Detection of hepatitis C virus NS5 protein and genome in Chinese carcinoma of the extrahepatic bile duct and its significance

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Abstract

AIM To investigate the hepatitis C virus (HCV) infection in the tissues of carcinoma of extrahepatic bile duct and study their correlation.

METHODS HCV NS5 protein and HCV RNA were detected by labeled streptavidin biotin (LSAB) method and in situ reverse transcription polymerase chain reaction (IS-RT-PCR) in sections of 51 cases of carcinoma of extrahepatic bile duct and 34 cases of control group (without malignant biliary disease).

RESULTS In 51 cases of carcinoma of extrahepatic bile duct, HCV NS5 protein was detected in 14 (27.5%), which was clearly stained in the cytoplasm of cancer cell but not in the nucleus or cell membrane. HCV RNA was detected in 18 (35.4%), which was located in the nucleus of cancer cell in 12 cases and in the cytoplasm in 6 cases. HCV NS5 protein and RNA coexistence was found in 2 cases. In 34 cases of control group, HCV RNA was detected in 2 (5.9%). HCV NS5 protein and RNA positive cells were found either scattered or in clusters.

CONCLUSION The prevalence of hepatitis C viral infection in the tissues of carcinoma of extrahepatic bile duct was significantly higher than in control group ($\chi^2=9.808$, $P=0.002$). The findings suggest a correlation between HCV infection and carcinoma of extrahepatic bile duct, which is different from the traditional viewpoint. HCV infection might be involved in the development of carcinoma of extrahepatic bile duct.

INTRODUCTION

Cancer of bile duct arises from malignant transformation of the epithelia of bile duct. It is even less common than gallbladder carcinoma, and seen in 0.01%-0.46% of all autopsies[1] and its pathogenesis has not been fully understood. Cancer of bile duct is different from hepatocellular carcinoma (HCC) in etiologic factors, the former is not correlated to HBV or HCV infection and liver cirrhosis, traditionally[2]. It has been reported that only 10%-20% patients with bile duct cancer accompany liver cirrhosis, but 70%-90% patients with HCC are associated with liver cirrhosis[3-5]. There is a high incidence of bile duct cancer in the southeast Asia, and liver fluke infection due to clonorchis sinensis and opisthorchis viverrini is the most frequently cited cause of bile duct cancer[6-7]. Now the development of bile duct cancer has been linked to hepatolithiasis, clonorchis sinensis, congenital dilatation of bile duct, and chronic inflammatory bowel disease[8-11]. In China, 0.33%-9.7%, patients with hepatolithiasis[12,13], 2.1%-21% with the choledochal cyst[14-17] and 0.22% with clonorchis sinensis infestation[18] are simultaneously complicated with bile duct cancer. Bile duct cancer simultaneously complicated with gallstone, choledochal cyst, and clonorchis sinensis infestation accounts for 6.15% - 16.9%[19-22], 7%[19] and 6.4%[23] in total bile duct cancer of the corresponding period, respectively. But the incidence of bile duct cancer complicated with ulcerative colitis simultaneously is not estimated in literature in China now. These investigations indicated, therefore, that Chinese patients with bile duct cancer suffered from above-mentioned diseases before account for one third or a half of the...
total patients with bile duct cancer. In extrahepatic biliary carcinoma in China, carcinoma of extrahepatic bile duct covered 75.2%[24], the incidence of carcinoma of extrahepatic bile duct tends to increase over the past decade, but its cause is still unclear[24].

Hepatitis C virus (HCV) is a RNA virus with a genomic size of 9.6kb, and now known to be the chief cause of transfusion-associated non-A, non-B hepatitis, which has been reported to occur in 7%-10% patients who received transfusion[25,26]. More than 50% of individuals exposed to HCV develop chronic infection. Of those chronically infected individuals, about 20%-30% will develop liver cirrhosis and/or HCC when followed for 20 or 30 years[27].

In China, the prevalence of HCV antibodies in blood donors as measured by the second or third generation assays is about 0%-4.6%[28,29], and in a rural population the HCV infection rate is up to 15.3%[30], which indicates that China is a relatively high incidence area of HCV infection. In situ reverse-transcription polymerase chain (IS-RT-PCR) has been successfully applied to the detection and localization of HCV RNA in formalin-fixed paraffin-embedded liver sections[31,32], indicating that it becomes easy to detect the low level of HCV RNA. This study aims to investigate the HCV infection in the tissues of carcinoma of extrahepatic bile duct (51 cases) and control group specimens (34 cases) by detecting HCV NS5 protein and RNA using labeled streptavidin biotin (LSAB) method and IS-RT-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 34 specimens as control group (including 10 cases of choledochal cyst, 8 cases of hepatolithiasis, 2 cases of congenital dilatation of the intrahepatic bile duct, 2 cases of cystadenoma and 2 cases of adenoma of common bile duct, and 10 cases of wall of extrahepatic bile duct near gallstone) were collected from Department of Hepatobiliary Surgery, General Hospital of People’s Liberation Army (PLA). All specimens were resected from 1995 to 1998, and fixed and embedded routinely. All carcinomas of extrahepatic bile duct were diagnosed as adenocarcinoma by the Department of Pathology, General Hospital of PLA. Five-micrometer thick for malin-fixed paraffin-embedded sections were prepared.

Primers and probe preparation
Primers and probe were all located at the highly conserved 5’-non-coding region of the HCV genome. The oligonucleotide primers and probe were synthesized, and the probe was labeled with biotin (Sangon Co.Ltd). The sequences of outer primers are: sense, 5’-GGCGACACTCCACCATAGATC 3’ (1-21nt), antisense, 5’-GGTGCACGGTC-TACGAGACCT 3’ (304-324nt). The sequences of inner primers are: sense, 5’-CTTGAGGAACT-TACTGTCCTTC 3’ (28-48nt), antisense, 5’-CCCTATCAGGAGTACCACAA 3’ (264-284nt). Probe sequence is: 5’-ACACCGAATTGGC-CAGGACGACCGGTTCTTCTTG 3’(142-177nt).

HCV NS5 protein detection by LSAB method
The sections were dewaxed and rehydrated routinely, and then treated for 5min with 0.03% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. Antigen of the tissues was prepared in microwave oven. Sections were incubated with normal goat serum for 5min, and then incubated with anti-HCV-NS5 IgG (diluted 1:50) at 4°C overnight. After washed with phosphate buffered saline (PBS), sections were incubated with biotin-labeled goat anti-mouse IgG (diluted 1:200) for 45 min at 37°C. Sections were then incubated with horseradish peroxidase streptavidin (S-A/HRP) for 45min at 37°C. After washed with PBS, peroxidase activity was developed using 3,3’-diaminobenzidine (DAB, 0.3g/L) for 15min. The DBA detection method yields a yellow precipitate. Sections were subsequently counterstained with hematoxylin.

Negative control groups
PBS substituting for anti-HCV-NS5 IgG, biotin-labeled goat anti-mouse IgG, S-A/HRP, and omitting DBA in procedure served as negative controls, respectively.

HCV RNA detection by is RT-PCR method
The sections were deparaffinized with fresh xylene and graded alcohols, followed by PBS for 5min. The tissues were digested with protease K (30mg/L, 37°C, Sigma) for 15min and rinsed with DEPC-treated PBS three times, and then treated with Rnase-free Dnase I(700U/mL, Promega) at room temperature overnight or 37°C 2h in a humidified chamber. The sections were then fixed twice in 95% and 100% alcohol each for 3min. RT was achieved with 30µL RT solution for each section (1×RT buffer, dNTP 250µM each, antisense of outer primer 1µm, Rnasin 1U/µL, AMV reverse transcriptase 0.4µ/mL Promega) in a humidified chamber at 42°C for 60min. The reaction solution was dripped away and was hed with DEPC-treated PBS twice each for 5min, then fixed twice in 95% and 100% alcohol each for 2min, and the 50µL PCR solution for each section was applied which consisted of MgCl2 2.5mM, 1×PCR buffer, each primer 1µM, dNTP 250µM, Taq DNA polymerase 4U/50µL and BSA 3g/L. 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 4min followed by 20 cycles of denaturation at 94°C for 2min, annealing at 55°C for 1.5min, and final extension of 72°C for 3min. The cover slip was removed. The sections were washed with PBS for 5min and fixed in 100% alcohol for 10min, then second PCR amplification was made. Except inner primers substituted for outer primers, the remaining steps were the same as the initial PCR amplification. The cover slip was removed. The sections were washed with PBS, and fixed in 100% alcohol for 10min. Sections in PBS were heated at 80°C for 10min, and put on ice. Then hybridization solution (probe 2.5mg/L, 50% deionized formamide, 5xSSC, 1xDenhardt’s solution, sssDNA 100mg/L) was 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 45min. DAB solution was added in slides at 37°C for 15min. The sections were incubated in the dark and checked at a 5min interval. The DBA detection method yields a yellow precipitate. After development, the sections were counterstained with hematoxylin. Positive cells and their histological distribution were examined.

**Negative control groups**
Hepatitis B liver tissues; HCV RNA positive specimens digested by Rnase (10g/L) at 37°C for 1h; HCV RNA positive specimens omitted AMV reverse transcription; HCV RNA positive specimens omitted Taq polymerase; and no probe control.

**RESULTS**
In 51 cases of carcinoma of extrahepatic bile duct, HCV NS5 protein was detected in 14 (27.5%), which was clearly stained in the cytoplasm of cancer cell but not in the nucleus or cell membrane. The positive signals of NS5 protein were distributed diffusely in the cytoplasm (Figure 1). The positive immunochemical reaction was not obtained in the same section when PBS substituted for anti-HCV-NS5 IgG, as did omission of the primary antibody from the staining procedure.

HCV RNA sequence was detected in 18 (35.4%) of 51 cases of carcinoma of extrahepatic bile duct. HCV RNA was located in the nucleus in 12 cases (Figure 2), and in the cytoplasm in 6 cases (Figure 3). In 34 cases of control group, HCV RNA sequences were detected in 2 (5.9%) (Figure 4). 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, no sections showed HCV RNA positive signal.

HCV NS5 protein and RNA coexistence were found in 2 cases. HCV NS5 protein and RNA positive cells were found to be either scattered or in clusters. In the cytoplasm, some positive signals of HCV NS5 protein and RNA were so strong that it might be difficult in determining nucleic positive.

**Figure 1** HCV NS5 protein located in the cytoplasm of cells of carcinoma of extrahepatic bile duct. LSAB×400
**Figure 2** HCV RNA located in the nucleus of cells of carcinoma of extrahepatic bile duct. IS-RT-PCR×400
**Figure 3** HCV RNA located in the cytoplasm of cells of carcinoma of extrahepatic bile duct. IS-RT-PCR×400
**Figure 4** HCV RNA located in the cytoplasm of cells of choledochal cyst. IS-RT-PCR×400
DISCUSSION

Although HCV is considered essentially to be hepatotropic, some studies indicate that it can also be found in the extrahepatic tissue, such as peripheral blood mononuclear cells[33-37], kidney tissue and salivary glands[38]. In the present study, HCV NS5 protein and RNA were found in cells of carcinoma of extrahepatic bile duct, which further demonstrates that HCV has wide host cells, and the main nucleic localization of HCV RNA in cells of carcinoma of extrahepatic bile duct was resembled the localization of HCV RNA in HCC[39]. The incidence of HCV infection in the tissues of carcinoma of extrahepatic bile duct is significantly higher than in control group ($\chi^2 = 9.808, P = 0.002$). This study indicates a correlation between HCV infection and carcinoma of extrahepatic bile duct, which is different from the traditional viewpoint-HCV infection is not correlated to bile duct cancer[2]. It is inferred that HCV infection, being similar to hepatolithiasis, choleodextral cyst, etc., may be one of the risk factors involved in the development of carcinoma of extrahepatic bile duct.

HCV is a plus-strand RNA virus[40]. HCV RNA and proteins can be detected in cells of HCC[41-46], and infection with the HCV is now known to be a major risk factor for the development of HCC[47-51]. But HCV genome does not integrate into the genome of infected cells[52,53]. For hepatitis B virus (HBV), integration of HBV DNA into host chromosomes raises the possibility of a direct carcinogenic effect of HBV through interaction with oncogenes, growth factors, or tumor suppressor genes[54-58]. The mechanism of carcinogenesis of HCV is not fully understood 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[59], inhibit cisplatin-mediated apoptosis in human cervical epithelial cells and apoptosis induced by the overexpression of c-myc in Chinese hamster ovarian cells[60], and repress transcriptional activity of P53 promoter[61]. Translocated expression of HCV core protein may also inhibit apoptosis in the tissue of HCC[62]. REF cells cotransfected with HCV core and H-ras genes became transformed and exhibited rapid proliferation, anchor-independent growth, and tumor formation in athymic nude mice[63]. 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[64]. HCV NS3 protein may exert its hepatocarcinogenic effect in early stage on host cells by endogenous pathway which may bring about mutation of p53 gene and transformation of hepatocytes[65]. NS5 protein from HCV-1b ORF includes NSSA and NSSB. Recently, NSSA protein is reported to be a potent transcriptional activator[66], and can repress the interferon-induced protein kinase through direct interaction with each other[67]. 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-infected cells. But how HCV infection is involved in the development of carcinoma of extrahepatic bile duct needs further research.

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