10^7/ml) viable tumor cells from mice pancreas by s.c. in the left flank. The development of pancreatic carcinoma was observed in every mice.

Immunotherapeutic effect of DCs vaccine 30 mice from the mice model of pancreatic carcinoma with average tumor size 0.3×0.5 cm^2 were at random divided into five groups (described above), which were respectively injected in the right flank with 2×10^4 DC-IL18-Lysate,DC-Lysate,DC-IL18, DC, PBS, respectively, twice at 7 days. The size of the tumors was recorded twice weekly by measuring the largest perpendicular diameters (PD) and transverse diameter (TD) with calipers. The weight of the tumor was calculated by the formula[(PD×TD^2)/2].

Cytokine analysis After 7 days of the mice with pancreatic carcinoma immunized (described above), serum was obtained from the carotid artery of mice. The concentration of IL-18 and IFN-γ in the serum were measured by ELISA.

Statistical analysis

Data were expressed as the mean ±SEM and were analyzed by *t* test or ANOVA. Differation were considered significant when *P* was <0.05.Tests were performed using SAS (Statistical Analysis Software).

RESULTS

Tumor-specific CTL in vivo

Spleen cells obtained from mice at 7 days after the final immunization were cocultured with pancreatic carcinoma cells for 5 days. T cells of DC-IL18-Lysate group, DC-Lysate group and DC-IL18 groups were able to efficiently lyse the pancreatic carcinoma cells. The lytic efficiency increases following the rise of E/T ratio. The lytic efficiency of DC-IL18-Lysate group was the best, DC-Lysate group was the subsequence, and DC-IL18 group was the final. But T cells from additional groups obviously lack this ability (*P*<0.01, Figure 1).

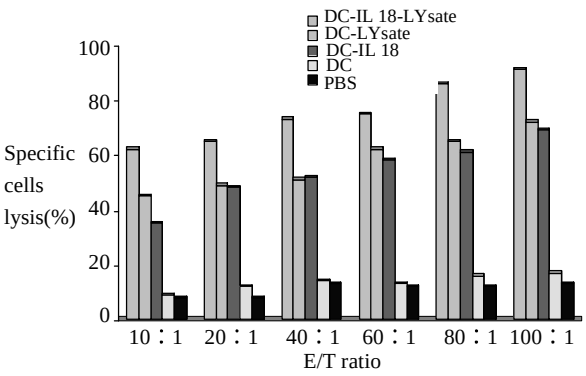

Figure 1 T cells were tested for cytolytic activity in a standard 4-hr ^{51}Cr-release assay. Effector-to target (E/T) ratio were from 20:1 to 100:1.

Cytokine analysis

After 7 days of the mice immunized, the concentration of IL-18 and IFN-γ in the serum were measured by ELISA. The concentration of IL-18 and IFN-γ were 2161±439 ng • L^{-1} and 435±72 ng • L^{-1} in DC-IL18-Lysate group.There were significant differences in DC-IL18-Lysate group versus other groups (*P*<0.05, *P*<0.01,Figure 2a-2b).

Immunological protection of DCs vaccine

The transplanted tumors were not observed in DC-IL18-Lysate group after the viable tumor cells from mice pancreas injected into the left flank 50 days. After 30 days, the transplanted tumors were observed in DC-Lysate groups. The first time was 16 days the transplanted tumor observed in DC-IL18 groups after the tumor cells injected into mice. Additional

groups all were observed the development of transplanted tumors from 3 to 9 days (P<0.01, Figure 3).

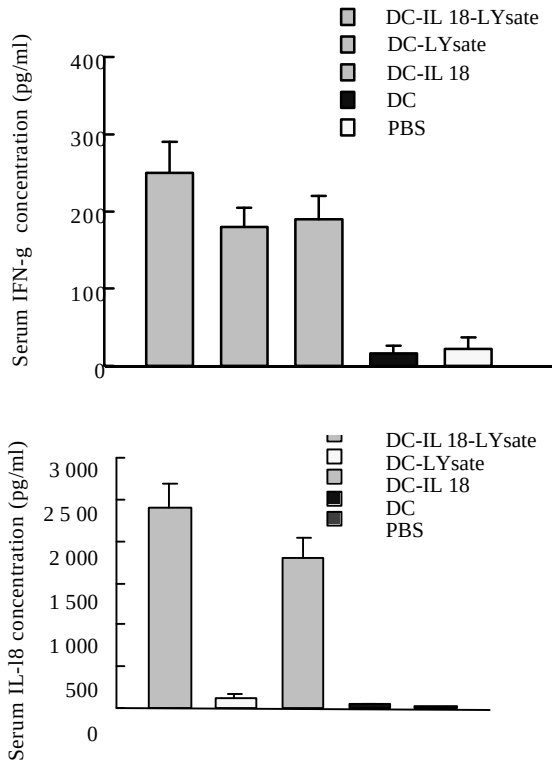

Figure 2a,2b The concentration of IL-18 and IFN-γ in the serum were measured by ELISA

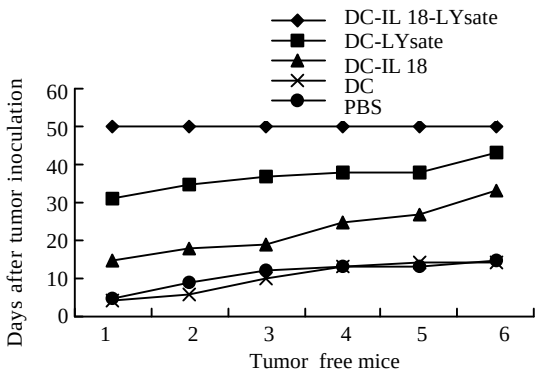

Figure 3 The development of the transplanted tumors was observed in every day

Immunotherapeutic effect of DCs vaccine

The growth velocity of tumor was observed obviously slowing in the mice immunized with DC-IL18-Lysate, DC-Lysate and DC-IL18. There were 3 cases in DC-IL18-Lysate group and 1 case in DC-Lysate group observed almost complete regression of the tumors. In contrast, the tumor cells displayed infiltrating style and adherent with nearby tissue in the mice immunized with PBS and DC. There was significant difference in the weight of tumor between the DC-IL18-Lysate group and the other groups (P<0.01,versus PBS group, DC group and DC-IL18 group; P<0.05, versus DC-Lysate group).The median survival exceeds 62 days in DC-IL18-Lysate group. But the median survival was only 48.6 days in DC-Lysate group, 33 days in DC-IL18 group, 17 days in PBS group (Table 1).

Table 1 Immunotherapeutic Effect of DCs Vaccine in Mice with Pancreatic Carcinoma

	DC-IL18-Lysate Ⅰ group	DC-Lysate Ⅱ group	DC-IL18 Ⅲ group	DC Ⅳ group	PBS Ⅴ group
Tumor Weight (g)	0.22±0.083[a]	1.45±0.74[b,c]	1.89±1.34[d]	3.0±1.6	2.9±2.0
Median Survival (d)	>62[a]	48.6±9.4[b,c]	33±12.3[d]	17±3.2	19.6±5.4

[a]P<0.01, *vs* the other groups; [b]P<0.05, *vs* Ⅲ group; [c]P<0.01, *vs* Ⅳ and Ⅴ groups; [d]P<0.05, *vs* Ⅳ and Ⅴ group

DISCUSSION

Dendritic cells (DCs) play an important role as primary antigen-presenting cell to initiate and maintain T-cell responses[23-25]. In our study, we designed an *in vivo* model to estimate the effect of a therapeutic vaccine against pancreatic carcinoma based on DCs modified with tumor lysate and Interleukin-18 gene. It was observed that DCs were pulsed with tumor lysate could obviously increase the efficiency against the pancreatic carcinoma cells.

At present, potential targets for the immunotherapy of pancreatic carcinoma are antigens such as carcinoembryonic antigen[26], HER-2/neu[27], mutant ras[28], p53[29]. However, vaccinating a single antigen has disadvantages, because it is unknown which of the identified antigens was the potential to induce an effective antitumor immune response. Furthermore, immunity against a single antigen maybe ineffective in tumors with pancreatic carcinoma and carries the risk of inducing tumor antigen escape variant[30,31]. In addition, this strategy is restricted to those patients with a specific HLA type.

In our research, we selected the tumor lysate as the target antigent because these unfractionated tumor-derived antigens could circumvent these disadvantages. Tumor lysates contain multiple known as well as unknown antigens that could be presented to T cells by MHC class I- and class II-pathways[32-35]. Therefore, lysate-load DCs are more likely to induce a polyclonal expansion of T cells, including MHC class II restricted T-helper cells. These have been recognized to play an important role in the activation of CTLs, which were probably the most important cells in antitumor immune response. The generation of CTL clones with multiple specificities may be an advantage in heterogeneous tumors and could also reduce the risk of tumor escape variants. Furthermore, lysate from the autologous tumor can be used independently of the HLA type the patient. A major drawback of unfractionated tumor antigens is the possibility of inducing an autoimmune reactivity to epitopes that are shared by normal tissues[36]. However, in clinical trials using lysate as the source of antigen, no clinically relevant autoimmune responses were detected[37-44].

IL-18 is a recently discovered cytokine cloned from mice with fluminant hepatitis induced by challenge with propionibacterium acnes and subsequent administration of LPS[14]. IL-18 lacks a signal sequence and is processed into the mature form by an IL-1β-converting enzyme (ICE)[15]. IL-18 is produced by cells of monocyte lineage, augments NK cytolytic activity, and enhances proliferation of T cells. IL-18 also promotes NK and T cells to secrete IFN-γ and GM-CSF. Based on those findings, IL-18 was demonstrated to confer a superior antitumor activity in some murine tumor systems. However, the systemic administration of recombinant IL-18 proteins, though effectively inhibited the tumor growth, resulted in death of all animals because of toxicity[45-47].

Systemic administration of IL-18 also was associated with severe dose-dependent toxicity in patient during the first human trial. The transfer of cytokine genes may circumvent the toxicity of systemic IL-18,at same time, may delivery and provide adequate local cytokine levels for immune cell activation.

In our study, the IL-18 gene was transfected into DCs by recombinant adenoviral vector and we observed cytokine releases in serum of the mice were immunized with DCs modified with IL-18 and tumor lysate by ELISA. After 7 days of the mice immunized, the concentration of IL-18 and IFN-γ was obviously increased in DC-IL18-Lysate group. This indicated DCs transfected with the cytokines gene exhibited significant levels of IL-18 production, At same time, augmented the production of IFN-γ.

In our research, the effect of immunological protection and immunotherapy of DCs vaccines was obviously increased when DCs were modified with IL-18 gene and tumor lysate than IL-18 gene alone, tumor lysate alone. Especially, from the results of our study we found DCs modified with IL-18 gene alone induced a limited antitumor immunologic reaction. These findings strongly suggest that IL-18, tumor lysate and DC interacted and there were congenerous effect against pancreatic carcinoma. At first, IL-18 enhanced NK cytolytic activity to induce more frequent and effective tumor cell death and promoted NK and T cells to secrete IFN-γ. Zitvoget *et al*[48] reported the antitumor effect of DC-based vaccination was dependent on production of Th1-associated cytokines such as IFN-γ. Therefore, IL-18 may play an important role in antitumor activity of DCs through enhancing the production of IFN-γ[49]. In turn, DCs sever as effective antigen-presenting cells to induce potent and specific immunologic reaction. At same time, DCs expressed more ICE that was required when the preprotein of IL-18 was processed into the biological activity form. As shown in a recent study, the functions of DCs were affected by contact with tumor cells. Dynamic changes in chemokine receptor expression (up-regulation of CCR7) were identified on DCs following contact apoptotic tumor cells[50]. Fumiaki *et al*[51] also demonstrated that direct contact with DCs and tumor cell could be important for generating CTLs. Thus, the implications are that IL-18/Tumor cell/DC plays a critical inductive and interactive role in promoting the efficiency of immunotherpy against pancreatic carinoma.

In summary, DC vaccines modified with tumor lysate and Interleukin-18 gene can induce a specific and effective immune response against pancreatic carcinoma cells. However, the feasibility and security of this DCs vaccine still need to be observed in additional experiments.

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• BASIC RESEARCH •

Synthesis of an enzyme-dependent prodrug and evaluation of its potential for colon targeting

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Abstract

AIM: To synthesize dexamethasone-succinate-dextran (DSD) conjugate and to evaluate the potentiality of DSD for the treatment of inflammatory bowel diseases.

METHODS: Dexamethasone was attached to dextran (average molecular weight=70 400 Dalton) using succinate anhydride in an anhydrous environment catalyzed by 4-dimethylaminopyridine and 1, 1'-carbonyldiimidazole. The chemical structure of DSD was identified by UV, IR and NMR, and the *in vivo* drug release behavior of this prodrug was investigated after oral administration of DSD suspension.

RESULTS: The DSD conjugate was obtained in two steps and the content of dexamethasone in DSD was 11.28 %. The dextran prodrug was stable in rat stomach and small intestine and negligibly absorbed from these tracts. Four to nine hours after the oral administration, most of the prodrug (>95 %) had moved to the cecum and colon, and was easily hydrolyzed by an endodextranase. Recover of dexamethasone from colon and cecum after administration of DSD conjugate was 6-12 folds higher than the recovery after administration of unmodified dexamethasone(t=2.74, P<0.05). The preferential release of free dexamethasone in cecum and colon over that in the small intestine was statistically significant (t=2.27, P<0.05).

CONCLUSION: The results of this study indicate that dextran conjugates may be useful in selectively delivering glucocorticoids to the colon.

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INTRODUCTION

Inflammatory bowel diseases, which include ulcerative colitis and Crohn's disease are currently treated with glucocoticoids and other anti-inflammatory agents[1,2]. For a steroidal anti-inflammatory drug, e.g. dexamethasone or prednisolone, a long-term administration would produce systemic side effects, including adrenosuppression, Cushinoid symptoms, immunosuppression, and diabetes. In this case, it is desirable to localize the release of dexamethasone insofar as possible to the afflicted sites in the colon. Release of drug in the proximal GI tract should be avoided to circumvent absorption from the small intestine, and consequent drug wastage and systemic side effects[2]. Because of the unique physiological characteristics of the large intestine, drug delivery to the colon can be achieved in different ways, including pH dependent approaches utilizing the changes in pH along the GI tract[3-12], coated dosage forms[13-17], time-controlled or pulsatile release systems[18-24], pressure-controlled colon delivery systems[25-30], coating drugs with bacterially degradable polymers[31-40], and delivery of drugs as prodrugs[41-47].

The bacterial count in the colon is higher than that in the preceding sections of the GI tract by many orders of magnitude in humans and other animals. Enzymes of the colonic bacteria can specifically degrade some kind of polysaccharides and azo-polymers or break the chemical bonds between the parent drug and the carrier, and then the pharmacological active component can be released from natural and synthetic prodrugs. The most important issue for this approach is a selection of the functional groups that can survive the passage through stomach and small intestine, but are degraded by enzymes of the colonic microflora thus specifically releasing the drug into the colon[2].

This project used dexamethasone as the model drug to synthesize a prodrug via a succinate tetracarbon-bridge that links the parent drug to the dextran carrier. Compared with unmodified dexamethasone, dexamethasone-succinate-dextran conjugate is more hydrophilic and has a larger molecular weight, which may decrease its possibility of being absorbed into the systemic circulation through the small intestinal epithelial cells. When it arrives to the colon, the dextran structure is hydrolyzed quickly by endogenous dextranase and then the esterase breaks the ester bond to release the dexamethasone. Distributions of dexamethasone in plasma and luminal contents were investigated after gastric intubation of DSD suspension or equivalent dose of dexamethasone to male SD rats.

MATERIALS AND METHODS

Material and apparatus

Dexamethasone was purchased from Tianjin Pharmacy Ltd., China. 4-dimethylaminopyridine (DMPA), 1,1'-carbonyldiimidazole and dextran (weight-average molecular weight=70 400 Dalton) were obtained from Sigma Chemical Company, St. Louis, MO. Succinate anhydride was purchased from Beijing Medicine Corporation, China. Molecular sieve (5Å) was obtained from Shitian Chemical Ind. China.

Methods

Synthesis of dexamethasone-dextran conjugate Dexamethasone 3.98 g, succinate anhydride 1.27 g and 4-dimethylaminopyridine 1.55 g were dissolved in 400 ml anhydrous acetone over 5Å molecular sieves. The reaction solution was stirred at 25 ℃ for 30 minutes, and the resulting solution was evaporated in a rotary evaporator to produce light

yellow solid. After the solid was dissolved in anhydrous ethanol, distilled water was added to achieve a solution of ethanol and water (29:71v/v). The solution was kept at -4 ℃ for 48 h to crystallize and filtered under reduced pressure. The resulting crystals were dried in a P_2O_5 drying pistol with refluxing of 95 % ethanol under vacuum (10 mmHg) for 24 h to produce dexamethasone succinate hemiester (DS). The yield is 85.28±4.57 %.

3.08 g of DS and 1.78 g of 1,1' -carbonyldiimidazole were dissolved in 15 ml of anhydrous dimethyl sulfoxide (DMSO). The reaction was run at 25 ℃ with stirring for 30 min. Then a solution of dextran in anhydrous DMSO(200 ml) and triethylamine(17.5 ml) was added, and the mixture was stirred at 25 ℃ for 21 h. The dextran conjugate was precipitated by adding 300 ml of ethanol/ether (50:50v/v) to the DMSO solution with stirring. The resulting polymer was dispersed in ethanol again and filtered under a stream of dry nitrogen. The precipitate was collected by filtration under reduced pressure, then washed with anhydrous ether three times to produce DSD white powder (yield: 81.27±5.09 %): UV λmax: 242 nm (ε14 500); IR(KBr): 3420(OH), 2930(CH_2),1740(C=O), 1660 (C=C), 1020(C-O-C) 898 cm^{-1}; ^{1}HNMR (DEXO-d_6): δ7.310, 7.285(d,1H,C-1), 6.233, 6.207(d,1H,C-2), 6.002 (s,1H, C-4), 3.488, 3.508, 3.623, 3.742,4.668 (s,1H,C-5' ,C-4' ,C-3' ,C-2' , C-1'), 3.202(s,2H,C-6'), 2.054(s,2H, C-21), 1.464 (s,3H,C-19), 0.860(s,3H, C-18), 0.758, 0.774 (d,3H,C-16).

The content of dexamethasone in DSD was measured by HPLC after alkaline hydrolysis.

Preparation of DSD granules and DSC test To evaluate the potential colon specificity of DSD *in vivo* test, granules of DSD or dexamethasone were prepared with the following ingredients: DSD or dexamethasone, cornstarch and lactose (5:50:45). A wet granulation method was applied. The granules were partially dissolved and suspended in water before dosing. Before granulation, all the ingredients were subjected to Differential Scanning Calorimetry (DSC). Measurements were performed on a calorimeter DSC7 connected to a Thermal Analysis Data Station 3 700 (Perkin-Elmer, Germany). Five mg of bulk materials were accurately weighed into standard aluminum pans. Thermograms were recorded from 303 to 573 K at a heating rate of 10 K • min^{-1}.

***In vivo* test** Male SD rats (weighing about 150 g) were fed a standard diet (R-2, Chengdu) and were fasted for 18 h prior to drug administration with free access to drinking water. The rats were divided into the test group and control group randomly. Each group was subdivided further into seven subgroups. The test groups were administered with suspension of DSD (equivalent to 3 mg of dexamethasone per Kg of rat body weight) by gastric intubation, and the control groups were administered with suspension of dexamethasone. After the drug administration, blood samples of the test subgroups and the control subgroups were collected at each predetermined time (1,3,4,5,6,7,9 h). Then the rats were sacrificed by decapitation and the stomach, proximal small intestine (PSI), distal small intestine (DSI), cecum and colon were removed. The contents of the GI tract were removed by gently squeezing the GI segments. The separated contents and tissues were quickly frozen to -20 ℃ and stored until analysis. Rat blood samples were collected and centrifuged at 700 g for 10 min. The plasma was frozen to -20 ℃ and stored until analysis was performed.

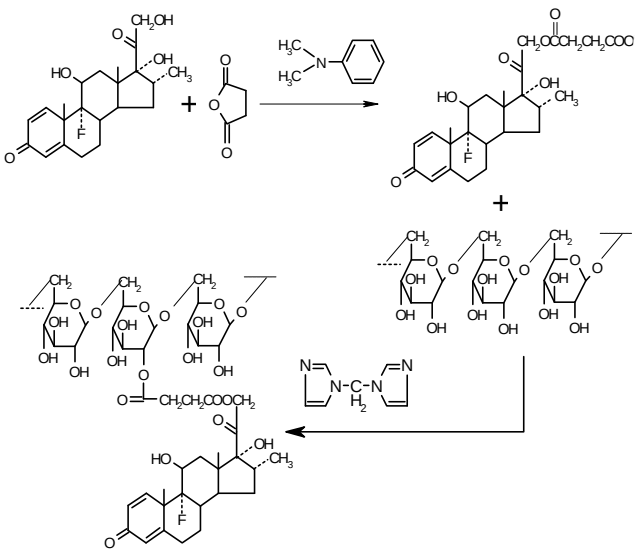
Analysis The frozen intestinal contents were thawed, weighed, and diluted to 50 %(w/v) with phosphate buffer (pH6.8). The suspended samples were homogenized by vortexing, and then 0.5 g of the diluted contents was placed in a 5-ml centrifuge tube. 200 μl of isotonic phosphate buffer (pH2), 100 μl of internal standard solution (0.1336 mg • ml^{-1}) and dexamethasone solution in different concentrations were added to the 5-ml centrifuge tube. The samples were extracted with acetic ester (3 ml) by vortexing for 2 min. After centrifuging for 10 min at 1000 g, 2 ml of the organic phase was removed and evaporate at 45 ℃ under vacuum. The residue was redissolved in mobile phase solution and centrifuged for 10 min at 1000 g. 20 μl of the supernatant fluid was subjected to HPLC analysis under the following conditions: Shimadzu CTO-10A system controller, LC-10AT pumps, SPD-10A variable wavelength detector, a Shimpack CLC C18 column(5 μm,4.6×150 mm). The mobile phase consisted of 35 % acetonitrile and 65 % buffer (50 mM trisodium citrate adjusted to pH4.6 with phosphoric acid). A flow rate of 1 ml.min^{-1} and a detection wavelength of 241nm were used. Prednisolone acetate was used as the internal standard. The plasma samples were treated by the same method described above.

RESULTS

Chemistry

DSD was prepared in two steps with a modified Mcleod reaction[2]. It is essential to keep the reaction continuing under the anhydrous condition to ensure high yield. Succinate anhydride was coupled to the dexamethasone hydroxyl group in anhydrous acetone in the presence of 4-dimethylaminopyridine to produce hemiester. Then the hemiester was coupled to dextran in DMSO using 1,1' -carbonyldiimidazole as catalyzer (Scheme 1).

The chemical structure was identified by ^{1}HNMR and IR, confirming the procedure of scheme 1. The content of dexamethasone in DSD was 11.28 equals to about 20 dexamethasone (molecular weight=392 Dalton) molecules were coupled to one dextran molecule (average molecular weight=70 400 Dalton).



Scheme 1 Preparation of DSD

Differential scanning calorimetry (DSC)

Study on interaction between the supplementary ingredients of suspension and DSD was performed by DSC. The thermogram displayed two transition peaks at 343K and 561K corresponding to DSD, and another two peaks at 420K and 486K corresponding to mixed ingredients. No new transition peak was observed when the physical mixture of DSD and ingredients were subjected to DSC, indicating that there was no interaction between DSD and the supplementary ingredients.

In vivo testing

The recovery of free dexamethasone from rat blood and GI tract at various times following oral administration of DSD suspensions is shown in Table 1. During the whole observation period (0-9 h), no dexamethasone was detected in blood. This observation indicated that DSD conjugate was so stable that it could not be degraded in upper GI tract and could not be absorbed into blood. Three hours after dosing, only very small amount (<3 % of total recovery) of dexamethasone was detected in small intestine in spite of the high level of esterase in small intestine. After 6h, the recovery of dexamethasone in small intestine further decreased. At the same time, a large portion (>95 % of total recovery) of the prodrug reached the cecum and colon intact.

Control experiments in which unmodified dexamethasone was administered showed that dexamethasone was absorbed primarily from the small intestine and the blood concentration of dexamethasone was much higher than test groups. Meanwhile, very small amount of dexamethasone was observed either in the cecum or in the colon (Table 2).

Table 1 Recovery of free dexamethasone from the rat blood and GI tract at various times after administration of DSD suspensions [a, b] (equivalent to 15mg of dexmethasone per Kg body weight)

t	T	Recovery of dexamethasone(%)					
h	μg	B	S	PSI	DSI	Ce	Co
1	5.2	nd	74	26	nd	nd	nd
3	7.3	nd	54	nd	46	nd	nd
4	14	nd	8.8	6.6	10.2	54	20.5
5	27.6	nd	9.2	5.0	13.4	54.4	18.0
6	133	nd	4.0	0.27	2.2	83	10.2
7	128	nd	4.5	nd	0.557	80	15.2
9	105	nd	12.4	3.49	3.72	62	18.2

Table 2 Recovery of free dexamethasone from the rat blood and GI tract at various times after administration of dexamethasone suspensions[a,b] (equivalent to 15 mg of dexmethasone per Kg body weight)

t	T	Recovery of dexamethasone (%)					
h	μg	B	S	PSI	DSI	Ce	Co
1	1076	8.9	54	15.8	20.8	0.05	nd
3	857	4.58	94.6	0.05	1.08	nd	nd
4	390	26	58	10.2	5.0	0.40	nd
5	230	36	22	1.19	4.2	16	2.56
6	203	33	30	6.7	3.63	24.7	2.25
7	139	39	49	2.02	5.6	3.74	0.79
9	134	40	42	1.52	7.8	6.0	3.1

[a]Values represent the average of three animals. [b]t, Time; T, Total recovery; B, Blood; S, Stomach; PSI, Proximal small intestine; DSI, Distal small intestine; Ce, Cecum; Co, Colon. nd: Not detected

The dexamethasone recoveries from cecum and colon after the oral administration of DSD suspensions or dexamethasone suspensions were also shown graphically (Figure 1 and Figure 2). It was obvious that the recovery of test groups from cecum and colon after administration were higher than that of control groups by 6-12 folds (t=2.74, P<0.05).

The specificity of dexamethasone release was further evaluated by comparing the amount of free dexamethasone recovered in the small intestine with that in the colon in the test group. A paired t-test indicated that the preferential release of free dexamethasone in cecum and colon over that in the small intestine was statistically significant (t=2.27, P<0.05). Meanwhile, a similar analysis in the control group showed that the difference between the dexamethasone concentration in the colon and that in the small intestine was not statistically significant.

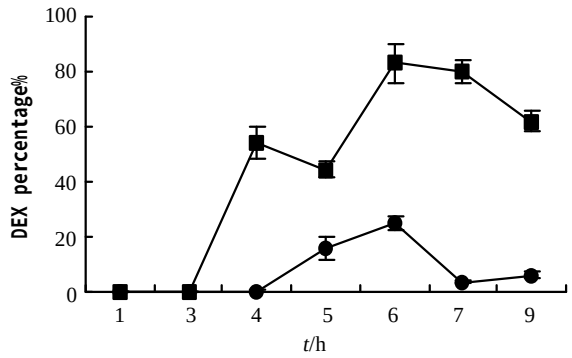


Figure 1 The dexamethasone contents-time curves in cecum after the oral administration dexamethasone suspensions (●-●) and DSD suspensions (■-■)

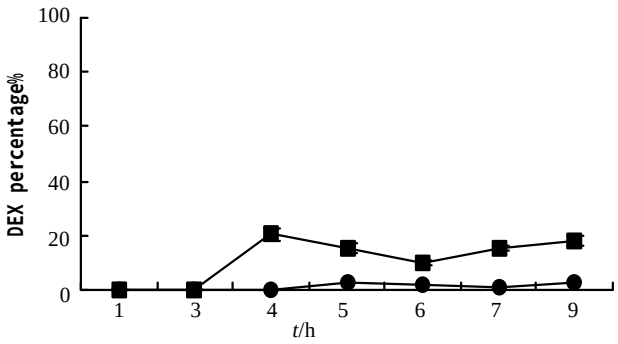


Figure 2 The dexamethasone contents-time curves in colon after the oral administration dexamethasone suspensions (●-●) and DSD suspensions (■-■)

DISCUSSION

The bacterial count in the colon is much higher than that in upper GI tract[2]. The colonic micro flora produce a variety of enzymes, including azoreductase, various glycosidases and amidases, which are not present in the stomach or the small intestine. Therefore, enzyme dependent drug release, which relies on the existence of enzyme-producing microorganisms in the colon, could be used to deliver drug to the colon after enzymatic cleavage of degradable carrier bonds and premature drug release does not occur in this case.

Besides treating inflammatory bowel diseases, colon-specific drug delivery system might be useful in other situations. The delivery of certain antineoplastic agents to the

colon might be beneficial in controlling colon cancer[48]. Enzyme prodrug gene therapy for colon cancer is also investigated by several researchers[49,50]. Antibiotics might be delivered specifically to the colon via cyclodextrin carriers[51-53]. In each of these cases, colon-specific delivery would allow the use of higher doses of potent agents with fewer systemic side effects.

The present results showed that the ester type prodrugs of dexamethasone/dextran release dexamethasone preferentially on cecal and colonic contents after the hydrolysis of dextran to small oligosaccharides, suggesting that dextran could serve as a new class of colon-specific drug carrier. The dextran conjugate survives the passage through upper GI tract although the high level of esterase in small intestine, indicating that dextran protects ester bond from hydrolysis by esterase. This result, together with the observation mentioned above, suggests that bacterial enzymes in the colon are responsible for hydrolysis of dextran conjugates. When DSD reached the colon, dextran was completely hydrolyzed into smaller oligosaccharides and exposed the ester bonds to esterase, which led to the rapid release of dexamethasone.

In summary, a colon-specific drug-delivery system has been developed based on drug-dextran conjugation and the unique glycosidase activity of the colonic microflora. Colonic drug delivery can be achieved with carriers by making prodrugs that survive the passage through stomach and small intestine, but the active moiety is released by enzymes specifically produced in colon.

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• BASIC RESEARCH •

The pre-synaptic blocker toosendanin does not inhibit secretion in exocrine cells

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Abstract

AIM: Toosendanin is a pre-synaptic blocker at the neuromuscular junction and its inhibitory effect is divided into an initial facilitative/stimulatory phase followed by a prolonged inhibitory phase. The present study investigated whether the subsequent inhibitory phase was due to exhaustion of the secretory machinery as a result of extensive stimulation during the initial facilitative phase. More specifically, this paper examined whether toosendanin could directly inhibit the secretory machinery in exocrine cells.

METHODS: Rat pancreatic acinar cells were isolated by collagenase digestion. Secretion was assessed by measuring the amount of amylase released into the extracellular medium as a percentage of the total present in the cells before stimulation. Cholecystokinin (CCK)-induced increases in intracellular calcium in single cells were measured with fura-2 microfluorometry.

RESULTS: Effects of toosendanin on CCK-induced amylase secretion and calcium oscillations were investigated. Toosendanin of 87-870 μM had no effect on 10 pM-100 nM CCK-stimulated amylase secretion, nor did 8.7-870 μM toosendanin inhibit 5 pM CCK-induced calcium oscillations. In contrast, 10 nM CCK_1 receptor antagonist FK 480 completely blocked 5 pM CCK-induced calcium oscillations.

CONCLUSION: The pre-synaptic "blocker" toosendanin is a selective activator of the voltage-dependent calcium channels, but does not interfere with the secretory machinery itself.

Cui ZJ, He XH. The pre-synaptic blocker toosendanin does not inhibit secretion in exocrine cells. *World J Gastroenterol* 2002; 8(5):918-922

INTRODUCTION

Toosendanin is a tetracyclic triterpenoid isolated from the seeds and barks of *Melia toosendan Seib. et Zucc* and *Melia azedarach L.* It has been used as an anthelmintic for many centuries, and has also been found to have pesticidal effects[1-5], and have anti-botulismic and other effects in whole animals[6, 7]. Work on nerve-muscle and other preparations (neuromuscular junction)[8-14] has established that toosendanin is a potent, long-lasting pre-synaptic inhibitor. The neuromuscular blocking effect of toosendanin is divided into two phases: an early stimulatory phase that is due to direct activation of voltage-dependent calcium channels[8, 12-18], and a delayed inhibition[9, 14]. However, it is not known whether or not the delayed blockade of the neuromuscular transmission is due to direct interference with the secretory machinery involved in neurotransmitter release.

Whether toosendanin has any direct effect on the secretory machinery can only be examined in secretory cells with no voltage-dependent calcium channels. If indeed the delayed blocking effect of toosendanin is due to direct inhibition of the secretory machinery, only will an inhibitory phase be observed in secretory cells which lack voltage-dependent calcium channels. The pancreatic acinar cell is an ideal model in which to address this question. The molecular mechanisms of secretion in pancreatic acinar cells are well elucidated[19-24], and these non-excitable cells have no voltage-dependent calcium channels[25, 26]. Therefore, this study examined if toosendanin has any inhibitory effect on secretion induced by a physiological secretagogue, cholecystokinin (CCK), in freshly isolated rat pancreatic acinar cells.

MATERIALS AND METHODS

Materials

CCK octapeptide, α-amylase and amylose azure were purchased from Sigma (St. Louis, MO, USA), Cell-Tak was purchased from Collaborative Biomedicals (Bedford, MA, USA). Toosendanin was a gift from Professor He LI (Department of Chemistry, Beijing Normal University). Collagenase P was bought from Boehringer Mannheim (Mannheim, Germany). Fura-2 AM was purchased from Molecular Probes (Eugene, OR, USA). (s)-N-[1-(2-fluorophenyl)-3,4,6,7-tetrahydro-4-oxo-pyrrolo[3,2,1-jk][1,4] benzodiazepin-3yl]-1H-indole-2-carboximide (FK480) was donated by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). Toosendanin and FK480 were both dissolved in DMSO as stock solutions before dilution to final concentration.

Isolation of rat pancreatic acini

Pancreatic acini were isolated from male Sprague-Dawley rats with body weight ranged from 170 g to 250 g according to the method reported previously[23, 27, 28].

Measurement of amylase secretion

Isolated acini were aliquoted into 2 ml portions and stimulated at 37 ℃ in a shaking water bath (50 cycle • min^{-1}) for 30 min. The amylase secreted into the buffer was assayed according to the procedures reported previously[29- 32] and expressed as percentage of the total present in the acini before stimulation.

Measurement of intracellular calcium $[Ca^{2+}]_i$

Ten microlitre of 1mM Fura-2 AM was added to1 ml of isolated acini (final concentration 10 μM) and the mixture was incubated in a shaking water bath at 37 ℃ and 50 cycle • min^{-1} for 40 min.

Fura-2-loaded acini were attached to the cover-slip of a Sykus-Moore chamber and perfused on the stage of an Olympus fluorescence miscroscope (IX70) attached to a microfluorometric calcium measurement system (M40, Photon Technology International, NJ, USA). Calcium increases were expressed as fluorescence ratios measured at 510 nm (F340/F380)[23, 27, 33-35].

Standard buffer used in this work was composed of (all in mM) NaCl 118, KCl 4.7, $MgCl_2$ 1.16, $CaCl_2$ 2.5, NaH_2PO_4 1.16, glucose 5.6, bovine serum albumin 2 mg • ml^{-1}, soybean trypsin inhibitor 0.1 mg • ml^{-1}, N-(2-hydroxyethyl) piperazine-N' -(2-ethanesulfonic acid) (HEPES) 10, MEM amino acid mixture (GIBCOBRL, Grand Island, NY, USA) 2 % and glutamine 2. The buffer was adjusted for pH to 7.4 with 4 mM NaOH and oxygenated with O_2 for 30 min before use.

RESULTS

Effects of toosendanin on CCK-induced amylase secretion

CCK stimulated amylase secretion from the freshly isolated rat pancreatic acini in a concentration-dependent manner (Figure 1). The maximum stimulation was achieved at CCK concentration of 100 pM, with a percentage secretion of 33± 1.6 (*n*=6). This bell-shaped dose response curve is consistent with previous report[36].

In separate experiments, acini were first incubated with toosendanin at 870 μM or 87 μM for 10 min before stimulation with CCK for a further 30 min in the continued presence of toosendanin (Figure 2). When toosendanin was added at 87 μM, the maximum stimulating CCK concentration shifted from 100 pM (18.2±1.6, *n*= 6) to 1 nM (19.7±1.2, *n*=6). With the addition of toosendanin at 870 μM, the maximum CCK concentration also shifted from 100 pM (18.2±1.6, *n*=6) to 1 nM (19.2±1.9, *n*=4). The slight rightward shift indicates a mild but statistically insignificance inhibition (Student' s *t* test, *P*>0.05). In control experiments, neither 0.01 % solvent DMSO nor toosendanin at each concentration used had any effect on amylase secretion (data not shown).

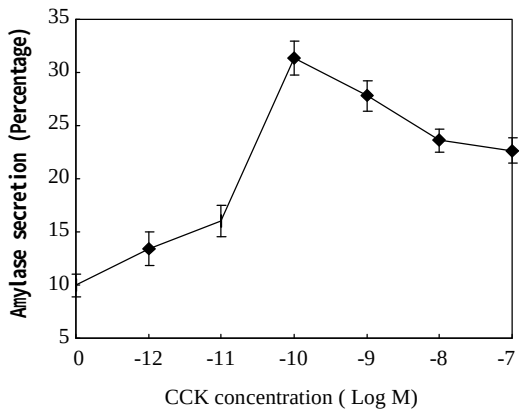
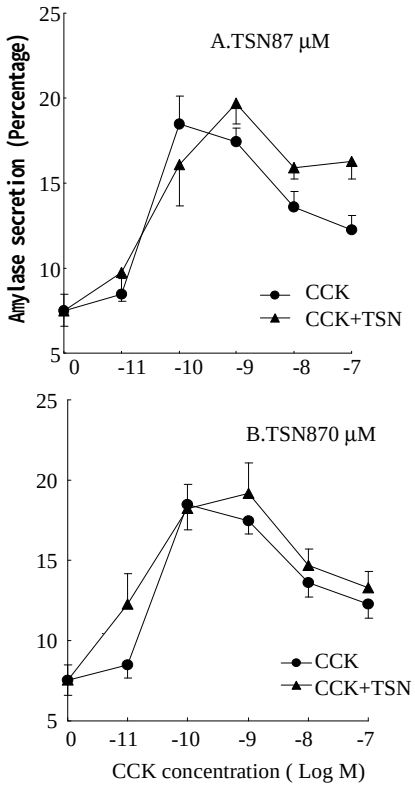


Figure 1 Concentration dependence of cholecystokinin (CCK)-stimulated amylase secretion. Note that maximum secretion was achieved at 100 pM of CCK. *n*=6

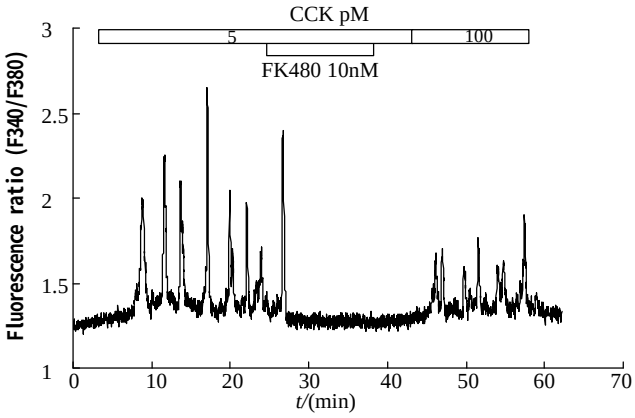
Effects of toosendanin on CCK-induced $[Ca^{2+}]_i$ oscillations

CCK of 5 pM induced regular calcium oscillations in perfused rat pancreatic acinar cells (Figure 3), which is consistent with previous reports[27, 28]. Addition of toosendanin at 8.7 μM to the CCK-stimulated acinar cells for 10-20 min had no apparent effect on CCK-induced calcium oscillations (Figure 3A, *n*=4). Even when toosendanin was increased to 87 μM, there was still no obvious inhibition observed (Figure 3B, *n*=7). At 870 μM, toosendanin induced a very mild inhibition: a single spike appeared missing in the trace shown (Figure 3C, *n*=8).

For comparison, FK480, an antagonist for CCK_1 receptors[37-39], produced immediate and complete inhibition of 5 pM CCK-induced calcium oscillations (Figure 4, *n*=5). FK480 at 10 nM abolished 5 pM CCK-induced calcium oscillations immediately upon addition, reducing the calcium to pre-stimulation level (Figure 4). After washout of FK480, calcium oscillations did not re-appear immediately, indicating that FK480 might bind to the acinar cells very tightly. It was possible, however, to re-introduce calcium oscillations when CCK was increased to 100 pM. At a lower FK480 concentration of 1 nM, a much longer time was needed before complete abolition of 5 pM CCK-induced Ca^{2+} oscillations was observed (data not shown).



igure 2 Effect of toosendanin (TSN) on CCK-stimulated amy-ıse secretion. TSN was added to the pancreatic acini 10 min efore stimulation with CCK which was maintained for an-ther 30 min. A. TSN 87 μM, *n*=6. B. TSN 870 μM, *n*=4. Note ıat, for clarity, the same CCK dose-response curve (*n*=6) was



the horizontal bars. *n*=5.

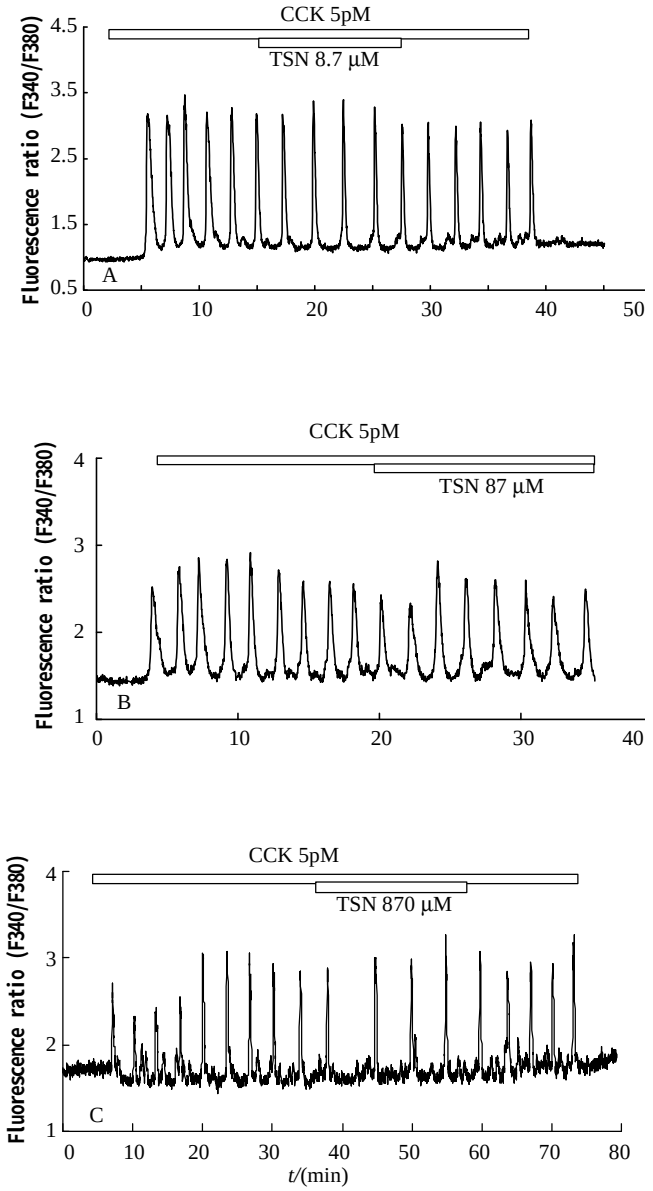


Figure 3 Effect of toosendanin on 5 pM CCK-induced calcium oscillations. CCK and TSN were added as indicated by the horizontal bars. A. TSN 8.7 μM, *n*=4. B. TSN 87 μM, *n*=7. C. TSN 870 μM, *n*=8.

DISCUSSION

The present work demonstrated that toosendanin, a pre-synaptic inhibitor of the neuromuscular transmission[7, 9, 13, 14], had little effect on CCK-stimulated amylase secretion. Although treatment with toosendanin at both concentrations tested (87 μM, 870 μM) resulted in a rightward shift in concentration of CCK that was needed to induce a maximum stimulation, i.e. from 100 pM in untreated cells to 1 nM in treated cells (Figure 2)[36], the toosendanin inhibition was not statistically significant for each CCK concentration (P>0.05).

Toosendanin of 8.7- 87 μM had no effect on 5 pM CCK-induced calcium oscillations. At a much higher concentration of 870 μM, a mild inhibition was observed because one spike seemed missing (Figure 4). This lack of marked inhibition of toosendanin on CCK-induced calcium oscillations is in sharp contrast with the complete blockade of CCK-induced calcium oscillations by FK480, a CCK_1 receptor antagonist [38, 40, 41]. Toosendanin at the neuromuscular junction had dual effects, a fast-onset stimulation which lasts about 40 min followed by a long-lasting inhibition[7-9, 12-14]. The fast phase has been postulated to be due to activation of voltage-dependent calcium channels[8, 12-18]. However, toosendanin had no effect on CCK-induced calcium oscillations in rat pancreatic acinar cells. This indicates that toosendanin had no effect on stores-operated calcium channels as they are the only calcium channels existing in the freshly isolated pancreatic acinar cells[20, 26] and are important for the continued presence of calcium oscillations[23, 27].

In view of the above findings, the pre-synaptic blocking effect of toosendanin should be looked at under a new light. The delayed inhibition of neuromuscular transmission could just be due to massive stimulation of synaptic vesicle fusion after activation of the voltage-dependent calcium channels at the nerve terminal, resulting in the depletion of synaptic vesicles and delayed depression. The fact that toosendanin administration in rat leads to a decrease in synaptic vesicles[42, 43] strongly supports this hypothesis. The initial strong stimulation of voltage-dependent calcium channels would afford the early stimulatory effects of toosendanin, providing an antidote to botulism[6, 18].

An activator for voltage-dependent calcium channels as toosendanin may be, a long-lasting inhibition of the neuromuscular junction[7- 9,12-14] or an extended period of synaptic vesicle depletion would require the stimulatory effect of toosendanin to be long-lasting. This stimulatory effect has indeed been found to last 40 min in nerve-muscle preparations[7-9, 12-14]. It is well known that voltage-dependent calcium channels inactivate rather quickly after opening, although with different kinetics[44-49]. Therefore, the long-lasting effect must be due to something other than constant opening of the calcium channels. It is known that intracellular calcium signals are subsequently encoded into activation of calcium/calmodulin-dependent protein kinases[50-52], and short-duration calcium signals could be transformed into long-lasting activation of calcium/ calmodulin-dependent protein kinase II[53], therefore it would be interesting in the future to further investigate the possible effect of toosendanin on calcium oscillations in excitable cells, and on oscillation-associated activation of calcium/calmodulin-dependent protein kinase II. It would also be interesting to identify which types of voltage-dependent calcium channels are activated by toosendanin since a number of them are involved in neurotransmitter release[54-56]. In this conjunction, it is important to note that decreased calcium influx into motor nerve terminals has been found to recruit additional neuromuscular junctions during the synapse elimination period [57].

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• BASIC RESEARCH •

Intestinal damage mediated by Kupffer cells in rats with endotoxemia

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Abstract

AIM: To determine the *in vivo* effects of phagocytic blockade of Kupffer cell (KC) on the release of proinflammatory cytokines in small intestinal lesion and on the integrity of intestinal tract by using gadolinium chloride ($GdCl_3$) during early endotoxemia.

METHODS: Wistar rats were divided into three groups: Group A, rats were injected with endotoxin (*E. coli* O111:B_4, a dose of 12 $mg.kg^{-1}$) only; Group B, rats were pretreated intravenously with 25 mg of $GdCl_3$ per kg 24 h are given endotoxin; and Group C, sham operation only. All animals were sacrificed 4 h after endotoxin injection. In portion of the rats of three groups, bile duct was cannulated, which the bile was collected externally. Morphological changes of ileum were observed under light microscopy and electronic microscopy. The KC were isolated from rats by collagenase perfusion and in KC, expression of TNF-α and IL-6 mRNA were determined by RT-PCR analysis. Plasma and bile TNF-α and IL-6 Levels were determined by enzyme-linked immunosorbent assay (ELISA).

RESULTS: In group A, there were neutrophil infiltration and superficial epithelial necrosis of the ileal villi, sloughing of mucosal epithelium, and disappearance of some villi. In group B, the ileal mucosal damage was much reduced. which in group C, no significant morphological changes were seen. $GdCl_3$ pretreatment decreased significantly the expression of TNF-α and IL-6 mRNA in group B (4.32±0.47 and 4.05 ±0.43) when compared to group A (9.46±1.21 and 9.04± 1.09) (P<0.05). There was no significant expression of TNF-α and IL-6 mRNA in group C (1.03±0.14 and 10.4±0.13). In rats of group A, the levels of TNF-α and IL-6 in bile and plasma were 207±29 $ng \cdot L^{-1}$, 1032±107 $ng \cdot L^{-1}$, 213±33 $ng \cdot L^{-1}$, and 1185±127 $ng \cdot L^{-1}$, respectively. In group B, they were 113±18 $ng \cdot L^{-1}$, 521±76 $ng \cdot L^{-1}$, 147±22 $ng \cdot L^{-1}$, and 572±54 $ng \cdot L^{-1}$, respectively. In group C, they were 67±10 $ng \cdot L^{-1}$, 72±13 $ng \cdot L^{-1}$, 109±18 $ng \cdot L^{-1}$, and 118±22 $ng \cdot L^{-1}$ respectively. There were significant difference between the three group (P<0.05).

CONCLUSION: KC release cytokines TNF-α and IL-6 causing damage to the integrity of intestinal epithelium and play a crucial role in the initiation and progression of intestinal mucosal damage during early endotoxemia.

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INTRODUCTION

LPS (lipopolysaccharide) is now considered to be a potent inducer of a series of inflammatory mediators[1-6]. In the early as infection, the main symptoms can be ascribed in part to the inflammatory cytokines as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6 and platelet-activating factor[7-9]. Recently, Jackson *et al*[10] reported that intestinal damage is mediated by factors derived from the bile. KC (Kupffer cell) constitute 80-90 % of the fixed tissue macrophages of the reticuloendothelial system (RES) and their activation and subsequent release of cytokines have great influence on the systemic response during sepsis[11-14]. KC has been shown to be a major source of released IL-6 following traumatic or hemorrhagic shock and after resuscitation[15]. Since LPS has been removed from the blood by the liver, we speculated the cytokines from KC in the bile may likely cause the damage to the intestinal during endotoxemia. Therefore, the aim of this study was to determine whether the *in vivo* phagocytic blockade of KC by using gadolinium chloride ($GdCl_3$) had any effects on intestinal integrity during early endotoxemia and whether the proinflammatory cytokines played crucial role in small intestinal lesions.

MATERIALS AND METHODS

Animals model

Wistar rats 10 wk old, weighing 220 g to 250 g were used in this experiment. The rats were obtained from Laboratory Animal Center of Chongqing University of Medical Science. These animals were divided into three groups: Group A, rats injected endotoxin only; Group B, rats pretreated with $GdCl_3$ and given endtoxin; and group C, sham operation only.

In vivo blockade of Kupffer cells by gadolinium chloride

In group B, animals were treated intravenously with 25 mg of $GdCl_3$ per kg 24 h prior to the operation as described by Koo *et al*[15]. In the preliminary experiment, the optimum dose and time of administration of $GdCl_3$ were determined by assessment of loss of KC labeled with India ink. In some of animals, in order to collect the bile, the animals were fasted overnight with free access to water. Bile duct was cannulated in rats of the three groups under ether anesthesia as described by Jackson *et al*[10]. Briefly, the abdomen was opened through a midline incision of 1.5-2.0 cm immediately below the sternum. The

bile duct was located, and approximately 1 to 1.5 cm of a 25 cm polyethylene cannulation tube (external diameter, 0.8 mm; internal diameter, 0.4 mm) was inserted into bile duct and tied in place, the other end of the tube was exteriorized through an opening in the right flank by an 18-gauge needle, allowing bile to be collected externally. The abdominal incision was then sutured, and the rats were held in restraining cages. Rats from group A and B were then injected iv with LPS from *E. coli* serotype O111: B4 (Sigma, St. Louis, Mo. USA), using a dose of 12 mg • kg^{-1}. Animals from group C were infused with some dose of pyrogen-free sterile saline. Plasma were separated from tail vein blood, and bile was collected 4 h after LPS administration when these rats were killed and the macroscopic appearance of the whole small instestine was assessed.

Morphological observation of ileum

One-to two-centimeter segments of the ileum from each rats were fixed in 100 ml • L^{-1} buffered formalin or 25 g • L^{-1} glutaraldehyde immediately. For light microscopy, the tissue blocks were embedded in paraffin, and the 5-um sections were stained with HE. For electronic microscopy, the tissue blocks were embedded in Epon 618 resin and ultrathin sections stained with uranyl acetate and lead citrate. A H-2000 transmission electronmicroscope was used.

Kupffer cell isolation

KC were isolated as described previously[16,17]. In brief, the livers were removed after a portal vein perfusion with Hanks' balanced salt solution (HBSS) and the homogenate was digested in a solution of 5 g • L^{-1} collagenase (Type IV, Sigma). The digest was washed thoroughly and plated on plastic dishes in RPMI medium containing 50 ml • L^{-1} fetal calf serum (FCS). After 3 h incubation at 37 ℃ in O_2 and CO_2 (0.95/0.05), nonadherent cells were removed with pipet. The adherent cells were collected with a rubber policeman. 70 % to 90 % viable KC was obtained in this manner. $GdCl_3$-treated and control cells exhibited similar viability, morphology, and *in vitro* phagocytosis, although the livers from $GdCl_3$-treated rats yielded 10-20 % fewer KC than saline-injected rats.

Expression of TNF-a and IL-1 mRNA in kupffer cell

RNA isolation and C-complementary DNA synthesis Total RNA was isolated from KC by using the Trizol Reagent (Life Technologies, USA). The quality of RNA was controlled by the intactness of ribosomal RNA bands. A total of 0.5 mg of each intact total RNA samples was reverse-transcribed to complementary DNA (cDNA) by using the reverse transcription polymerase chain reaction(RT-PCR) kit(Roche, USA). cDNA was stored at -70 ℃ till polymerase chain reaction (PCR) analysis.

Determination of TNF-a **and IL-6 mRNA by RT-PCR** The PCR primers used were TNF-α: sense (5'-CACCATGAGCACGGA AAGCA-3'), antisense (5'-GCAATGACTCCAAAGTAGAC-3'); IL-6: sense (5'-CTTCCAGCCAGTTGCCTTCT-3'), anti-sense (5'-AGCCAGAGTCATTCAGAGCA-3'); β-actin: sense (5'-ACCACAGCTGAGAGGGAAATCG-3'), antisense (5'-AGAGGTCTTTACGGATGTCAACG-3'). The sizes of the amplified PCR products were 692 bp for TNF-α, 309 bp for IL-6, and 281 bp for β-actin. The conditions for amplification were as follows: denaturation at 94 ℃ for 1 min, annealing at 57 ℃ for 2 min, and extension at 72 ℃ for 2 min for 31 cycles. The PCR products were electrophoresed in 15 g.L^{-1} % agarose gels, and the gels were ethidium bromide stained and video photographed on an ultraviolet transilluminator.

Bioassay for cytokines in plasma and bile

Plasma and bile levels of TNF-α and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacture's instructions and guidelines (Biosource International, Camarillo, CA).

Statistical analysis

All data were expressed as $\bar{x} \pm s$ and compared by one-way analysis of variance (ANOVA). Differences in values were considered significant if $P \leqslant 0.05$.

RESULTS

Effect of phagocytotic blockade of Kupffer cells on the intestinal integrity

We investigated the effect of phagocytotic blockade of Kupffer cells by $GdCl_3$ on the histological appearance of the ileum. 4 h after inoculation with LPS, macroscopic examination showed marked hyperemia, edema, and fluid accumulation within the lumen of small bowel in rats of group A. In contrast, in animals of group B, there was substantial reduction in hyperemia and no fluid accumulation. There was neither hyperemia nor fluid accumulation in group C.

In group A, there was marked tissue damage. Under light microscope, there were neutrophil infiltration in the villi, severely damaged epithelial layer with superficial necrosis and sloughing, some villi completely disappeared (Figure 1A). In contrast, the rats of group B, intestinal mucosal damage were much reduced, there was no evidence of hyperemia, vasodilatation or edema in the lamina propria, and the epithelial layer was intact, only modest hyperemia in the submucosa, only occasional edematous apex at the villi (Figure 1B). In group C, The histological appearance was normal (Figure 1C).

Figure 1A Superficial epithelial necrosis of villi and complete absence of some villi (HE×198).

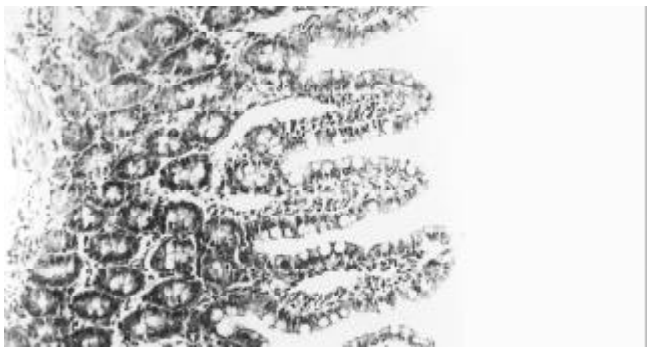

Figure 1B In intestinal mucosal damage much reduced (HE×198)

Figure 1C Normal appearance of intestinal mucosa (HE×198).

Effect of $GdCl_3$ on expression of TNF-α and IL-6 mRNA in Kupffer cells

$GdCl_3$ clearly resulted in sustained loss of phagocytic activity of KCs diminishing concentrations of TNF-α and IL-6 in bile and plasma. We found that $GdCl_3$ pretreatment decreased KCs cytokines transcripts after injection of LPS so that expression of TNF-α and IL-6 mRNA in group B were significantly less than those in group A ($P<0.05$). There were no expression of TNF-α and IL-6 genes in rats of group C (Figure 2).

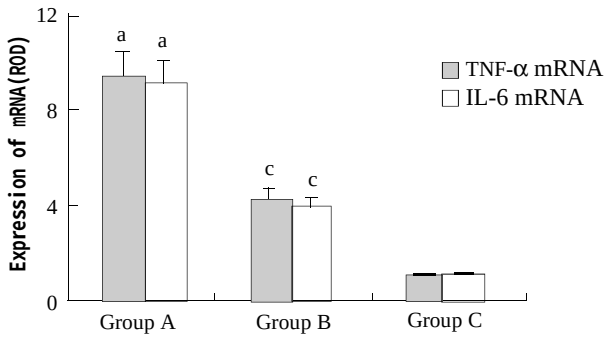

Figure 2 Expression of TNF-α and IL-6 mRNA. $^aP<0.05$, *vs* other two groups, $^cP<0.05$, *vs* group C.

Cytokines production in bile and plasma

TNF-α and IL-6 in the bile and plasma of above three groups were measured by bioassay (Table 1). In rats of group A, there were significant increase of TNF-α and IL-6 in bile and plasma, the concentration of the two cytokines in the bile were over 5-fold and 4-fold higher than those at in the plasma, respectively. In animals of group B, $GdCl_3$ given 24 h prior to LPS markedly reduced the levels of TNF-α and IL-6 in bile and plasma when compared to those in group A ($P<0.05$). Low levels of TNF-α and IL-6 were detectable in bile and plasma in rats of group C.

Table 1 Plasma and bile TNF-α and IL-6 levels of in rats ($\bar{x}\pm s$, n=7)

Group	TNF-α (ng.L^{-1})		IL-6 (ng.L^{-1})	
	Plasma	Bile	Plasma	Bile
A	207±29^a	1032±107^a	213±33^a	1185±127^a
B	113±18^c	521±76^c	147±22^c	572±54^c
C	67±10	72±13	109±18	118±22

$^aP<0.05$, *vs* other two groups; $^cP<0.05$, *vs* Group C

DISCUSSION

The role of cytokines in the bile causing gastrointestinal damage during sepsis has received little attention to date, and the bile continues to be viewed upon as having a purely digestive function. Other investigators reported after the relief of obstructive jaundice by internal drainage, endotoxemia could be reversed. But, the ratio of villous height to crypt depth in the mucosa of the terminal ileum was deceased in the rats with external drainage. The absence of bile from the gut may promote bacterial translocation to visceral organs[10,18]. These findings implicate the use of bile to prevent intestinal mucosal damage and reduce postoperative complications of jaundiced patient with endotoxemia. In fact, a growing body of evidence points to the presence of inflammatory mediators and other factors in bile. These factors include cytokines IL-1, IL-6, TNF-α, and epidermal growth factor, complement components, and acute-phase proteins, which are released by KC and other macrophages[13,19-27]. Recently, Jackson *et al*[10] reported the involvement of bile in gastrointestinal tract damage in endotoxemia. External drainage of bile or bile duct occlusion markedly reduced the effects of LPS to the intestine and prolonged survival of the animals[£Û18£Ý]. These studies indicate that after treatment with LPS, the bile contains substances that are capable of mediating intestinal damage. Our results showed there were higher concentrations of TNF-α and IL-6 in rats with endotoxemia than those in the control rats, and there were 5-fold and 6-fold higher levels of TNF-α and IL-6 in bile than in plasma. The intestinal mucosal epithelia of these animals were severely damaged. These findings suggest the luminal cytokines derived from the bile, contribute to the intestinal damage during early endotoxemia.

Where do these cytokines in bile come from The precise molecular mechanism of the cytokines in bile is uncertain. There are several reasons to believe that cytokines in the bile originate from synthesis by KC in the bile rather than extracted from the circulating blood. First, The KC are the main site for the clearance of endotoxin, they constitute about 80-90 % of sessile macrophages of the RES. LPS is taken up by KC and leads to production of cytokines by these cells[12,15-16,19,21,28,29]. Second, the concentration of TNF-α and IL-6 in the bile was more markedly increased than those in the plasma. Finally, the levels of these cytokines were significantly reduced by treatment with $GdCl_3$ which preferentially inhibited the KC function, again supporting the concept of production of cytokines by the hepatic KC. A number of studies had been conducted to examine the role of KC function during endotoxemia and other circulatory conditions. Studies dealing with the blockade of KCs phagocytosis in lethal endotoxic shock models had shown increased animal survival that underwent $GdCl_3$ pretreatment[30-32]. Moreover, utilizing KC blockade by $GdCl_3$ revealed the mediators released by KC during sepsis played a significant role[33]. Although studies had shown that intestinal damage occured during sepsis, it remained unknown whether blockade of KC prior to the onset of endotoxemia could prevent the occurrence of intestinal damage. The present study was designed to determine whether the *in vivo* phagocytic blockade of KC by $GdCl_3$ prior to the onset of sepsis had any salutary effects on intestinal integrity and expression of proinflammatory cytokines such as TNF-α and IL-6, their genes and proteins. Our results indicated that expression of TNF-α and IL-6 mRNA significantly increased 4 h after the induction of LPS. However, rats that underwent $GdCl_3$ treatment prior to LPS demonstrated significantly reduced expression of the genes and proteins of these cytokines in bile and plasma. Thus, KC can play a crucial role in the

initiation and progression of intestinal damage following endotoxemia[34-38].

The role of cytokines in intestinal pathology has not been welle lucidated. But, cytokines in intestinal tract could promote bacterial association with the epithelium, or might act upon luminal microorganism to enhance their invasive characteristics[39-43]. There are evidences that TNF-α is able to affect directly the virulent properties of some organisms. For example, TNF-α enhances the invasion of cultured cells, promoted by cytokines. IL-1 has been shown to act as a growth factor for pathogenic *E.coli*. Fluid accumulation in the intestine of LPS-treated rats indicates presence of altered permeability. Furthermore, TNF-α can affect directly the permeability of epithelial barriers, and loosening of the tight junction so as to facilitate the early entry of bacteria into the mesenteric lymph nodes and other target organs. TNF-α can induce expression of adhesion molecules ICAM-1 and LFA-3 in human intestinal epithelial cell lines. It can also influence cytokines production and proliferation in intestinal cell lines[44-48]. These findings reveals the presence of specific receptors on such cells, the TNF-α receptors are predominantly located on the basolateral aspect of intestinal epithelial cells, as are receptors for IL-1. After LPS stimulation, macrophages release cytokines damaging the intestinal epithelial integrity, allowing luminal TNF-α nd IL-6 to gain assiss to their receptors on the basolateral aspect of the epithelial cells and increase epithelial permeability, finally cause the intestinal damage.

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• BASIC RESEARCH •

Epidemiological survey of Blastocystis hominis in Huainan City, Anhui Province, China

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Abstract

AIM: To provide scientific evidence for prevention and controlling of blastocystosis, the infection of *Blastocystis homonis* and to study its clinical significance in Huainan City, Anhui Province, China.

METHODS: Blastocystis homonis in fresh stools taken from 100 infants, 100 pupils, 100 middle school students and 403 patients with diarrhea was smeared and detected with method of iodine staining and hematoxylin staining. After preliminary direct microscopy, the shape and size of Blastocystis homonis were observed with high power lens. The cellular immune function of the patients with blastocystosis was detected with biotin-streptavidin (BSA).

RESULTS: The positive rates of Blastocystis homonis in fresh stools taken from the infants, pupils, middle school students and the patients with diarrhea, were 1.0 % (1/100), 1.0 % (1/100), 0 % (0/100) and 5.96 % (24/403) respectively. Furthermore, the positive rates of Blastocystis homonis in the stool samples taken from the patients with mild diarrhea, intermediate diarrhea, severe diarrhea and obstinate diarrhea were 6.03 % (14/232), 2.25 % (2/89), 0 % (0/17) and 12.31 % (8/65) respectively. The positive rates of *Blastocystis homonis* in fresh stools of male and female patients with diarrhea were 7.52 % (17/226) and 3.95 % (7/177) respectively, and those of patients in urban and rural areas were 4.56 % (11/241) and 8.02 % (13/162) respectively. There was no significant difference between them (P>0.05). The positive rates of CD_3^+, CD_4^+, CD_8^+ in serum of Blastocystis homonis-positive and-negative individuals were 0.64±0.06, 0.44±0.06, 0.28±0.04 and 0.60±0.05, 0.40±0.05 and 0.30±0.05 respectively, and the ratio of CD_4^+/CD_8^+ of the two groups were 1.53±0.34 and 1.27±0.22. There was significant difference between the two groups (P<0.05, P<0.01).

CONCLUSION: The prevalence of *Blastocystis hominis* as an enteric pathogen in human seems not to be associated with gender and living environment, and that *Blastocystis hominis* is more common in stool samples of the patients with diarrhea, especially with chronic diarrhea or obstinate diarrhea. When patients with diarrhea infected by *Blastocystis hominis* , their cellular immune function decreases, which make it more difficult to be cured.

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INTRODUCTION

Blastocystis homonis (B.h) is increasingly recognized to be a cause of human enteric disease. Its presence has been reported in a wide variety of intestinal disorders resembling irritable bowel syndrome (IBS) such as bloating, flatulence, mild to moderate diarrhea, abdominal pain, and nausea[1-35].The geographic distribution of *Blastocystis homonis* appears to be global, with infections common in tropical, subtropical and developing countries[36-40]. In general, studies from developed countries report approximately a 1.5 % to 10 % overall prevalence of *Blastocystis homonis*[41-45]. However, few reports of the prevalence and the importance of the protozoan *Blastocystis homonis* as an intestinal pathogen in China have been found. In order to explore the epidemiological characteristics and clinical significance of blastocystosis in population of the city of Huainan, a prospective study was carried out from July to August in 2001.

MATERIALS AND METHODS

Population

The study was performed in the following groups of the population in Huainan: in a healthy population (n=300, normal group), including infants in day-care centers (n=100), pupils (n=100) and middle school students (n=100), and in outpatients with diarrhea (n=403, male 226 and female 177, aged from 6 to 52 years). In addition, we paid more attention to the patients with intractable diarrhea.

Methods

A questionnaire, administered by a nurse, was used to collect detailed information of each subject investigated. Information was collected by means of in-person, telephone and interview, including age, gender, history of present illness, anamnesis, symptomatology (i.e. fever, upper respiratory tract infection, nausea, diarrhea, abdominal cramps, bloating, steatorrhea), date of symptom onset, duration of symptoms, personal health habits, and living environmental condition and the date of stool sample collected.

Stool examination All individuals were asked to provide one stool sample in disposable stool boxes for analysis. Samples were sent to the Department of Etiology and Immunology, School of Medicine, Anhui University of Science & Technology in Huainan for *Blastocystis homonis*. Then the sample was smeared to semitransparent feces membrane on the surface of sheet slides. After these smears were left to dry naturally and fixed with methanol, iodine solution and hematoxylin staining were made, and examination under microscope was carried out. The shape and size of *Blastocystis homonis* were observed.

Detection of T lymphocyte subsets To investigate possible changes of cellular immune function in Blastocystis homonis-infected individuals, the level of CD_3^+, CD_4^+,CD_8^+ and CD_4^+/CD_8^+ in peripheral blood of Blastocystis homonis-positive individuals were tested with biotin-streptavidin (BSA) method. Firstly, peripheral venous blood of subjects was withdrawn, anticoagulated with heparin, and diluted with fluid free of Ca^{2+}, Mg^{2+}. Secondly, peripheral blood mononuclear cells were separated with lymphocytes separating medium, cleaned, and the number of cells was adjusted to $(1\text{-}3)\times10^9$/L of which 10 μl was taken and smeared in an acid-proof varnish circle on the surface of the slides. When it dried naturally, McAb of anti-CD_3^+, anti-CD_4^+ and anti-CD_8^+ and sheep anti-guineapig IgG, SA- HRP were added into the circle. After development with DAB, the slides were observed under microscope. Only brown cytomembrane staining was regarded as positive, otherwise, as negative specimen. A total of 200 cells were counted, and the positive percentage of cells were analyzed respectively.

Statistical analysis

The positive rates were expressed as percentage, and the statistical analysis was carried out by using χ^2 and *t*-test. A probability value of less than 0.05 was considered statistically significant.

RESULTS

Stool examination

Of the 703 stool samples examined, 3.70 % (26/703) were found to be positive for *Blastocystis hominis*. Furthermore, the positive rate of *Blastocystis hominis* in 300 stools of healthy people was 0.67 % (2/300); and those of infants, pupils and middle school students were 1.00 % (1/100), 0 (0/100) and 1.00 % (1/100) respectively. In addition, The positive rates of *Blastocystis hominis* in the stools taken from the outpatients with mild diarrhea, intermediate diarrhea, severe diarrhea and obstinate diarrhea were 6.03 % (14/232), 2.25 % (2/89), 0 % (0/17) and 12.31 % (8/65) respectively. There was significant difference in the positive rates between each type of patients (P<0.05). The detailed results are showed in Table 1.

Table 1 The detective results of *B.h* in fresh feces (*n*, %)

Group	*n*	*B.h* positive	
		n	rate
[b]Normal	300	2	0.67
Infants	100	1	1.00
Pupils	100	1	1.00
Middle school students	100	0	0.00
[b]Diarrheic outpatients	403	24	5.96
[a]Mild	232	14	6.03
[a]Intermediate	89	2	2.25
[a]Severe	17	0	0.00
[a]Obstinate	65	8	12.31

[a]P<0.05, χ^2=7.9475; [b]P<0.01, χ^2=13.5181 *vs*: comparison with normal and abnormal and different diarrhea

Relationship between gender and infection of Blastocystis hominis

Of the 403 outpatients, the positive rates of Blastocystis hominis in male and female patients were 7.52 % (17/ 226) and 3.95 % (7/177) respectively. Statistics found no significant difference in positive rate between male and female.

Relationship between living place and infection of Blastocystis hominis

The positive rates of *Blastocystis hominis* in stools taken from patients with diarrhea living in urban and in rural areas were 7.52 % (17/226) and 3.95 % (7/177) respectively. There was no significant difference between the two groups (P>0.05).

Relationship between types of diarrhea and infection of Blastocystis hominis

The positive rate of *Blastocystis hominis* in stools of healthy people was 0.67 % (2/300), while that of diarrheic patients was 5.96 % (24/403). Among the patients with diarrhea, the positive rates of *Blastocystis hominis* in loose stools ,watery stools and mucopurulent bloody stools were 3.70 % (21/305), 4.23 % (3/81) and 0 % (0/17) respectively. There was no significant difference between each type of patients (P>0.05). Results are showed in Table 2.

Table 2 Relationship between types of diarrhea and infection of B.h (*n*, %)

Group	*n*	*B.h* positive	
		n	rate
Normal	300	2	0.67
Diarrhea	403	24	5.96
Loose stool	305	21	3.70
Watery stool	81	3	4.23
Mucopurulent bloody stool	17	0	0.00

P>0.05, χ^2=2.2767 *vs*: comparison with different diarrhea

Changes of cellular immune function in Blastocystis hominis-infected individuals

Compared with the negative group, the level of CD_3^+ ,CD_4^+ and CD_4^+/CD_8^+ of *Blastocystis hominis*-infected individuals decreased, but that of CD_8^+ did not change.

Table 3 Tlymphocyte subsets of patients with *B.h* in faeces ($\bar{x}\pm s$, number fraction)

B.h	*n*	CD_3^+	CD_4^+	CD_8^+	CD_4^+/CD_8^+
Positive	26	0.64±0.06	0.44±0.06[a]	0.28±4.44	1.53±0.34[b]
Negative	30	0.60±0.05	0.40±0.05[a]	0.30±5.12	1.27±0.22[b]

[a]P<0.05, [b]P<0.01, *vs* negative

DISCUSSION

Results from this study showed that *Blastocystis hominis* as an intestinal pathogen in humans was found in Huainan area by stool examination, and the prevalence was not related to gender

and living circumstances, and that statistically significant association was observed between the presence of diarrhea and infection with *Blastocystis hominis*.

In this study, *Blastocystis hominis* was found in 26 (3.70 %) of the 703 stool specimens examined. The positive rates of male was similar to that of female, and there is no significant difference in the positive rates between the diarrhea patients living in urban areas and those in rural areas (P>0.05), which showed the prevalence of the organism was not related to gender and living environment of the individuals examined.

The results of this study supported the idea that *Blastocystis hominis* was associated with diarrhea. The positive rates of *Blastocystis hominis* in stools of the healthy people was 0.67 % (2/300), while that of the diarrheic patients was 5.96 % (24/403), and the difference between them was significant (P<0.05). To be exact, the positive rates of *Blastocystis hominis* was high in stools of the patients with mild diarrhea, intermediate diarrhea and obstinate diarrhea, but there was no *Blastocystis hominis* found in stools of patients with severe diarrhea. In accordance with other reports[46-49], vacuolar *Blastocystis hominis* were found in stools of patients with diarrhea with iodine solution and hematoxylin staining. This finding suggested that vacuolar *Blastocystis hominis* might be the main type of *Blastocystis hominis* causing diarrhea. Although the reasons why the organism had been found in both symptomatic and asymptomatic individuals have been largely unknown[50-56], one possibility was that it was due to infection time, infection dose, poly-infection with bacteria and the ability of host immunity that might decide whether the symptom turned up or not, because only over 24 h could the cysts of *Blastocystis hominis* develop into a large number of vacuolar forms[57-58].

In addition, this experiment demonstrated that the hematoxylin staining offered a very convenient and easy method to differentiate the various stages of *Blastocystis hominis*. As a matter of fact, there is high affinity between hematoxylin and *Blastocystis hominis*. By hematoxylin staining, the walls, nucleus, chromatoid bodies and other structures of *Blastocystis hominis* can be observed clearly, and vacuolar, granular, metamorphotic *Blastocystis hominis* can be easily differentiated from small amebae which do not cause any disease[59-61].

Our study provided evidence for the changes of cellular immune function in *Blastocystis hominis*-infected individuals. In this paper, the level of CD_3^+,CD_4^+,and CD_4^+/CD_8^+ decreased in *Blastocystis hominis*-infected individuals , but that of CD_8^+ was normal. Compared with the *Blastocystis hominis* negative group, the difference was significant (P<0.05).Recent advances in *Blastocystis hominis* found that in subjects suffering from immunodepression *Blastocystis hominis* showed a significant association with gastrointestinal symptoms[62-71]. All of these showed that the infection of *Blastocystis hominis* was related to the hosts' cellular immune function.

The level of CD_4^+/CD_8^+ is key to immunoregulation. When decreased, it suggested that T helper lymphocytes took part in the course of diarrhea caused by *Blastocystis hominis*. Indeed, both the ability of humoral immunity and that of cellular immunity decreased in the patients with low level of CD_4^+/CD_8^+, which made it difficult to cure diarrhea[72-75]. Because of low ability of immunological kill mediated by CD_8^+ cell, the cellular immunity of human bodies played an important role in the course of diarrhea.

In conclusion, Blastocystis hominis should be kept in mind of parasitologists and physicians when dealing with patients with diarrhea. Blastocystis hominis has long been described as a non-pathogenic protozoan parasite until recently, when claims have been made that it can result in pathogenic conditions[76-78]. Many labs do not know that it is now considered harmful to human bodies, or do not know how to test for it. Moreover, because of absence of specific symptoms, the disease was easily confused with other intestinal diseases and was easily misdiagnosed. The authors suggested that stool examination should be carried out on patients with diarrhea in order to decide whether or not the patients were infected by *Blastocystis hominis*, and the stool samples should be collected more than once from patients showing clinical signs and symptoms.

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• CLINICAL RESEARCH •

Pancreatic microcirculatory impairment in experimental acute pancreatitis in rats

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Abstract

AIM: To study the feature of pancreatic microcirculatory impairment, especially the initial changes, in caerulein-induced experimental acute pancreatitis (AP).

METHODS: The pancreatic microcirculation of caerulein-induced AP model was studied by intravital fluorescence microscopy with FITC-labeled erythrocytes (FITC-RBC), scanning electron microscopy of vascular corrosion casts, and light microscopy of Chinese ink-injected/cleared tissues.

RESULTS: Animals in caerulein-treated group showed hyperamylemia (×2), pancreatic oedema, infiltration of inflammatory cells in pancreas. Constrictions of intralobular arteriolar sphincters, presence of vacuoles in all layers of sphincter, and gross irregularity in capillary network of acini were found in the AP specimens. The decrease of pancreatic capillary blood flow (0.34±0.10 nl.min^{-1} *vs* 0.91±0.06 nl.min^{-1} of control, $P<0.001$), reduction of functional capillary density(277±13 cm^{-1} *vs* 349±8 cm^{-1} of control, $P<0.001$), and irregular intermittent perfusion were observed in caerulein-induced groups.

CONCLUSION: Impairment and constriction of pancreatic intralobular arteriolar sphincter are the initial microcirculatory lesions in the early phase of acute pancreatitis, and play a key role in the pancreatic ischaemia and pancreatic microvascular failure in acute pancreatitis.

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INTRODUCTION

Etiopathology of acute pancreatitis (AP) is not fully understood[1-19]. Microcirculatory impairment has long been recognized as one of the etiological factors of acute pancreatitis[20]. Pancreatic microcirculatory disturbance may act as initiating factor or aggravating/continuing factor. However, the mechanism of microcirculatory impairment in acute pancreatitis is complex; there are questions concerning local pancreatic microcirculatory change in acute pancreatitis and the features of pancreatic microcirculatory disturbance in various stages of AP remain subject to further study[21-28]. To investigate the feature of the pancreatic microcirculatory impairment in the early-stage of caerulein-induced experimental acute pancreatitis, dynamic method of microcirculatory research combined with static method had been carried out in this study.

MATERIALS AND METHODS

Animals

48 adult male Wistar rats, weighing 250-350 g, were randomly assigned to 4 groups: (1) control group (group 1, n=12). (2) intravital study group, panceatic microcirculation observed with FITC-labeled RBC and intravital fluorescence microscope (group 2, n=12). (3) light microscopy and scanning electron microscopy study group, pancreatic microvasculature perfused with ink and methylmethacrylate (group 3, n=12). (4) histocellular study group (group 4, n=12).

Experimental pancreatitis

Caerulein used to induce acute pancreatitis was obtained from Sigma Co.. All experimental groups were injected caerulein subcutaneously 5.5 and 7.5 μg.kg^{-1} 1 and 2 h after the beginning of experiment respectively, while control group was injected physiological saline solution subcutaneously. All groups were observed 4 after the beginning of the experiment.

Erythocytes labeling

Erythocytes were labeled by fluorescein isothiocyanate (FITC, purchased from Sigma Co.) using a combined approach of the procedures of Klar (1995). The labeled cells were stored a maximum of 24h before use.

In vivo microscopy

The pancreas of the studied animal was exteriorized on a stage, then FITC-labeled RBC was intravenously injected and intravital fluorescence microscope (Olympus X-70) were used to dynamically observe the pancreatic microcirculatory indices, and the images were simultaneously picked up by high-resolution video cassette recorder.

Morphology of microvasculature

Thoracic aortas of the studied animal were cannulated for perfusion. After flushing the vessels with warmed heparinized physiological saline solution, a diluted resin mixture or China ink was injected through the cannula with an injection pressure of 12-16kPa, until the portal vein and inferior vena cava was filled with the injected resin or ink.

The pancreas of resin-injected animal was corroded overnight or longer in a hot 300-400 g.L^{-1} KOH solution, washed in running water and rinsed again several times in distilled

water, air-dried, coated with gold in a vacuum evaporator, and observed in a scanning electron miscroscope.

The pancreas of ink-injected animal was fixed overnight or longer in Bouins solution, cleared in trichloromethane, embedded in paraffin, serially sectioned (thin sections of 5-7 μm for observation of the relationship between capillaries and cells, thick sections of 50-100 μm for observation of the vessel continuation), and observed with an Olympus X-60/50 light microscope. Serial reconstruction was carried out, camera lucida tracings of photographs were made at x330 final magnification on transparent sheets and superimposed for analysis.

Assays

Serum amylase level was determined and adopted as an indicator of AP. The increase in water content of pancreatic tissue served to indicate the formation and severity of pancreatic edema. The wet weight/dry weight ratio was expressed in per cent. Pancreatic tissue blocks of all groups were routinely paraffin wax-embedded, sliced, stained with hematoxylin/eosin and toluidine blue/basic fuchsin, then the sections were microscopically studied.

Statistical analysis

The results were expressed in mean ± standard deviation, and *t*-test was used to evaluate differences between control and AP groups. Difference was considered significant at the $P<0.05$ level.

RESULTS

Pancreatic edema

Gross appearance of pancreatic tissue of control group remained normal, and presented 72 % of water content. In comparison, pancreatic edema gradually appeared in Group2, 3 and 4 four hours after subcutaneous injection of caerulein, in parallel with an increase in pancreatic tissue volume. Edema of pancreatic head and body was much prominent, and the water content increased to 75 %. Inflammatory exudate accumulated in the anterior pararenal space and lesser omental sac in 50 % cases.

Morphology

Injury of intralobular arteriolar sphincter became visible 4 h after animal model established, and numerous cytoplasmic vacuoles formed; massive interstitial edema and inflammatory cell infiltration gradually emerged at 6 h. While in control group, pancreatic acini, tubules and blood vessels were normal microscopically.

Serum amylase

Serum amylase measurement in control group presented normal level (20.8 $\mu kat \cdot L^{-1}$). Serum amylase in all AP groups showed hyperamylasemia (45.0 $\mu kat \cdot L^{-1}$), significant higher than that of control group ($P<0.01$).

Light microscopy and scanning electron microscopy

Animals in the caerulein-treated group showed constriction of intralobular arteriolar sphincter 4 h after beginning of the experiment, presence of vacuoles in all the layers of sphincter, gross irregularity in capillary network of acini, reduction of capillary density, and blebs protruded from the surface of casts reflecting a substantial increase in capillary permeability.

In vivo fluorescence microscopy

Comparing with the control group, 4 hours after the start of experiment in AP groups the pancreatic microcirculatory in the caerulein-treated group showed the reduction of the velocity of FITC-labeled RBC, decrease of pancreatic capillary blood flow ($P<0.01$, Table 1), reduction of functional capillary density and arterioles diameter ($P<0.05$), and irregular intermittent perfusion of capillary network ($P<0.05$). Arterioles of pancreatic lobules and capillary density experienced significant changes at 6 h. The calibers of venules and capillaries showed no marked change in 6 h, while there was significant change by 8 h ($P<0.05$, Table 2).

Table 1 Intravital fluorescence microscopy of pancreatic microcirculation with FITC-labeled erythrocytes

t/h	Group	d(FITC-RBC)/ ($\times 10^9$cell/L)	Velocity of FITC-RBC/ (cell.min^{-1})	RBC flow/ (nl.min^{-1})	Microcirculatory blood flow/ (nl.min^{-1})
4	Control	113±5	86±3	0.28±0.01	0.88±0.06
	AP[b]	85±9	43±2	0.12±0.03	0.56±0.09
6	Control	104±4	81±4	0.31±0.02	0.99±0.07
	AP[b]	68±7	36±5	0.09±0.03	0.45±0.12
8	Control	96±6	84±5	0.29±0.04	0.91±0.06
	AP[b]	59±9	30±5	0.07±0.03	0.34±0.10

[b]$P<0.001$ *vs* control.

Table 2 Intravital observation of pancreatic microcirculation

t/h	Group	D(arteriole) /μm	D(venule) /μm	D(capillary) /μm	d(Capillary) /cm^{-1}	Capillary perfusion
4	Control	23.5±8	28±3	6.7±1.5	394±7	Stable
	AP	20.2±5.1	29.1±2	7±1.4	381±9	Unstable
6	Control	24.1±8	28±2.7	6.9±1.48	400±5.8	Stable
	AP	16.4±3.1[a]	27.5±3	6±0.3	291±16[a]	Intermittent & irregular
8	Control	23.2±5.5	27.4±1.6	7.3±1	349±8	Relatively stable
	AP	18.2±3.5[a]	29±1.5[a]	5.2±0.3[b]	277±13[b]	Intermittent & irregular

[a]$P<0.05$, [b]$P<0.001$ *vs* control.

DISCUSSION

In 1862 Panum demonstrated that acute hemorrhagic pancreatitis could be induced with wax droplets injected into pancreatic arteries. From then on, the etiological role which pancreatic ischaemia and tissue hypoperfusion plays in AP has been extensively discussed[29]. Many researches suggested that local microcirculatory disturbance, not insufficient blood flow in peripheral circulation, was responsible for perfusion failure of pancreatic tissue. In recent years, various animal models such as hemorrhagic shock, embolization of pancreatic microvasculature by minute particles and ligation of pancreatic arteries, have been used to verify that microcirculatory impairment of pancreas is the initial stage of AP. But the following questions haven't been answered conclusively: whether all types of AP are initiated by pancreatic microcirculatory impairment? What are the characteristics of early-stage pancreatic microcirculatory change? And what are the features of pancreatic microcirculatory disturbance in the natural process of AP? Insights into all these areas are crucial to the development of prevention and treatment measures.

Animal model

Sodium taurocholate-induced experimental pancreatitis was used by many authors to investigate microcirculatory change of AP; this model can reflect soundly the pathological features of acute necrotizing pancreatitis. Since direct injury to pancreatic ductules, acini and blood vessels may happen in several minutes, the gradual evolution of early-stage pathological change of pancreas in AP cannot be explored. In addition, modulation of intraductal pressure in the process of retrograde pancreatobiliary injection of sodium taurocholate also poses a real challenge. In this study, caerulein-induced experimental pancreatitis was chosen to investigate the features of early-stage pancreatic microcirculatory change. Subcutaneous administration of caerulein is easy to operate, and can result in acute edematous pancreatitis similar to that induced by intravenous injection of caerulein. In this model, the pathological changes develop slowly and gradually, the microcirculatory and histological changes of pancreas become prominent 4 h after the beginning of experiment, and pancreatic edema reaches its zenith by 8 h. This gradual development course allows us to study the triggering factor and the features of early-stage pancreatic microcirculatory impairment without haste.

Study techniques

For decades, pancreatic microcirculatory study heavily depended on the following techniques: injection of minute particles, Indian ink and methylthionine chloride into pancreatic arteries; measurement of pancreatic blood flow through pancreatoduodenal arteries and veins; measurements of relative blood flow and tissue perfusion of pancreas with intravenous injection of nuclide Rb-86, etc. Since acute necrotizing pancreatitis is characterized with progressive regional or focal necrosis of pancreatic tissue, observation with a single method has the following disadvantages: (1) dynamic and direct observation of local microcirculatory change of pancreas is impossible, since the animal must be sacrificed at a specific time; (2) observation of local blood flow of pancreas and tissue perfusion cannot be made simultaneously on the same specimen; (3) as to traditional intravital observation of pancreatic microcirculation, quantitative study cannot be effective due to dim image. This experiment has solved the above problems by developing a new approach; this approach combined intravital microcirculation observation technique, using selective blood element fluorescent marker, with another technique-maintaining dynamic and tissue message on static specimen.

Pancreatic microcirculatory impairment in AP

In recent years, applied basic researches on the morphology of pancreatic microcirculation revealed that the blood supply of pancreatic lobule, in most cases, is provided by a single intralobular arteriole. This arteriole sends forth tree-like branches when entering pancreatic lobule; it has no anastomosis with adjacent intralobular arterioles and their branches, and can be considered end-artery[30]. This characteristic suggested that pancreatic lobules are susceptible to ischaemic injury due to spasm of intralobular arterioles, embolization of arterioles by emboli, formation of microthrombi or compression by interstitial edema. However, causative factors of early-stage ischaemia and the precise triggering factor of local microcirculatory disturbance are not evident.

This study showed that, manifested as lasting spasm of arteriolar sphincter and multiple cytoplasmic vacuoles within smooth muscle cells of sphincter, the main feature of early-stage pancreatic microcirculatory impairment of AP is injury of sphincter of pancreatic intralobular arteriole. This experiment also demonstrated that among many factors causing early-stage ischaemia, the key one is injury and spasm of sphincter of pancreatic intralobular arteriole. In this study, injury of arteriolar sphincter occurred earlier than microcirculatory impairment, which reflected that injury of intralobular arteriolar sphincter was the initial stage of pancreatic perfusion failure and local microcirculatory disturbance. Microcirculatory hypoperfusion happened almost simultaneously with injury and spasm of arteriolar sphincter, indicating that pancreatic tissue is highly sensitive to ischaemic stress and has no compensatory reserve. Since sphincter of pancreatic intralobular arteriole serves as main lockgate to control blood flow to pancreatic lobule, and intralobular arteriole has characteristics of end-artery, even sphincter spasm of very short time will quickly evoke obvious pancreatic microcirculatory impairment. Other factors causing ischaemia[31-37], such as compression from interstitial edema, microemboli or obstruction due to thombosis, tend to be secondary ones, which may happen gradually in the course of pathological change of AP. These traumatic factors help to sustain and aggravate pancreatic microcirculatory impairment. To clarify relationship between traumatic factors of pancreatic microcirculatory impairment and pathological evolution of AP has guiding value in making treatment plans for clinical AP cases of various development stages. Features of early-stage microcirculatory change of experimental pancreatitis suggested that early adoption of spasm relieving and counter-injury measures are of vital importance in prevention and treatment of local microcirculatory disturbance of AP.

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• CLINICAL RESEARCH •

A ten-year study on non-surgical treatment of postoperative bile leakage

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Abstract

AIM: To summarize systematically our ten-year experience in non-surgical treatment of postoperative bile leakage, and explore its methods and indications.

METHODS: The clinical data of 57 patients with postoperative bile leakage treated non-surgically from January 1991 to December 2000 were reviewed retrospectively.

RESULTS: The site of the leakage was mainly the disrupted or damaged fistulous tracts of T tube in 25 patients (43.9 %), the fossae of gallbladder in 14 cases (24.6 %) , the cut surface of liver in 7 cases(12.3 %), and it was undetectable in the other 2 cases. Besides bile leakage, the wrong ligation of bile ducts was found in 3 patients, residual stones of the distal bile duct in 5 patients, benign papillary strictures in 3, and biloma resulting from bile collections in 2. The diagnoses were made according to the history of surgery, clinical situation, abdominal paracentesis, ultrasonography, ERCP, PTC, MRI/MRCP, gastroscopy and percutaneous fistulography. All 57 patients were treated non-surgically at the beginning of bile leakage. The non-surgical methods included keeping original drainage unobstructed, percutaneous abdominal paracentesis or drainage, percutaneous transhepatic cholangial/biliary drainage (PTCD/PTBD),endoscopic management, traditional Chinese medicine and so on. Of the 57 patients,2 patients died,5 were converted to reoperation later, the other 50 were directly cured by non-surgical methods without any complication. The cure rate of the non-surgery was 82.5 %(50/57).

CONCLUSION: Many nonoperative methods are available to treat postoperative bile leakage. Non-surgical treatment may serve as the first choice for the treatment of bile leakage for its advantages in higher cure rate, convenience and safety in practice. It is important to choose the specific non-surgical method according to the volume, site of bile leakage and patient's condition.

Chen XP, Peng SY, Peng CH, Liu YB, Shi LB, Jiang XC, Shen HW, Xu YL, Fang SB, Rui J, Xia XH, Zhao GH. A ten-year study on non-surgical treatment of postoperative bile leakage. *World J Gastroenterol* 2002; 8(5):937-942

INTRODUCTION

Bile leakage is one of the most common and serious complications after hepatobiliary surgery. In recent years, the treatment strategy of bile leakage has generated fundamental changes due to the excellent effect of non-surgical therapy and its minimally invasive intervention[1-6]. Non-surgical treatment has been regarded as the first choice in the management of postoperative bile leakage in many cases. Only when there is no essential condition or no effect by non-surgery, will operation be considered[1,2]. Although there are many nonoperative approaches that could be used to cure bile leakage, most literatures only reported one of these methods[7-53], being deficient in systematization and comprehensiveness. Thus, surgeons could not often apply them synthetically and reasonably. Over the past decade from 1991 to 2000, we treated 57 patients with postoperative bile leakage non-surgically in our two hospitals. In this article, we summarized the methods and indications of the non-surgical treatment for postoperative bile leakage.

MATERIALS AND METHODS

Patients

From January 1991 to December 2000, a total of 57 patients with postoperative bile leakage, including 23 males and 34 females, were treated non-surgically in the Department of Surgery of the Second Affiliated Hospital, Medical School of Zhejiang University and the Affiliated Yijishan Hospital of Wannan Medical College. The mean age of the patients was 46 years, with a range from 25 to 73 years. All of them belonged to postoperative bile leakage. The original operations were performed with simple open cholecystectomy (OC) in 21 cases, OC plus choledochotomy with T tube drainage in 25, simple hepatectomy in 7, hepatectomy plus Roux-en-Y cholangiojejunostomy in 3, and repair of bile duct injury plus choledochoduodenostomy in 1, respectively. The clinical presentations were diverse. Most patients were presented with sudden or gradual abdominal pain after operation, and T tube removal/ slippage or U tube exchange. Almost half of the cases had much bile-like liquid outflowing from the original drainage tubes or the fistulous tracts of T tube. Twelve patients had light to moderate fever, four hyperpyrexia, six jaundice, and two had nausea and vomiting.

Diagnosis

The diagnoses were made according to the operational history, clinical situation, abdominal paracentesis (21 person times), ultrasonography (17), ERCP (8), PTC (5), MRI/MRCP (3), gastroscopy (3), and percutaneous fistulography (2). The site of the leakage was the cystic duct in 3 cases, the fossae of gallbladder in 14 (Figure 1), the disrupted or damaged fistulous tracts of T tube in 25 (Figures 2 and 3), the damaged bile duct in 3 (Figures 4 and 5), the cut surface of liver in 7 (Figure 6) , the stomas of bilioenteric anastomosis in 3 and it was

undetectable in the other 2 cases. Besides bile leakage, the wrong ligation of bile ducts beyond the leakages was also found in 3 patients with common bile duct injury. Of the 25 patients with bile leakage following T tube removal, residual stones of the distal bile duct were also found in 5 cases (Figures 3 and 7) and benign papillary strictures were detected in 3 (Figure 5). Bile collections in 2 patients developed biloma, which oppressed pylorus, resulting in pyloric obstruction. Bile collections at two different sites, including hepatorenal recess and hepatogastric interspace with only a slender and bent tract (diameter <0.5 cm) between them, were confirmed in one patient undergoing hepatectomy, whose original subphrenic drainage was removed owing to occlusion before the bile leakage was found. Although the drainage on the anterior abdominal wall was still retained, it only drained bile from hepatogastric interspace.

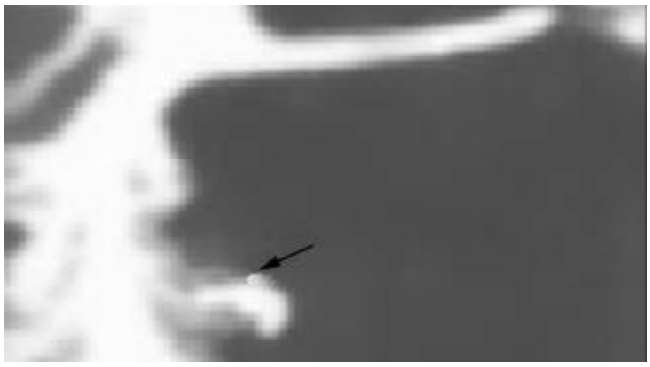

Figure 1 ERCP showed a bile leak from the fossae of gallbladder (↑).

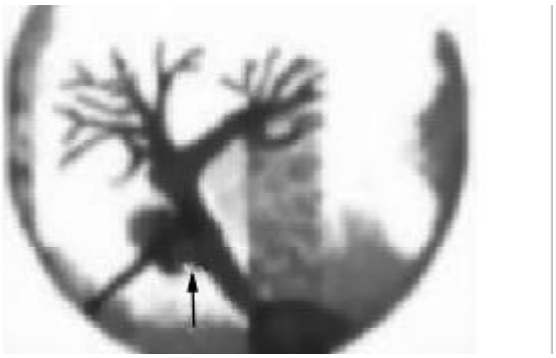

Figure 2 Cholangiography through T tube showed a leak from common bile duct (↑).

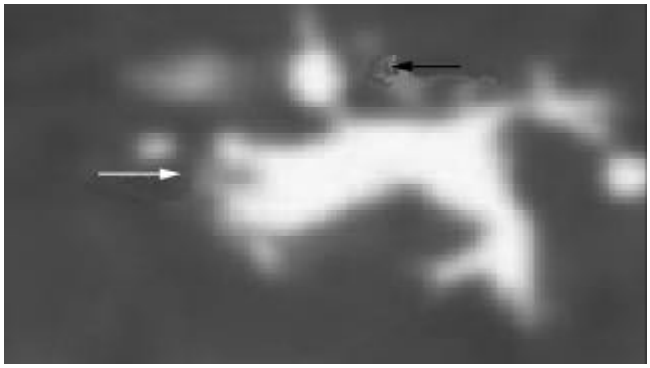

Figure 3 ERCP showed a leak from the fistulous tract of T tube (↑) and stones at distal duct (⇧).

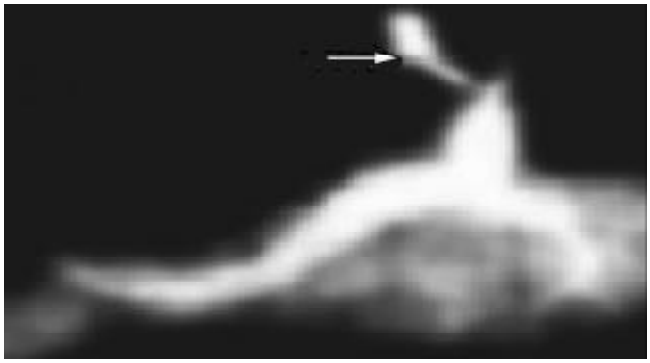

Figure 4 ERCP showed a leak from right hepatic duct (⇧).

Figure 5 MRCP showed stricture at distal duct (↑) and liquid collection in the right subhepatic region (⇧).

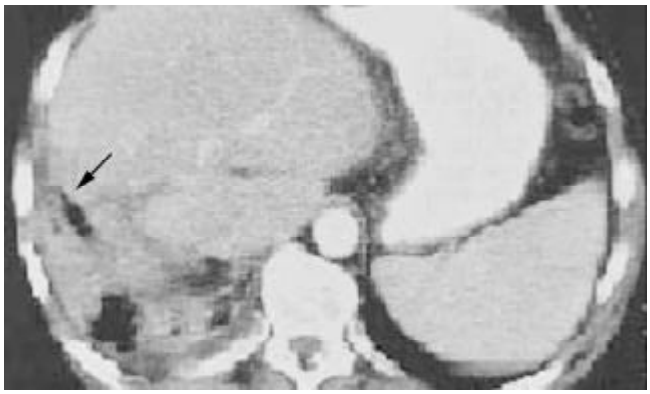

Figure 6 CT showed bile accumulating in the cut surface of right liver (↑).

Figure 7 The distal duct stones was removed with choledochoscope (⇧).

Management

When patients revealed the symptoms of bile leakage or biliary peritonitis,some essential disposal measures were immediately used such as right lateral decubitus position, semi-reclining position, gastrointestinal decompression, complementing water-electrolyte, antibiotic and appropriate nutritional support[1,2]. After that, specific non-surgical methods were chosen according to the volume, site of bile leakage and patient's condition. The original drainage in 19 patients undergoing OC or hepatectomy were kept unobstructed because they still produced some effect of drainage. The cannulae were changed in 5 cases of OC or hepatectomy due to their occlusion. In addition, washing with antibiotic was performed through the cannulae. In all the 25 patients with bile leakage following T tube removal or slippage, a new drainage was once again placed into the fistulous tract of T tube. Repeated abdominal cavity suction and drainage with a percutaneous catheter were performed by percutaneous abdominal paracentesis in 3 of 25 patients while percutaneous transhepatic cholangial drainage (PTCD) or percutaneous transhepatic biliary drainage (PTBD) were performed in 2 patients whose intrahepatic bile ducts were dilated. The residual ductal stones in 5 patients were eliminated by choledochoscope (Figure 7), endoscopic sphincterotomy (EST) and the traditional Chinese medicine, respectively. The traditional Chinese medicines included bitter orange, costusroot, mongolian milkvetch, rhubarb, christina loosestrife herb, etc. In order to maintain the pressure of bile duct and facilitate stones passage, it was necessary to obstruct the T tube at intervals when the Chinese medicines were administered. The bile duct stricture in 3 cases was relieved by choledochoscope and EST, respectively. Percutaneous catheter drainages guided by ultrasound were used in one patient with biloma and 3 patients with common bile leakage. As to the patient with subhepatic fluid accumulations at two different sites detected by ultrasonography, MRI and percutaneous fistulography, it was inappropriate to reoperate on him or her due to the postoperative weak health, and it was very dangerous to apply abdominal paracentesis because the hepatorenal recess was just near the right thoracic cavity. A new drain tube was first placed into hepatogastric interspace and afterwards into the bent tract and hepatorenal recess through the fistulous tract on the anterior abdominal wall. The whole process was guided by gastroscope and X-ray. Flushing with antibiotic was performed everyday.

RESULTS

In the early stage of our study, one patient with stoma leakage died of infectious shock, and one with post-hepatectomy bile leakage died of multiple organ failure (MOF). After 1996, no patient died of bile leakage. Five patients were converted to operation due to ineffective non-surgical management, presentation of jaundice or concomitant wrong ligation of bile duct. All the 5 patients were cured by late reoperation. Of the 5 cases, one with biloma belonged to the early stage of our study. Owing to the absence of experience in the non-surgical treatment at that time (1993), he only received some essential disposals except percutaneous catheter drainage at the beginning of bile leakage. He underwent exploratory laparotomy plus drainage a week later because nausea and vomiting were not relieved. Two patients with bile leakage from cystic duct stump underwent exploratory laparotomy plus re-ligation of cystic duct after 2-3 days drainage. The other two patients all underwent hepatojejunostomy 2-3 months later due to concomitant wrong ligation of bile duct. The other 50 patients were directly cured by non-surgical treatment without correlated complications. The mean closure time of bile leakage was 9 days, with a range from 5 days to 3 months. The cure rate of non-surgical treatment was 82.5 % (50/57).

DISCUSSION

When bile leakage occurs, it is necessary to adopt some essential disposals such as gastrointestinal decompression, fluid replacement, antibiotic therapy, etc. However, it is important to choose operation or nonoperation according to the volume of bile leakage and patient' s condition. In recent years, the treatment strategy of bile leakage has generated fundamental changes due to the invention or finding of some new non-surgical methods, which have further improved the therapeutic effect of bile leakage with minimal invasive intervention or no trauma. Non-surgical treatment has been regarded as the first choice in the management of postoperative bile leakage in many cases, and it is acceptable by such patients. Only when there is no essential condition for non-surgery, or no effect, will operation be considered[1-6].

Non-surgical methods

In our study, the 57 patients with postoperative bile leakage were all managed by non-surgical treatment at the beginning of bile leakage. Fifty patients were cured except that two died and five were converted to reoperation later. The cure rate of non-surgical treatment was 82.5 % (50/57). The total therapeutic efficacy is satisfactory. Based on our data, the following non-surgical methods could be used in the treatment of bile leakage.(1)Keep the original drain tube unobstructed or chang it[1,2,5]. The original drainage should be maintained when it still drains some bile, and should be changed for a new drain tube when obstructed. In addition, we think it is equally of importance to wash with antibiotic through the cannulae[9], because it could increase the local concentration of antibiotic and improve anti-infection effect. It was difficult for the bile leakage to heal in patients of older age, malnutrition, complicated diabetes and using hormone. Besides drainage, some related therapy should be continued in these cases, the hormone should be cancelled, and the time of drainage be properly prolonged. (2) Insert a new catheter through the fistulous tract of T tube[1,5,16,17]. It is necessary to place a new catheter immediately through the fistulous tract of T tube if the bile leakage occurs after a T tube removal or slippage.The procedure should be performed as early as possible to prevent the occlusion of fistulous tract. Suction with negative pressure could be additionally used when necessary[1,16,18]. (3) Percutaneous abdominal paracentesis[1,5,6,8,10-13]**.** As an important nonoperative method, abdominal paracentesis can be applied because it does not need special device except an injection syringe. If the site of leakage was deep or near important organs, abdominal paracentesis should be guided by ultrasound. It is helpful to not only the diagnosis of bile leakage but also the abdominal cavity suctions or percutaneous catheter drainage. In this group, abdominal paracentesis was performed in five patients (4 with bile leakage after T tube removal and 1 with post-cholecystectomy biloma). Among them, the biloma was punctured under the guidance of ultrasound[1]. All 5 were cured. (4) PTCD/PTBD or placement of a percutaneous transhepatic stent[1,14,15]. Owing to some dangers(including hemorrhage, new bile leakage and so on),it should be generally performed under the guidance of ultrasound in patients with intrahepatic cholangiectasis[54]. PTCD is helpful to the healing of bile leakage because it may reduce the volume of leakage. In our

study, PTCDs were used in 2 cases because both abdominal drainages were insufficient. Two weeks later, both patients were cured. (5) Endoscopic management[1,6,8,24-53].As a new progression in the nonoperative treatment of bile leakage, endoscopic management generally means EST, endoscopic nasobiliary tube drainage (ENBD) and endoscopic biliary stent (EBS)[32,33,55], which are all finished by duodenoscope. It is mainly applied to the patients with bile duct stone, stricture or biloma[32,33,55]. These endoscopic methods could effectively remove the residual ductal stones, relieve the stricture of bile duct and facilitate the healing of leakage. However, endoscopes include choledochoscope and gastroscope besides duodenoscope. Because choledochoscope may not only gets rid of the residual stones but also dilate the narrow bile duct, it should also play an important role in the nonoperative treatment of bile leakage, and should not be neglected. In our study, choledochoscopic treatments were given to 4 patients (2 with residual stones, 2 with bile duct stenosis). All were cured. Gastroscope may occasionally be used in the nonoperative treatment of bile leakage. In this group, the new drain tube in the patient with bile accumulations at two different sites was placed under the guidance of gastroscope and X-ray. The leakage closed 3 months later. Thus, we should not confine our sights to the duodenoscope when using endoscopic treatment. Besides duodenoscope, gastroscope and choledochoscope may also be applied.(6)Traditional Chinese medicines[1]**.** It is mainly applied to the patients with bile duct stone. Some Chinese medicines may faciliate the passage of residual stones, resulting in accelerated healing of leakage. In this group, after Chinese medicines were administered to 2 patients for 5-7 days, the residual stones were all precluded that was confirmed by ultrasound and contrast examination. Both leakages closed within 2 weeks. In addition, there are some other non-surgical methods including laparoscopy[19-22], ethanol ablation[23] and so on.

Indications

Non-surgical treatment therapy plays an important role in the management of bile leakage due to its many advantages. First, the cure rate is high[33,39]. Binmoeller *et al*[33] reported that the total cure rate of endoscopic management was 86 % .In Fuji's study, all 8 postoperative bile leakages were cured by endoscopic treatment[39]**.** In our study, 57 patients were treated with various non-surgical methods, 50 were directly cured, the rate being 82.5 %. Next, non-surgical treatment is convenient, safe[1,10,40,41], atraumatic or micro-traumatic[43]. If only it is properly used, reoperation could be avoided in most patients with postoperative bile leakage, and the pain and costs of patients could be reduced. Thus, it is acceptable by patients, and fits the general trend and direction of modern surgery. The non-surgical therapy is especially suitable for the elderly patients who are physically weak, poor in common state, complicated by other serious diseases, unbearable or unwilling to accept reoperation in a short time. In this group, more than half of the patients belonged to such cases. Owing to correct choice of treatment, they not only avoided reoperation, but also safely passed the dangerous period. As for the patients who need surgery but could not be operated on at the early stage of bile leakage due to their conditions, non-surgical treatment can not only serves as interim measure before operation but also wins precious time for preoperative preparation. It was quite obvious that such condition was present in the 5 patients who were converted to late reoperation. When the patients with bile leakage were treated non-surgically, its indications should be strictly controlled. Based on our data, we think that nonoperative therapy could be first attempted in the following cases[1]. (1) Patients with early bile leakage[2](within 4-6 hours),relatively minor leakage, rather light or local peritonitis and no infectious shock or following shock by estimation; (2) Postoperative early patients who are poor in general state, complicated with other serious diseases, unbearable or unwilling to accept reoperation in a short time; (3) Patients whose original subhepatic drainages were still reserved and unobstructed; (4) Patients who suffer from bile leakages after T tubes removal or slippage and can be implanted with new drain tubes through the fistulous tracts of T tubes; (5) Patients who can be treated with percutaneous abdominal paracentesis or drainage; (6) Patients with intrahepatic bile duct dilataltion who can be managed by PTCD/PTBD; (7) Patients whose residual bile duct stones or strictures can be cured by endoscopic management or traditional Chinese Medicine. Of course, it is essential to pay close attention to the change of patient' s condition and complications during nonoperative treatment, and it also necessary to be simultaneously ready to reoperate at any time. Surgery should be considered as soon as possible if the patient' s condition has not improved even aggravated such as abdominal pain degree worsening, scope expanding, temperature apparently rising and so on[1,10]. Reoperation should also be considered if only the patient' s condition goes beyond the limit of nonoperative therapy such as relatively major bile leakage, serious peritonitis[1,2], persistent leakage or concomitant wrong ligation of bile duct. In our study, 5 patients were converted to reoperation later due to inefficiency of non-surgical management, occurrence of jaundice or wrong ligation of bile duct.

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• CLINICAL RESEARCH •

Multivariate statistical analysis of clinicopathologic factors influencing survival of patients with bile duct carcinoma

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Abstract

AIM: To evaluate the influence of various clinicopathologic factors on survival of patients with bile duct carcinoma after curative resection.

METHODS: A retrospective analysis was made for 86 cases of bile duct carcinoma treated from January 1981 to September 1995. Fifteen clinicopathologic factors possibly influencing survival were selected. Independent variables were first analyzed by univariate methods. Survival for variable was estimated by the method of Kaplan and Meier. The variables that were statistically significant by univariate analysis were included in a multivariate analysis, which were confirmed using the Cox stepwise proportion hazard model with the help of SPSS 10.0 for Windows software.

RESULTS: The overall cumulative survival rate was 72.6 % at 1 year, 32.4 % at 3 years, and 18.7 % at 5 years. The results of univariate analysis showed that the major significant prognostic factors influencing survival of these patients were histological type of lesion, lymph node metastasis, pancreatic invasion, duodenal invasion, perineural invasion, macroscopic vessel involvement, resected surgical margin and depth of cancer invasion (P=0.02, 0.02, 0.004, 0.005, 0.01, 0.43, 0.03 and 0.04). Age, sex, location of tumor, size of tumor, macroscopic type of lesions, hepatic metastasis, and hepatic invasion were not significantly associated with prognosis (P>0.05). Pancreatic invasion, perineural invasion and lymph node metastases were the three most important prognostic factors by multivariate analysis using the Cox proportional hazards model.

CONCLUSION: Pancreatic invasion, perineural invasion and lymph node metastases are the most important prognostic factors for bile duct carcinoma after curative resection.

He P, Shi JS, Chen WK, Wang ZR, Ren H, Li H. Multivariate statistical analysis of clinicopathologic factors influencing survival of patients with bile duct carcinoma. *World J Gastroenterol* 2002; 8(5):943-946

INTRODUCTION

With the recent improvement of surgical techniques in hepatobiliary surgery, a curative surgical resection of bile duct carcinoma can be accomplished with acceptable morbidity and mortality[1-8]. However, the prognosis for such patients is frustrating, although this tumor is small, grows slowly and metastasizes late[7-13]. In the present article, an effort is made to evaluate the influence of various clinicopathologic factors on survival of patients with bile duct carcinoma using the Cox proportional hazards model. The results of these analyses were used when surgical treatment was performed for patients with bile duct carcinoma.

MATERIALS AND METHODS

General data

Eighty-six cases of bile duct carcinomas were resected in the Department of Hepatobiliary Surgery, First Hospital of Xi' an Jiaotong University from January 1981 through September 1995. The resected specimens were examined pathologically, and the relation between clinicopathologic findings and patient survival was studied.

Variables

The following clinicopathologic variables were considered for prognosis: age, sex, location of primary tumor, size of the tumor, macroscopic type of lesion (papillary, nodular, infiltrating), histological type of lesion (papillary adenocarcinoma, well-differentiated, moderately differentiated, and poorly differentiated adenocarcinoma, and adenosquamous cell carcinoma), hepatic metastasis, lymph node metastasis, hepatic invasion, pancreatic invasion, duodenal invasion, perineural invasion, vascular invasion, resected margin of the bile duct, depth of cancer invasion (invasion limited to fibromuscular layer, to adventitia and subserosal layer, to and beyond the serosal exposure).

Analysis

Independent variables were first analyzed by univariate methods. Statistical significance of the variables was determined by t-test and Chi-square test. Survival for variable was estimated by the method of Kaplan and Meier. The variables with statistical significance in univariate analysis were included in a multivariate analysis, which were further confirmed using the Cox stepwise proportion hazard model with the help of SPSS 10.0 for Windows software.

RESULTS

Clinical findings

Of the 86 surgically treated patients, 51 were male and 35 female aged from 33 to 78 years, averaging 58.6 years. The patients aged from 50 to 78 years made up 66.5 %. Of the lesions, 40 (47 %) were upper bile duct cancer, 13 (15.2 %) were middle bile duct cancer, and 33 (38.8 %) lower bile duct cancer. All the lesions were resected at operation. The type of operation depends on the site and extent of tumor. Bile duct resection was done with cholangiojejunostomy in 17 patients,

bile duct resection in 26 with irregular hepatectomy and cholangiojejunostomy, pancreatduodenectomy in 42 patients, and hepatopancreatoduodenctomy in one.

Overall survival

The overall cumulative survival rates were 72.6 % at 1 year, 32.4 % at 3 years, and 18.7 % at 5 years. Fifteen clinicopathologic factors were analyzed, and the prognoses were significantly related to 8 of the 15 variables analyzed by univariate method (Table 1).

Table 1 Univariate analysis of the clinicopathologic factors for the survival of 86 patients with bile duct carcinoma

Factors	No. of patients	*P* value
Sex		0.90
Male	51	
Female	35	
Age (yrs)		0.33
<50	29	
⩾50	57	
Location of tumor		0.15
Upper	40	
Middle	13	
Lower	33	
Size of tumor		0.21
<2cm	11	
2 - 4cm	62	
>4cm	13	
Macroscopic type of lesions		0.43
Papillary	17	
Nodular	32	
Infiltrating	37	
Histological type of lesion		0.02
Papillary adenocarcinoma	7	
Well differentiated adenocarcinoma	27	
Moderately differentiated adenocarcinoma	36	
Poorly differentiated adenocarcinoma	14	
Adenosquamous cell carcinoma	2	
Hepatic metastasis		0.88
Present	2	
Absent	84	
Lymph node metastasis		0.02
Present	37	
Absent	49	
Hepatic invasion		0.36
Present	29	
Absent	57	
Pancreatic invasion		0.004
Present	21	
Absent	65	
Duodenal invasion		0.005
Present	14	
Absent	72	
Resected margin of the bile duct		0.03
Present	19	
Absent	67	
Perineural invasion		0.01
Present	65	
Absent	21	
Vascular invasion		0.04
Present	17	
Absent	69	
Depth of cancer invasion		0.04
Invasion limited to fibromuscular layer	9	
Invasion limited to adventitia and subserosal layer	59	
Invasion to and beyond the serosal exposure	18	

The significant variables were lymph node metastasis, duodenal invasion, pancreatic invasion, perineural invasion, vascular invasion, resected margin of the bile duct, histological type of lesion, and depth of cancer invasion. The following factors were not significantly associated with prognosis: age, sex, location of tumor, size of tumor, macroscopic type of lesions, hepatic metastasis, and hepatic invasion.

Multivariate analysis using the Cox proportional hazards model involving the 8 significant factors determined by univariate analysis identified the three prognostic variables (Table 2). They were the pancreatic invasion, the perineural invasion and the lymph node metastasis. Pancreatic invasion was observed in 21(24.4 %) of the 86 patients with bile duct carcinoma. The 5-year survival rates for patients with negative and positive pancreatic invasion were 36 % and 2 %, respectively. A statistically significant difference in survival could be observed between the patient with positive and negative pancreatic invasion (P=0.005). Perineural invasion was seen in 75.6 % of the patients with bile duct cancer. Univariate analysis showed a statistically significant difference of survival between the perineural invasion and perineural noninvasion groups (P= 0.01) (Table 1). The 5-year survival rate was 47 % for patients without perineural invasion, whereas 13 % for the perineural invasion-positive patients. Lymph node metastasis was observed in 37(43 %) of the 86 patients with bile duct carcinoma. The 5-year survival rate was 44 % for patients without lymph node metastasis, and 11 % for patients with lymph node metastasis.

Table 2 Relative values of three prognostic variables derived from Cox stepwise proportional hazards model

Variables	β	SE	Sig(*P*)	Exp(B)	95%CI for Exp(B)
Pancreatic invasion	0.226	0.084	0.007[b]	1.254	(1.064-1.479)
Perineural invasion	0.691	0.236	0.012[a]	2.408	(1.221-4.753)
Lymph node metastasis	0.894	0.489	0.023[a]	2.762	(1.164-6.557)

[a]P<0.05, [b]P<0.01, *vs* control

DISCUSSION

With the continuing progress of diagnostic and surgical techniques in biliary surgery, a great deal of biliary cancers can be resected with acceptable morbidity and mortality. However, the 5-year survival was only 10-20 %, and only one-third of the patients could be treated surgically at the time of diagnosis[14-18]. The local recurrence of bile duct cancer is relatively high even after curative resection of this lesion. Therefore, a proper surgical procedure should be considered for preventing this undesirable outcome. It is important to know what prognostic factors relate to the survival of the patients with bile duct cancer.

In our study, the overall cumulative survival rates for 86 patients with bile duct carcinoma were 72.6 % at 1 year, 32.4 % at 3 years, and 18.7 % at 5 years. This study showed that the prognoses for patients with bile duct cancer were significantly associated with pancreatic invasion, perineural invasion, duodenal invasion, histological type of lesion, lymph node metastasis, vascular invasion, resected margin of the bile duct, and depth of cancer invasion (P<0.05). Age, sex, location and size of tumor, macroscopic type of lesions, hepatic metastasis, and hepatic invasion were not significantly associated with survival (P>0.05).

Our study also showed that pancreatic invasion, perineural invasion and lymph node metastases were the three most important prognostic factors by multivariate analysis using the Cox proportional hazards model (Table 2). Todoroki *et al* revealed that the primary tumor and tumor node metastasis (TNM) stage were independent predictors of survival using multivariate analysis of 67 patients with bile duct cancer[19]. Havlik *et al* found that lymph nodes, vascular invasion, advanced tumor stage, positive tumor margins, and p53 mutation were associated with poor survival by multivariate analyses[20]. Inoue *et al* identified that surgical margin, lymph node metastasis, lymph node dissection, vascular invasion, and left-side location of the main tumor were significant risk factors for overall survival using univariate analysis and confirmed that surgical margin, lymph node metastasis, and vascular invasion were independently significant variables for overall survival using multivariate analysis[21]. All of them did not mention pancreatic and perineural invasion were prognostic factors for the survival of patients with bile duct carcinoma. Other scholars[22,23] and we, however, have all observed a significant correlation between perineural invasion and postoperative survival.

Pancreatic invasion is the first prognostic variable (Table 2). Patients with negative pancreatic invasion survived significantly longer than those with positive pancreatic invasion after resection of the lesion. Our findings show that the 5-year survival rate for patients with negative pancreatic invasion was 36 %, whereas it was 2 % for patients with positive pancreatic invasion. This poor prognosis might be due to the fact that when the bile duct cancer invades pancreatic tissue it behaves like a primary pancreatic cancer, and the 5-year survival rate was only around 6 %[24-28], leading to a worse prognosis. Since bile duct cancer possess biological characteristic of the invasive growth and anatomical location, lower bile duct carcinoma mostly invade pancreas, making that the 5-year survival rate for postoperative patients with lower bile duct carcinoma less than 10 %[29].

Perineural invasion and lymph node metastasis were also determined to be the independent prognostic factors for survival by the multivariate analysis (Table 2). Some scholars had studied extensively the clinicopathologic significance of perineural invasion, and the results of this study substantiated these findings[22,23]. In our study, the 5-year survival rate for patients with negative perineural invasion was 47 %, whereas it was 13 % for patients with positive perineural invasion. It is well accepted that lymph node metastasis is an independent prognostic factor for bile duct cancer patients[19-21,30]. According to our study, the 5-year survival rate was 44 % for patients without lymph node metastasis, and 11 % for patients with lymph node metastasis. As a result of abundant lymphatic, blood vessel, nerve fibers and loose connective tissue around the bile duct, the cancer cells provided with the way of ‘ jump model’ growth. The excessive metastasis fashion results in the inevitable local recrudescence postoperatively. Consequently, we emphasize the need for dissection of autonomic nerve fibers and plexuses around the hepatic and celiac arteries and the portal vein during operation. In addition the lymph nodes, lymphatic vessels, and connective tissues must be dissected for radical operation on bile duct carcinoma.

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• CLINICAL RESEARCH •

Abdominal pain among children re-evaluation of a diagnostic algorithm

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Abstract

AIM: To re-evaluate the algorithm that has been used for over 40 years for diagnosis of acute abdominal pain among children.

METHODS: Among the 937 cases admitted to the surgical emergency ward in 2000, 656 cases of acute appendicitis were studied to evaluate the usefulness of the present algorithm for its calculated accuracy, false positive and false negative rates, the sensitivity and specificity in the instant diagnosis of various types of acute appendicitis in different age groups. The algorithm used was established in 1958 and revised for this study in 1999. It includes a 3-step analysis of clinical presentations, i.e.: firstly, a diagnosis of surgical pain by definite organic abdominal signs; then a diagnosis of the subgroup of surgical condition by special signs; and finally the diagnosis of the present disease by specific signs. A footnote describes a "comparative technique" of abdominal examination in non-cooperative children.

RESULTS: The general accuracy of diagnosis was 92.8 %, overall mortality 0.1 % among 973 cases of abdominal pain in 2000. 373 attending surgeons and 241 residents including trainees joined the diagnosis and treatment with no remarkable difference in the results. The incidence of acute appendicitis, 656 in 973 cases, was 67.4 % representing the majority of abdominal pain. In the series of 656 cases, the accuracy of diagnosis of acute appendicitis was 93.6 %, false positive 6.4 %, false negative 0.9 %, sensitivity at first visit 82.7 %, specificity for appendicitis 98.0 %, no death or documentary complication.

CONCLUSION: The present algorithm used for diagnosis of acute abdominal pain is effective and preferable in reducing misdiagnosis and maltreatment at emergency. The use of some modern technology should be further explored.

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INTRODUCTION

In recent literature, the incidence of perforated appendicitis seems to be rising, and most of the late cases are due to misdiagnosis[1-3] In China, we have not yet had the similar poor impression[4]. For many years, we' ve achieved good results in the management of acute abdomen in pediatric surgery[5]. The best results as reported by Beijing Children' s Hospital (BCH) are: no death in 20 years in 10 000 consecutive cases of acute appendicitis[6], no death in 3 years in 100 consecutive cases of strangulated intestinal obstruction including all those cases that came in shock state[7], and over 90 % of cases of early intussusception that were reduced without emergency surgery even in county hospitals[8,9]. In 1958, a systematical analysis method, the algorithm, for diagnosis of acute abdominal pain among children was published[10]. and popularized in many places of China. It has been generally followed, with timely revisions, for over 40 years. However, with the rapid progress of the modern diagnostic tools in recent years, it is necessary to have a re-evaluation of the above mentioned diagnostic algorithm for abdominal pain among children and try to find out some reasonable modern technology to make it further improved and popularized.

MATERIALS AND METHODS

This is a prospective study of the diagnostic algorithm, which was established in 1958. With the improvement of medical sciences, and changes in the incidence of common diseases in pediatric surgical emergency clinic, the algorithm has been revised many times. Generally, for over 40 years, it has been proved helpful in the diagnosis of acute abdomen of children. For this study, the algorithm was revised again in 1999 as shown in Table 1.

Table 1 Algorithm for diagnosis of abdominal pain

Abdominal Pain		
Acute abdomen (long time pain definite signs)		Non-acute abdomen (short pain no definite sign)
1 Focal inflammation	2 Intestinal obstruction	1 Primary enterospasm (allergic,idiopathic)
(1)local tenderness (appendicitis, etc.)	(1)intestinal pattern (adhesive obstruction)	(1)weaning colic
(2)tender mass (torsion ovarian cyst)	(2)movable mass (intussusception)	(2)school-boy colic
3 generalized peritonitis (full and silent abdomen)		2 secondary enterospasm (organic, non-acute)
(1)spreading peritonitis (inflamed organ)	(2)gangrenous peritonitis (strangulation)	(1) chronic G-I ulcers (2) pancreticobiliary colic
(3)perforating peritonitis (peptic ulcer, typhoid)	(4)primary peritonitis (bacteremia,vaginitis)	(3) chronic gastritis (4) cerebrospinal colic (5) hemo-vascular disease (6) metabolic disease (7) auto-immue disorder (8) intoxication

*Footnote: (1) "comparative technique" of abdominal palpation, watching different reaction of the child on different part of abdomen. (2) "dynamic comparison" of clinical presentation, is today better or worse than yesterday?

It consists of a 3-step analysis of clinical presentations. The first step is to make the diagnosis of a surgical abdomen by finding definite abdominal signs indicating organic lesion, i.e. tenderness, spasm, intestinal pattern and mass. The second step is to make the diagnosis of the subgroup of surgical abdomen, namely organ inflammation, intestinal obstruction and generalized peritonitis by their special signs respectively. The third step is to make the diagnosis of the present disease, like acute appendicitis or intussusception by their specific cardinal signs. In the footnote, the "comparative technique" of abdominal examination for non-cooperative children and its "comparative dynamic evaluation" are described.

Strictly following the above algorithm, 973 cases of acute abdominal pain admitted to the surgical emergency ward of BCH in 2000 (Table 2) were reviewed. The overall rate of misdiagnosis and a general status of the diagnosis and result of treatment were studied (Table 3). In order to make a differential evaluation, 614 cases of acute appendicitis which is the representative disease occupying 68.0 % in 2000 (Table 4) were investigated in detail. The accuracy of diagnosis, the false positives, false negatives, the sensitivity and the specificity of the use of the diagnostic algorithm were calculated accordingly. Different-level doctors concerned in making diagnosis were also recorded.

Table 2 Came with abdominal pain to emergency ward, BCH, 2000

Category	*n*	%
Organ inflammation[a]	637	65.5
Intestinal obstruction[b]	160	16.5
Traumatic abdomen	28	2.9
GI hemorrhage	38	3.9
Primary peritonitis	5	0.5
Perforation peritonitis	8	0.8
Tumor twisting or rupturing	14	1.4
Torsion testes or appendage	8	0.8
Other organic lesion	5	0.5
Non organic pain	70	7.2
Total	973	100.0

[a] Included 614 appendicitis [b] Included 57 intussusception

Table 3 Outcome of 973 cases of acute abdominal pain admitted to surgical emergency ward in 2000

Outcome	*n*	%
Cure	894	91.9
Improved[a]	67	6.9
Unimproved[a]	11	1.1
Died	1	0.1
Total	973	100.0

[a]Most are chronic traumatic, inflammatory, and malignancy cases with acute pain

Table 4 Number of operated patients of common surgical abdomen, 2000

Disease	*n*	%
Acute appendicitis	614	68.0
Intussusception[a]	57	6.3
Incarcerated hernia	45	5.0
Traumatic abdomen	28	3.1
Adhesive obstruction	25	2.8
Fecalith obstruction	19	2.1
Miscellaneous	115	12.7
Total	903	100.0

[a]The other 90 % of early cases cured in OPD by rectal insufflation

RESULTS

Among 973 cases in 2000, the overall mortality was 0.1 %. It was a boy aged 6 yr, who died of strangulation perforation of stomach in uncontrolled Crohn' s disease. There was still no death of acute appendicitis. The general accuracy of diagnosis of acute abdomen was 94.5 %. Concerning the 656 cases of acute appendicitis in 2000, the following figures were calculated: Diagnosis at discharge (614)/Diagnosis on admission (656)=93.6 % overall accuracy; Non-surgical abdomen (42)/Appendicitis admitted (656)=6.4 % false positive at admission; Admission after 2nd visit (196)/ Appendicitis admitted (656)=29.9 % false negative at the 1st visit, mostly delayed in other hospital due to transfer; Second visits in BCH surgery (6)/Appendicitis admitted (656)=0.9 % false negative BCH; Admitted by 1st visit (258)/Appendicitis in 24 h (312)=82.7 % sensitivity (It means, 82.7 % of early appendicitis within 24 h can be diagnosed at first visit); Proved appendicitis (542)/Cases operated (553)=98.0 % specificity (It means, 98.0 % of appendicitis can be differentiated from other surgical conditions); Ratio between cases diagnosed at first visit by surgeons of high level and low level=373:241 cases (in BCH, surgeons under 3 years of training are not allowed to manage on-call emergency); Acute appendicitis (656)/ Abdominal pain (973)=67.4 % incidence at admission. There was no mortality, no documentary complication, but 5 perforated cases in moderate postoperative morbidity. Pathology of 614 case of acute appendicitis in 2000 is shown in Table 5.

Table 5 Pathology of 614 cases of operated appendicitis (2000)

Pathology	Simple	Suppurative	Gangrenous	Perforative	Recurrent	Abscess	Infiltrative	Total
Cases	166	254	7	103	38	42[a]	4	614
%	27.0	41.4	1.1	16.8	6.2	6.8	0.7	100.0

[a]Simple drainage only

DISCUSSION

Theoretic basis of the diagnostic algorithm

Because the basic pathology of surgical conditions must be an organic lesion inside the abdomen, the chief symptoms and signs must be persistent and definite[11]. Acute pain shorter than

6 hours is hard to rule out from the more common abdominal pain due to functional intestinal spasm unless some specific abdominal signs are found[12,13]. By the term "definite", it means definite pain, definite location and definite area involved. Therefore, the child has to be examined many times in an appropriate period of time. In BCH, people usually do the 1st time examination at the first visit, the 2nd examination after laboratory tests, and the 3rd repeat of examination before sending the patient to the ward or back home. Full agreement of the positive findings in the 3 examinations will make the final diagnosis. If negative sign appears in any one examination, further observation and re-examination should be made. The common positive abdominal signs indicating surgical condition are local tenderness, muscular spasm, palpable intestinal pattern and mass. By a soft flat abdomen without the above mentioned signs, people can rule out acute surgical abdomen.

According to common presentations of surgical abdomen, it can be classified into 3 sub-groups: (1) Definite local tenderness and spasm usually indicate a focal inflammation. (2) A palpable distended intestinal loop or a movable solid sausage mass indicates intestinal obstruction. (3) A resistant and silent abdomen indicates generalized peritonitis. These practically cover up all common diseases seen in children. (1) In the sub-group of focal inflammation, the diagnosis depends on the location of tenderness, e.g. in RLQ of abdomen which is usually acute appendicitis; pelvic rectal tenderness with mass is usually torsion of ovarian tumor; flank tenderness is usually renal colic or perinephretic abscess; (2) In the sub-group of intestinal obstruction, a distended intestinal pattern indicates extraluminal obstruction of intestine, e.g. adhesional strangulation, or a solid mass usually indicates intraluminal obstruction, e.g. intussusception or ascaris bezoar; (3) In the sub-group of generalized peritonitis: a marked tender point indicates the focus of spreading peritonitis, e.g. perforated appendicitis; a palpable loop or mass indicates gangrenous peritonitis, e.g. strangulated intestinal obstruction. Pneumoperitoneum by percussion or X-ray indicates perforating peritonitis, e.g. peptic ulcer perforation; thin pus by abdominal puncture aspiration indicates most probably primary peritonitis.

After 3-step analysis, the final diagnosis of a surgical disease is usually made. And then, a comparison between the cardinal signs of the disease and the signs found in this particular patient should be made as a feed-back checking. Any disagreement must be carefully investigated or the diagnosis be reconsidered otherwise.

Method of abdominal assessment in non-cooperative children

Examination of abdomen needs cooperation of the patient. Answering tenderness must be accurate. A crying kid always makes the palpation confused. Even the school-boy may not be able to give an accurate answer to abdominal examination. So the surgeon has to try all means to search for cooperation of a bigger child and to evaluate the findings carefully by repeated examinations. At the same time, he has to observe every action of the kid carefully. Quick squatting or jumping up and down from the examination table without hesitation may rule out abdominal tenderness[14]. For examination of crying kids, a 3-step "comparative technique" of abdominal palpation has been practiced satisfactory in BCH. (1) The 1st step: the mother stands by the head to beguile the baby and hold both its hands. The surgeon stands by the right side of the examination table and palpates the abdomen from LLQ, LUQ, RUQ to RLQ subsequently. Carefully watching the reaction of baby, a high pitch crying might be the answer of tenderness. (2) The 2nd step: let baby' s left hand free, surgeon presses the abdomen with two hands on RLQ and LLQ. Baby naturally uses his free hand to push away the hand in the tender point, e. g. RLQ. (3) The 3rd step: surgeon makes a finger press at the tender area, e.g. McBurney' s point. Baby will try all means to get rid of the finger. By this time, the surgeon may press all other parts of abdomen, to make sure no tenderness beside McBurney' s point or elsewhere.

By the same way, surgeon' s two hands press on both sides of the baby' s abdomen. After several cryings, people may find the hand on left side having pressed much deeper than that on the right side if there is spasm in RLQ of abdomen.

In difficult cases, a dose of short action sedative can be used, e.g. 10 % choral hydrate by dose 1 ml/mos of age. This is limited only to the last examination. Under sedation, deep palpation in six parts (4 quadrants, periumbilical and pelvis) can be made. In order to feel the deep structures in abdomen, the abdominal aorta or other landmarks of posterior abdominal wall should be reached. In rectal pelvic bimanual examination, infiltration or abscess of peri-appendiceal structure can be felt in right iliac fossa and incarcerated inguinal hernia can be felt at the middle along the inguinal ligament from interior by the endo-rectal examination finger[15,16]. It must be kept in mind that the pathology of an acute abdomen is continuously changing. Therefore dynamic comparative observation and evaluation are essential. For making an instant diagnosis in emergency clinic, the information from mother' s impression about baby' s appetite and general activity, whether today is better or worse than yesterday, will be very helpful in making decision of emergency surgery[17].

Supplementary diagnostic techniques (Table 5)

By quick plane X-ray film of erect position, especially in suspected intestinal obstruction, preferably with low pressure barium enema, perforating peritonitis, intestinal obstruction and intussusception can be well demonstrated (Figure 1, 2)[18]. By ultrasound, acute appendicitis and abscess, intussusception, peritoncal fluid, and ovarian cyst or tumor, and perinephretic lesions may be demonstrated (Figure 3, 4)[19-32]. By paracentesis, the abdominal puncture fluid may tell the peritonitis, strangulation of intestine, perforation of intestine or gall bladder[33,34]. Bloody aspirate always calls for an immediate surgical exploration. In case of fine needle puncture getting into intestine under pressure, don' t hurry, keeping on aspiration until negative pressure will prevent intestinal leakage after withdrawal of needle. CT, MRI, Scintiscanning and other fantastic modern tools are used only occasionally[35-38]. But dramatic improvement of the diagnosis of acute abdomen in children must depend on further exploration for the use of high technology. e.g. laparoscopy and portable ultrasound computerized[39-44].

Table 6 Confirmatory diagnostic method used in 973 admissions, 2000

Diagnostic method	*n*	%
Physical exam	630	64.7
Paracentesis[a]	58	6.0
Imaging	285	29.3
Plane film	93	
Rectal insufflation[b]	48	
Barium enema	8	
Barium meal[a]	33	
Sonography	90	
Tc^{99}scintiscan[a]	13	
Total	973	100.0

[a]After admission [b]Reduction failed cases in O.P.D.

Figure 1 Small empty colon

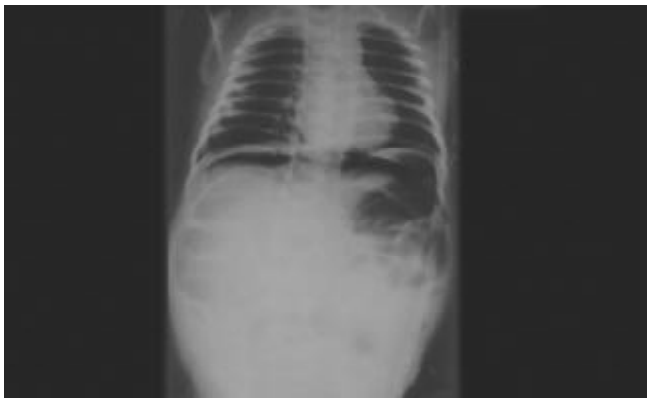

Figure 2 Pneumoperitoneum

Figure 3 Appendiceal abscess

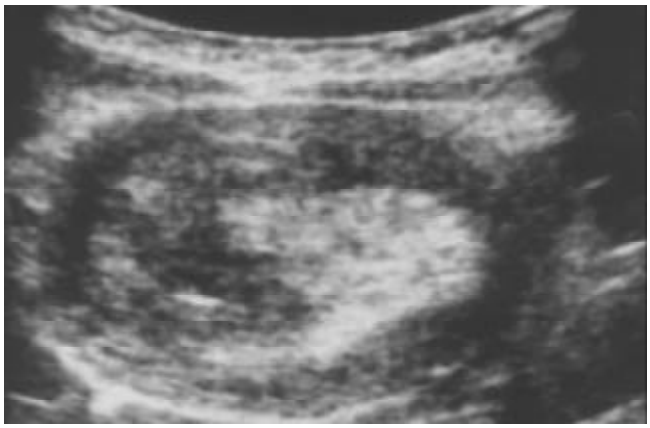

Figure 4 Intussusception-target sign

We conclude that the algorithm has been used and accepted for more than 40 years in thousands of patients by hundreds of surgeons of different levels. It proves helpful in handling acute abdominal pain among children. The prospective study of appendicitis in 2000, has proved its accuracy, false positive, false negative, specificity and sensitivity, all being of acceptable value. However, there are still doubtful cases needing observation, especially in cases with 6 hrs duration without definite signs, partial intestinal obstruction without toxic reactions, and late appendicitis may not need operation. Besides, in the other half of the algorithm (Table1), non-surgical abdominal pain, it includes a large number of diseases needing systemic examination and investigation. However none of them need immediate surgical operation. The most common "school-boy colic" (or primary intestinal spasm) characterized by short pain in attacks, normal appetite and activities in intervals, repeated attacks for months without impairment of nutrition or growth occupies about 60 % of school-boy abdominal pain. Keep this in mind, and it may reduce the false positive diagnosis of acute abdomen.

PROSPECTIVE TECHNOLOGY

(1)Since this algorithm is simple and regular, it can be put into a software of computer to up-grade it into high technology; (2)Portable ultrasonography machine to be used on emergencies examination table could take place of manual palpation of abdomen for uncooperative kids, making the examination more objective; (3)Further improvement of laparoscopy, exploratory laparotomy in acute abdominal pain may be unnecessary.

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• CLINICAL RESEARCH •

Study on the function of pharynx & upper esophageal sphincter in globus hystericus

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Abstract

AIM: Globus pharyngeus is not an uncommon symptom. Presently, its unclear dated pathophysiology remains unclear and the disease can not be evaluated correctly with routine diagnostic methods. The objective of this study is to establish the normal values of pharyngeal and UES pressure, pharyngeal transit time in healthy volunteers and to compare the differences between healthy volunteers and patients with globus pharyngeus.

METHODS: Twenty-four healthy volunteers and thirty-two patients clinically diagnosed as globus pharyngeus entered the study. Pressures of pharynx and UES were measured. Pharyngeal transit time was measured by videofluoroscopic procedure.

RESULTS: Normal pressure of pharynx, and normal resting pressure of UES were 157.81±63.86 mmHg and 68.33±37.56 mmHg, respectively. The corresponding values in the patients were 175.50±93.47 mmHg and 71.38±41.42 mmHg. The pharyngeal transit time was 1.44±0.30 s in normal control group, among them there were 4 cases with stasis of barium in the valleculae and one in the piriform sinus. No laryngeal penetration or aspiration was found. In the patient group, the pharyngeal transit time was 1.37 ±0.41 s, among them there were 6 cases with stasis of barium in the valleculae and 5 in the piriform sinus. 9 cases had laryngeal penetration and 2 had aspiration. There were no statistical differences of pressures of pharynx, UES and the pharyngeal transit time between the two groups. But there was an association between laryngeal penetration and globus pharyngeus.

CONCLUSION: Radiographic examination of the pharynx show specific findings of pharyngeal dysfunction in patients with globus pharyngeus. UES pressure is normal in most patients. Hence, we find no role for UES hypertonicity as an etiologic factor in globus pharyngeus.

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INTRODUCTION

Globus sensation (globus pharyngitis, globus pharyngeus) is best described as a constant sensation of something stuck or a lump or tightness in the throat. Typically, a lump, or crumb-like (foreign body) sensation, a constriction or choking[1-3] is complained. Nearly half of the general population has had intermittent symptoms resembling globus[4-6]. More severe and distressing symptoms comprise up to 4 % of referrals to otolaryngological specialists[4]. In addition to emotional stress, mood, and personality disorders, it has been attributed to a high upper esophageal sphincter (UES) resting pressure, gastroesophageal reflux (GER)[7,8], esophageal dysmotility, hiatal hernia, kinematic pharyngeal transit time and local anatomic abnormalities[9-16]. Although the associations are diverse and broadly investigated, the etiology of globus still remains unclear [4].

The aim of our study was to investigate the association of globus pharyngeus with UES resting pressure and kinematic pharyngeal transit time.

MATERIALS AND METHODS

Study subject

Two groups of persons were studied: globus pharyngeus patients and the control group. The globus pharyngeus group comprised thirty-two consecutive patients presenting themselves to the otolaryngology and gastroenterology departments with predominant symptom of globus pharyngeus with no abnormality on examination of neck, larynx, or pharynx between December 2000 and March 2001. The diagnostic criteria of the globus sensation is in accordance with Rome Ⅱ criteria: At least 12 weeks, which need not be consecutive, in the preceding 12months of: (1) The persistent or intermittent sensation of a lump or foreign body in the throat;(2) Occurrence of the sensation between meals; (3) Absence of pathologic gastroesophageal reflux, achalasia, or other motility disorder with a recognized pathologic basis as scleroderma of the esophagas. The control group consisted of twenty-four healthy volunteers. The male: female ratio in the globus pharyngeus group was 1:1.29, as conpared with 1:1.4 in the control group. The mean age in the globus pharyngeus group was 47±11 years (range: 28-73 years), as compared with 49±17 years (range: 21-77 years). These differences were not significant. A single investigator examined all patients by videofluoroscopy and esophageal manometry.

Esophageal manometry

After an overnight fast, manometry was performed using a solid-state intraluminal transducer assembly (Konigsberg Instruments, Inc., Pasadena, USA). The catheter consists of a flexible tube containing four pressure microtransducers. The distal two are circumferential transducers that record the average pressure over 360°. The circumferential transducer consists of a 4.6-mm-diameter silastic annulus enclosing a small transducer within a castor oil-filled chamber. The two

proximal transducers with a single sensor oriented in one direction. The transducers, starting at the distal end, are separated by 3, 2 and 5 cm. Pressure data are collected on-line by means of a computerized motility system and analyzed using Polygram equipment and software [Synectics (Medtronics), Shoreview, Minnesoda].

Before the examination, the transducers were calibrated at 0 mmHg and 50 mmHg after balneum in 37 ℃ water 10 minutes. Manometry of the UES and pharynx was performed in the upright position. The catheter was passed through a nostril into the stomach. Then it was withdrawn in 1-cm increments every 20 seconds back into the esophagus. UES resting pressure was assessed using a station pull-through of the distal transducer with at least 15-sec intervals for stabilization between each 1-cm movement of the catheter, since the UES is somewhat reactive to the catheter movement. After identifying the high-pressure zone during this slow pull-through, at least 15-sec is allowed to elapse before measuring the resting UES pressure. Maximal stable pressure is recorded in millimeters of mercury using esophageal pressure just distal to the UES as zone baseline. Five 5-ml water (room temperature) swallows were used to assess UES relaxation, pharyngeal contraction, and UES/pharyngeal coordination. Swallows were 30 seconds apart. The swallows were performed with the circumferential transducer placed just proximal to the high-pressure zone of the UES to allow accurate recording of the pressure throughout the cycle of elevation of the sphincter during deglutition. Placement of the circumferential transducer proximal to the high-pressure zone of the sphincter compensates for orad movement during swallowing.

Mamometry helped clarify the specific timing of pressure events during pharyngeal contraction and UES relaxation[17].

Videofluoroscopy

Detailed sequential analysis of videofluoroscopic images recorded at 25 frames/s clarifies timing of the events that occur during pharyngeal contraction and UES opening. It clearly defines the key role of muscle contraction occuring early in the swallowing sequences that produce the elevation of the hyoid and larynx, which is an essential element in the opening of the UES[18-20].

Kinematic pharyngeal transit time is defined as the time from the first movement of the bolus, which results in a complete swallow, to the time the epiglottis returns to its initial position. The definition excludes oral preparation time and tongue movements that do not result in a complete swallow[21].

Videofluoroscopy was completed in both anteroposterior and lateral projections with 5-ml thin barium at room temperature[22-23]. To standardize the evaluations in our study, timing measurements were completed in the lateral projection.

The following data were collected by one physician evaluator: (1) quantitative kinematic pharyngeal transit times including the time of arrival of the head of the bolus reaching the level of the posterior inferior border of C_2 verterbral body (C_2L), the inferior level of the valleculae (BV), the posterior inferior border of C_4 verterbral body (C_4L), and the time at which the epiglottis returned to its original position (Em); These data points were selected to provide more easily identifiable radiologic landmarks for measurement[21,24-26]. (2) The presence or absence of vallecular or piriform pooling, and (3) the presence or absence of penetration of part of the bolus to or through the true vocal cord[27-29].

Statistical analysis

Fisher *P* test was used to compare patients and controls, with statistical significance set at the level of 0.05.

RESULTS

Kinematic pharyngeal transit time

The data of kinematic pharyngeal transit time from the control group and globus pharyngeus group were presented in Table 1.

Table 1 Kinematic pharyngeal transit time

	Control group(n=24)	Globus sensation group(n=32)	P
C_2L	0.50±0.27	0.45±0.33	>0.05
BV	0.57±0.26	0.51±0.34	>0.05
C_4L	0.67±0.27	0.63±0.36	>0.05
EM	1.44±0.30	1.37±0.41	>0.05

Note: Times are given in seconds.

There was no statistically significant difference between the control group and globus pharyngeus group, which showed ^aP>0.05. There were four cases of volunteers presenting stasis of barium in the valleculae and one in the piriform sinus. No laryngeal penetration and aspiration were found. Meanwhile, there were six and five of patients representing stasis of barium in the valleculae and piriform sinus respectively. Nine and two of patients had laryngeal penetration and aspiration. The results of valleculae and piriform sinus stasis and airway aspiration had no statistically significant differences between the two groups. But there was an association between laryngeal penetration and globus pharyngeus.

Esophageal manometry

The data of esophageal manometry from the control group and globus pharyngeus group were presented in Table 2.

Table 2 Data of esophageal mamometry

	Control group (n=24)	Globus pharyngeus group(n=32)
UES		
Resting pressure	68.33±37.56mmHg	71.38±41.42mmHg
Residual pressure	-7.30±6.48mmHg	-11.04±6.90mmHg
Relaxation time (from the beginning of relaxation to the nadir)	0.27±0.06s	0.28±0.15s
Duration (time from beginning of relaxation to the end)	0.54±0.08s	0.59±0.15s
Recovery (time from nadir of relaxation to the end)	0.27±0.08s	0.33±0.15s
Pharynx		
Peak pressure	157.81±63.86mmHg	175.50±93.47mmHg
contraction time (from the beginning of contraction to the nadir)	0.20±0.06s	0.22±0.08s
duration (time from beginning of contraction to the end)	0.42±0.08s	0.44±0.12s
Recovery (time from peak of contraction to the end)	0.22±0.05s	0.22±0.06s

There was no significant difference between the two groups (P>0.05). And also no difference in gender in the two groups (Table 3)

Table 3 Data of male and female in two groups

	Control group		Globus pharyngeus group	
	Male	Female	Male	Female
UES resting pressure(mmHg)	65.55±34.02	70.32±38.49	67.61±34.39	73.65±44.00
UES residual pressure (mmHg)	-9.33±6.80	-5.84±5.82	-8.80±6.98	-12.39±6.49
Pharyngeal peak pressure(mmHg)	165.72±69.19	151.45±57.36	163.19±105.47	182.89±81.99

DISCUSSION

Globus pharyngeus is a common disorder, accounting for 3-10 % of otolaryngologic outpatient referrals[1,4]. The cause is unknown, and is likely to be of multifactorial origin. Abnormal function of the upper esophageal sphincter (UES) may produce the globus sensation symptom[14,30]. Disorders of the UES include the hypertonsic and hypotonsic sphincter and various abnormalities of relaxation. Globus sensation has been associated with hypertonsic upper esophageal sphincter and a persistent cricopharyngeal impression (i.e., bar), and Zenker diverticulum may be seen in patients with abnormal sphincter relaxation[9-13,30-31].

Hypertonicity of the UES was suggested as an etiologic factor in the genesis of globus sensation by Watson and Sullivan[32] in a study involving only nine patients suggested hypertonicity of the UES as an etiologic factor in the genesis of globus pharyngeus. This observation was in contradiction with a previous controlled study by Calderelli *et al*[33], who found normal UES pressures in their patients with globus sensation. The techniques used for UES manometry in both studies might have questionable accuracy, as they did not count for the radial asymmetry of the UES pressure profile. In a more recent study, Cook *et al*[34] failed to demonstrate any difference in resting UES pressure between normal controls and those with a history of globus sensation. Our study, using computerized solid-state manometry with a circumferentially recording transducer has not shown increased prevalence of UES hypertonicity in patients with globus sensation.

Through studies using multi-lumen catheters, radial asymmetry of the UES has been demonstrated over a number of years[35,36]. Pressures recorded in the anterior and posterior directions were usually two or three times greater than pressures recorded laterally. This was believed to result from the anatomical disposition of the cricopharyngeus, but might be due in some extent to the compressive effect of the posterior lamina of the cricoid cartilage against the vertebral bodies of the cervical spine. So we believe that values for UES pressure are best obtained using a circumferential sensing solid-state transducer[37]. In addition to radial asymmetry, oral movement of the sphincter during deglutition complicates the manometric assessment of the UES. With the transducer located in the sphincter high-pressure zone, a recording artifact will usually occur. As the swallow is initiated, the sphincter moves oral, leaving the transducer recording esophageal pressure. This fall in pressure may be erroneously interpreted as sphincter relaxation. If, however, the transducer is located proximal to the high pressure zone, when the subject swallows and the larynx elevates, the UES (which has not yet relaxed) moves onto the transducer, which then records a rise in pressure followed by a fall in pressure as the sphincter relaxes. The pressure rises again as the sphincter regains tone and falls as the larynx returns to its original position, moving the sphincter distal to the transducer. The manometric recording in the UES that results from this sequence of events resembles the letter 'M' and this configuration can be used to ascertain correct transducer placement[38-41].

The manometric findings in our study did not demonstrate any possible origin for the globus sensation.Detailed sequential analyses of videofluoroscopic images recorded at 25 frames/s have clarified timing of the events that occur during pharyngeal contraction and UES opening[42,43]. These studies have clearly defined the key role of muscle contractions occurring early in the swallowing sequence that produce the elevation of the hyoid and larynx which is an essential element in the opening of the UES[44,45]. Videofluoroscopy was completed in both anteroposterior and lateral projections with 5 ml 250 % barium sulfate liquid[27].

The initiating event in our kinemic times is approximately at the beginning of the oropharynx. Consequently, BV in our study represents transit time through the oropharynx and mesopharynx. Pharyngeal dysfunction was defined as the presence of one or more of the following findings: (1) poor laryngeal elevation and epiglottic motion, (2) laryngeal penetration or aspiration, and (3) stasis of barium in the vallecula and piriform sinuses[46,47].

Radiographic evidence of pharyngeal dysfunction such as stasis and aspiration was seen in patients, but these findings were not specific for upper esophageal sphincter dysfunction. Batch and Wilson et al also found that pharyngeal abnormalities were rarely seen at radiography in patients with globus pharyngeus[48-50]. Thus, radiographic findings suggestive of upper esophageal sphincter dysfunction were rarely present in patients with globus sensation[30].

In conclusion, findings by radiographic examination of the pharynx may show specific findings of pharyngeal dysfunction in patients with globus pharyngeus. Results of the pressures of the UES are normal in most patients with this symptom. So we find no role for UES hypertonicity as an etiologic factor for globus, but a strong association between laryngeal penetration and globus pharyngeus.

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• CLINICAL RESEARCH •

Combined small bowel and reduced auxiliary liver transplantation: case report

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Abstract

AIM: To present a case of combined small bowel and reduced auxiliary liver transplantation.

METHODS: A 55-year-old patient with short bowel syndrome and TPN-related liver dysfunction received small bowel transplantation combined with a reduced auxiliary liver graft. A liver was added to restore the patient's liver function and to protect the intestinal allograft from rejection. His own liver was not removed.

RESULTS: Without donor pretreatment and by conventional immunosuppresive therapy following transplantation, the patient experienced had only one episode of mild intestinal rejection, which was easily reversed by treatment with Methylprednisolone. No liver rejection occurred. Unfortunately, the patient died of heart and lung failure 30d after transplantation, despite successful graft replacement. Histopathologic examination of specimens after death demonstrated normal structure in both intestinal and liver grafts.

CONCLUSION: The auxiliary liver graft might play a role in preventing intestinal allograft rejection. However, the observation period in this case is short. Further study is needed to determine the risks, effect on the protecting the small-bowel from rejection, and feasibility of general application of this procedure.

Zhang WJ, Liu DG, Ye QF, Sha B, Zhen FJ, Guo H, Xia SS. Combined small bowel and reduced auxiliary liver transplantation: case report. *World J Gastroenterol* 2002; 8(5):956-960

INTRODUCTION

Small bowel transplantation is a possible choice of treatment for patients with irreversible failure of the intestine[1-8]. Compared with the success in other solid organ allografts, attempts at small bowel transplantation in human have got poor results in terms of patient and graft survival[9-17]. Rejection, immunosuppression-related infections and graft-versus-host reaction (GVHR) are the main obstacles to clinical application[18-30].

In 1990, Grant *et al*[31] first reported a case of successful small-bowel transplantation combined with a liver graft. This patient had only one episode of mild intestinal rejection, which was easily reversed by treatment with OKT_3. She had maintained normal nutrition for more than 2 years after surgery. The authors considered that the lack of serious intestinal rejection in this case may be due to immunological protection provided by the liver graft. Subsequently, some investigators demonstrated the same observations[32-38], others reported that combined liver-bowel transplantation has no immunologic advantage over bowel transplantation alone[39-41]. Furthermore, it has been shown that auxiliary liver transplantation had a slight protective effect on simultaneously transplanted small bowel, and it was not as strong as has been observed with orthotopic liver transplantation[42].

We report a case of short-bowel syndrome and secondary TPN-related hepatic dysfunction who received small-bowel transplantation combined with a reduced auxiliary liver graft in our institute. After operation, only one episode of mild intestinal rejection occurred without liver rejection.

CASE REPORT

Case history

A 55 year old patient has had the short-bowel syndrome since February 1999 after the resection of whole small bowel and right colon because of thrombosis of the superior mesenteric artery. He was then alive on total parenteral nutrition (TPN), but was not discharged from hospital due to uncontrollable diarrhea. Besides, the TPN-related liver impairment developed. After extensive discussion with the patient and his family, small-bowel transplantation was performed on April 15, 1999. An auxilliary liver was simultaneously transplanted for the purpose of both restoring his liver function and protecting the intestinal graft from rejection. We did not remove his own liver.

Transplantation procedures

The donor is a brain death adult. Both donor and recipient were blood group O. The donor's HLA phenotype was A11,-; B75, -; DR12, 15 and DQ6 (1),-. The recipient's HLA phenotype was A2,23;B44,62;DR7,- and DQ2,5. The lymphocytotoxic crossmatch was negative. No pretreatment was given to alter the graft immunogenicity with antilymphocyte or other modalities.

To reduce the volume of donor liver, left lateral lobectomy and right frontal lobectomy were performed. The reduced liver and small bowel including the duodenum, jejunum and partial ileum were grafted into the abdominal cavity of the recipient. The donor's abdominal aorta duct containing the origins of the superior mesenteric artery and coeliac artery was anastomosed end-to-side to the recipient's infrarenal aorta. The donor's infrahepatic vena cava was anastomosed end-to-side to the recipient's infrarenal vena cava. The end of the donor

jejunum was anastomosed to recipient' s duodemum; intestinal continuity was restored with an end-to-side ileocolic anastmosis. The end of the donor' s ileum was exteriorized as an ileostomy (Figure 1).

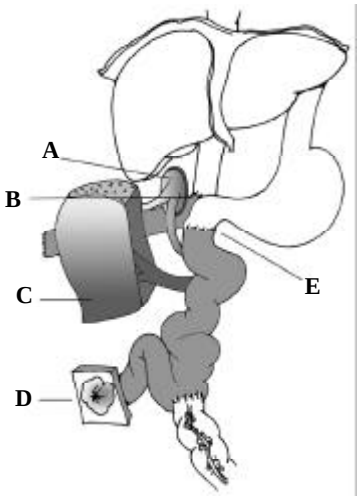

Figure 1 Small-bowel and auxiliary liver allograft. A. Carrel patch containing the origin of the superior mesenteric artery and the coeliac artery is anastomosed to the recipient's aorta; B. Anastomosis of end of the donor infrahepatic vena cava to the side of recipient's vena cava; C. The reduced liver; D. Ilesotomy; E. Anastomosis of donor jejunum to the recipient's duodenum

Immunosuppression management

Methylprednisolone was given intravenously 30 min before graft revascularization (first dose of 500mg bolus) and rapidly tapered to 20 mg· d^{-1} over the next 10 days. Cyclosporin A by continuous intravenous infusion was begun intraoperatively (3mg· kg· d^{-1}) to maintain the whole blood concentration of 350-450 μg· L^{-1} by monoclonal radioimmunoassay. Cyclophosphamide 100 mg was also given intravenously daily for the first 3 days. Prostaglandin E_1 (600 μg· d^{-1}) was began intraoperatively and continued for 20 days.

Postoperative course

Detection of graft rejection was based primarily on clinical observations and mucosal biopsies. During his postoperative course, the bowel graft developed only one histologic evidence of rejection. Mucosal biopsy on the seventh postoperative day showed lymphocyte infiltration in epithelium, slight fattening of the villi, decreased numbers of goblet cellls, but the mucosal destruction and necrosis were not observed (Figure 2). The rejection was successfully reversed by a 3 day course of methylprednisolone bolus (15mg· kg· d^{-1} per day in tapering doses).

There was no clinical or histological evidence of liver rejection. The liver function including ALT, AST, Tbil, cholesterol, triglyceride returned to the normal range 5 days after surgery. On the 9th and 23rd postoperative day, laparotomy was performed because of surgical complications. During the operation, the liver was biopsied, and a normal histological appearance was found (Figure 3).

The patient did not receive any specific treatment for preventing graft-versus-host disease (GVHD) other than the immunosuppression therapy previously described. No sign of GVHD developed. Unfortunately, some severe complications occurred including intestinal fistula, stress ulcer and bleeding, ARDS, pulmonary and abdominal infection. The patient died of heart and lungs failure 30 days after transplantation despite successful graft replacement. The histopathologic examination of specimens after death demonstrated normal structure in both intestinal and liver grafts.

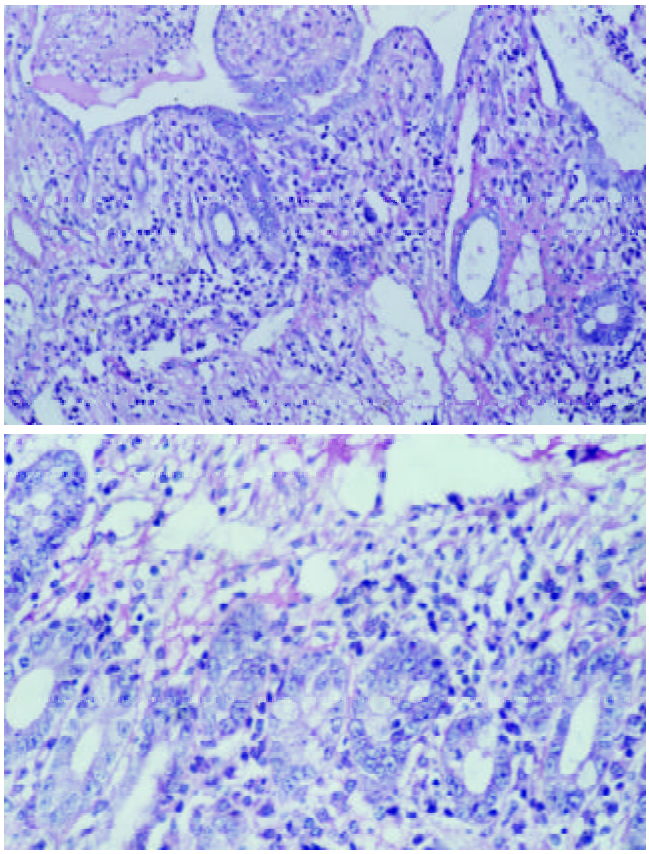

Figure 2 Photomicrographs showing acute rejection with lymphocytic crytitis on the 7 th postoperative day, the mucosal destruction and necrosis were not observed (H&E, A, ×200; B. ×400).

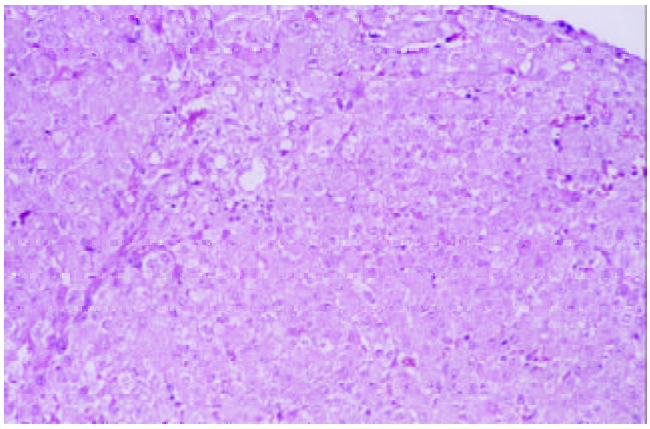

Figure 3 Liver biopsy specimens on the 9th postoperative day showed normal appearance of the allograft (×200), no inflammatory infiltrate in the portal tract.

DISCUSSION

Liver dysfunction is a well-recognized complication of intestinal failure. Advances in TPN have allowed the patient

suffering from short bowel syndrome to survive. However, in many instances total parenteral nutrition causes severe liver damage leading to cirrhosis. Thus, combined liver and small bowel transplantation becomes an established life-saving therapy for the treatment of liver disease and intestinal failure[43-46]. In the general, an orthotopic liver and small bowel are transplanted. To our knowledge, this case is the first report of transplantation of combined auxiliary reduced liver and small bowel in human. We tried to restore the patient' s liver function and to protect the intestinal allograft from rejection. For these reasons, an auxiliary liver was simultaneously transplanted. The auxiliary reduced liver-small bowel transplantation model represents a new, less aggressive possibility for multiorgan transplantation[31,47,48]. It offers some advantages over multivisceral transplants, including simplicity and less mortality than the combined orthotopic liver-intestinal transplantation. This procedure is useful for the patients with reversible hepatopathy associated with intestinal insufficiency because it can offer temporal or definitive hepatic support.

Without donor pretreatment and under conventional immunosuppresive therapy, this patient had only one episode of mild intestinal rejection, which was easily reversed by treatment with Methylprednisolone. These data indicate a possible role of the auxiliary liver graft in preventing intestinal allograft rejection. In fact, the immunoprotecting effect of the liver was first described by Calne in 1969 in a porcine model[49]. The animal can reject skin, kidney and hearts rapidly. However, orthotopic and accessory heterotopic liver allografts can protect preferentially from rejection grafts of donor specific skin, kidney and heart. Injected soluble liver antigen may also protect donor specific tissue from rejection. It suggested that allogeneic liver can induce immunological tolerance in immunologically mature pigs[49]. Subsequent studies demonstrate that specific tolerance can be achieved in combined liver/small bowel transplantation after a transient rejection crisis[32-38].

The mechanism of immunological protection of the liver is not very clearly until now[50-52]. Apoptosis of T lymphocytes may be involved in graft rejection and tolerance induction[50]. Apoptosis is a mechanism for eliminating autoreactive cells during T cell maturation in the thymus. T cells themselves use apoptosis to eliminate alloantigen-expressing donor cells during rejection responses. Apoptosis of parenchymal cells in the grafted livers correlated directly with interleukin-2 receptor expression of the infiltrating T cells. In the late phase of rejection, a peak of apoptosis in the lymphocyte infiltrate was demonstrated, characterized as predominantly apoptotic $CD8^+$ T lymphocytes. T cell inactivation seems to result in apoptosis of cytotoxic T cell and tolerance[50]. In addition, microchimerism is associated with long-term graft acceptance in combined liver/small bowel transplantation[51,52]. Donor specific leucocytes could be detected immunhistochemically in the combined liver/small bowel group and isolated liver group in spleen, host Peyer patches, and mesenteric lymph nodes. Particularly in the liver sinusoids investigators[52] found a great number of persisting donor leukocytes in all long-term survivors in combined liver/small bowel rats. The persisting leucocytes obviously originate from the initially transplanted white cell population of the liver. The liver as constant source of antigen plus a persisting and obviously active leukocyte popuolation may provide the basis for a long-term survival of the liver graft and any cotransplanted organ.

However, the observation period in this case was short and it is difficult to extrapolate that the complex immune responses between the donor and recipient are affirmatively associated with adding a liver graft. Moreover, other studies found that liver grafting failed to prevent intestinal rejection in human and large animal model[39-41]. Further studies are needed to determine the risks, effect on the protecting the small bowel from rejection, and feasibility of general application of this procedure.

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