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TT virus infection among hemodialysis patients at a medical center in Taiwan

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Although the association between TT virus (TTV) infection and hepatitis is controversial, the high prevalence of TTV infection in healthy blood donors and even higher rate among frequently transfused patients poses a potential threat to public health and clinical care. In addition, there is a lack of data concerning the prevalence and mode of transmission of TTV infection in different subpopulations in Taiwan. In the present study, we investigated the prevalence of TTV infection in 111 uremic patients receiving regular hemodialysis in a single hospital in Taiwan. Blood samples were collected and analyzed using a seminested polymerase chain reaction (PCR) designed to amplify a 271 base-pair DNA fragment. The results show that the overall TTV positive rate in uremic patients in our hospital was 61% (68/111), which was much higher than the reported TTV prevalence rate among the normal population (ranging from 1%-12%). The results of analysis of the demographic and clinical characteristics of the patients indicate that blood transfusion may play an important role in TTV transmission ($p < 0.05$). In addition, the hepatitis B positive rate was significantly lower in TTV positive patients. However, liver function tests were not significantly different between TTV positive and TTV negative patients. The results of the present study suggest that blood transfusion plays an important role in TTV transmission in uremic patients.

Key words: TT virus (TTV), hemodialysis, blood transfusion, hepatitis B virus (HBV)

In December 1997, a novel viral clone (N22) was isolated using representational difference analysis in the serum of a patient with post-transfusion hepatitis not caused by the known hepatitis viruses (non-A to -G hepatitis) [1,2]. Comparison of the DNA and deduced protein sequences of the N22 clone with the data sequences of the Japanese National Institute of Genetics indicate that the N22 clone originated in the genome of a novel virus, which was named the TT virus (TTV) after the patient. TTV was originally classified as a member of the parvoviruses, with unenveloped single-stranded DNA, 3739 bases, and high buoyant density [1,3,4]. However, recent studies reclassified this virus as a member of a novel family, Circinoviridae, with an additional 113 base pairs in the terminal regions [5,6].

Several studies have been conducted to determine the prevalence and the potential role of TTV infection among patients with different disease entities including chronic liver diseases, hemophilia, and among different population groups including abusers of intravenous drugs, normal blood donors, and healthy individuals [4,7-9]. Although TTV was first reported in Japan, it was later found to have a worldwide distribution [1]. The prevalence of TTV DNA in the general population appears to be high, ranging from 1% to 12% [4,7]. Okamoto *et al* reported that TTV DNA was detected by polymerase chain reaction (PCR) in 47% and 46% of patients with acute and chronic hepatitis of non-A to -G etiology, respectively. In comparison, the rate in blood donors was 12% [4]. In addition, TTV DNA has been reported to be common in certain disease entities and populations a higher risk of blood-borne viral infections, such as in hemophilia (68%), patients under dialysis (46%), and intravenous drug abusers (40%). In the North American population, TTV infection has been reported to be present in 1% of blood donors, 18% of patients with a history of exposure to blood products, and 4% of patients without parenteral risk factors [7]. The relative risk of TTV infection among individuals

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with a history of exposure to blood products was 4.5. However, another study conducted in the United Kingdom detected TTV DNA in 25% (18/72) of patients with chronic liver disease, which was not statistically different from the 10% prevalence in the normal population [8]. Although parenteral transmission of TTV was highly suggested by statistical analysis, no direct evidence of TTV-induced post-transfusion hepatitis has been documented. Several recent studies also suggested possible oral-fecal transmission of TTV by demonstrating identical TTV DNA sequences in fecal excretion and sera and a high prevalence in the normal population in certain areas [3,10,11]. Although TTV has been shown to be a common virus in the serum of the general population, its role in human diseases and mode of transmission remain unclear.

Since information on the prevalence of TTV infection in Taiwan is limited, we examined the prevalence of TTV infection at our hospital in December 1998 in uremic patients who were receiving regular hemodialysis, due to the higher risk of blood-borne infections in this group. We also sought to determine the possible route of transmission of TTV infection in these patients.

Materials and Methods

Patients and sample preparation

A total of 111 uremic patients receiving regular dialysis therapy at our OPD (Taipei VGH) were screened for TTV infection in December 1998 by obtaining 3 mL of whole blood in EDTA-containing tubes (Vacutainer, 0.57 mL of 15% EDTA). There were 58 male and 53 female patients, with age ranging from 25 to 86 years old (mean 58 years old). All of the patients had received hemodialysis over the period ranging from 5 to 276 months (Table 1). Liver function and hepatitis B and C markers were also checked at the same time. Transfusion histories were obtained both by patient inquiry and data collection from special dialysis charts. The blood samples were centrifuged at 800 x *g* for 5 min (Kubota 5100, Japan) and the plasma and buffy-coat layers were separated. All the samples were stored at -20 °C for future analyses.

Table 1. Demographic and clinical characteristics of patients with and without TTV DNA

Characteristic	TTV DNA		*p*
	Positive (n = 68)	Negative (n = 43)	
Age (years)	59.0 ±14.9	57.9 ±14.6	0.705
Male	38 (55.9%)	20 (46.5%)	0.443
Duration of HD (months)	57.4 ±51.5	55.1 ±48.8	0.823
HCV positive rate	21/68 (30.9%)	7/43 (16.3%)	0.084
HBV positive rate	2/68 (2.94%)	9/43 (20.9%)	0.003
AST	22.3 ±23.8	16.6 ±11.1	0.091
ALT	23.1 ±14.9	19.7 ±8.5	0.130

Abbreviations: HD = hemodialysis; TTV = TT virus; DNA = deoxynucleic acid; HCV = hepatitis C virus; HBV = hepatitis B virus; AST = aspartate aminotransferase; ALT = alanine aminotransferase

DNA isolation from patient blood samples

DNA isolation was performed using a commercial kit (Wizard Genomic DNA Purification kit, Promega, Madison, WI, USA.) according to the manufacturer's instructions. Briefly, 300 µL of Cell Lysis Solution was added to a sterile 1.5 mL microcentrifuge tube containing 100 µL sample volume. The contents of the tube were then mixed by inverting the tube five to six times and the mixture was incubated for 10 min to lyse the red blood cells. The mixture was centrifuged at 13,000 x *g* for 20 sec at room temperature and the supernatant was removed. The previous procedure was repeated by adding 150 µL of Cell Lysis Solution. The tube was vortexed vigorously until the white blood cells dispersed evenly. One hundred microliters of Nuclei Lysis Solution was added to the tube and the mixture was incubated at 37 °C with 0.5 µL RNase Solution for 15 min, and then was cooled to room temperature. Thirty-three microliters of Protein Precipitation Solution was added to the lysate and the tube was vortexed for 20 sec. The mixture was centrifuged at 13,000 x *g* for 3 min at room temperature and the supernatant (130 µL) was transferred to a clean 1.5 mL microcentrifuge tube containing 100 µL of room temperature isopropanol. The solution was mixed by gently inverting the tube and centrifuged at 13,000 x *g* for 1 min at room temperature. The supernatant was decanted and one sample volume (100 µL) of 70% ethanol was added at room temperature. The tube was gently inverted and centrifuged at 13,000 x *g* for 1 min. The ethanol was then carefully removed. The pellet was exposed to open air for 10 min and 33 µL DNA Rehydration Solution was then added. The DNA was stored at 2 to 8 °C until used in further experiments.

Seminested PCR detection of TTV

Five microliters of TTV DNA were added to a first round of PCR containing 1 x PCR reaction buffer, 0.1 mM dNTP mixture, 1.5 mM Mg^{2+}, 0.5 µM primer mix, and *Taq* DNA Polymerase (Viogene, Sunnyvale, CA, USA) in a final volume of 50 µL. The first-round of PCR was carried out for 30 cycles (94 °C, 30 sec; 55 °C, 25 sec; 72 °C, 45 sec; with the initial

cycle at 94 °C, 5 min; and an additional 72 °C, 7 min in the last cycle) with TTVF1 primer (sense: 5'-ACAGACAGAGGAGAAGGMAACATG-3') and TTVB1 primer (antisense: 5'-CTGGCATTTTAC CATTTCCAAAGT3') using a Perkin Elmer thermal cycler 480. The second-round PCR was carried out for 35 cycles (94 °C, 30 sec; 62 °C, 25 sec; 74 °C, 30 sec; with initial 94 °C, 5 min) using 0.5 µL of the first-round PCR product with the TTVF2 (sense: 5'-GGCAACATGYTRTGGATAGACTGG-3') and TTVB1 (antisense: 5'-CTGGCATTTTACCATTTCCAAAGT3') primer pair. The amplification products of the second round PCR measured 271 base pairs (bp) and were electrophoresed in 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

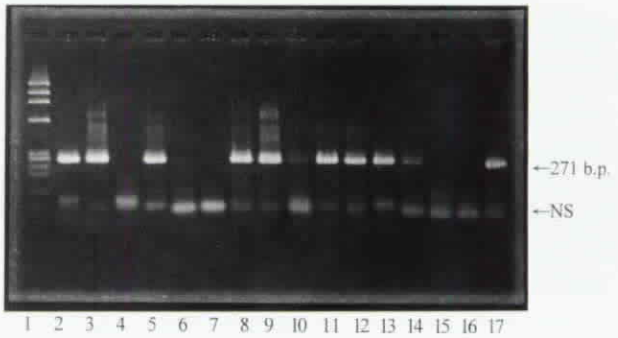

Fig. 1. Detection of TTV DNA in uremic patients by semi-nested PCR. Lane 1 = DNA marker; Lane 2 = positive control; Lanes 3-17 = seminested PCR results from 15 uremic patients; NS = nonspecific bands.

Statistical analyses

Student's t-test was used to evaluate differences in age, duration of hemodialysis, aspartate aminotransferase (AST) level, and alanine aminotransferase (ALT) level between patients with and without TTV infection. We used the chi-square test with Yates' correction to evaluate the influence of blood transfusion on TTV positive rate and Fisher's exact test to evaluate the relation between TTV infection and hepatitis B virus (HBV) and hepatitis C virus (HCV) infection rates. A p value of less than 0.05 was considered statistically significant.

Results

Detection of TTV in uremic patients receiving regular hemodialysis

A specific 271 base pair DNA fragment was demonstrated after the second round of the seminested PCR in all TTV infected patients (Fig. 1). Some of the DNA fragments were subsequently cloned into plasmids and sequenced. The sequencing data showed high levels of homology to the published TTV sequences (data not shown). The initial results showed a high (61%, 68/111) prevalence of TTV infection among these uremic patients. In contrast, the prevalence of TTV infection among healthy individuals was approximately 16% (Table 2). The demographic, biochemical, and clinical data of the 111 patients with and without TTV DNA are shown in table 1.

Blood transfusion and TTV infection

The clinical and demographic characteristics of the 68 uremic patients with TTV infection and the 43 uninfected patients are shown in table 1. To our surprise, there was a significant negative correlation between HBV positivity and TTV infection ($p = 0.003$). There was no significant difference in age, sex, months of hemodialysis, HCV positive rate, and AST/ALT levels between patients with and without TTV infection. To further evaluate the possible risk factors for TTV infection, we performed chi-square analysis of the clinical and demographic characteristics of the 110 patients with definite transfusion history. As shown in table 2, of the 95 patients who had previously received transfusion, there were 62 TTV positive patients, and the TTV positive rate in patients with a previous transfusion (65.3%) was significantly higher than that (33.3%) of the 15 patients who had not received any prior transfusion. This finding indicates that transfusion is an important risk factor for TTV transmission.

Table 2. Incidence of TTV infection in patients receiving HD with and without prior blood transfusion

Feature	Total no. of patients	Age (year) mean ±SD	Male (%)	TTV positive rate No. of patient (%)
Healthy individuals	236	38.5 ±13.3	88 (37.3)	37 (15.7)
Patients receiving HD[a]	110	58.5 ±14.8	58 (52.7)	67 (60.9)
With blood transfusion	95	57.1 ±14.7	48 (50.5)	62 (65.3)[b]
Without blood transfusion	15	67.7 ±12.0	10 (66.7)	5 (33.3)[b]

[a]HD = hemodialysis, [b]$p < 0.05$

Discussion

Our results indicate that the positive rate for TTV DNA in uremic patients was unexpectedly high (61%). Previous studies have reported a TTV virus infection rate of 1% to 12% in normal blood donors. A higher rate of TTV infection has been reported in chronic hepatitis patients of non-A to -G etiology (46%) compared to the normal population [4]. A recent report showed a high TTV prevalence (62%) in healthy people in Brazil [10]. A screening for TTV infection in Taiwan by Jaw-Chin Wu (Section of Gastroenterology, Taipei VGH) found that approximately 15% of healthy people were TTV positive (personal communication). The positive rate for TTV in uremic patients in this study was four-fold higher than Wu's finding for the general population.

TTV was originally isolated from a non-A to -G cryptogenic hepatitis patient and was thought to be the etiology of the hepatitis [1]. However, several other studies were not able to confirm a pathogenic role of this virus [10-13]. In addition, the original assumption that TTV belonged to a family of parvovirus was not well accepted. Recent studies have reclassified TTV into a new family, the Circinoviridae [6]. In the present study, patients with a positive finding for TTV virus showed no significant differences in liver function tests compared with TTV negative patients. However, a significantly lower incidence of HBV infection was found in TTV positive patients. There is no previously reported information concerning any interaction between TTV and HBV. The data of the present study suggest that TTV might inhibit HBV gene expression or that TTV infection might interfere with later HBV infection. Determination of the clinical implication of the lower incidence of HBV infection in TTV positive patients will require further study.

The results of this study showed that blood transfusion was significantly associated with TTV transmission ($p < 0.05$). However, several previous studies have suggested that a parenteral TTV transmission may not be the only mode of TTV transmission based on the findings of TTV in bile and feces and the high prevalence rate in the normal population in certain geographical areas such as Brazil and Columbia [3,10,11]. However, the major mode of transmission could be parenteral and this may have accounted for the high rate of TTV infection among the uremic patients in this study, although we can not rule out the possibility of other modes of transmission. The existence of a nonparenteral mode of TTV transmission is supported by the observation in this study that 33% of uremic patients with no history of blood transfusion were infected with TTV.

The long interval between the discovery of parvovirus B19 and recognition of its role in aplastic anemia may explain the similar delays in discovering a role of TTV infection [14,18]. The B19 virus, discovered in the mid-1970s by Yvonne Cossart while investigating laboratory assays for hepatitis B using an immunoelectrophoretic technique, was the first pathogenic human parvovirus to be discovered [16]. The "false-positive" reactions identified turned out to be caused by a coincident parvovirus infection in the hepatitis patient. Although the clinical relevance of Cossart's discovery was not immediately obvious and most of the B19 viremic blood donors were healthy, Pattison reexamined the significance of B19 infection in 1981 by screening 800 stored sera and found antigenemia or seroconversion in six similar patients with transient aplastic crisis [14]. The pathogenic role of B19 in fifth disease, transient aplastic crisis, and hydrops fetalis was further confirmed by later investigations and laboratory experiments.

At least two and up to six genotypes of TTV have been determined by different methods. However, no specific connection between the different genotypes and their clinical presentations has been deduced. Several reports suggested an association of TTV virus with arthritis [16] and a lack of association of TTV with non-B, non-C hepatocellular carcinoma [17]. A recent report suggests that TTV variants with phylogenetic differences can infect the same individual, and that some variants may have a predilection for peripheral blood mononuclear cells [18]. In the present study, we identified three different TTV genotypes using a simple restriction digestion of PCR products.

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