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Detection of Hepatitis B and G Virus Genomes in Sera from Patients with Acute Non-A, Non-B, Non-C Hepatitis

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ABSTRACT

In this study we used the polymerase chain reaction (PCR) method to detect hepatitis B virus (HBV) or hepatitis G virus (HGV) genomes in the sera of patients with non-A, non-B, non-C hepatitis. Stock sera of 10 patients with acute non-A, non-B, non-C hepatitis who had been admitted to our hospital were used to amplify viral genomes. Bands amplified with PCR for the precore (pre-C) region of HBV were detected in 8 of the 10 patients. By direct sequencing, PCR products of 7 patients showed the same sequence as the DNA fragment at the pre-C region and a point mutation that changed codon 28 (TGG) of the pre-C region to a stop codon (TAG) was recognized in 1 patient with fulminant hepatitis. However, the HGV genome was not amplified in any of the patients. These results suggest that HBV is a cause of non-A, non-B, non-C hepatitis. More cases must be studied to clarify the pathogenicity of "silent" HBV.

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Key words : polymerase chain reaction, hepatitis B virus, hepatitis G virus, non-A, non-B, non-C hepatitis, pre-C region

INTRODUCTION

Recently introduced diagnostic kits for hepatitis C have shown that 70% to 80% of cases of non-A, non-B hepatitis in Japan are due to hepatitis C virus (HCV). However, the cause of acute hepatitis in the remaining patients is not clear. In 1996 Linnen et al. used the polymerase chain reaction (PCR) method to detect hepatitis G virus (HGV) in the sera of patients with non-A, non-B, non-C hepatitis and suggested that HGV might be a new hepatotrophic virus[1]. However, Uchida et al. frequently found genome fragments of hepatitis B virus (HBV) in patients with acute or chronic hepatitis without serologic viral markers and called such cases "silent" HBV[2].

In this study, we used PCR to examine the sera of patients with acute non-A, non-B, non-C hepatitis for the presence of the HBV or HGV genome.

PATIENTS AND METHODS

1. *Patients*

The subjects were 10 patients with acute hepatitis who had been admitted to our hospital from 1990

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Table 1. Age, sex, clinical forms, laboratory data, and outcome of patients with acute non-A, non-B, non-C hepatitis

patient	Age	Sex	Clinical form	ALT	PT	Outcome
1	24	M	Acute	1,064	62	Alive
2	49	F	Acute	1,279	67	Alive
3	20	F	Fulminant	3,550	<10	Dead
4	47	F	Acute	1,334	90	Alive
5	64	F	Acute	981	60	Alive
6	38	M	Acute (prolonged)	907	98	Alive
7	27	F	Acute	1,732	66	Alive
8	41	F	Fulminant	1,762	19	Dead
9	52	M	Acute	1,568	84	Alive
10	45	F	Acute	425	100	Alive

ALT : alanine aminotransferase (mU/ml)
PT : prothrombin activity (%)

through 1995 (Table 1). Serological markers for hepatitis A virus (HAV), HBV, and HCV were negative. None of the patients had a history of drug-induced, alcoholic, or autoimmune hepatitis or chronic liver disease. Two patients with fulminant hepatitis died soon after admission despite intensive treatment. The stock sera obtained from the patients at admission were used to amplify viral genomes.

2. Assay for serological markers of hepatitis viruses and HCV-RNA detection

Immunoglobulin (Ig) M antibodies against HAV and hepatitis B core antigen (anti-HBc) were analyzed with radioimmunoassay (Dinabott, Tokyo). Hepatitis B surface antigen (HBsAg) and antibodies against HBsAg (anti-HBs) and HCV (anti-HCV) were analyzed with enzyme immunoassay (AxSYM autoanalyzer ; Dinabott). Reverse transcription (RT)-PCR to detect HCV-RNA was performed with a PCR diagnostic kit (Amplicore ; Roche, Chiba). Hepatitis due to hepatitis E virus (HEV) is rare in Japan and almost always occurs as an imported infection. Therefore, diagnostic assays for antibodies against HEV are rarely done in Japan. Because no patients in this study had traveled abroad recently, sera were not examined for antibodies against HEV.

3. Extraction, amplification, and sequencing of HBV DNA and HGV RNA

To extract HBV DNA, 20 μl of each sample was placed in 0.5-ml microtubes with 20 μl of 0.2 N NaOH. The DNA was precipitated with centrifugation at 1,000 to 3,000 revolutions per minute (rpm) for 10 to 30 seconds. After incubation for 1 hour at 37°C, 20 μl of 0.2 N HCl was added to each tube without pipetting and the samples were centrifuged at 10,000 rpm for 5 minutes. Supernatants were collected to amplify HBV DNA. To extract HGV RNA, 1 ml of RNA extraction reagent (Isogen ; Nippon Gene, Tokyo) was added to 100 μl of each sample. After mixing with Vortex and the addition of 0.2 ml of chloroform, the samples were centrifuged at 12,000 rpm for 15 minutes. Twenty microliters of glycogen and 600 μl of 2-propanol were added to the collected supernatants. After stabilization at −80°C for 15 minutes, the samples were centrifuged at 15,000 rpm for 15 minutes and the supernatants were removed. One milliliter of 75% ethanol was added to the precipitants, which were then centrifuged at 12,000 rpm for 15 minutes ; the supernatants were then removed. The samples were dissolved in 20 μl of distilled water and stored at −80°C until assay.

Nested PCR for HBV DNA at the precore (pre-C) region was performed with the method of Omata et al.[3], and RT-PCR for HGV was performed with the method of Schlueter et al.[4]. For the first step of

nested PCR of the pre-C region of HBV DNA, oligonucleotides 5′ GGGAGGAGATTAGGTTAA 3′ (position 174) and 5′ GGCAAAAAAGAGAGTAACTC 3′ (position 1959) were synthesized as forward and reverse primers. For the second step, 5′ TAGGAGGCTGTAGGCATAA 3′ (position 1774) and 5′ GTCCAAATTCTTTATA 3′ (position 1932) were synthesized as forward and reverse primers. RT-PCR of HGV was performed at the 5′ untranslated region (UTR) and at the NS5A region. For the 5′ UTR of HGV, oligonucleotides 5′ CGGCCAAAAGGTGGTGCATG 3′ (position 100) and 5′ CGACGAGCCTGACGTCGGG 3′ (position 285) were synthesized as forward and reverse primers. For NS5A, 5′ CTCTTTGTGGTAGTAGCCGAGAGAT 3′ (position 6904) and 5′ CGAATGAGTCAGAGGACGGGGTAT 3′ (position 7059) were synthesized as forward and reverse primers. The PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide fluorescence.

Amplified PCR products were analyzed with the dideoxynucleotide chain termination method using a Thermo Sequenase premixed cycle sequencing kit (Molecular Dynamics, Sunnyvale, CA, USA)[5]. Fragments of labeled DNA were separated with electrophoresis and sequenced with fluorescence DNA sequencer (SQ-5500 ; Hitachi, Tokyo).

For positive or negative control of amplification and sequencing of HBV genomes, sera of patients with acute hepatitis B or acute hepatitis A were used.

Results

Results of examinations for IgM anti-HAV, HBsAg, anti-HBs, IgM anti-HBc, anti-HCV, and HCV-RNA were negative in all patients. Therefore, all patients were confirmed to have non-A, non-B, non-C hepatitis.

Bands amplified with PCR for the pre-C region of HBV were detected in 8 of the 10 patients (Fig. 1). In 7 of these patients, bands appeared at the same position as in positive controls. However, a product of patient 1 differed in size from other products, and the same band appeared again when electrophoresis was repeated.

Direct sequencing was successfully performed in 8 patients. The PCR products of 7 patients properly showed the same sequence as the DNA fragment at the pre-C region of control HBV (Fig. 2 and Table 2). However, the PCR product of patient 1 which differed in size showed the same sequence as the DNA fragment of part of the core region of the HBV genome.

A point mutation that changed codon 28 (TGG) of the pre-C region to a stop codon (TAG) was recog-

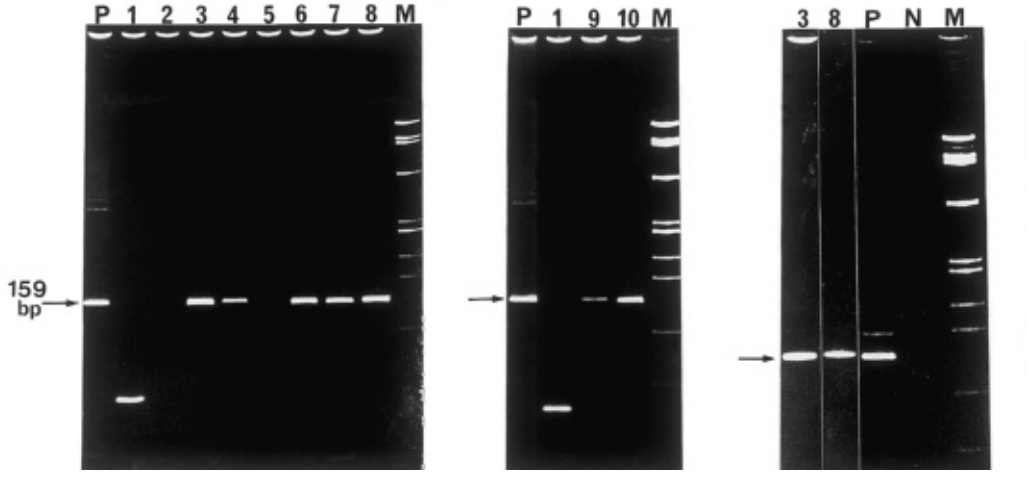

Fig. 1. Amplification of HBV-DNA at the pre-C region with PCR.
P : positive control. N : negative control. M : size marker. No. 1-10 : samples from patients with acute hepatitis. No. 3 and 8 : samples from patients with fulminant hepatitis.

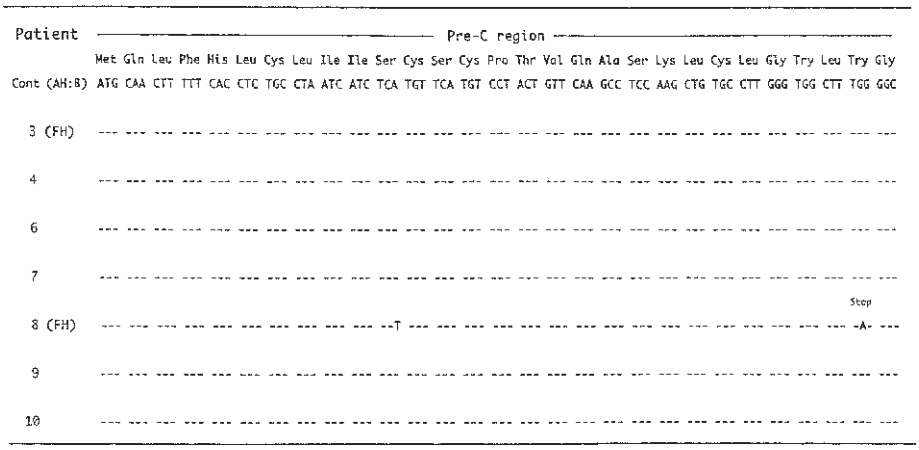

Fig. 2. Nucleotide and deduced amino acid sequences of HBV-DNA as determined with the direct sequencing method. AH : B : acute hepatitis B. FH : fulminant hepatitis.

Table 2. HBV DNA detection with PCR and mutation analyzed with direct sequencing, and HGV RNA detected with RT-PCR

Patient	HBV-DNA pre-C	mutation codon 28	HGV-RNA 5′UTR	HGV-RNA NS5A
1	+[a]	NA	−	−
2	−	NA	−	−
3	+	−	−	−
4	+	−	−	−
5	−	NA	−	−
6	+	−	−	−
7	+	−	−	−
8	+	+	−	−
9	+	−	−	−
10	+	−	−	−

[a]core-region fragment
NA=not available

nized in 1 patient with fulminant hepatitis.

The HGV genome was not amplified in any of the patients.

Discussion

Since its discovery in 1989, HCV has been shown to be the cause of most cases of non-A, non-B hepatitis. After diagnostic kits for HCV were introduced, the incidence of posttransfusion hepatitis decreased markedly in Japan. However, sporadic cases of acute hepatitis still occur, and we often encounter patients with acute or chronic hepatitis of unknown etiology. Therefore, the causes of these remaining cases of hepatitis must be clarified.

Wright et al.[6] have suggested that HBV plays a major etiologic role in fulminant non-A, non-B hepatitis because HBV DNA is often detected in liver tissue. In 1994 Uchida et al. detected fragments of the HBV genome with PCR in the sera of many patients with acute or chronic non-A, non-B, non-C hepatitis[2] and named this condition "silent" HBV owing to its heterotypic nature. Simonds et al.[7] in 1995 and Linnen et al.[1] in 1996 used PCR to detect a new hepatotrophic RNA virus in the sera of patient with non-A, non-B, non-C hepatitis. These viruses are usually described as the same virus (GBV-C/HGV) because of the near identity of their amino acids (94.7%).

"Silent" HBV or GBV-C/HGV is diagnosed only by the presence of viral genome without serologic markers. However, the presence of a certain viral genome in serum or liver tissue does not always cause hepatitis. Yoshiba et al detected the genome of GBV-C in the sera of 3 of 6 patients with fulminant hepatitis of unknown etiology and suggested that GBV-C was the responsible virus[8], a conclusion that was contradicted by several other workers[9,10]. Although GBV-C/HGV RNA was found in patients with hepatitis of unknown etiology, it was also found in almost the same percentage of healthy persons. Furthermore, the clinical features of patients with GBV-C/HGV were similar to those of patients without GBV-C/HGV.

In this study we used PCR to examine the sera of patients with acute non-A, non-B, non-C hepatitis for

the presence of the HBV genome. Fragments of the HBV genome were amplified with PCR in 8 of the 10 patients, whereas the HGV genome was not amplified in any of the patients. Direct sequencing showed that the fragments of the HBV genome were from the pre-C region in 7 patients and from the core region in 1 patient. Genomic contaminations were denied because of the presence of different sequences as point mutations. Therefore, we could confirm that fragments of the HBV genome were present in the sera of our patients with non-A, non-B, non-C hepatitis. Thus, we thought that HBV DNA fragments might be detected in many cases of non-A, non-B, non-C acute hepatitis. For this reason, clarifying the clinical significance of the presence of HBV DNA fragments in patients with non-A, non-B, non-C hepatitis remains important.

Why all serological markers for HBV were negative in our patients despite the presence of the HBV genome is unclear. We believe that the virus responsible for some cases of non-A, non-B, non-C hepatitis may be an HBV that has critical genomic mutations. Uchida et al. have speculated that such negative serologic results are due to mutations of the X gene of HBV[14]. To clarify the hepatotrophic activity of "silent" HBV, we must prove the presence of several genomic regions of HBV (such as S, C, and X), analyze the sequences of those fragments, and compare them with the sequences of HBV DNA in sera or in hepatocytes of patients with non-A, non-B, non-C hepatitis.

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