INVESTIGATION ON HEPATITIS C AND B VIRUS INFECTION IN CARCINOMA OF THE EXTRAHEPATIC BILE DUCT IN CHINA

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ABSTRACT

Objective: The incidence of carcinoma of extrahepatic bile duct tends to increase during recent decade in China, but its cause is unclear. This study is to investigate the Hepatitis C virus (HCV) and Hepatitis B virus (HBV) infection in the tissues of carcinoma of extrahepatic bile duct and study their correlation. Methods: HCV RNA and HBV DNA was detected by *in situ* polymerase chain reaction (IS-PCR) in sections of 51 cases of carcinoma of extrahepatic bile duct and 34 cases of control group. Results: Of 51 carcinoma of extrahepatic bile duct, HCV RNA was detected in 18 (35.4%), HBV DNA in 8 (15.9%). In 34 cases of control group, HCV RNA was detected in 2 (5.9%), and HBV DNA in 3 (8.8%). Conclusion:The prevalence of hepatitis C and B viral infection in the tissues of carcinoma of extrahepatic bile duct was significantly higher than in control group. The findings suggest a correlation between HCV, HBV infection and carcinoma of extrahepatic bile duct, inferring HCV and HBV might be involved in the development of carcinoma of extrahepatic bile duct.

Key words: Hepatitis C virus, Hepatitis B virus, Carcinoma of extrahepatic bile duct, *In situ* polymerase chain reaction

Cancer of bile ducts, arising from malignant transformation of the epithelium of bile duct, is even less common than gallbladder carcinoma and is seen in 0.01%–0.46% of all autopsies,[1] and its pathogenesis is still unclear completely. The development of bile duct cancer has been linked to hepatolithiasis, clonorchis sinensis, congenital dilatation of bile duct, and chronic inflammatory bowel disease.[2–5] In China, 0.33% patients with Hepatolithiasis,[6] 2.1%–21% with the choledochal cyst[7–10] and 0.22% with clonorchis sinensis infestation[11] are simultaneously complicated by bile duct cancer, and bile duct cancer being simultaneously complicated by gallstone, choledochal cyst, clonorchis sinensis infestation account for 6.15%–16%,[12–14] 7%,[8] 6.4%[15] in total bile duct cancer of the corresponding period, respectively. But how many bile duct cancers are complicated by ulcerative colitis simultaneously has not been estimated in literature in China. These investigations indicated, therefore, that Chinese patients with bile duct cancer suffered from above-mention diseases before only account for one third or one second in total bile duct cancer. In extrahepatic biliary carcinoma in China, carcinoma of extrahepatic bile duct accounted for 75.2%.[16] The incidence of carcinoma of extrahepatic bile duct tends to increase during recent decade in China, but its cause is unclear .[16]

Hepatitis C virus (HCV) is an RNA virus with a genomic size of 9.6 kb. More than 50% of individuals exposed to HCV develop chronic infection. Of those individuals chronically infected, approximately 20% to 30% will develop liver cirrhosis and/or hepatocellular carcinoma when followed for twenty or thirty years.[17] In China, the prevalence of HCV antibodies in blood donors as measured by second or third generation assays is about 0%–4.6%.[18,19] HCV RNA has been successfully detected and located in formalin-fixed paraffin-embedded liver sections by *in situ* reverse-transcription polymerase chain (IS-RT-PCR).[20,21] Hepatitis B virus (HBV) is one of the smallest human viruses known with a genome size of

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only 3.2 kb. In China, the prevalence of serum HBsAg is about 10%. HBV DNA has been detected in formalin-fixed paraffin-embedded liver sections greatly using *in situ* hybridization, due to its high copy number in liver cells.

This study aims to investigate the HCV and HBV infection in the tissues of carcinoma of extrahepatic bile duct (51 cases) and control group specimens (34 cases) by detecting HCV RNA and HBV DNA using IS-PCR, and to determine their correlation.

MATERIALS AND METHODS

Carcinoma of Extrahepatic Bile Duct tissues

Fifty-one cases of carcinoma of extrahepatic bile duct, and thirty-four specimens as control group (including ten cases of choledochal cyst, eight cases of hepatolithiasis, two cases of congenital dilatation of the intrahepatic bile duct, two cases of cystadenoma and two cases of adenoma of common bile duct, and ten cases of wall of extrahepatic bile duct) were collected from Department of Hepatobiliary Surgery, General Hospital of the People's Liberation Army (PLA). All specimens were resected from 1995 to 1998, and fixed and embedded routinely. All Carcinoma of extrahepatic bile duct were diagnosed as adenocarcinoma by Department of Pathology, General Hospital of PLA. Five-micrometer thick formalin-fixed paraffin-embedded sections were prepared.

Primers and Probes Preparation

The oligonucleotide primers and probes were synthesized, and probe was labeled with biotin (Sangon.Co.Ltd). HCV primer was all located at the highly conserved 5'non-coding region of the HCV genome. The sequences of outer primers are: sense, 5' GGCGACACTCCACCATAGATC 3' (1–21 nt), antisense, 5'GGTGCACGGTCTACGAGACCT 3' (304–324 nt). The sequences of inner primers are: sense, 5' CTGTGA-GGAACTACTGTCTTC 3' (28–48 nt), antisense, 5' CCCTATCAGGCAGTACCACAA 3' (264–284 nt). Probe sequences are: 5' ACACCGGAATTGCCAGG-ACGACCGGGTCCTTTCTTG 3' (142–177 nt). HBV primer was located at the pre-C and C region of HBV genome. The sequences of HBV primers are: sense, 5' GCCTTGGGTG-GCTTTGGGGC 3' (1881–1900 nt), antisense: 5' CCTG-AGTGCTGTATGGTGA 3' (2050–2068nt), the sequence of probe is: 5' GAGATCTCCTT-GACACCGCCTCTGC 3' (1983–2007 nt).

IS-RT-PCR Detecting HCV RNA

The sections were deparaffinized with fresh xylene and graded alcohols, followed by phosphate buffered saline (PBS) for 5 min. The tissues were digested with protinease K (30 μg/ml, Sigma) for 15 min and rinsed with DEPC-treated PBS three times. The tissues were then treated with Rnase-free Dnase I (700 U/ml, Promega) at room temperature overnight or 37°C 2 h in a humidified chamber. Then the sections were fixed twice in 95% and 100% alcohol for two 3 min. RT was achieved with 30 μl RT solution for each section (1 × RT buffer, dNTP 250 μM each, antisense of outer primer 1 μl, Rnasin 1 U/μl, AMV reverse transcriptase 0.4 U/μl [Promega]) in a humidified chamber at 42°C for 60 min. The reaction solution was dripped away and washed with DEPC-treated PBS for two 5 min. Then fixed twice in 95% and 100% alcohol for two 2 min, and the 50 μl PCR solution for each section was applied which consisted of $MgCl_2$ 2.5mM, 1 × PCR buffer, each primer 1 μM, dNTP 250 μM, Taq DNA polymerase 4U/50 μl, BSA 3 mg/ml. The "hot-start" approach was employed during which Taq DNA polymerase was added at 80°C. The *in situ* amplification of target sequences was performed in a thermal cycler (GeneAmp *in situ* PCR System 1000 [Perkin Elmer]), using two primer pairs. The cycling conditions used were: the initial denaturation step at 94°C for 4 min followed by 20 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 1.5 min, and final extension of 72°C for 3 min. The cover slip was then removed. The sections were washed with PBS two 5 min and fixed in 100% alcohol 10 min. Then processing secondary PCR amplification. Except that inner primers replaced outer primers, the remaining steps were as the same as the initial PCR amplification. The cover slip was removed. The section was washed with PBS, fixed in 100% alcohol 10 min. Sections in PBS were heated at 80°C for 10 min, and put on ice. Then hybridization solution (probe 2.5 μg/ml, 50% deionized formamide, 5×SSC, 1× Denhardt's solution, sssDNA 100 μg/ml) added on the slides at 37°C overnight. The section was washed with serial SSC, and covered with 10% normal sheep serum. The specimens covered with S-A/HRP at 37°C for 45 min. DAB solution added in slides at 37°C for 15 min. The sections were incubated in the dark and checked at 5-min intervals. The DBA detection method yields a yellow precipitate. After development, the sections were counterstained with hematoxylin. Positive cells and its histological distribution were examined.

Negative Control Groups

Including: (1) hepatitis B liver tissues, (2) HCV RNA positive specimens digested by RNase (10 mg/ml) at 37°C for 1 h, (3) HCV RNA positive specimens omitted reverse transcription, (4) HCV RNA positive specimens omitted Taq polymerase, and (5) no probe control.

IS-PCR Detecting HBV DNA

Except for omitting RNase-free DNase I treatment and RT step, and using a pair of primer with 25 PCR cycles, other steps were done as the same as detecting HCV RNA.

Negative Control Groups

Including: (1) hepatitis C liver tissues, (2) HBV DNA positive specimens omitted Taq polymerase, and (3) no probe control.

RESU LTS

In 51 cases of carcinoma of extrahepatic bile duct, HCV RNA sequence was detected in 18 (35.4%). HCV RNA was located in the nucleus in 12 cases (Figure 1), and in the cytoplasm in 6 cases (Figure 2). In 34 cases of control group, HCV RNA sequences were detected in 2 (5.9%) (Figure 3). The HCV RNA positive signal was located occasionally in the mononuclear cells. After treated by RNase or omitting AMV, Taq polymerase and probe in the procedure, all positive sections showed no HCV RNA positive signal.

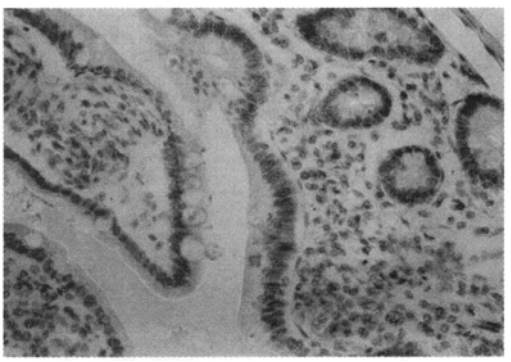

Fig. 1. HCV RNA located in the nucleus of cells of carcinoma of extrahepatic bile duct. IS-RT-PCR × 400

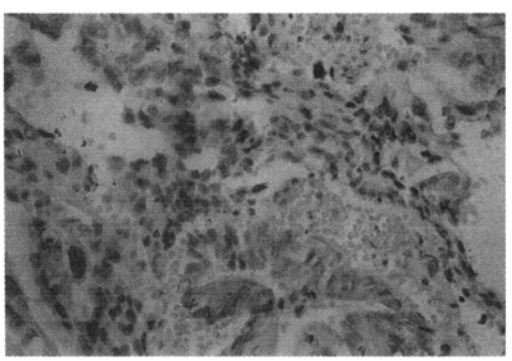

Fig. 2. HCV RNA located in the cytoplasm of cells of carcinoma of extrahepatic bile duct. IS-RT-PCR × 400

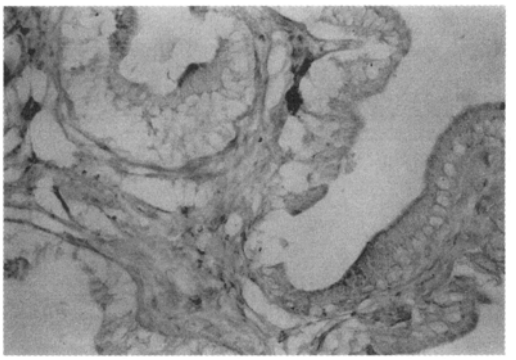

Fig. 3. HCV RNA located in the cytoplasm of cells of choledochal cyst. IS-RT-PCR × 400

Of 51 carcinoma of extrahepatic bile duct, HBV DNA sequence was detected in 8 (15.9%). HBV DNA was confined to the cytoplasm of carcinoma cells in 6 cases (Figure 4) and the nucleus in 2 cases (Figure 5). In 34 cases of control group, HBV DNA sequence was detected in 3 (8.8%) (Figure 6). After omitting Taq polymerase and probe in the procedure, all positive sections showed no HBV DNA positive signal.

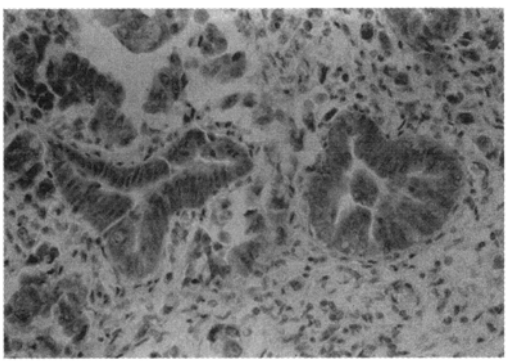

Fig. 4. HBV DNA located in the cytoplasm of cells of carcinoma of extrahepatic bile duct. IS-PCR × 400

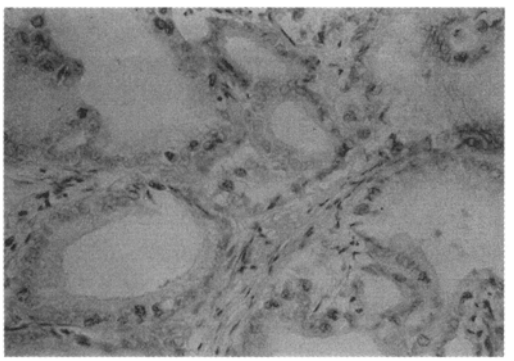

Fig. 5. HBV DNA located in the nucleus of cells of carcinoma of extrahepatic bile duct. IS-PCR × 400

HCV RNA and HBV DNA were simultaneously detected in 1 case.

HCV RNA and HBV DNA positive cells were found to be either scattered or in clusters. In the cytoplasm, some positive signals of HCV RNA and HBV DNA were so strong that it might be difficult in determining the nucleic positive.

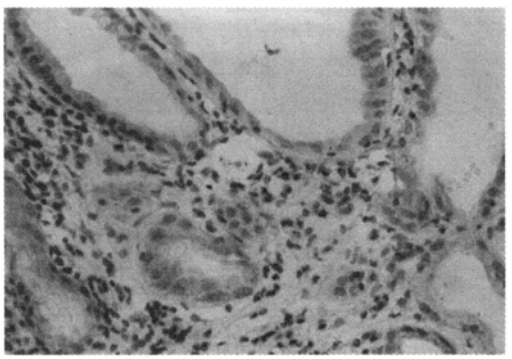

Fig. 6. HBV DNA located in the cytoplasm of cells of choledochal cyst. IS-PCR × 400

DISCUSSION

Although HCV and HBV are considered to be essentially hepatotropic, some studies indicate that it can also be found in extrahepatic tissues, such as peripheral blood mononuclear cells, kidney tissue and salivary glands.[22, 23] In the present study, HCV RNA and HBV DNA were found in cells of carcinoma of extrahepatic bile duct, which further demonstrates HCV and HBV have a wide host cells.

HCV Infection and Carcinoma of Extrahepatic Bile Duct

HCV infection is now known to be a major risk factor for the development of hepatocellular carcinoma (HCC), in which HCV RNA and proteins can be detected.[24] In cells of carcinoma of extrahepatic bile duct, HCV RNA was mainly located in the nucleus, which was resembled the localization of HCV RNA in cells of HCC.[25] The incidence of HCV infection in the tissues of carcinoma of extrahepatic bile duct is significantly higher than in control group, which indicates that both have a correlation. It is inferred that HCV infection, being similar to hepatolithiasis, choledochal cyst, etc., may be one of risk factors being involved in the development of carcinoma of extrahepatic bile duct.

HCV genome does not integrated into the genome of infected cells.[26, 27] The mechanism of carcinogenesis of HCV is unclear completely now, which may be involved in proteins HCV gene encoding. It has been noted recently that the HCV core protein demonstrates diverse biological functions, including the regulation of cellular and unrelated viral genes at the transcriptional level, and has some potential direct carcinogenic effects *in vitro*. HCV core protein could activate human c-*myc*, early promoter of SV10, Rous sarcoma virus LTR and HIV-1 LTR,[28] inhibit cisplatin-mediated apoptosis in human cervical epithelial cells and apoptosis induced by the overexpression of c-*myc* in Chinese hamster ovarian cell,[29] and repress transcriptional activity of P53 promoter.[30] REF cells co-transfected with HCV core and H-*ras* genes became transformed and exhibited rapid proliferation, anchor-independed growth, and tumor formation in athymic nude mice.[31] Transformation of NIH3T3 cells to the tumorigenic phenotype by the nonstructural protein NS3 of HCV was demonstrated and the proteinase activity associated with this protein was suggested as the cause of transformation.[32] NS5 protein from HCV-1b ORF includes NS5A and NS5B. Recently, NS5A protein is reported to be a potent transcriptional activator,[33] and can repress the interferon-induced protein kinase, PKR, through direct interaction with each other.[34] The experimental data suggest that HCV gene products have a function of gene regulation, and can modulate cell growth and differentiation, and may be directly involved in the malignant transformation of HCV-infecting cells. But how HCV infection is involved in the development of carcinoma of extrahepatic bile duct needs search further.

HBV Infection and Carcinoma of Extrahepatic Bile Duct

In the present study, Pre-C and C region sequence of HBV genome can be detected in the tissue of carcinoma of extrahepatic bile duct. It was found that HBV DNA was mainly located in the cytoplasm of cancerous cell, which was "free" HBV DNA. As we know, this is different from HBV DNA location in the nucleus of cancer cell of HCC, which means HBV DNA integration into host cell. In fact, HBV DNA was more detected in the tissue of carcinoma of extrahepatic bile duct as compared with in control group, which therefore indicates that HBV infection may be involved in the development of part of carcinoma of extrahepatic bile duct, particularly when HBV DNA located at the nucleus.

As the same as HCV, HBV is also thought as a major risk factor of the development of HCC, but the exact role of HBV in the development of HCC is not known, which research focus on the carcinogenesis of HBxAg, HBsAg and integration of HBV DNA.

It is found that the integration of HBV DNA fragments into host DNA adjacent to or within oncogenes and/or tumor suppressor protein-encoding genes may alter their function in *cis*, and raise the possibility of a

direct carcinogenic effect of HBV.[35] For example, HBV DNA has been found integrated near the V-erb-A, cyclin A, the mevalonate kinase, or the retnoic acid receptor genes,[36–40] suggesting that the deregulated expression of these genes may contribute to cancer. In addition, HBV DNA integration has been reported near the P53 gene,[41] and appears to be associated with loss of heterozygosity on chromosome 17b, where P53 resides,[42] which suggests that the integration may indirectly inactivate the P53 gene. Besides, there is considerable evidence that the products of the integrated viral DBA fragments contributes importantly to hepatocarcinogenesis in *trans*. Integrated HBV DNA fragments have been shown to encode truncated HBsAg and HBxAg polypeptides with *trans*-activation properties, which may alter the patterns of host gene expression important to the development of HCC.[43] *Trans*-activation activity of truncated surface antigen polypeptides appears to involve stimulation of the protein kinase C (PKC), AP-1, AP-2, and nuclear factor kappa B (NFkB) pathways.[43–45]

HBxAg was mainly located in the cytoplasm of host cells,[46, 47] but also was found in the nucleus of host cells.[47] Mechanistic studies have shown that HBxAg with *trans*-activation activity may alter patterns of gene express by binding to transcription factors in the nucleus,[48, 49] such as the TATA binding protein (TBP),[50] ATF-2, CREB,[51] and by altering the activity of several signal transduction pathways in the cytoplasm,[52] such as the ras[44, 53, 54] and or NFkB[55] signal transduction pathways. HBx gene of HBV has induced liver cancer in transgenic mice successfully, which indicates that HBV is directly involved in the development of liver cancer.[56] But if HBV, through all-mentioned ways, may be involved in the development of part of carcinoma of extrahepatic bile duct needs study further.

In sum, in this study, 49% (25/51) carcinoma of extrahepatic bile duct suffered from HCV and HBV infection, which suggests that there is correlation between carcinoma of extrahepatic bile duct and hepatitis virus, particularly HCV, in molecular epidemology. But how HCV and HBV are involved in the development of carcinoma of extrahepatic bile duct needs search further.

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Advances in Brief

DETECTION OF CPG METHYLATIONS IN HUMAN MISMATCH REPAIR GENE HMLH1 PROMOTER BY DENATURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (DHPLC)

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ABSTRACT

Objectives: To develop a novel method to detect CpG methylation by DHPLC. Methods: After DNA was treated with sodium bisulfite, mismatch repair gene hMLH1 promoter was amplified by polymerase chain reaction (PCR). DHPLC was used to separate the PCR products at their partially denaturing temperatures. BstUI digestion assay was also used for comparison study. Results: A 294bp band was obtained by PCR after each DNA samples of colon cancer cell line RKO and gastric cancer cell line PACM82. These two bands could be separated completely by DHPLC at 53°C (retention time 6.7 min for RKO vs. 6.2 min for PACM82). We concluded that the hMLH1 promoter in RKO cells is methylated, while PACM82 is not methylated, since methylation can protect the conversion of C to T and keep higher C/G content after bisulfite treatment, leading to the delayed time. These results consistent with those from BstUI digestion assay. Conclusion: Methylation in CpG islands of hMLH1 could be detected conveniently by DHPLC after bisulfite modification.

Key words: hMLH1,CpG islands, Methylation, DHPLC

Methylation of these CpG islands is associated with silencing of gene transcription and imprinting of genes. Detection of CpG methylation is important for understanding the expression status of target genes. The current major approaches to detect methylation have many defects. We try to detect the methylation with denaturing high-performance liquid chromatography (DHPLC).

MATERIALS AND METHODS

Cell Lines and DNA

Colorectal carcinoma cell line RKO with the silenced hMLH1 was kindly provided by University of California San Francisco. RKO and gastric carcinoma cell line PACM82 were cultured in DMEM medium (Gibco) containing 10% FBS at 37°C with 5% CO_2. Genomic DNA of these cells and one surgical specimen of gastric carcinoma were isolated with phenol/chloroform and modified by sodium bisulfite as described [1,2].

Primers and PCR

Two pairs of primers were designed according to the sequences of the hMLH1 promoter. Upstream and downstream primers in each pair were from the sequences of –318 (1 was indicated as translate start site) to -293 and –48 to –25, respectively. One pair of primers was used to amplify the bisulfite modified hMLH1 (PCR for treated template, tPCR). The sequences of the primer pair are: t-sense, 5'gtatttttgtttttattggttggata; t-antisense, 5'aataccttcaaccaatcac-ctcaata. Another pair was used to amplify the unmodified samples (PCR for wild template, wPCR). Their sequences are: w-sense, 5'gcatctctgctcctattggctggata and w-antisense, 5'agtgccttcagccaatcacctcagtg. Hot-started temperature-decreasing PCR was used (from 65°C to 45°C for tPCR and from 75°C to 58°C for wPCR, -1.0°C per cycle, 35 cycles).

BstUI Digestion Assay for Methylation

1μg PCR of products was digested with 5U of BstUI (New England Biolabs) in 30 μl of total volume at 60°C for 3h. 2.5% agarose gel was used to separate the digested fragments.

Analysis for Methylation by DHPLC

DHPLC was performed with WAVE™ DNA Fragment Analysis System (Transgenomic, Inc.) to detect point mutation in wPCR products and methylation in tPCR products. Chroma-togramphic peaks were detected by UV-detector at 260 nm.

RESULTS AND DISCUSSIONS

A single 294bp band of the hMLH1promoter was observed in both wPCR and tPCR products of templates from RKO and PACM82 cell lines and the tissue sample of gastric cancer. That means the quality of two kinds of PCR products meets the requirement for DHPLC analysis.

Two fragments (88bp and 206bp) were obtained from the 294 bp wPCR products of both the cell lines in the BstUI digestion assay. This agreed with that the hMLH1promoter has a BstUI cleaving site CGCG. It was reported that the methylated

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