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Multiple Infection of *TT Virus* (*TTV*) with Different Genotypes in Japanese Hemophiliacs

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To clarify the persistent *TT virus* (*TTV*) infection, we studied a possibility of multiple TTV infection by genotype analysis of isolated TTV obtained from seven Japanese hemophiliacs. The nucleotide sequences including 222 bp in the open reading frame 1 (ORF1) region of 10 TTV isolates from each patient were analyzed and classified into various TTV genotypes such as G1 to G6 by phylogenetic analysis using a N-J method. Multiple TTV genotypes were observed in all the hemophiliacs: three different TTV genotypes were found in three patients, whereas four different TTV genotypes were observed in the other three patients. The remaining patient was also infected with TTV of five different genotypes. Moreover, new TTV genotypes were found in these seven patients and tentatively designated as G7. The present findings indicate that multiple TTV infection with different TTV genotypes has occurred in Japanese hemophiliacs. They also provide valuable information to understand persistent TTV infection.

A newly discovered DNA virus, *TT virus* (*TTV*) (1), was isolated in 1997 from a patient with cryptogenic posttransfusion hepatitis without A to G virus infection by means of representational difference analysis (2). TTV is supposed to belong to the *Parvoviridae* family (3). Initially, 4 TTV (sub) genotypes such as G1a, G1b, G2a, and G2b (3) have been reported. In more recently, one subgenotype of G2c (4) and 4 genotypes of G3 to G6 (5) have been further identified. The high prevalence rates of TTV DNA in parental risk-like hemophiliacs (67–75%), intravenous drug users (40%), and hemodialysis patients (45.6%) indicate that TTV is a blood-transmissible virus (3, 6). TTV infection in general population has been reported to be 12, 1.9–10, and 1% in Japan (3), UK (7, 8), and USA (9), respectively. Since Japanese hemophiliacs had been treated with non-virus-inactivated plasma products produced from several countries before 1985 and/or 1986, they had been exposed to blood-transmissible infectious viruses, such as hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis G virus (HGV) and human immunodeficiency virus (HIV) (10). With respect to TTV, on the other hand, we have already reported that many hemophiliacs was persistently infected with TTV (4). As found for the cases with multiple infection of HCV (11), HIV (12), etc., these findings suggest that Japanese hemophiliacs may also be multiple-infected with plural TTV genotypes. However, there have been no information on TTV infection. To understand TTV infection, in this study, we studied whether or not multiple TTV infection with different genotypes is caused by analysis of TTV genotype in sera from 7 TTV-positive Japanese hemophiliacs.

MATERIALS AND METHODS

Serum samples. Seven TTV DNA-positive serum samples from Japanese severe hemophiliacs (A, 1; B, 6) in 1995 were used in this study. All of these hemophiliacs had a history of treatment with non-virus-inactivated clotting factor concentrates prepared in the USA (mainly), Austria, Germany, and Japan before 1985. The serum samples were freshly obtained and were stored at −80°C until use. TTV infection was repeatedly confirmed by a seminested PCR assay in our previous study (6).

Serological backgrounds of the seven hemophiliacs were briefly described as below. Patient 1 (21 years old, hemophilia B) was hepatitis B virus surface antibody (HBsAb) (+), HCV RNA(−), second generation HCV antibody (HCV II-Ab) (+), HGV RNA(−), hepatitis G virus envelope 2 antibody (HGV E2 Ab) (−), human immunodeficiency virus antibody (HIV Ab) (+), and 25 of alanine-aminotransferase (ALT) level (IU/L). Patient 2 (28 years old,

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Abbreviations used: TTV, TT virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HGV, hepatitis G virus; HIV, human immunodeficiency virus; ALT, alanine-aminotransferase.

hemophilia A; HA) showed HBsAb(+), HCV RNA(+), HGV RNA(+) and 57 of ALT level. Patient 3 (20 years old, HA) had hepatitis B virus surface antigen (HBsAg) (+), HCV RNA(−), HCV II-Ab(−), HGV RNA(−), HGV E2 Ab(+) and 15 of ALT level. Patient 4 (29 years old, HA) had HBsAb(+), HCV RNA(−), HCV II-Ab(+), HGV RNA(−), HGV E2 Ab(−) and 9 of ALT level. Patient 5 (32 years old, HA) was HBsAg (−), HBsAb(−), HCV RNA(−), HCV II-Ab(+), HGV RNA(−), HGV E2 Ab(+) and 50 of ALT level. Patient 6 (14 years old, HA) was HBsAg(−), HBsAb(−), HCV RNA(+), HGV RNA(−), HGV E2 Ab(+) and 45 of ALT level. Patient 7 (29 years old, HA) showed HBsAg(−), HBsAb(−), HCV RNA(+), HGV RNA(−), HGV E2 Ab(+) and 24 of ALT level. None of these 7 patients had received vaccination against HBV.

Nucleotide sequencing of TTV isolates. By the seminested PCR assay (6), the PCR products (271 bp) of the open reading frame (ORF) 1 region (nt. 1915-2185) of the TTV genome from 7 TTV DNA-positive patients were obtained and cloned into a pGEM-T Vector System (Promega, Madison, WI). The nucleotide sequences including 222 bp (nt 1939–2160) in 10 TTV isolates from each patient were determined by a Dye termination method (Model 310, PE Applied Systems, Foster City, CA) and classified into G1a to G6 TTV genotype (3–5) by a phylogenetic analysis using a neighbor-joining (N-J) method. The nucleotide positions were numbered according to the sequence of TA278 (DDBJ, EMBL, and GenBank Accession No. AB008394) (3).

Statistics. A phylogenetic tree was constructed using GenetyxMac (ver. 8.0) software (Genetix Computer Group, MA).

RESULTS

By analysis of 10 TTV isolates from each of 7 TTV DNA-positive hemophiliacs, the nucleotide sequences of 222 bp (nt 1939–2160) of the ORF 1 region of the TTV were determined. Genotyping of their nucleotide sequences by phylogenetic analysis using a N-J method is shown in Fig. 1. The intratypical homology of the nucleotide sequences of TTV isolates from P4-1 (TT001; Accession No. AB021806), P5-1 (MS501; AB021809), and P7-1 (MM509; AB021817) belonged to genotype G1a was found to be 97.3–100%; mean 98.5% (91.9–100%; mean 96.7% at the amino acid level), whereas those of P2-1 (IS007; AB021798) belonged to genotype G1b was 94.1–98.2%; mean 95.8% (90.5–94.6; mean 92.8% at the amino acid level). The intratypical homology of P2-2 (IS505; AB021799) belonged to genotype G2a was 87.6–98.6%; mean 91.3% (89.2–100%; mean 92.8% at the amino acid level), whereas those of P1-1 (KY501; AB021795), P3-1 (GN006; AB021801), P4-2 (TT004; AB021807), P5-2 (MS505; AB021810), P6-1 (MH501; AB021813), and P7-2 (MM503; AB021818) belonged to genotype G2b was 92.2–99.1%; mean 95.5% (91.9–100%; mean 96.7% at the amino acid level). P1-2 (KY507; AB021796), P3-2 (GN008; AB021802), P5-3 (MS510; AB021811), P6-2 (MH502; AB021814) and P7-3 (MM505; AB021819) belonged to genotype G2c showed homology at 86.9–99.5%; mean 91.9% (89.2–100%; mean 94.7% at the amino acid level). P2-3 (IS004; AB021800), P3-3 (GN001; AB021803), P4-3 (TT006; AB021808) and P6-3 (MH504; AB021815) belonged to genotype G3 showed homology at 94.7–99.1%; mean 97.2% (91.3–100.0%; mean 96.3% at the amino acid level). P6-4 (MH508; AB021816) and P7-4 (MM501; AB021820) belonged to genotype G4 showed homology at 97.1–98.6%; mean 97.2% (91.3–100%; mean 96.3% at the amino acid level), whereas P5-4 (MS506; AB021812) belonged to genotype G6 showed homology at 86.0–88.2%; mean 87.5% (93.2%; mean 93.2% at the amino acid level).

Some nucleotide sequences which did not belong to any of previously reported TTV genotypes were also found in TTV isolates from 7 patients. Although the TTV clones of P1-3 (KY502; AB021797), P3-4 (GN002; AB021804), and P3-5 (GN003; AB021805) were distributed nearby the representative genotype G5 according to the phylogenetic tree analysis, the intertypical homology between these three TTV isolates and the G5 genotypes (JaM21 and JaNBNC10[5]) was found to be 53.7–58.5%; mean 56.7% (35.2–38.0%; mean 36.1% at the amino acid level). Since the nucleotide sequence homology of these three TTV isolates was found to be 78.3–80.9%; mean 79.8% (76.5–82.4%; mean 79.4% at the amino acid level), two unclassified TTV isolates such as P1-3 (KY502) and P3-4 (GN002) were tentatively designated as G7a, whereas P3-5 (GN003) was named as G7b.

Various TTV genotypes were observed in sera from 7 TTV-positive hemophiliacs who had been treated with non-virus-inactivated plasma concentrates. The multiple TTV infection with different genotypes was observed in all of them. The TTV genotypes in 10 TTV isolates from Patient 1 were 3 types with G2b (4 isolates), G2c (3 isolates) and tentatively designated G7a (3 isolates) although he was co-infected with HIV, whereas those of Patient 2 were 3 genotypes with G1b (one isolate), G2a (6 isolates), and G3 (3 isolates). Patient 3 had 5 genotypes with G2b (one isolate), G2c (one isolate), G3 (2 isolates), tentatively designated G7a (2 isolates), and G7b genotype (4 isolates). Patient 4 also showed 3 genotypes with G1a (4 isolates), G2b (2 isolates), and G3 (4 isolates). The genotypes of Patient 5 were 4 types with G1a (2 isolates), G2b (one isolate), G2c (one isolate), and G6 (6 isolates), whereas those of Patient 6 were 4 types with G2b (4 isolates), G2c (4 isolates), G3 (one isolate), and G4 (one isolate). Patient 7 showed 4 genotypes with G1a (2 isolates), G2b (3 isolates), G2c (one isolate), G4 (4 isolates), respectively.

DISCUSSION

We have previously reported that TTV infection frequently occurred in Japanese hemophiliacs who had been treated with non-virus-inactivated plasma concentrates (6). TTV DNA was repeatedly detected in sera from Japanese hemophiliacs, suggesting that TTV may cause persistent infection like HCV. It has been found that multiple infection of HCV in patients with hemophilia occurred by different HCV genotypes (11). Various TTV genotypes such as G1a, G1b, G2a, G2b

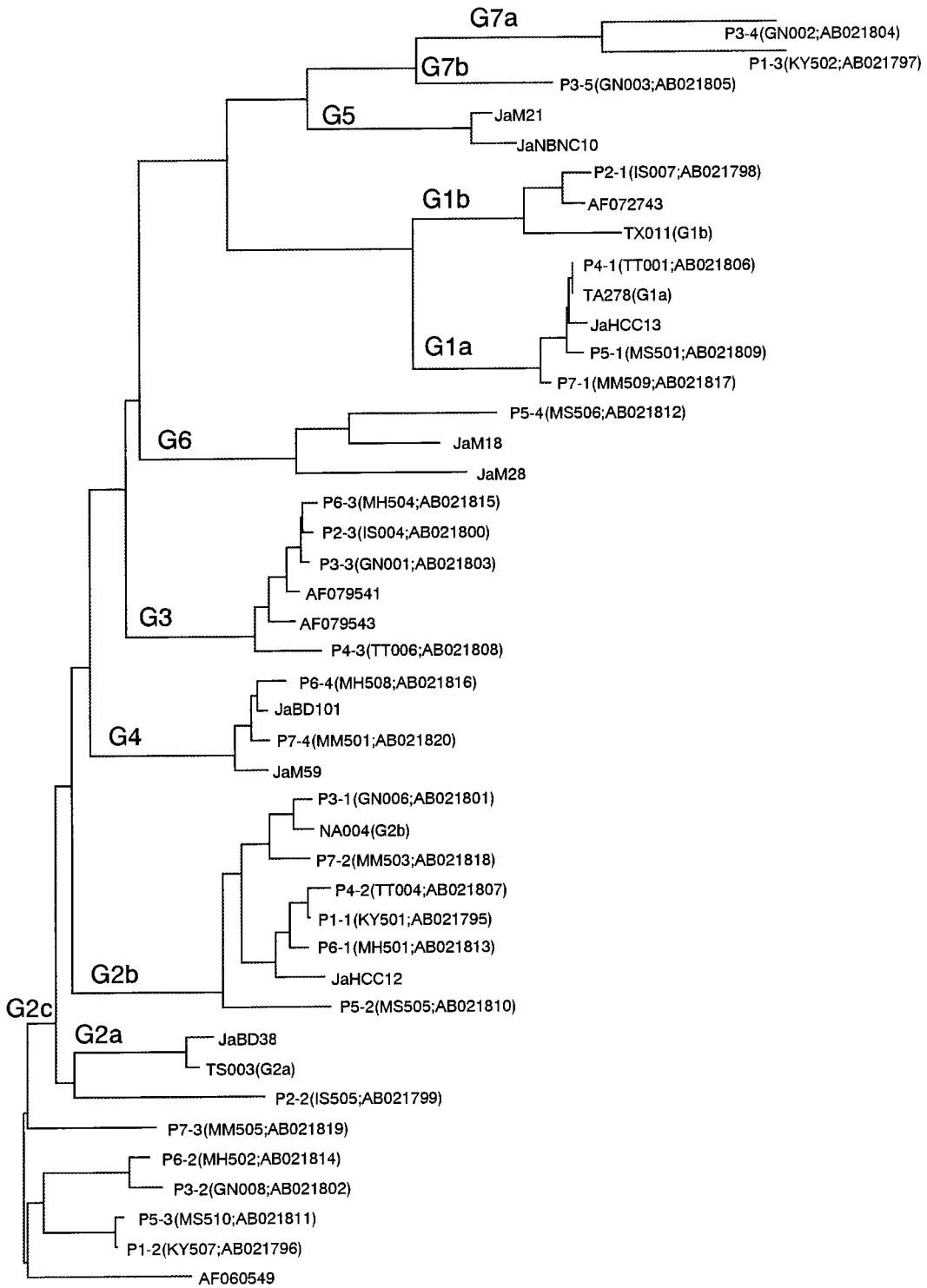

FIG. 1. Phylogenetic tree analysis on the basis of 222 bp (nt 1939–2160) of partial ORF1 region of the TTV by neighbor-joining (N-J) method. The representative TTV genotypes of G1a, TA278 (AB008394)[3] and JaHCC13 (AB018890)[5]; G1b, TX011 (AB017769)[3] and AF072743[7]; G2a, TS003 (AB017770)[3] and JaBD38 (AB018906)[5]; G2b, NA004 (AB017771)[3] and JaHCC12 (AB018889)[5]; G2c, AF060549[4]; G3, AF079541 and AF079543[7]; G4, JABD101 (AB018919) and JaM59 (AB017891); G5, JaM21(AB017887) and JaNBNC10 (AB018961); and G6, JaM18 (AB017886) and JaM28 (AB017888)[5] were quoted from DDBJ/GenBank/EMBL. The nucleotide sequences (AB021795–AB021820) of P1-1 to P7-4 TTV isolates obtained from seven patients with TTV DNA-positive hemophilia were classified. Accession numbers are in parentheses.

(3), G2c (4), and G3 to G6 (5) have been recently reported on the basis of the partial open reading frame 1 (ORF 1) nucleotide sequence of TTV. To understand persistent TTV infection, therefore, it is helpful to study multiple TTV infection with different genotypes.

By analysis of nucleotide sequences including 222 bp in the ORF1 region of the TTV genome, various TTV genotypes and unclassified genotype (tentatively designated as G7) were also identified in the sera. The constructed phylogenetic tree was different from that reported by Tanaka *et al.* (5) although the same N-J method was used in the present and previous studies. Such discrepancy may be accounted for by the difference in analyzing parameters. Although the intratypical homologies of the nucleotide sequences in each TTV genotype were 83.0–100.0%, the tentatively designated genotype G7 showed lower intratypical homology at 79.8–80.0% (76.5–82.9% at the deduced amino acid level). The low homology in nucleotide sequences among the genotype G7 may suggest additional unknown TTV genotypes which is responsible for the widely spread diversity of TTV genus.

Simmonds *et al.* (7) have reported that TTV DNA was frequently detected in several batches of factor VIII and IX concentrates prepared before 1986. Although they showed that viral inactivation efficacy was different between the factor concentrates with and without a pasteurization step, they concluded that TTV infectivity of the factor concentrates remarkably decreased after 1992. In the present study, there was no difference in TTV genotypes between the patients with hemophilia A and B. Similar results were also obtained in the sera isolated in 1995. Thus, different genotypes of TTV detected in each patient strongly suggest that multiple TTV infection may occur as found for HCV infection reported previously (11).

TTV genotypes in sera from patients without hemophilia in Japan have been reported to mainly be G1 (97%) (3). In contrast, TTV genotypes belonged to G1 were less frequently found in sera from 7 hemophiliacs and TTV genotypes detected in 7 Japanese hemophiliacs were found to be quite different. Since geographical distribution of TTV genotypes has not yet been established, the multiple TTV genotypes detected in serum from each patient may be reflected by use of non-virus-inactivated clotting factor concentrates produced from several countries before 1986. Thus, the present findings will be helpful to understand persistent TTV infection although the clinical implication of TTV infection leading to liver dysfunction is still unclear.

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