Original Report

Lack of Association between Acquisition of TT Virus and Risk Behavior for HIV and HCV Infection in Vietnam

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ABSTRACT

Background: The search for the cause of chronic hepatitis among individuals with non-A to G hepatitis has led to the discovery of a post-transfusion hepatitis-related DNA virus, designated TT virus (TTV), which, based on viral sequences, belongs to a new virus family. The principal modes of infection with TTV are poorly understood, and its role in human immunodeficiency virus type 1 (HIV-1) infection is unclear.

Objective: To determine if injection drug use (IDU) and high-risk heterosexual activity (HRHA), principal modes of acquiring HIV-1 infection, place individuals at greater risk of acquiring TTV.

Methods: The authors analyzed DNA, extracted from sera or filter paper-blotted whole blood, obtained during August 1997 and June 1998 from 324 Vietnamese (148 male; 176 female), for TTV sequences by hot-start, heminested polymerase chain reaction.

Results: Prevalence of TTV viremia was similar among individuals engaging in IDU or HRHA (23.4% vs. 20.2%; $P > 0.5$), with no age- or gender-specific differences. No association was found between TTV viremia and co-infection with HIV-1 or hepatitis C virus (HCV). Phylogenetic analysis of 30 TTV sequences revealed two distinct genotypes and four subtypes that did not segregate according to gender, HIV-1 and HCV risk behaviors, or geographic residence.

Conclusions: Among HIV-1- or HCV-infected Vietnamese, who presumably acquired their infection by either the parenteral or nonparenteral route, the data indicate no clear association between acquisition of TTV infection and risk behavior for HIV-1 or HCV infection, suggesting that the usual route of TTV transmission in Vietnam is other than parenteral or sexual.

Key Words: Circoviridae, *circovirus, HCV, hepatitis, HIV, IDU, nucleotide sequence, phylogeny, post-transfusion*

Int J Infect Dis 1999; 3:181–185.

Sensitive molecular techniques, particularly representational difference analysis, have led to the discovery of the Kaposi sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8) and a non-A to E hepatitis-associated flavivirus, GB virus C/hepatitis G virus (GBV-C/HGV).[1-3] More recently, pursuit of the cause of chronic hepatitis among individuals with non-A to G hepatitis has resulted in the detection of a novel, single-stranded DNA virus, designated TT virus (TTV), from a patient with post-transfusion elevation of serum alanine aminotransferase.[4] Based on viral sequences and buoyant density, TTV is related to but distinct from circoviruses.[5-8]

High prevalences of TTV infection and multiple genotypes of TTV recently have been reported among blood donors and patients with liver disease.[4,7-15] However, as with GBV-C/HGV, the disease potential of TTV and its principal modes of transmission are unclear. In particular, few data are available on TTV infection among individuals who are infected with or are at risk of infection with human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV).[16,17] The principal objective of this study was to ascertain if HIV-1 risk behaviors, such as injection drug use (IDU) and high-risk heterosexual activity (HRHA), were operative in the acquisition and dissemination of TTV.

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Presented in part at the 36th Annual Meeting of the Infectious Diseases Society of America, Denver, Colorado, November 12–15, 1998, and at the Sixth RCMI International AIDS Symposium, San Juan, Puerto Rico, November 15–17, 1998.

Supported by United States Public Health Service grants from the Research Centers in Minority Institutions (RCMI) Program (G12RR/AI-03061) and the Fogarty International Center (D43TW00013 and R3TW00866A) of the National Institutes of Health.

Received: April 30, 1999; Accepted: July 13, 1999.

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MATERIALS AND METHODS

Study Participants

Through the Sentinel Surveillance Program of the Vietnam National AIDS Committee, coded sera or filter paper-blotted whole blood were obtained during August 1997 and June 1998 from 324 individuals (148 males; 176 females), who engaged in IDU or HRHA. Informed consent was obtained from all study participants and the study was approved by the Committee on Human Subjects of the University of Hawaii at Manoa. Ages were available on 137 males (range, 17–60 y; median, 35 y) and 150 females (range, 16–55 y; median, 28 y). Of the 324 study participants, 217 (66.9%) (131 males; 86 females) were infected with HIV-1 and 107 (33.1%) (17 males; 90 females) were uninfected. The presumed mode of HIV-1 infection was HRHA in 106 (24 males; 82 females) and IDU in 111 (107 males; 4 females). All 107 HIV-1-uninfected individuals engaging in HRHA were commercial sex workers or their clients and partners, and all denied the use of injection drugs. Human immunodeficiency virus type-1 infection status was ascertained serologically by enzyme-linked immunoassay (ELISA; Vironostika HIV Uni-Form II plus O, Organon Teknika, The Netherlands), with confirmation by Western blot (New LAV blot 1, Sanofi Diagnostics Pasteur, Marnes la Coquette, France).

DNA Extraction

DNA was extracted from 200 to 280 μL of serum from 285 study participants using either the QIAamp DNA or Viral RNA Extraction kit (QIAGEN, Chatsworth, CA). DNA extraction from filter paper-blotted whole blood samples from 39 individuals was accomplished using a previously described phenol-chloroform protocol.[18,19] Each DNA sample was eluted in 50 μL of diethylpyrocarbonate-treated distilled water and was stored at -30°C until use.

Detection of TTV DNA

Oligonucleotide primers for polymerase chain reaction (PCR) were derived from the highly conserved open reading frame 1 (ORF1) of TTV (GenBank accession number AB008394); outer primers: A5430, bases 1901 to 1923, 5′-CAGACAGAGGAGAAGGCAACATG-3′, and RD052, bases 2257 to 2238, 5′-GTACTTCTTGCTGGTGAAAT-3′; heminested inner primers: A5430, as stated above, and primer B, bases 2186 to 2161, 5′-CTGGCATTTTACCATTTCCAAAGTT-3′.[4,10,12] Primers were used at a concentration of 0.1 μM in a 25-μL reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.8 mM $MgCl_2$, 0.2 μM each dNTP and 5 μL DNA. Using a DNA thermal cycler, the outer reaction mixtures were initially denatured at 94°C for 60 seconds, then cycled 10 times at 94°C for 15 seconds, 45°C for 30 seconds, and 72°C for 90 seconds, followed by 35 cycles at 94°C for 15 seconds, 50°C for 30 seconds, and 72°C for 90 seconds, with an extension of 7 minutes at 72°C, before storing at 4°C. Subsequently, hot-start, heminested PCR was performed in a 20-μL reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.8 mM $MgCl_2$, 0.1 μM each inner primers and 0.2 μM each dNTP. Each mixture was overlaid with a bead of PCRGem 50 Ampliwax (Perkin-Elmer, Branchville, NJ), heated to 80°C for 5 minutes, and cooled at room temperature. A 30-μL reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 U *Thermus aquaticus* DNA polymerase (Perkin-Elmer), and 5 μL of the outer product was added on top of the solidified wax. The cycling conditions for heminested PCR were similar to conditions for the outer PCR, except the template was annealed at 50°C for 30 seconds and cycled 45 times. Enzymatically amplified DNA was size fractionated by electrophoresis on 2% agarose gels to ascertain the presence of the 285-bp product.

Amplicons, purified using the Microcon column YM-100 (Millipore Corp., Bedford, MA) were sequenced directly using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) with inner PCR primers (A5430 and Primer B) on an automated sequencer (model 373A; Applied Biosystems).[18]

Nucleotide sequences derived from the TTV ORF1 of 30 Vietnamese (24 HIV-1-infected and 6 uninfected) were aligned and compared with TTV sequences amplified from patients with and without liver diseases from various geographic regions. Sequence alignment was facilitated using the software package available on the VAX computer system, as part of the Genetics Computer Group (GCG), as described previously.[18]

Diagnosis of HCV Infection

Serum IgG antibodies against HCV core and nonstructural proteins were detected by ELISA, using the HCV version 3.0 ELISA kit (Ortho Diagnostics, Raritan, NJ). For detection of HCV RNA in plasma, the 5′-untranslated region was amplified by reverse transcription PCR.[19] Presence of either HCV RNA or antibodies to HCV was considered evidence for HCV infection.

Statistical Data Analysis

Epidemiologic data were analyzed using Epi Info version 6.02a and SAS version 6.12 (Cary, NC).[20] Odds ratios were used to estimate associations between dichotomous variables, such as TTV infection. Chi-square P-values were used to test statistical significance. Mantel-Haenszel summary chi-square P-values and weighted odds ratios were used in stratified analyses of categoric variables. Student's t-test was used to test differences in means of continuous variables between groups.

RESULTS

TTV Prevalence among Individuals Infected with or At Risk of HIV-1 Infection

Of the 324 Vietnamese who were infected or at risk of infection with HIV-1, 69 (21.3%) had detectable TTV sequences in blood or serum, as determined by PCR. Based on the presumed mode of HIV-1 infection, no difference in TTV prevalences was evident among individuals practicing IDU and HRHA. Although HIV-1-infected men practicing HRHA had a higher prevalence of TTV compared to HIV-1-uninfected men (29.2% vs. 11.8%; P > 0.18; OR = 3.09), the biologic significance of this finding is unclear; HIV-1-infected and -uninfected women practicing HRHA did not mimic this finding.

No gender-specific differences in TTV prevalence was found (Table 1), and the median ages of TTV-positive (n = 65) and TTV-negative (n = 222) individuals were nearly identical (32 vs. 31 y). Moreover, age-specific prevalences of TTV infection were similar after stratification by 10-year age groups: 5 of 15 (33.3%) in the age group 16 to 19 years; 17 of 85 (20.0%) in the 20 to 29 years group; 19 of 70 (27.1%) in the 30 to 39 years group; 10 of 47 (21.3%) in the 40 to 49 years group; and 2 of 7 (28.5%) in the 50 years and older group. Furthermore, age-specific prevalences did not vary by gender or by geographic residence; that is, TTV prevalences in northern Vietnam (Hanoi, Haiphong, Lang Son, and Quang Ninh) and in southern and south-central Vietnam (Ho Chi Minh City, An Giang, Can Tho, Khanh Hoa, Kien Giang, and Da Nang) were nearly equal (8/32 [25%] and 61/292 [21%], respectively).

TTV prevalences among HCV-infected IDU and individuals practicing HRHA were similar (27.0% vs. 24.2%). Six (20.7%) of the 29 individuals co-infected with HCV, HIV-1, and TTV engaged in HRHA, whereas all 15 infected only with TTV did so.

HCV Prevalence among Individuals At Risk of HIV-1 Infection

Of the 278 participants (120 males; 158 females) from whom serum specimens were available for HCV testing, 118 (42.4%) (90 males; 28 females) were infected with HCV. Of the 118 HCV-infected individuals, 85 (72.0%) (84 males; 1 female) were IDUs and 33 (28.0%) (6 males; 27 females) practiced HRHA. All but one HCV-uninfected individual engaged in HRHA (30 males; 129 females). Of the 107 (88 males; 19 females) individuals co-infected with HCV and HIV-1, 85 (79.4%) were IDUs; the remaining 22 (20.6%) (4 males; 18 females) practiced HRHA. Similarly, all 96 (15 males; 81 females) individuals who were negative for both HCV and HIV-1 engaged in HRHA.

Table 1. Prevalence of TTV Infection among At-risk Individuals in Vietnam

Variable	*n*	*Positive (%)*	*Odds Ratio*	*P-Value*
Male	148	34 (23.0)		
Female	176	35 (19.9)	1.2	>0.5
Intravenous drug user	111	26 (23.4)		
High-risk heterosexual activity	213	43 (20.2)	1.2	>0.5
HIV-1 positive	217	52 (24.0)		
HIV-1 negative	107	17 (15.9)	1.67	>0.1
HCV positive	118	31 (26.3)		
HCV negative	160	31 (19.4)	1.48	>0.17
HCV and HIV-1 positive	107	29 (27.1)		
HCV and HIV-1 negative	95	15 (15.8)	1.98	0.052

HIV = human immunodeficiency virus; HCV = hepatitis C virus.

Genetic and Phylogenetic Analyses of TTV

Since sequencing was performed directly, the viral sequences presented here represent those of the predominant virus population in vivo and do not reflect selection artifacts from cloning. Alignment and comparison of the 238-base pair (bp) TTV ORF1 from 30 (43.5%) of the 69 TTV-infected Vietnamese with representative TTV strains from other geographic regions indicated multiple base substitutions. TTV genotype 1 (1a = 40.0%; 1b = 53.3%) was predominant among these 30 study participants. Of these 30 individuals, 12 (40.0%) and 18 (62.1%) were IDUs and individuals practicing HRHA, respectively. At the nucleotide sequence level, the Vietnamese TTV genotypes 1a and 1b were 88.7% to 97.9% (median, 96.0%) and 92.9 to 98.3% (median, 97.1%) similar to the prototype Japanese TTV genotype 1a (N22 clone; GenBank accession no. AB017767) and genotype 1b (CS5),[4] respectively.

The median nucleotide sequence divergence between the Vietnamese TTV genotype 1a sequence compared to TTV sequences from China (Ch1; AF055897) and Thailand (TTV33; AF078114) were 3.1% and 10.3%, respectively. The intra-genotype nucleotide sequence diversity of TTV sequences from Vietnam ranged from 0.4% to 17.2% for genotype 1a (n = 12; median, 8.0%) and from 0% to 11.3% for genotype 1b (n = 17; median, 5.0%). The inter-genotype 1a and 1b nucleotide sequence divergence was 13.0% to 26.0% (n = 29; median, 16.8%). Similarly, the inter-genotype 2a and 2b sequence divergence was 20.1%. TTV nucleotide sequences were 99.6% similar in a CSW and her partner, both of whom were from An Giang province and were HIV-1 and HCV uninfected. Based on the phylogenetic analysis, TTV sequences from Vietnam segregated into two genotypes (genotype 1 and 2) and four subtypes (subtypes 1a, 1b, 2a, and 2b) with high bootstrap values (Figure 1). TTV sequences did not segregate according to gender, HIV-1 and HCV risk behaviors, or geographic residence.

DISCUSSION

Recent data indicate high prevalences of TTV infection among healthy individuals or blood donors from Japan, Thailand, the United Kingdom, and the United

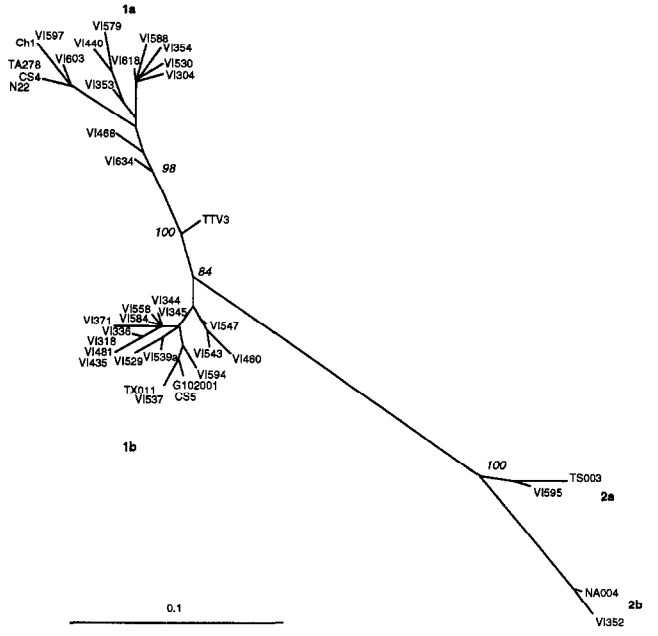

Figure 1. Representative phylogenetic tree, constructed by the CLUSTAL W program, using the neighbor-joining method. The tree is based on the 238-bp open reading frame 1 of TTV genome. TTV strains from Vietnam are designated with the prefix VI. The phylogenetic positions of TTV subtype 1a from Japan (N22 [AB017767], CS4,[4] TA278 [AB008394]), China (Ch1 [AF055897]), and Thailand (TTV3 [AF078114]); subtype 1b from Japan (TX011 [AB017769], CS5,[4] G102001 [AB011488]); subtype 2 from Japan (NA004 [AB017771] and TS003 [AB017770]) are shown. Branch lengths are drawn in proportion to the number of nucleotide substitutions per site, and bootstrap probabilities (1000 iterations) exceeding 80% for each node are noted in italics. GenBank accession numbers for Vietnam TTV strains are: AF133540 to AF133567.

States.[8-10,12,13] This cross-sectional study on 324 individuals infected with or at risk of infection with HIV-1 in Vietnam similarly indicates high prevalences of TTV infection. Since serodiagnostic assays for TTV currently are unavailable, serum antibodies against TTV were not assessed in this study. Moreover, since the duration of detectable TTV viremia is not known, TTV prevalences based on PCR must be viewed as minimal rates.

The initial report of Nishizawa and co-workers suggested TTV transmission by the parenteral route.[4] Simmonds and co-workers demonstrated TTV contamination in 50% of 34 batches of factor VIII and IX concentrates manufactured from remunerated and non-remunerated donors.[12] Viral inactivation procedures have been shown to reduce the frequency of TTV detection in clotting factor concentrates. Although the number of virally inactivated concentrates was small, the removal of TTV by solvent or detergent treatment appeared less effective than pasteurization at 60°C for 10 hours. In this regard, a higher frequency of TTV infection has been detected in hemophiliac patients receiving non-virally inactivated concentrates when compared to those receiving virally inactivated concentrates (27% vs. 0.5%). Also, the frequency of TTV infection increased with severity of hemophilia or with the amount of clotting factor received. Moreover, a TTV prevalence of 18% (2/11) has been reported among patients with a history of exposure to blood products who were awaiting liver transplantation.[9] Collectively, these data suggest a high risk of TTV transmission by transfusion of blood products.

Among HIV-1-infected or HCV-infected Vietnamese, who presumably acquired their infection by either the parenteral or nonparenteral route, the data in this study indicate no clear association between acquisition of TTV infection and risk behavior for HIV-1 or HCV infection.

In addition to transfusion-acquired TTV infection, nonparenteral modes of transmission have been suggested.[11,12] In five patients with non-A to G hepatitis in whom TTV was detected in serum specimens, Okamoto and co-workers demonstrated TTV in fecal specimens of three patients.[11] In two patients, excretion of TTV was correlated with higher viral titers in serum. TTV from feces and serum banded at a similar peak density and the nucleotide sequences in three pairs of feces and serum were identical. All patients had hepatocellular carcinoma, suggesting an older patient population with markers for hepatitis B surface antigen (HBsAg) or antibodies to HCV. Simmonds and co-workers have hypothesized that the high frequency of TTV infection in blood donors in the United Kingdom is consistent with TTV transmission by nonparenteral route(s).[12] This hypothesis is supported by the significantly older age distribution of TTV-infected donors (mean age, 53 y), compared with donors drawn from the same population who were infected with parenterally transmitted viruses, such as HCV (mean age, 32 y, $P < 0.0001$) or GBV-C/HGV (mean age, 36 y; $P = 0.001$).[12] By contrast, the mean age of TTV- or HCV-infected individuals in Vietnam was 32.4 years and 34.4 years, respectively. Nonparenteral transmission of TTV is supported by approximately 10% TTV prevalence reported among patients with sexually transmitted disease in the United Kingdom and Thailand.[16,17] Charlton and co-workers have demonstrated TTV prevalence of 4% (1/25) among patients without a history of parenteral risk factors,[9] and Naoumov and co-workers suggest nonparenteral transmission of TTV based on 10% and 38% prevalence among a general population and individuals with "community-acquired infection," respectively.[10] The present data on non-drug injecting HIV-1-infected and -uninfected individuals practicing HRHA demonstrated TTV prevalences of 24.5% and 15.9%, respectively. Collectively, these data support, in addition to the parenteral route, a nonparenteral route of TTV transmission.

Analysis of a 238-nucleotide region of TTV from three cases of post-transfusion hepatitis showed diversity of 1 to 9% at the nucleotide sequence level, suggesting distinct genotypes, such as HCV.[4] Further analysis of 78 sequences in the same region, from patients in Japan with or without liver disease, distinctly clustered TTV into two

groups, differing by 30%.[7] Of the 78 sequences, 97.4% belonged to group 1 and the remaining to group 2. Sequences in the two groups were further divided into two subgroups differing by 11 to 15%. Thus, TTV strains in group 1 were subgrouped into G1a (subtype 1a), consisting of 52 strains, and the remaining 24 were subgrouped in G1b (subtype 1b). The two strains in group 2, which differed by 14%, were subgrouped into G2a (subtype 2a) and G2b (subtype 2b). The intra-subtype similarity was 93.3 to 100%. Additionally, divergent sequences have been identified in the United Kingdom and Japan.[12] Recently, Tanaka and co-workers reported six new genotypes of TTV, based on 72 serum specimens obtained from Asia, Africa, and South America, but were unable to find any relation between liver disease and TTV genotypes.[14] To date, none of the studies have systematically linked these subtypes to biochemical or histologic parameters of liver disease or disease progression.[13-15]

Nucleotide sequence analysis of TTV based on a 238-bp region from patients in Japan with or without markers for hepatitis infection revealed no segregation of TTV genotypes according to the underlying disease,[7,8] but did demonstrate segregation according to the two major genotypes. Similar to the observation of Okamoto and co-workers,[8] more than 95% of the Japanese TTV sequences and approximately 93% of Vietnamese TTV sequences belonged to genotype 1a or 1b.[7] TTV sequences from individuals at risk for HIV-1 infection in Vietnam did not cluster according to the route of acquisition of HIV-1 or gender, but rather segregated based on genotype as observed among the Japanese patients. Based on this short fragment of TTV genome, the intra- and inter-genotype nucleotide sequence distance between Vietnamese and Japanese, Thai, and Chinese patients were similar, further confirming that the TTV sequences segregate by the underlying genotype, and genotype may have little impact, if any, in the underlying disease.

ACKNOWLEDGMENTS

The authors thank Dr. Quoc Cuong Nguyen, Dr. Thang Hong Pham, Dr. Thanh Cong Duong, and Ms. Tran Phung for their assistance in specimen collection and processing.

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