RAPID COMMUNICATION Jpn. J. Cancer Res. 81, 862–865, September 1990

Detection of Hepatitis C Virus RNA in Sera and Liver Tissues of Non-A, Non-B Hepatitis Patients Using the Polymerase Chain Reaction

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Sera obtained from patients with non-A, non-B hepatitis were examined for the presence of hepatitis C virus (HCV) genome by using the reverse transcription-polymerase chain reaction assay, as well as for antibody to HCV (anti-HCV) by using an enzyme-linked immunosorbent assay (ELISA). We also examined the presence of HCV RNA in liver tissue obtained by surgical resection of hepatocellular carcinoma. Among 33 patients, HCV RNA was detectable in 21 (64%), and the antibody was also positive in 21 (64%). Eighteen (55%) patients were positive for both assays. The two assays gave inconsistent results in 3 patients who were positive for HCV RNA but negative for anti-HCV, and in 3 other patients who were negative for HCV RNA and positive for anti-HCV. HCV RNA was also detected in 6 out of 10 non-cancerous liver tissue specimens and in 3 out of 7 tumor tissue specimens. Using the polymerase chain reaction, the HCV genome was detected directly in many specimens obtained from patients with non-A, non-B hepatitis, suggesting the presence of replicating virus in patients positive for anti-HCV. In addition, the differing results of the two assay systems suggest that the application of both is important for evaluation of the status of HCV infection.

Key words: Hepatitis C virus (HCV) — Reverse transcription-polymerase chain reaction — Anti-HCV

Non-A, non-B hepatitis is considered to cause a high proportion of chronic hepatitis and liver cirrhosis.[1)] Recently, the genome of a virus that is apparently responsible for a large part of clinical non-A, non-B hepatitis was molecularly cloned from infected chimpanzee plasma, and was named hepatitis C virus (HCV).[2)] HCV contains a positive-strand RNA molecule of at least 10,000 nucleotides and is related to flaviviridae.[2)] Circulating antibody (anti-C100-3) against an antigen expressed by a portion of the genome can be detected in about 80% of post-transfusion hepatitis patients,[3,4)] as well as in more than 80% of patients with hepatitis B viral surface antigen (HBsAg)-negative hepatocellular carcinoma (HCC) in Japan.[5)]

The polymerase chain reaction (PCR) is an exquisitely sensitive test with which it is possible to analyze rare gene sequences.[6)] In combination with reverse transcription (RT-PCR assay), it can be applied to the detection of the cellular transcripts and genomes of RNA viruses.[7)] We previously isolated the partial genome corresponding to the putative non-structural protein 5 (NS5) region of HCV using an RT-PCR assay based on the published sequence of HCV.[8)] This genome was found in sera and in liver tissues obtained from Japanese patients with chronic hepatitis and HCC. We also found that the HCV sequence in Japan shows about 80% homology with the original isolate detected in the USA.[9)]

Since small sequence mismatches between primer and template can cause the failure of the PCR,[10,11)] we designed primers for the PCR based on the sequence of the Japanese variant of HCV (HCV-J). Using these primers for the RT-PCR, we then investigated the incidence of the HCV genome in Japanese patients with non-A, non-B hepatitis. Co-existence of the HCV genome sequence and anti-HCV was observed in 55% of the patients, but in some cases only the viral genome or the antibody was detectable.

Thirty-six serum specimens obtained from 33 patients with non-A, non-B hepatitis and 17 liver tissue specimens obtained from 11 patients by surgical resection were used. All patients were negative for HBsAg. The 33 patients from whom sera were obtained had a clinical diagnosis of chronic hepatitis in 23 cases, acute hepatitis in 8 cases, liver cirrhosis in 1 case, and HCC in 1 case

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Table I. Clinical Data of the Patients and the Results of the RT-PCR and Anti-HCV Assays

No.	Age	Sex	Diagnosis	BT[e]	HCV RNA	Anti-HCV
1	29	M	CH[a]	(+)	(+)	(+)
					(+)[f]	(+)[f]
2	63	F	HCC[b]	(−)	(+)	(+)
					(+)[g]	(+)[g]
3	61	F	CH	(+)	(+)	(+)
					(+)[h]	(+)[h]
4	59	F	CH	(−)	(+)	(+)
5	65	F	CH	(+)	(+)	(+)
6	58	F	CH	(+)	(+)	(+)
7	72	F	AH[c]	(+)	(+)	(+)
8	39	M	CH	(+)	(+)	(+)
9	58	F	CH	(−)	(+)	(+)
10	43	M	CH	(+)	(+)	(+)
11	19	M	CH	(+)	(+)	(+)
12	40	F	AH	(+)	(+)	(+)
13	68	F	AH	(+)	(+)	(+)
14	57	M	AH	(+)	(+)	(+)
15	59	F	CH	(+)	(+)	(+)
16	70	M	CH	(−)	(+)	(+)
17	56	M	CH	(−)	(+)	(+)
18	69	M	CH	(+)	(+)	(+)
19	65	M	CH	(+)	(+)	(−)
20	55	M	CH	(−)	(+)	(−)
21	58	F	LC[d]	(+)	(+)	(−)
22	64	M	CH	(−)	(−)	(+)
23	55	F	CH	(+)	(−)	(+)
24	77	M	CH	(+)	(−)	(+)
25	22	F	CH	(−)	(−)	(−)
26	19	M	CH	(−)	(−)	(−)
27	52	M	CH	(+)	(−)	(−)
28	80	M	AH	(+)	(−)	(−)
29	50	F	AH	(+)	(−)	(−)
30	18	F	AH	(−)	(−)	(−)
31	60	F	AH	(+)	(−)	(−)
32	83	F	AH	(−)	(−)	(−)
33	49	F	CH	(−)	(−)	(−)

a) Chronic hepatitis.
b) Hepatocellular carcinoma.
c) Acute hepatitis.
d) Liver cirrhosis.
e) Blood transfusion.
f), *g*), *h*) Sera obtained at different periods of time were assayed.

(Table I). The age of the patients ranged from 18 to 83, and the male:female ratio was 14:19. In 3 patients (1, 2 and 3), a blood sample was obtained twice at different periods of time and analyzed independently. Twenty-one patients (64%) had a history of blood transfusion. Of the 17 tissue specimens which were available for the RT-PCR, 7 were tumor tissues and 10 were non-tumor liver tissues. Histological examination shows HCC in 10 cases, and metastatic liver tumor in one case in tumor tissues, and liver cirrhosis in 5, chronic hepatitis in 4, precirrhosis in one and normal liver in one in non-tumor liver tissues (Table II).

Table II. Detection of HCV RNA in Liver Tumor Specimens and Non-tumor Liver Tissues

No.	Age	Sex	Histology		HCV RNA	
			NT[a]	T[b]	NT	T
1	64	M	LC[c]	HCC[g]	(+)	NT
2	59	M	LC	HCC	(+)	NT
3	65	M	CH[d]	HCC	(+)	NT
4	63	M	PC[e]	HCC	NT[i]	(−)
5	63	M	LC	HCC	(−)	(−)
6	75	F	LC	HCC	(+)	(−)
7	74	M	NL[f]	MT[h]	(−)	NT
8	70	M	CH	HCC	(−)	(−)
9	60	M	CH	HCC	(+)	(+)
10	64	F	CH	HCC	(+)	(+)
11	53	M	LC	HCC	(−)	(+)

a) Non-tumor portion of liver tissue.
b) Tumor portion of liver tissue.
c) Liver cirrhosis.
d) Chronic hepatitis.
e) Precirrhosis.
f) Normal liver.
g) Hepatocellular carcinoma.
h) Metastatic tumor.
i) Not tested.

RNA from plasma was prepared by the method previously described.[12] Briefly, 1 ml of plasma was diluted 1:1 with phosphate-buffered saline (PBS) and centrifuged at 50,000 rpm for 5 h. The pellet was dissolved in a solution containing 4 *M* guanidium isothiocyanate, 25 m*M* sodium citrate, 0.5% (w/v) sodium lauroylsarcosine, and 0.1% (v/v) β-mercaptoethanol. After phenol-chloroform extraction, RNA was precipitated by adding isopropanol. The RNA pellet was dissolved in water, re-extracted using phenol-chloroform followed by ethanol precipitation, and stored at −70°C until use. RNA from liver tissue was prepared by the lithium chloride-urea method.[13]

The RT-PCR assay was carried out as previously described.[7] RNA was annealed with 10 ng of antisense primer (HCV 36R) in a total volume of 10 μl. Reverse transcription was then performed with 200 units of Moloney murine leukemia virus reverse transcriptase at 37°C for an hour. The PCR was carried out in a DNA thermal cycler (Perkin-Elmer, Cetus) for 35 cycles, as described previously.[6] After the reaction, the PCR products were analyzed by Southern blot hybridization

probed with ^{32}P-labeled 234 bp fragment (nucleotides 6,822 to 7,055) of the molecularly cloned DNA of HCV-J.[9] The filter was washed with 1×SSC/0.5% (w/v) $NaDodSO_4$ twice at 55°C for 30 min (1×SSC is 0.15 *M* NaCl/15 m*M* sodium citrate, pH 7.0), and exposed to Kodak X-AR film for 24 h at −80°C.

Oligonucleotide primers used for the RT-PCR were synthesized with a DNA synthesizer (Applied Biosystems). The primers used were HCV 42 (5′-AAGTGGCGTGCTGACGACTA-3′), and HCV 36R (5′-CACGGGTGAGGGAGTAGACCCT-3′), which have sequences derived from HCV-J. These primers respectively correspond to nucleotides 6,755 to 6,774 and are complementary to nucleotides 7,050 to 7,071 of the published sequence.[8, 9]

Anti-HCV was detected by using an enzyme-linked immunosorbent assay (ELISA) system with the recombinant HCV C100-3 antigen[3] in the solid phase (Ortho Diagnostic Systems, Tokyo). The ELISA was performed according to the manufacturer's instructions and antibody levels were measured as the absorbance at 490 nm determined with a MicroELISA Auto Reader (Dynatec Instruments, USA). HBsAg was measured by the passive hemagglutination test.

The results of the RT-PCR assay followed by hybridization with the 234 bp fragment probe are summarized in Table I, and some representative data are shown in Fig. 1. Amplified bands were visible in agarose gel stained with ethidium bromide in some cases (data not shown). All signals migrated to the expected positions (317 bp). The HCV genome and/or anti-HCV was detected in 24 (73%) of the patients. Eighteen (55%) were positive for both assays, and 9 (27%) were negative for both. Six (18%) patients showed positive results in only one of the two assays, with three showing positive results for each. Sera obtained at different times from 3 patients (cases 1, 2, and 3, Table I) were positive at both times in all 3 patients. Plasma from 10 healthy individuals were shown to be negative for both anti-HCV and HCV-RNA (data not shown). Analysis of HCV RNA by RT-PCR was carefully conducted so that laboratory contamination was excluded. In fact, samples which were negative for HCV RNA gave no positive signal in RT-PCR during the course of this experiment.

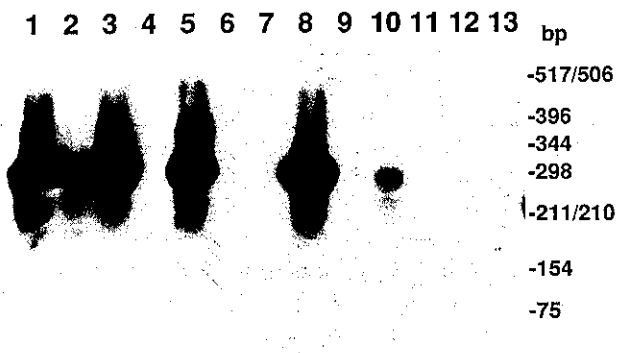

Fig. 1. Detection of HCV RNA in sera from patients with non-A, non-B hepatitis by the RT-PCR assay. RNA extraction and the PCR were carried out as described in the text. A 10-μl aliquot from 50-μl reaction volume was subjected to agarose gel electrophoresis and analyzed by Southern blot hybridization. Data of 13 representative cases are shown. The positions of the DNA fragments obtained by *Hinf* I digestion of pBR322 are shown on the right.

The results of RT-PCR assays of liver tissue obtained at surgical resection are shown in Table II. HCV RNA was detectable in 3 of the 7 (43%) tumor specimens and in 6 of the 10 (60%) non-tumor liver tissue specimens. Among 6 patients from whom both types of tissue could be obtained, 2 were positive in both tissues and 2 each were positive in only one type of tissue.

HCV is considered to be a probable causative agent of non-A, non-B hepatitis, although it has not been proven that organisms which contain this genetic material are actually infectious. Retrospective investigation has suggested that blood samples positive for anti-HCV can cause post-transfusion hepatitis,[4] and the decrease of post-transfusion hepatitis produced by blood screened for anti-HCV[14] suggests that the presence of this antibody is closely associated with the presence of infectious HCV. Frequent detection of both anti-HCV and HCV RNA in the same patients with hepatic diseases indicates a strong association of virus infection with serum antibody (anti-C100-3) to HCV. A similar result was observed by Kaneko *et al.*[15]

Use of other methods in addition, such as screening for viral antigen or the viral genome, may be useful to minimize false-positive or false-negative results, as suggested in this study.

Using the PCR method to detect the viral genome in plasma gave results inconsistent with those of ELISA for anti-HCV antibody. A significant percentage (9%) of patients with hepatitis or liver cirrhosis were negative for the anti-HCV ELISA but positive for the RT-PCR assay. This inconsistency has also been noted by another group.[16] The failure to detect anti-HCV in sera positive for the HCV genome may be due to a low immune response to the C100-3 antigen in such individuals or may be due to differences of amino acid sequence of the epitope between the C100-3 antigen and some proportion of HCV-J.[9] In 3 patients we failed to detect HCV RNA, despite the presence of anti-HCV. A possible explanation for this would be failure of the PCR due to a sequence mismatch between the primer and viral template because of variation of the HCV sequence,[9] or else the presence of too little viral RNA in the specimens.

HCV RNA was detectable in both liver tumor specimens and non-tumor liver tissue. Although we do not have any direct evidence showing that the viral RNA was derived from liver cells, and not from plasma which may have contaminated the liver specimens, this result suggests that HCV can persist for long periods in liver cells and may be involved in the process of their malignant transformation.

(Received June 8, 1990/Accepted June 25, 1990)

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