Clinical Outcome of Frequent Exposure to Torque Teno Virus (TTV) Through Blood Transfusion in Thalassemia Patients With or Without Hepatitis C Virus (HCV) Infection

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As a consequence of the high prevalence of TorqueTeno virus (TTV) in blood donors, thalassemia patients frequently acquire various genotypes of this virus through therapeutic blood transfusions. At present, the clinical consequences of TTV infection remain indeterminate for these patients. Here, several hundred thalassemia patients were tested for the presence of TTV and its genotypes using a combination of PCR and clone-based DNA sequencing. Approximately 10% (12/118) of the patients aged 2–20 years remained negative for TTV including eight genotypes of SENV. Ferritin, aspartate-aminotransferase (AST) and alanine-aminotransferase (ALT) levels were invariably lower in TTV-negative patients (P=0.02, <0.01, and 0.06, respectively) than in TTV-positive patients. Patients with TTV–HCV co-infection showed elevated ferritin and ALT levels compared with patients with TTV infection alone (P< 0.02 and P< 0.01). AST and ALT levels were within the normal range for all TTV-negative patients, whereas abnormal levels of AST and ALT were seen in a significant proportion of TTV-positive patients (30.7% and 33.6%, respectively) and patients with TTV–HCV co-infections (70.0% and 56.6%, respectively). Only TTV-positive patients (28.0%) and patients with TTV–HCV co-infections (36.3%) had hyper-ferritin levels (≥3,000 ng/ml). The genotype(s) of TTV responsible for the liver dysfunction could not be determined. However, high levels of AST and ALT were found to be correlated with detection of a higher number of TTV genotypes in the patients. The data suggests that frequent and persistent TTV infection through blood transfusion is associated with

hepatic dysfunction and/or damage in transfusion dependent thalassemia patients. *J. Med. Virol. 80:365–371, 2008.* © 2007 Wiley-Liss, Inc.

KEY WORDS: TTV; HCV thalassemia patient; liver disease

INTRODUCTION

Hepatic dysfunction and/or damage are frequent complications in thalassemia patients who depend on blood transfusions that are associated with a high frequency of exposure to viruses. Accordingly, the incidence of transfusion related hepatitis for this group of patients has been markedly reduced since the implementation of blood screening for hepatitis B virus (HBV) and hepatitis C virus (HCV) nucleic acid and antibodies. However, over one third (37%) of thalassemia patients without infection by known hepatitis viruses still have an abnormal alanine-aminotransferase (ALT) pattern [Chen et al., 1999]. The exact cause of the ALT abnormality in those patients remains unknown [Okamoto and Mayumi, 2001].

A virus with a small single-stranded DNA genome was identified by Nishizawa et al. [1997] in Japan from patients with non-A-E transfusion acquired hepatitis in

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1997. With reference to the index patient, the virus was originally named TT virus however it is currently renamed as Torque Teno virus (TTV) the type species of the genus Anellovirus, in an unassigned family that is most closely related to the Circoviridae [Hino, 2002]. TTV is widely distributed geographically with a high rate of viremia within the general population [Simmonds, 1998; Cossart, 2000]. Mixed genotype infections are therefore common, particularly in frequently transfused patients such as thalassemia patients where 80% of them carry more than one genotype of TTV [Chen et al., 1999; Okamoto et al., 1999b]. This is presumably due to the high frequency of viral transmission through blood transfusion and the persistent nature of TTV viral infections [Chen et al., 1999; Gallian et al., 1999; Kanda et al., 1999; Kobayashi et al., 1999; Matsumoto et al., 1999; Oguchi et al., 1999; Prati et al., 1999]. TTV viruses have been classified into at least five groups consisting of more than 27 genotypes as a result of the extremely wide range of sequence divergence observed among TTV isolates [Okamoto et al., 1998; Tanaka et al., 1998; Takayama et al., 1999; Worobey, 2000; Okamoto and Mayumi, 2001]. Early studies indicate that TTV might cause some forms of cryptogenetic hepatitis, post transfusion hepatitis and/or other diseases, however these observations have not been confirmed in most subsequent studies although it has been suggested that certain TTV groups or genotypes (e.g., group 4, genotype 1 and genotype 21) might be especially pathogenic and associated with liver or other diseases [Simons et al., 1995; Okamura et al., 2000; Sugiyama et al., 2000; Bendinelli et al., 2001; Maggi et al., 2003; Pifferi et al., 2005; Szladek et al., 2005]. The clinical significance of TTV infection thus remains controversial. [Okamura et al., 2000; Sugiyama et al., 2000; Maggi et al., 2003; Pifferi et al., 2005; Szladek et al., 2005]. Considering that thalassemia patients frequently acquire multiple genotypes of TTV through repeated blood transfusion administered throughout their lifetime, the role of this virus in the development of clinical disease in this group of patients cannot be excluded [Chen et al., 1999]. In addition to TTV, a substantial proportion of thalassemia patients have acquired HCV infection through blood transfusion. It remains uncertain if HCV–TTV co-infections result in more severe biochemical and histological changes compared to TTV infection alone [Charlton et al., 1998; Watanabe et al., 1999; Yuki et al., 1999; Zein et al., 1999; Cleavinger et al., 2000; Meng et al., 2001; Tokita et al., 2002].

Since TTV cannot be cultivated in vitro, PCR is the only available tool for detection of TTV. It has been difficult to determine the clinical significance of TTV infections because diagnostic systems using one or two sets primers for PCR are unable to detect the entire spectrum of TTV genotypes and their variants that exist in individuals [Okamoto et al., 1999b, 2000; Maggi et al., 2003]. Obviously, this has impeded a proper assessment of TTV viral pathogenesis. We recently found that all TTV genotypes (except genotype 21), and all SENV genotypes (A–H) can be detected using three TTV

primer sets [Hu et al., 2005]. This finding has provided a more accurate and efficient tool for TTV diagnosis. In this study, we used this efficient primer system combined with clone-based DNA sequencing to investigate the prevalence of various genotypes of TTV with respect to clinical outcome in various age groups of blood transfusion dependent thalassemia patients with or without HCV co-infection.

MATERIALS AND METHODS

Study Groups

A total of 197 thalassemia patients from the United Arab Emirates (UAE) who had received regular blood transfusions (13–18/year) were enrolled in this study, including 54 with hepatitis C virus (HCV) infection. Among these patients a younger group of 118 thalassemia patients (49 female and 69 male) ranging in age from 2 to 20 years (with a median age of 10.8 years) was tested for blood ferritin, AST and ALT levels to study the clinical outcome of TTV and HCV infections. The remaining thalassemia patients (n = 79: aged 21–53 years) were only tested for ALT in this study. All samples were negative in standard donor-screening tests including HIV, Human T Cell Leukemia Viruses, and hepatitis A, B and G.

Isolation of Viral DNA

Plasma (100 µl) was used for isolation of viral DNA with a silica gel based membrane while using microspin technology as described in the QIAamp blood kit (QIAGEN, Inc., Mississauga, Ontario).

Amplification and Detection of TTV DNA

Purified TTV DNA was amplified using three sets of nested primers derived from the conserved regions in 5′UTR and ORFs of the TTV genome following the procedure as previously described [Okamoto et al., 1999b; Hu et al., 2005]. They include RD037-038 plus RD051-052 [Okamoto et al., 1998], TT6-7 plus TT8-9 [Hohne et al., 1998], and NG 5′UTR based nested primers (NG054-147 plus NG133-132) [Okamoto et al., 1999a]. TTMV (TTV-like mini virus) was also tested in the thalassemia patients using two sets of TTMV specific primers as described previously [Hu et al., 2005]. The sensitivity of PCR used in this study had previously been evaluated by both DNA dilution and real-time PCR methods. The sensitivity was determined to be ≤10 copies/ml. To confirm that thalassemia patients testing negative for TTV were truly negative, the 12 TTV negative samples were subjected to additional PCR reaction conditions including different and lower stringency primer annealing temperatures and additional reaction cycles. PCR amplified TTV DNA was detected on a 1.5% agarose gel using ethidium bromide staining.

Direct DNA Sequencing

Direct DNA sequencing was performed using an automated DNA sequencer (Visible Genetics). A 5′

Cy5.5 labeled sense primer, RD051, was used to produce a sequence of approximately 150 bases from RD primer set PCR fragments. For the PCR products amplified using TT primers, a 5′ Cy5.5 labeled sense primer, TT8, was used to produce a sequence of approximately 150 bases in length. 5′UTR based primers NG133F and NG132R were used for sequencing the PCR products amplified by 5′UTR based primers.

Cloning Based DNA Sequencing

Clonal based DNA sequencing was performed using TOPO- TA™ vector (Amersham Pharmacia Biotech, USA) for cloning of purified PCR products. The TTV sequence was amplified from colonies of *Escherichia coli* using M13 primers (Amersham Pharmacia Biotech, USA). Fifteen clones from each individual were sequenced. Re-amplified PCR products were sequenced using the same procedure as for direct DNA sequencing.

Genotyping and Computer Analysis of Nucleotide Sequences

DNA sequences derived from DNA sequencing were compared using an on line database for the best possible match using the Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information home page (http://www.ncbi.nlm.nih.gov/). All further sequence analyses and comparisons were performed using the DNA-STAR Lasergene '99 software package (DNAStar, Madison, WI).

Statistical Analysis

The mean differences in AST, ALT and ferritin between three clinical groups were compared using Student's t-tests. Since the sample size for both TTV-negative and TTV+HCV positive groups is small compared to that of the TTV positive group, we performed a matching comparison to increase the comparison power. Each case in the TTV-negative group (or TTV+HCV positive group) was matched to a case of the same age and gender in the TTV-positive group. Paired t-test was performed for the matching comparison. In addition, we categorized the AST, ALT and ferritin into normal and abnormal groups using the clinical normal range as the cut-off value, and compared the abnormal rates in the three clinical groups. Chi-square tests were used in the comparison of rates. The SAS software package was used for the statistical analyses.

RESULTS

Clinical Significance of TTV Infection

Ferritin, AST and ALT levels. For a thorough analysis of the clinical data, we first investigated the impact of age and gender on the levels of clinical parameters (Table I). Ferritin, AST and ALT levels were found to be higher in patients aged 11–20 years than in younger patients aged 2–10 years. However, the difference did not reach statistical significance ($P = 0.13$, 0.10 and 0.10 for ferritin, AST and ALT, respectively). All three parameters were slightly increased in male relative to female patients, but again no significant difference was found between different genders ($P = 0.46$, 0.19 and 0.43 for ferritin, AST and ALT, respectively). The data suggest that age and gender are not major factors that significantly contributed to liver disease in thalassemia patients aged 2–20 years. However, since all three parameters were slightly higher in older patients and male patients in comparison with younger patients and female patients, a minor impact of age and gender on liver dysfunction could not be completely excluded. To improve the accuracy of data analysis, we therefore decided to use both age-sex matching and non-age-sex matching methods for evaluating the clinical significance of TTV infection in thalassemia patients.

The results in Table II indicate that by using an exact age-sex matching method, the ferritin, AST and ALT levels were found to be invariably lower in TTV-negative patients compared to the TTV-positive patients ($P = 0.02$, $P < 0.01$ and $P < 0.06$, respectively). The trends of the results generated by non-age-sex matching were, in general, consistent with those derived using the age-sex matching approach, where ferritin, AST and ALT levels were shown to be higher in TTV-positive patients than in TTV-negative patients ($P < 0.02$, $P > 0.05$ and $P > 0.05$, respectively), but in this case only ferritin was significantly elevated. The clinical data regarding TTMV (TTV-like mini virus) negative samples (8.5%, 10/118) was also analyzed and compared to the TTMV positive samples by age-sex matching method. The levels of ALT, AST and ferritin were not significantly different between TTMV-negative and TTMV-positive patients (data not shown). Of the TTV negative patients, 91.7% (11/12) were positive for TTMV. These data further support the observation that TTV, but not TTMV causes liver dysfunction and/or damage. Using age-sex matched groups, the levels of both ferritin and

TABLE I. Ferritin, AST and ALT Levels in Different Age and Gender Groups of TTV Infected Thalassemia Patients

Patients (no.)	Ferritin (ng/ml) (P)	AST (IU/L) (P)	ALT (IU/L) (P)
Age			
2–10 (45)	2290.9 ± 1331.2	35.1 ± 20.7	37.3 ± 47.2
11–20 (47)	2706.3 ± 1274.5 (0.13)	42.9 ± 24.2 (0.10)	53.2 ± 44.8 (0.10)
Gender			
Male (52)	2566.4 ± 131318.5	42.2 ± 24.4	48.8 ± 47.4
Female (40)	2364.1 ± 1296.5 (0.46)	35.9 ± 21.4 (0.19)	41.2. ± 43.8 (0.43)

TABLE II. Comparison of Ferritin, AST and ALT Levels Between TTV Negative Patients, Patients With TTV Infection Alone (TTV+) and With TTV–HCV Co-Infections (TTV + HCV+)

Patients (no.)	Age	Ferritin (ng/ml) (P)	AST (U/L) (P)	ALT (U/L) (P)
Age-sex matching				
TTV– (12)	9.5 ± 5.6[a]	1470.3 ± 514.7	27.1 ± 7.1	22.1 ± 12.2
TTV+ (12)	9.5 ± 5.6	2340.0 ± 733.5 (0.02)	38.8 ± 18.1 (<0.01)	49.3 ± 45.3 (0.06)
No age-sex matching				
TTV– (12)	9.5 ± 5.6	1470.3 ± 514.7	27.1 ± 7.1	22.1 ± 12.2
TTV+ (92)	10.8 ± 4.6	2477.7 ± 1305.6 (0.02)	39.5 ± 23.3 (0.08)	45.5 ± 45.8 (0.11)
Age-sex matching				
TTV+ alone (11)	13.2 ± 4.4	2340.0 ± 733.5	36.2 ± 22.0	27.6 ± 17.0
TTV + HCV+ (11)	13.2 ± 4.4	3112.9 ± 1477.8 (0.02)	53.1 ± 27.6 (0.13)	55.4 ± 27.6 (<0.01)
No age-sex matching				
TTV+ alone (92)	10.8 ± 4.6	2477.7 ± 1305.6	39.5 ± 23.3	45.5 ± 45.8
TTV + HCV+ (11)	13.2 ± 4.4	3112.9 ± 1477.8 (0.08)	53.1 ± 27.6 (>0.05)	55.4 ± 27.6 (>0.05)

[a]Mean ± SD; significant differences are in bold.

ALT were found to be higher in patients with HCV–TTV co-infections than in patients with TTV infection alone ($P = 0.02$ and $P < 0.01$). It was expected that patients with TTV–HCV co-infections would have more severe liver disease than TTV alone as HCV is a serious liver pathogen. Non-age-sex matched analysis showed that all three clinical parameters were higher in patients with TTV–HCV co-infections than in patients with TTV infection alone, but no significant difference was found between the two groups of patients. This suggests that the use of age-sex matching approach is necessary for the clinical data analysis.

Abnormality rate of ferritin, AST and ALT. We found that all 12 TTV-negative patients aged 2–20 years had normal AST ($\leq$40 IU/L ranging from 14 to 40 IU/L) and ALT ($\leq$50 IU/L, ranging from 10 to 48 IU/L) levels. However, AST and ALT levels were elevated in nearly one-third of TTV-positive patients aged 2–20 years (30.7%, 27/88 for AST; and 33.6%, 48/143 for ALT; Fig. 1). Abnormal levels of AST (70%, 7/10) and ALT (56.6%, 30/53) was even higher in patients with TTV–HCV co-infections than patients with TTV infection alone. Data resulting from analysis of abnormalities in three clinical parameters agreed completely with those obtained from the assessment of actual differences in ferritin, AST and ALT levels. Although the ferritin levels were significantly higher ($P = 0.02$) with respect to TTV and HCV positivity for matched patient groups, the ferritin baseline level of the patient group is high due to transfusion treatments. The ferritin level in TTV-negative thalassemia patients was five times higher (1470.3 ± 514 ng/ml; Table II) than normal (10–300 ng/ml). Therefore, we used the hyper-ferritin level value, which is ten times the normal limit (3,000 ng/ml) to calculate the abnormality rate in thalassemia patients. None of the TTV-negative patients had ferritin levels over 3,000 ng/ml with the highest level being 2,440 ng/ml. However, about one third of patients with TTV infection alone (28.0%, 26/93) and patients with HCV–TTV co-infections (36.4%, 4/11) had hyper-ferritin levels (3,000 ng/ml ranging up to 6,250 ng/ml).

One might expect that the ALT abnormality rate should be highest in older patients due to the greater duration of transfusion treatment. In fact, the ALT abnormality rate was the highest (33.6%, 48/143) in young patients (11–20 years) compared to the youngest patients aged 2–10 years (21.2%, 14/66) and the older patient group, between the ages of 21 and 53 (20.9%, 9/43; Fig. 2). This indicates that ALT elevation in thalassemia patients was not dependent or directly attributable to the time span or number of transfusions alone.

TTV Prevalence and Pathogenesis of Various Genotypes

As reported previously [Hu et al., 2005], RD primers are known to specifically amplify genotype 1 (1a and 1b) and TT6/7/8/9 primers detect genotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 15, and 16; mainly in genogroups 1 and 3. TT6/7 primers specifically detect TTV genotypes 2 and 3. NG primers can detect almost all known TTV genotypes except genotype 21, including eight genotypes (A–H) of SENV. Use of the three nested sets of TTV primers
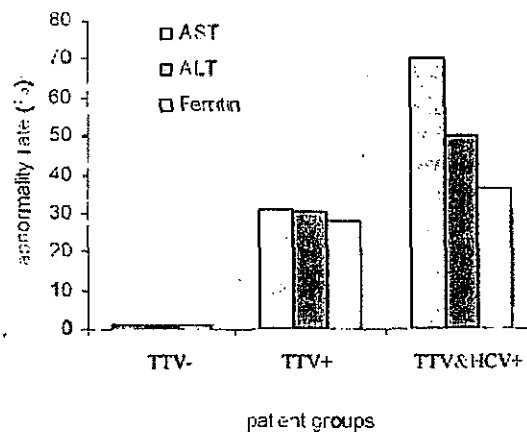

Fig. 1. Abnormality rate of ferritin, aspartate-aminotransferase (AST) and alanine-aminotransferase (ALT) levels in TTV-negative patients (TTV–), TTV-positive patients (TTV+) and patients with TTV–HCV co-infections (TTV and HCV+).
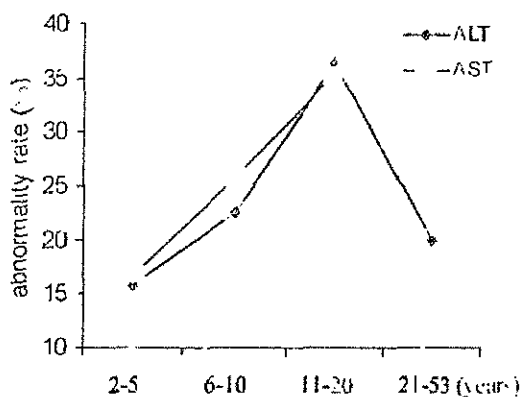
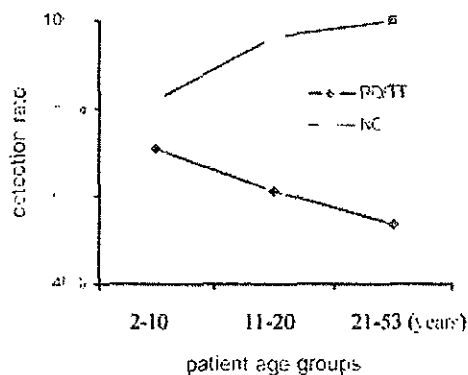Fig. 2. Abnormality rate of AST (■) and ALT (♦) at different ages in TTV-positive thalassemia patients.

Fig. 3. TTV-positive rates of various genotypes in different age groups of thalassemia patients. The graphs indicate the viremia rates (%) of NG primer-specific genotypes (■), RD/TT6/7/8/9-specific genotypes (♦). The ages of patient groups (2–10, 11–20 and 21–53 years) are plotted against rate of infection.

allowed us to assess the prevalence of various genotypes in different age groups of thalassemia patients. Figure 3 shows that the positivity rate for the genotypes detected by NG primers increased significantly with age from younger (2–10 years) to older groups of patients (ages 11–20 years as well as 21–53 years) with detection rates of 81.8%, 45/55; 96.2%, 50/52; and 100.0%, 30/30, respectively (trend test $P < 0.0001$). In contrast, the detection frequency for the most prevalent genotypes that are detectable by RD/TT6/7/8/9 primers (i.e., genotypes 1–10 and 14–16) declined significantly from younger to older groups. The TTV-positive rate was 71.0%, 27/38; 61.5%, 32/52; and 53.6%, 30/56; for the younger and the two older groups of patients, respectively (trend test $P < 0.001$). The genotypes of amplified TTV viruses were confirmed by clone-based sequencing (see Materials and Methods Section, data not shown). The shifting of predominant TTV genotypes over a patient's lifetime suggests that younger patients have a greater susceptibility to the most prevalent genotypes relative to older patients. Presumably this is due to the fact that some of the older patients may become immune due to prior exposure and subsequent clearance of infection. As a result, these patients are protected from re-infection by some genotypes such as the genotypes detectable by RD/TT6/7/8/9 primers. However, in addition to being exposed to more TTV genotypes, the older patients have also been exposed to less prevalent TTV genotypes over time because of increased exposure due to repeated transfusions. Overall, the TTV viremia rate is higher in older patients. The genotype shifting during a patient's life-time provides strong evidence to indicate that both self-limited and chronic TTV infections exist and that persistence of the virus (at least certain genotypes) may not be life-long in thalassemia patients. The declining prevalence of some genotypes in older patients was paralleled by a decrease in the ALT abnormality rate (see Figs. 2 and 3). This suggests that the most prevalent genotypes such as 1, 2 and 3 may play a more important role in the development of liver disease in thalassemia patients. However, the exact genotype(s) responsible for the elevation of ferritin, AST and ALT

levels could not be determined because the majority of TTV infected patients carried more than one genotype.

DISCUSSION

The role of TTV infection in liver disease has been the subject of much debate since the first TT virus was identified in 1997. In our present study, the data suggest that frequent and persistent TTV infection through blood transfusion is associated with abnormal AST, ALT and ferritin levels (seen in about one third of blood-dependent thalassemia patients). This supports the hypothesis that certain genotypes or variants of TTV cause disease when individuals are exposed to these genotypes or their variants for the first time and/or re-exposed to partially cross-reactive or non-cross reactive TTV strains [Bendinelli et al., 2001]. To further assess whether a specific genotype or group of TTV is associated with elevated ALT levels in thalassemiacs, we compared the genotypes infecting patients with abnormal ALT levels (>100 IU/L) and patients with normal ALT levels (<50 IU/L). A total of 480 clones from 32 samples (15 clones for each sample) were genotyped by DNA sequencing. We found that the majority (78.1%, 25/32) of the thalassemia patients were infected with more than one genotype. It was therefore difficult to judge which genotype was responsible for the elevation of ALT levels in patients with mixed genotype infections (data not shown). However, the number of mixed genotypes was found to be significantly increased in patients with abnormal ALT levels (three vs. two genotypes per patient, $P = 0.01$). It appears that ALT elevation is associated with a higher frequency of TTV mixed genotype infections, including transient and persistent infections through blood transfusion.

It is reasonable to propose that multiply transfused thalassemia patients are at a much greater risk of being infected by new and more pathogenic genotypes or strains than blood donors. In the UAE thalassemia patients average over 15 transfusion per year, where over one third of blood donors carry the virus. It would be expected that transfusion dependent thalassemia