TT Virus Infection in Japanese Children: Isolates from Genotype 1 are Overrepresented in Patients with Hepatic Dysfunction of Unknown Etiology

KOHACHIRO Sugiyama, KENJI Goto, TOSHIHIRO Ando, FUMIHKO Mizutani, KOJI Terabe, TAKAO Yokoyama and YOSHIRO Wada

Department of Pediatrics, Nagoya City University Medical School, Nagoya 467–8601

Sugiyama, K., Goto, K., Ando, T., Mizutani, F., Terabe, K., Yokoyama, T. and Wada, Y. TT Virus Infection in Japanese Children: Isolates from Genotype 1 are Overrepresented in Patients with Hepatic Dysfunction of Unknown Etiology. Tohoku J. Exp. Med., 2000, 191 (4), 233–239 — The pathogenicity of the TT virus (TTV) especially during childhood remains obscure. We investigated the prevalence of TTV in 40 patients with non-A to C hepatic dysfunction (non-A to C hepatic dysfunction group). Five patients with fulminant hepatitis of unknown etiology were enrolled in this group. We also examined 380 children without a history of transfusion or liver disease (control group). Subsequently, the genotypes of TTV strains isolated were analyzed in terms of their nucleotide sequences including 222 bp in the open reading frame 1 region. The prevalence of serum TTV DNA was 10/40 (25%) in the non-A to C hepatic dysfunction group and 25/380 (7%) in the control group. Sixty-six percent (23/35) of all examined cases exhibited either genotype 1 or 2. However, assessment of genotype in the non-A to C hepatic dysfunction group (10 cases) revealed a higher prevalence of genotype 1 than of all other genotypes (80% vs. 20%). This result differed significantly from that of the control group (25 cases; 32% vs. 68%). Such overrepresentation of genotype 1 suggests that this type of TTV strain is associated with the development of hepatic dysfunction of unknown etiology in Japanese children. ———— non-A to C hepatic dysfunction; gene analysis; TTV © 2000 Tohoku University Medical Press

The new viral agent, TT virus (TTV), was discovered in the serum of adult patients with posttransfusion hepatitis of unknown etiology (Nishizawa et al. 1997). TTV was detected in the serum of 41% of patients with non-A to E liver disease, but in only 12% of blood donors in Japan (Okamoto et al. 1998). As a consequence, TTV has been implicated as a potential cause of non-A to E
hepatitis. However, its clinical role remains obscure. To date, most studies have revealed that TTV lacks obvious pathogenic effects on hepatitis (Charlton et al. 1998; Naoumov et al. 1998; Simmonds et al. 1998; Takahashi et al. 1998; Kanda et al. 1999; Kato et al. 1999; Matsumoto et al. 1999), but the possibility exists that certain strains can cause liver disease while most others are non-pathogenic. Genotype 1 has been proposed to contain such pathogenic strains (Okamoto et al. 1999a). However, these studies focused on adult patients, without considering children. The purpose of this study was to determine whether a causal relationship exists in childhood by investigating the genotypes of TTV in Japanese children.

**Patients and Methods**

Initially, we investigated the prevalence of TTV in 40 patients who underwent a screening to eliminate clear cases of marker positive viral hepatitis (hepatitis A to C viruses, cytomegalovirus, rota virus), metabolic disease, congenital anomaly, and malignant tumor (non-A to C hepatic dysfunction group) (21 boys, 19 girls; mean age ± s.d., 53 ± 52 months; range 2-178 months; mean ALT value ± s.d., 465 ± 601 IU/liter; range, 83-2206 IU/liter). Five patients with fulminant hepatitis of unknown etiology were enrolled in this group. Most patients in this group presented with elevated serum alanine aminotransferase (ALT) levels only and liver dysfunction had resolved within 2 months of hospital admission. We also examined 380 children with normal ALT levels (205 boys, 175 girls; mean age ± s.d., 46 ± 46 months; range 0-180 months; ALT values were below 30 IU/liter) and these cases had no history of liver disease (control group). All serum samples in the two groups were obtained before transfusion of blood or blood products and were stored at −30°C until use. Parental consent was obtained prior to taking the blood samples.

For each patient, DNA was extracted from 100 μl of sera by the phenol-chroloform-isoamyl alcohol procedure as previously described (Miyake et al. 1996). Polymerase chain reaction (PCR) was carried out on the isolated DNA using primers synthesized according to a published TTV sequence (Okamoto et al. 1998). In this system, semi-nested PCR was carried out and the sense primer sequences used for first and second round PCR were 5′-A C A G A C A G A G G A G A G C A A C A T G - 3′ and 5′-GGCAACATGTATGGATAGACTGG-3′, respectively, while that of the anti-sense primer sequence was 5′-CTGGCATTTTACCATTCCAAAGTT-3′. First round PCR was carried out on 20 μl aliquots of the serum sample using Taq polymerase (Takara Shuzou, Otsu) for 35 cycles, each of which consisted of denaturation for 1 minutes at 94°C, annealing for 1 minute at 55°C, and extension for 2 minutes at 72°C (with an additional 7 minutes extension during the last cycle). Second round PCR was carried out for 30 cycles under the same conditions using 2 μl of a 10×-diluted solution of first round PCR products. PCR
products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and then observed under UV light. Sera were determined to be TTV DNA-positive by the presence of a 271 bp band.

Subsequently, the amplified products were then sequenced to assess genotype by phylogenetic analysis. For each sample, amplified DNA fragments were purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Tokyo) and the purified DNA was then sequenced using the BigDye Primer Cycle Sequencing, FS Ready Reaction Kit (Applied Biosystems, Chiba) and ABI PRISM™ 310 Genetic Analyser (Applied Biosystems, Chiba), according to the manufacturers’ instructions. In the sequencing reaction, the primers for second round PCR were used as the sequencing template.

Sequences were compared by multiple sequence alignment and a phylogenetic tree was constructed using the unweighted pair-group method with arithmetic mean. Genetic distances were calculated using ODEN version 2.0 software (Nei and Gojobori 1986; Ina 1994: ftp.dna.affrc.go.jp/pub/unix/oden/ODEN.tar.Z) for molecular evolutionary analysis.

Statistical analysis was performed using the chi-squared test for independence or Fisher’s exact probability test or t-test. Significance was established at the \( p < 0.05 \) level.

Results

The prevalence of serum TTV DNA in the non-A to C hepatic dysfunction group and the control group were 10/40 (25\%) and 25/380 (7\%), respectively. There were no significant differences in the mean age and sex ratio between the two groups. However, significant differences were observed in the prevalence of TTV DNA between the two groups \((p < 0.01)\). Table 1 shows differences between non-A to C hepatic dysfunction patients with and without TTV DNA. Clinically, no significant differences were observed in the age, sex ratio, or mean ALT value.

Of the 35 PCR products directly sequenced, none of the cases yielded more than 3\% nucleotide sequence ambiguities. Thus, we could identify the genotype of the TTV strain in each case by PCR-direct sequencing assay. An evolutionary tree-diagram of nucleotide sequences is shown in Fig. 1. All sequences were compared with TTV consensus sequences from GenBank (genotypes 1a [G1a,

<table>
<thead>
<tr>
<th>Table 1. Clinical characteris of the 40 patients with non-A to C hepatic dysfunction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Number of patients</td>
</tr>
<tr>
<td>Age (months, mean ± s.d., range)</td>
</tr>
<tr>
<td>Sex (male : female)</td>
</tr>
<tr>
<td>ALT (IU/liter, mean ± s.d., range)</td>
</tr>
</tbody>
</table>
Fig. 1. Phylogenetic analysis. An evolutionary tree-diagram was calculated by the unweighted pair-group method with arithmetic mean. Horizontal axis shows the number of nucleotide substitutions per site. UE, non-A to C hepatitis dysfunction group; C, control group. G1 to G6 indicate genotype 1 to 6. Sequences of G1a, G1b, G2a, G2b, and G3 to G6 from GenBank were used as consensus sequences.

GenBank accession number; TA017610], 1b [G1b; AB011493], 2a [G2a; AB017770], 2b [G2b; AB017771], 3 [G3; AB017774], 4 [G4; AB017775], 5 [G5; AB017776] and 6 [G6; AB017777]). Most TTV strains isolated were identical to those of G1 to G6 strains, whereas five TTV strains isolated did not resemble any of the 6 genotypes (Okamoto et al. 1999a, b). These strains also did not resemble any of the genotypes 7 to 16 strains (separated by a sequence difference of over 30% from G7 to G16 strains), (data not shown).

Table 2 shows genotypes for each group. The cases shown in the table include cases of subtypes among each genotype. The results showed 23 (72%) of
the 35 strains isolated exhibited either genotype 1 or 2. Two of the 5 fulminant hepatitis patients showed TTV DNA positivity, and their genotypes were genotype 1a and 1b, respectively. The prevalence of genotypes 1, 2, 3, 4, 5, 6 and a genotype other than genotypes 1 to 6 was: 8 (80%), 0 (0%), 0 (0%), 0 (0%), 0 (0%), 1 (10%) and 1 (10%), respectively, in the 10 cases of the non-A to C hepatic dysfunction group; 8 (32%), 7 (28%), 3 (12%), 1 (4%), 1 (4%), 0 (0%) and 5 (20%), respectively, in the 25 cases of the control group. The distribution of genotypes differed significantly among the two groups (chi-squared test for independence, \( p = 0.04 \)). Furthermore, the rate of genotype 1 was higher than that of all other genotypes in the non-A to C hepatic dysfunction group (80% vs. 20%), whereas it was lower in the control group (32% vs. 68%) (Fisher’s exact probability test, \( p < 0.05 \)).

**DISCUSSION**

Although TTV was first found in posttransfusion hepatitis patients of unknown etiology (Nishizawa et al. 1997), to date, the findings of most studies have been contradictory, but the consensus has been that TTV should not be considered a causative agent of liver disease (Charlton et al. 1998; Naoumov et al. 1998; Simmonds et al. 1998; Takahashi et al. 1998; Kanda et al. 1999; Kato et al. 1999; Matsumoto et al. 1999).

Okamoto et al. (1999a, b) have identified at least 16 TTV genotypes separated by a sequence difference, and further have determined that some of these strains (genotypes 7 to 16) show a large degree of heterogeneity from the original TTV strain sequences. Therefore, caution is needed when using primers deduced from a non-coding region, as in Takahashi’s procedure (Takahashi et al. 1998). Thus, when using such primers the prevalence of TTV is high in healthy Japanese adults (Takahashi et al. 1998; Okamoto et al. 1999b) and adult patients with liver disease of unknown etiology (Hijikata et al. 1999). In our previous study (Sugiyama et al. 2000), the results were determined by either Okamoto’s PCR primers (Okamoto et al. 1998) or Takahashi’s PCR primers (Takahashi et al. 1998). The prevalence determined by the latter PCR system tended to be 2–9 times higher than those determined using the former. Consequently, no significant difference in the prevalence of TTV infection was found in childhood between various liver disease groups and the control group when Takahashi’s primer system was employed. In this study (the same group of children as in the
previous study were included), to detect the original TTV strains, we used Okamoto's primer system (Okamoto et al. 1998). The detection rate of TTV using this procedure was 27% in the non-A to C hepatic dysfunction group and 7% in the control group, a finding which was consistent with that of previous reports (Sugiyama et al. 2000).

The present results showed that 66% (23/35) of all examined cases exhibited either genotype 1 or 2. However, a high rate of genotype 1 was only found in the non-A to C hepatic dysfunction group, including fulminant hepatitis of unknown etiology. Although most studies found no association between genotype 1 strains and hepatitis (Colombatto et al. 1999; Hijikata et al. 1999; Kao et al. 1999; Parquet et al. 1999; Tanaka et al. 1999), this overrepresentation in children with hepatic dysfunction of unknown etiology suggests that this TTV strain is associated to some degree with hepatic dysfunction in such cases. The precise reason for the difference between our data and data in the previous reports is unknown, but one contributing factor may be that the previous studies focused on adult patients whereas the present study focused on children. Perhaps the clinical nature of this disease is similar to that of human cytomegaloavirus infection in childhood. In many cases, human cytomegaloavirus infection is reported to be subclinical, but hepatic dysfunction is often diagnosed (Numazaki 1998). However, a larger number of samples must be analyzed to clarify the relationship between genotype 1 of TTV and non-A to C hepatic dysfunction in children.

References

7) Kato, T., Mizokami, M., Orito, E., Nakano, T., Tanaka, Y., Ueda, R., Hirashima, N.,


