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Hepatocellular Carcinomas Infected with the Novel TT DNA Virus Lack Viral Integration

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A novel DNA virus designated TT virus (TTV) was cloned from a patient with posttransfusion hepatitis and is thought to be a new hepatitis virus. At present, hepatitis B virus (HBV) and hepatitis C virus (HCV) are known to induce hepatocellular carcinoma (HCC). But, actually, in Japan approximately 5 to 10% of HCCs are in HBV-negative and HCV-negative (NBNC) patients. In order to study the possible role of TTV in hepatocarcinogenesis, we investigated the frequency of the TTV genome in liver tissue of 20 HCC patients. As a result, 3 of 8 NBNC HCC patients and 5 of 12 HBV- or HCV-associated HCC patients were TTV positive, and TTV was shown not to be specific for NBNC HCC. For all TTV-positive patients, we also confirmed that the TTV genome was not integrated into host hepatocyte DNA.

Key Words: **TT virus; hepatocellular carcinoma; hepatocarcinogenesis; integration.**

In 1997, Nishizawa et al. cloned a novel DNA virus designated as TT virus (TTV) from a patient with posttransfusion hepatitis (1). TTV is an unenveloped, single-stranded DNA virus consisting of 3739 bases with two possible open reading frames (2). The quantity of virus genome closely correlates with the patient's aminotransferase level (1). As for chronic liver diseases with unknown etiology, Okamoto et al. reported positivity for TTV in 47% of chronic hepatitis, 48% of liver cirrhosis and 39% of hepatocellular carcinoma (HCC), contrasting to 12% positivity in apparently normal blood donors. Their results suggested that TTV may be associated with chronic liver diseases, but it is not known whether TTV is associated with the development of HCC. In Japan, HCC is responsible for about 30,000 deaths a year (3). Most HCCs are induced by hepatitis C virus (HCV) or hepatitis B virus (HBV) infection, and approximately 5 to 10% of HCC in Japan occurs in HBV-negative and HCV-negative (NBNC) patients. Therefore, it is important to find out the cause of these NBNC HCCs. TTV and HBV share the same features; both are DNA viruses causing persistent infection in hepatocytes. The genome of HBV is frequently integrated into the host genome of HCC tissues (4, 5, 6, 7), but at present we do not know whether the genome of TTV is integrated. We studied here the prevalence of TTV DNA in 20 HCC cases. By PCR, TTV was detected in 37.5% (3/8), 50% (3/6), and 33% (2/6) of NBNC-, HBV(+)-, and HCV(+)-HCC patients, respectively, and it was suggested that TTV was not specific for NBNC HCC. TTV integration into host DNA was not detected in any HCC patient by Southern blotting.

MATERIALS AND METHODS

Patients and samples. HCC and nontumorous liver tissues were obtained surgically or by autopsy from 20 Japanese HCC patients between February 1995 and August 1997. In cases 1 to 8, serum markers for both HBsAg (enzyme immunoassay) and anti-HCV (second-generation enzyme-linked immunosorbent assay) were negative. In cases 9 to 14, HBsAg was positive and anti-HCV was negative. In cases 15 to 20, HBsAg was negative and anti-HCV was positive (Table 1). Serum samples from cases 1 to 7 collected within one month prior to surgery or autopsy were available for our PCR study, but those in other cases were not. Patient details and histological findings of nontumorous liver tissue are shown in Table 1. In all HBsAg negative patients, we applied PCR of HBV DNA to exclude more strictly the possibility of HBV infection, and results were all

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Abbreviations used: TTV, TT virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; NBNC, hepatitis B virus negative and hepatitis C virus negative; CH, chronic hepatitis; LC, liver cirrhosis; nt, nucleotide.

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TABLE 1

Clinical Characteristics of Patients

Case	Age	Sex	HBsAg	Anti-HCV	Histological finding of nontumorous liver tissue
1	68	M	−	−	LC
2	59	M	−	−	LC
3	56	M	−	−	CH
4	58	M	−	−	LC
5	56	M	−	−	LC
6	68	M	−	−	CH
7	63	M	−	−	CH
8	61	M	−	−	LC
9	51	M	+	−	LC
10	52	F	+	−	LC
11	64	M	+	−	LC
12	51	M	+	−	LC
13	61	M	+	−	LC
14	70	M	+	−	LC
15	51	M	−	+	LC
16	57	F	−	+	LC
17	62	F	−	+	LC
18	65	M	−	+	LC
19	49	M	−	+	LC
20	61	M	−	+	LC

Note. M, male; F, female; LC, liver cirrhosis; CH, chronic hepatitis.

negative (data not shown). The tissues and serum samples were stored at −80°C until use.

DNA extraction from liver tissue and serum samples. From frozen tissue, total cellular DNA was extracted using the standard proteinase K/SDS method. Serum DNA was extracted from 200 μl serum samples using a blood DNA extraction kit (QIAamp Blood Kit, QIAGEN Inc., Chatsworth, CA, USA).

Detection of TTV DNA. We applied the two-step PCR assay described by Okamoto et al. (2) to detect TTV DNA in liver tissue or in serum. As templates, we used one microgram of tissue-derived genomic DNA or one-twentieth of the DNA extracted from 200 μl serum. PCR reaction was carried out in a volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mixture, and 1 μM of NG059 (5′-ACA GAC AGA GGA GAA GGC AAC ATG-3′) as a sense primer, 1 μM of NG063 (5′-CTG GCA TTT TAC CAT TTC CAA AGT-3′) as an antisense primer, and 1.25 units of Ampli Taq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA) to amplify a 286-bp product. The second round PCR was done under the same conditions except that the template was 10 μl of the first PCR product, and that the sense primer was 1 μM of NG061 (5′-GGC AAC ATG YTR TGG ATA GAC TGG-3′). The size of the second round PCR product was 271 bp and the region of TTV amplified by this method (region M) is shown in Fig. 1. The other two regions of the TTV genome (regions U and L) were also examined by the same procedure using specific primers. Primers and the size of each PCR product are shown in Table 2. Each PCR was performed in a programmable thermal cycler. The program of PCR consisted of 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 45 seconds, followed by the final extension at 72°C for 7 minutes. Five microliters of PCR product was electrophoresed on 2% agarose gels and stained by ethidium bromide. The experiment was repeated at least three times, and reproducible results were obtained.

Nucleotide sequencing of the PCR product and determination of the genotype. To confirm that the PCR product consisted of a portion of TTV DNA, we purified it with the High Pure PCR Product Purification Kit (Boehringer Mannheim, Mannheim, Germany) and determined the nucleotide sequence by the direct sequencing method using oligomers 466U and 731R for region U, NG061 and NG063 for region M, and 2711U and 2992R for region L as primers. We examined the sequence homology between PCR products and TTV DNA genotype G1a (2).

Southern blotting. After digestion with *Bam*HI or *Eco*RI, 10 μg of tumor DNA was electrophoresed on a 1% agarose gel and transferred onto a nylon membrane (PALL, Biodyne B) in 0.4 N NaOH, then hybridized with a ^{32}P-labeled 1.2 kb fragment of TTV DNA containing clone N22 (1), in 0.2 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% bovine serum albumin, and 7% SDS at 65°C overnight. After being washed in 1x SSC and 0.1% SDS at 65°C for 30 minutes, the nylon membrane was exposed to Kodak XAR-5 film. As for the cases in which PCR successfully amplified the TTV DNA, Southern blot was repeated with 1% and 2% agarose gels using the PCR product specific for each patient as a probe.

RESULTS

In three of eight NBNC patients (cases 1, 2 and 6), PCR products were detected in tumor tissue (Fig. 2A). PCR products were also detected in the nontumorous tissues of these patients (data not shown). We also examined the existence of TTV DNA in serum samples in cases 1 to 7 by PCR, and amplified PCR products were detected in cases 1 and 6 (Fig. 2B). In case 2, TTV DNA was not detected in the serum sample, although it was detected in liver tissue. In five of twelve HBV or HCV-associated HCC patients, TTV DNA was detected in tumor tissues by PCR of region M. Among these, PCR products of regions U and L were not detected in cases 10 and 12. In case 18, a PCR product in region L was not detected (Fig. 2A). Concerning DNAs derived from nontumorous liver tissue, PCR products were not detected in cases 10 and 18 in which amplification in tumor tissue was successful. In other patients, the same results were obtained in tumor and nontumorous tissue (data not shown).

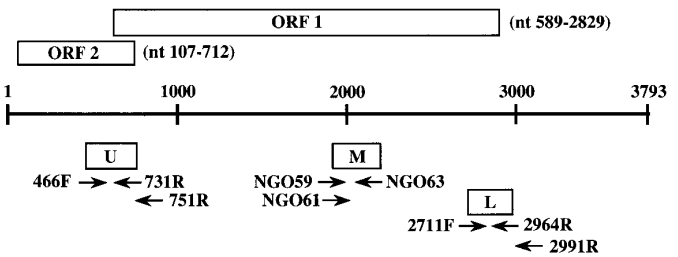

FIG. 1. TTV genomic structure and the location of primers. the two-step PCR assay was employed. In the first round of PCR, DNA of regions U, M, and L were amplified with primer sets 466F and 751R, NGO059 and NGO063, or 2711F and 2991R, respectively. Then primer sets 466F and 741R, NGO061 and NGO063, or 2711F and 2964R were used at the second round PCR for regions U, M, and L, respectively. The nucleotide sequence of primers and the size of PCR products are shown in Table 2. The horizontal solid line indicates TTV genomic DNA. Open reading frames (ORF 1 and ORF 2), region U (U), region M (M), and region L (L) are denoted in boxes. Numbering of the TTV genome is in accordance with Okamoto's report (2). nt, nucleotide.

TABLE 2

Nucleotide Sequence of PCR Primers

Region[a]	Primer	Nucleotide sequence	Size of PCR products (bp) First PCR	Second PCR
U	466F	5′-TCA TCT TGC TTC TGT GCT TCG TGC-3′	309	289
	731R	5′-TAT ATC TCC TCC TCC ACC TCC CTC-3′		
	751R	5′-CCT GCC CTT TCT TTT CCA TCT CCT-3′		
L	2711F	5′-AAA GCT CAG AGG AAG AGA CGC AGA-3′	304	276
	2964R	5′-GGG TCT GTC CCA TAT TCT ACA GGC-3′		
	2991R	5′-AGG GGT ATC TCT TAG GTT GCC TCT-3′		

[a] Regions of TTV genome and the location of primers are shown in Fig. 1.

We determined the nucleotide sequences of all amplified products and confirmed that they were really part of the TTV. The identity between nucleotide sequences of PCR products and TTV genotype G1a are shown in Table 3. In regions U and L, sequence homology showed high identity, more than 98%, between PCR products and genotype G1a. On the other hand, identities of region M were not always at a high percentage.

To confirm whether TTV integrates into the hepatocyte genome as does HBV, we employed Southern blotting. In addition to using a 1.2 kb fragment containing clone N22 for such confirmation, we used the subgenomic fragment of TTV which is specific for each patient to improve hybridization efficiency in Southern blotting. Representative data of cases 1, 12 and 16 are shown in Fig. 3. In all 20 cases, a band showing TTV DNA integration was not detected.

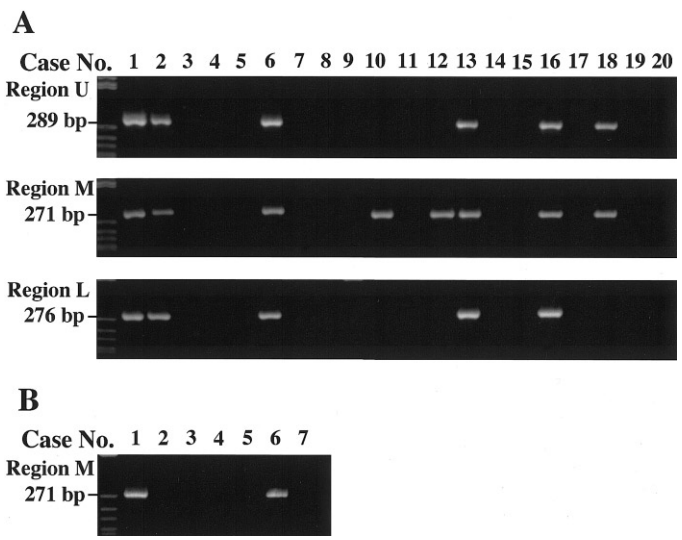

FIG. 2. Results of PCR for regions U, M, and L of the TTV genome. (A) DNAs derived from HCC tissue in cases 1 to 20 were amplified with region-specific primer sets. Results of PCR amplifying a 289-bp fragment of region U, a 271-bp fragment of region M, and a 276-bp fragment of region L are shown in the top, middle, and bottom panels, respectively. (B) DNAs extracted from serum samples in cases 1 to 7 were amplified with primers of region M.

DISCUSSION

In this study, the genome of TTV was detected by PCR at almost similar frequencies among NBNC-, HBV- or HCV-associated HCC and surrounding nontumorous tissues. It is less likely that TTV is a specific causative virus of NBNC liver disease, although we cannot deny the possibility that TTV can augment the intensity of hepatitis or accelerate the process of hepatocarcinogenesis. Simmonds et al. and Naoumov et al. recently reported the prevalence of TTV in patients with chronic liver diseases and in normal controls, and their results also did not support the role of TTV as the causative agent for NBNC hepatitis (8, 9).

The TTV genomes showed some heterogeneity among patients as shown in Table 3. In cases 1, 2, 6, 13, and 16, in whom all three regions (U, M, and L) were amplified, the sequences of U or L were more highly conserved than that of M. The results suggested that the genome around regions U or L may be more

TABLE 3

Nucleotide Sequence Homology (%) between PCR Products and TTV Genotype G1a (2)

Case	Regions[a] U	M	L
1	98.8	63.1	98.3
2	99.2	64.0	98.7
6	99.2	98.2	99.6
10	N[b]	59.5	N
12	N	86.2	N
13	99.2	93.2	99.6
16	98.8	98.2	99.1
18	98.8	98.2	N

[a] Regions of TTV genome are shown in Fig. 1.
[b] A region not amplified by PCR is indicated N.

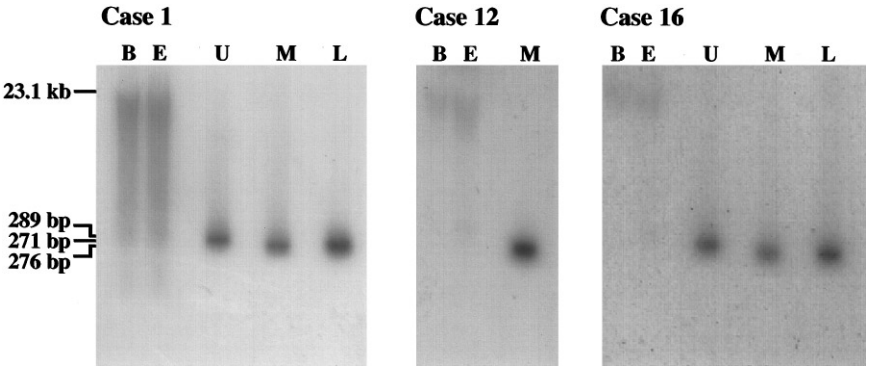

FIG. 3. Southern blot analysis of HCC DNA in cases 1, 12, and 16 probed with PCR products specific for each case. As a positive control, 20–30 pg of purified PCR products of regions U, M, and L was applied on lanes U, M, and L, respectively. On lanes B and E, 10 μg of *Bam*HI or *Eco*RI digested HCC DNA was applied, respectively. Mixures of regions U-, M-, and L- specific PCR products were used as a probe. The figures shown here are the results of Southern blotting using 2% agarose gels, and we also obtained the negative results using 1% agarose gels.

indispensable for virus life cycles than region M, although at present nothing is known about the functions of these regions we selected. As for the negative results of PCR, we cannot deny the possibility that the TTV genome was not amplified because of the misannealing of primers caused by sequence heterogeneity. We used three sets of primers to increase the detection rate in each patient.

In all cases with a positive TTV genome, the nucleotide sequence of the genome in serum was almost identical to that in liver tissue. We determined the sequence of the TTV genome by direct sequencing using autosequencers, and there were several nucleotides, on average one or two bases out of 200 bases, which were not read in each genome, probably reflecting the heterogeneity of the genome in each patient. At present, we do not know the details of the heterogeneity of the TTV genome in a single patient. For the comparison of the sequences shown in Table 3, equivocal nucleotides were included in non-homologous parts.

The genome of TTV is a single-stranded DNA. Although the replication cycle is not known, it is speculated that the TTV genome makes a double-stranded DNA as the replication intermediate in the nucleus of hepatocytes. Generally, exogenous DNA, if once entered into the nucleus, is apt to be integrated into the host genome, although the frequency is low. In an in vitro system, transfected DNA can be non-specifically integrated into chromosomal DNA. It is also known that the genome of DNA viruses such as papova viruses, EB virus or hepadnaviruses, which can cause persistent infection, integrate into the chromosome, and that this integration sometimes cause genomic rearrangement (10). TTV is also a DNA virus causing persistent infection in hepatocytes, and it is possible that its genomic DNA is integrated into the host genome with persistence in hepatocytes, and is involved in the process of hepatocarcinogenesis. Therefore, we studied the integration frequency of the TTV genome in 20 HCC cases, but integration was not detected in any case. We used as a probe of Southern blotting not only the prototype genomic DNA (clone N22) supplied by Dr. M Mayumi but also the viral genomic DNA specific for each patient in order to increase the efficiency of detection of integrated TTV DNA.

In summary, the TTV genome was detected not only in NBNC, but also in HBV- or HCV-associated HCC patients in similar frequencies by PCR of the liver or serum DNA, and TTV seemed to be not specific to NBNC liver diseases. The integration of TTV DNA was not detected by Southern blotting in any of the 20 HCC cases, suggesting that even if TTV is involved in hepatocarcinogenesis, the mechanism should be different from that related to HBV DNA, which is integrated into the host genome of patients with HCC at a very high frequency.

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