Detection of TT Virus DNA in Liver Biopsies by *in Situ* Hybridization

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A novel hepatitis-associated virus named TT virus (TTV) has been isolated. However, its hepatotropism has not been proven. We have retrospectively analyzed the presence of TTV-DNA by polymerase chain reaction (PCR) and *in situ* hybridization in liver biopsies from 30 patients with liver disease (15 TTV-DNA-positive and 15 TTV-DNA-negative in serum), and prospectively in serum and liver from eight patients with normal liver histology. TTV-DNA was detected by PCR in the liver from the 15 patients with serum TTV-DNA and in serum and liver of two of the eight patients without liver disease. TTV-DNA titers in liver were 10 times higher than in serum, although no correlation between TTV-DNA titers in serum and liver were observed. *In situ* hybridization shows positive signals in the hepatocytes of the 17 patients infected by TTV but in none of the TTV-DNA-negative patients by PCR. No morphological changes were observed in the hepatocytes showing hybridization signals. The percentage of positive hepatocytes ranged from 2.1% to 30% and correlated with the TTV-DNA titers in liver ($r = 0.54$; $P = 0.037$). In conclusion, our results show that TTV is able to infect liver cells although they do not support a role for TTV in causing liver disease. (*Am J Pathol* 2000, 156:1227–1234)

Although isolation of the hepatitis C and hepatitis E viruses has made it possible to identify these agents as being responsible for most cases of posttransfusional or community-acquired hepatitis, respectively,^{1,2} there are still posttransfusional or fulminant hepatitis cases of unknown etiology.³ The identification of GB virus C/hepatitis G virus (GBV-C/HGV)^{4,5} has not solved this problem because several studies have shown that GBV-C/HGV would not account for a significant part of hepatitis cases of unknown etiology.^{6,7} Recently, a novel virus called TTV has been molecularly cloned from the serum of a patient with posttransfusional non-A-G hepatitis.^{8–10} TTV is an

unenveloped virus whose genome consists in a circular and negative single-stranded DNA molecule of ~3.8 kb in length.^{11,12} Based on its genomic and physicochemical features, it has been proposed that TTV is a member of a new family of viruses named *Circinoviridae*.¹²

Whether there is any association of the TTV infection and liver disease is a matter of debate.^{13,14} Consequently, to establish that TTV has a role in causing liver damage, it must be demonstrated that this is a hepatotropic virus. Okamoto et al⁹ have reported the presence of TTV-DNA by polymerase chain reaction (PCR) in the liver of TTV-infected patients with titers 10 to 100 times higher than those in serum. Furthermore, TTV secretion into bile has been demonstrated.¹⁵ All these findings could indicate that the virus may be hepatotropic. To confirm this finding, morphological evidence of the presence of TTV in the liver cells from infected patients should be proven. However, there is still no information available about the detection of TTV within individual liver cells by *in situ* hybridization or by immunohistochemistry.

In the present study, we have applied nonisotopic *in situ* hybridization for the detection of TTV genome in liver biopsies to identify the cell tropism, the intracellular location of the virus, and its relationship with the liver disease.

Patients and Methods

The presence of TTV-DNA was retrospectively analyzed in frozen liver samples (by PCR) and in the corresponding paraffin-embedded liver biopsies (by *in situ* hybridization) from 30 patients (19 males, 11 females) with abnormal blood alanine aminotransferase levels (ALT) and histologically-proven liver disease.

Fifteen of the patients had TTV-DNA in a serum sample collected when the liver biopsy was obtained. Of them, seven patients had chronic hepatitis C, one had chronic hepatitis B, and five had nonviral liver diseases (three steatosis, one ductopenia, one siderosis), and two had cryptogenic hepatitis. The remaining 15 patients were TTV-DNA-negative in serum and were selected based on the criteria of having similar clinical, histological, and epidemiological features as the TTV-DNA-positive cases.

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Table 1. Main Clinical Characteristics of the 30 Patients Studied Retrospectively

Patient data	Serum TTV-positive	Serum TTV-negative
<i>n</i>	15	15
Age* (years)	50.80 ± 9.97	42.90 ± 10.18
ALT* (IU/L)	103.8 ± 66.90	120.3 ± 102.54
AST* (IU/L)	60.06 ± 38.60	74.06 ± 75.53
γ-GT* (IU/L)	83.73 ± 55.26	51.13 ± 34.77
Viral chronic hepatitis		
Chronic hepatitis B	1	2
Chronic hepatitis C	7	8
Nonviral		
Steatosis	3	3
Ductopenia	1	—
Siderosis	1	—
Cryptogenic hepatitis	2	2
Epidemiology		
Blood transfusion	3	3
Surgery	5	2
Intravenous drug users	—	1
Dental manipulation	—	1
Unknown	6	8
Homosexual	1	—

*Expressed as the mean ± SD.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyltransferase.

Eight of them presented with chronic hepatitis C, two with chronic hepatitis B, three with steatosis, and two with cryptogenic hepatitis. Cryptogenic hepatitis was diagnosed on the basis of its negativity to viral markers, autoimmunity, metabolic disease, alcohol intake, and hepatotoxic drug exposure. No differences were found with respect to age, gender, ALT, aspartate aminotransferase (AST), and γ-glutamyltransferase levels or epidemiological antecedents between TTV-positive and TTV-negative patients. The main clinical characteristics of the patients are summarized in Table 1.

In addition, the presence of TTV-DNA was prospectively analyzed in serum and in frozen and paraffin-embedded liver biopsies from eight patients with normal ALT levels and without evidence of liver disease. Liver biopsies from these patients were obtained at the time of cholecystectomy. The histological examination of these biopsies showed a normal liver histology in all cases.

This study was approved by the ethics committee of our hospital and was conducted in accordance with the guidelines of "Good Clinical Practice," which underwrites the principles of the Declaration of Helsinki on human experimentation. Written informed consent according to local legal requirements was obtained from each patient participating in the study.

Detection of TTV-DNA in Serum and Liver by PCR

Total DNA from 200 μl of serum was isolated by proteinase K (1 mg/ml) and 1% sodium dodecyl sulfate digestion at 37°C for 2 hours. After phenol extraction and ethanol precipitation, the nucleic acid pellet was resuspended in 20 μl of distilled water.

For TTV detection in frozen liver samples, liver biopsies were homogenized in the presence of 1 × TSE buffer (10 mmol/L Tris-HCl, pH 7.5; 10 mmol/L NaCl; 2 mmol/L ethylenediaminetetraacetic acid) and digested with proteinase K and sodium dodecyl sulfate as described above. After phenol extraction and ethanol precipitation, the total DNA pellet was resuspended in 50 μl of distilled water. DNA concentration was estimated by measuring its absorbance at 260 nm. Ten μl of total DNA obtained from the 200 μl of sera or 1 μg of total DNA from liver biopsies were amplified by PCR using primers from ORF-1 from the untranslated region. PCR reactions were performed exactly as described by Okamoto et al⁹ (ORF-1 primers) and by Takahashi et al¹⁶ (untranslated region primers). In all cases, distilled-water serum samples previously known as negative for TTV-DNA and DNA extracted from the human hepatoma cell line HepG2 were used as negative controls for the DNA extraction and PCR. To confirm the specificity of the detection of TTV in liver, all PCR products were directly sequenced using the ALF Express DNA Sequencing System (Pharmacia Biotech AB, Uppsala, Sweden).

TTV-DNA Semiquantitation

TTV-DNA titers in serum and liver were semiquantitated by PCR using the pCI-neo plasmid (Promega Co., Madison, WI) as external standard. A 1:10 dilution of each positive serum sample and 0.1 μg of total DNA from each positive liver sample were mixed with 1 pg of pCI-neo. All of the mixtures were amplified by PCR with α-³²P-dATP, using the primers TTSV (sense, nucleotide position: 1021–1045) and SV2 (antisense, nucleotide position: 1372–1395) deduced from the SV40 late polyA signal of pCI-neo and the primers from TTV ORF-1 and untranslated region, as described above. Primers for pCI-neo amplification were designed to have a similar length and melting temperature as those used for TTV-DNA amplification. The DNA fragments of 374 bp corresponding to pCI-neo and 271 or 199 bp corresponding to ORF-1 or untranslated region TTV-DNA amplification, respectively, were resolved by 6% polyacrylamide gel electrophoresis, followed by autoradiography of the dried gel. Quantitation of the signals was performed by densitometric analysis (MD ImageQuant Software version 3.3, Molecular Dynamics Ltd., Kemsing, UK). The TTV-DNA titers were expressed as the intensity of the TTV-PCR products, normalized with respect to the intensity of the pCI-neo-PCR products.

Sequence Analysis

The PCR products from ORF-1 obtained from serum and liver samples of selected patients were cloned into the pCR-II-TOPO vector (TOPO TA Cloning kit, Invitrogen, San Diego, CA). Several clones from each sample were automatically sequenced in both directions using the ALF Express DNA Sequencing System (Pharmacia Biotech AB).

The TTV nucleotide sequences were aligned with the Clustal X program¹⁷ and edited with the GeneDoc program version 2.5.000 (Nicholas KB, Nicholas HB Jr, GeneDoc: a tool for editing and annotating multiple sequence alignments. 1997, distributed by the authors). The nucleotide sequences of the TTV-DNA amplified from our patients were compared with the TTV-DNA sequences retrieved from GenBank, corresponding to all of the published TTV genotypes. Phylogenetic analysis was performed using the DNADIST, NEIGHBOR, SEQBOOT, and CONSENSE programs from the Phylip package version 3.5c.¹⁸ The genetic distances were estimated by the Kimura two-parameters method and the unrooted phylogenetic tree was constructed by the neighbor-joining method. The final output of the tree was obtained using the Tree View program version 1.5.2.¹⁹ Bootstrap values were determined on 1000 resamplings of the data set. Values >70% were considered statistically significant for the observed grouping.

Nucleotide Sequence Accession Numbers

The GenBank accession numbers of the sequences reported in this article are from AF216391 through AF216432.

In Situ Hybridization

A PCR product of 271 bp in size corresponding to TTV ORF-1 was directly cloned into the pCR II-TOPO vector to obtain the pCTTV plasmid following the instructions supplied by the manufacturer. Verification of the insert orientation and nucleotide sequence was performed by automatic sequencing. The pCTTV plasmid was purified using the Wizard Plus SV Minipreps kit (Promega) and labeled with digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, IN) by nick translation (Nick Translation System; GIBCO BRL, Grand Island, NY).

Paraffin-embedded liver sections (4 μ m) were dewaxed in xylol and rehydrated through a series of ethanol dilutions. After digestion with proteinase K (1 μ g/ml) at 37°C for 10 minutes, the sections were postfixed in a freshly-prepared solution of 4% paraformaldehyde in 0.1 mol/L phosphate-buffer at pH 7.0, dipped in distilled water, and acetylated in 0.5% (v/v) acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0) for 20 minutes at room temperature. Slides were rinsed in 2 $\times$ SSPE (20 $\times$ SSPE: 3 mol/L NaCl, 0.2 mol/L NaH₂PO₄, 16 mmol/L ethylenediaminetetraacetic acid, pH 7.4) and quickly dehydrated in ethanol. Hybridization was carried out in a solution consisting of 50% deionized formamide, 0.1 mol/L phosphate-buffer (pH 7.0), 4 $\times$ SSPE, yeast t-RNA (500 μ g/ μ l), and 10% dextran sulfate containing 5 ng of heat-denatured labeled DNA probe per slide. After 16 hours at 50°C, slides were washed for 1 hour at 50°C with 1.5 $\times$ SSPE followed by an additional 1-hour wash at 50°C in 0.75 $\times$ SSPE. The digoxigenin-labeled hybrids were detected with a digoxigenin-antibody alkaline-phosphatase conjugate and an enzyme-substrate chromogen (nitroblue tetrazolium salt in 70% (v/v) dimethylform-

amide/5-bromo-4-chloro-3-indolyl-phosphate in dimethylformamide) according to the instructions supplied by the manufacturer (DIG Nucleic Acid Detection kit, Boehringer Mannheim Biochemicals). The slides were counterstained with 0.2% safranin in 5% ethanol.

The specificity of the signal was assessed by: 1) hybridization with the labeled plasmid without TTV-derived sequences; 2) digestion of the liver sections with RNase A (0.2 mg/ml; Sigma Chemical Co., St. Louis, MO) and DNase I (20 U/ml; Sigma) for 2 hours at 37°C, before the *in situ* hybridization; 3) competitive hybridization in the presence of an excess of unlabeled TTV probe; and 4) omission of the probe in the hybridization mixture.

Visualization of the *in situ* hybridization signals was performed using a NIKON Eclipse E400 light microscope (Nikon, Tokyo, Japan) using 20 $\times$, 40 $\times$, and 100 $\times$ objectives. The images were captured using a high-resolution monochrome CCD camera (DIC-N; World Precision Instruments, Cambridge, UK).

The percentage of infected hepatocytes was determined by visual inspection, counting at least 2 $\times$ 10³ cells per sample.

Histological Diagnosis

Slides from the liver biopsies analyzed were stained with hematoxylin and eosin for the histological diagnosis. The histological activity index was estimated according to Knodell et al²⁰ and histological diagnosis was made according to Desmet et al.²¹

Statistical Analysis

Continuous variables were compared using the Student's *t*-test. A difference with a *P* < 0.05 was considered significant. The Spearman correlation was used for correlations. The statistical analyses were performed using the SPSS package (SPSS for Windows release 6.0, SPSS Inc., Chicago, IL).

Results

A PCR product of the expected size was observed after the amplification of the total DNA isolated from the frozen liver biopsies in the samples corresponding to the 15 patients with TTV-DNA-positive in serum but in none of the liver samples from the serum TTV-DNA-negative patients, using the primers from ORF-1 and untranslated region. Regarding the eight patients without evidence of liver disease, TTV-DNA was detected in both serum and liver in two patients, whereas the remaining six patients were negative in both compartments. The sequencing data from the PCR products confirmed the specificity of the TTV detection in liver biopsies. Semiquantitation of the TTV-DNA titer in serum and liver expressed as density units showed that the viral DNA titer was 10 times higher in liver ($3.2 \times 10^3 \pm 1.9 \times 10^3$ density units) than in serum ($3.2 \times 10^2 \pm 2.8 \times 10^2$ density units; Figure 1). No statistical differences in TTV-DNA titers in serum or in liver

Figure 1. Autoradiography of a 6% polyacrylamide gel showing the semi-quantitation of the TTV-DNA titers in liver (L) and serum (S) of six patients. M, ϕ X174 DNA digested with *Hinf*I (Promega).

were found between patients with liver disease coinfecting with HBV or HCV, patients with liver disease infected only by TTV and the two cases without evidence of liver disease but with TTV-DNA in serum and liver (Table 2). Furthermore, no statistical correlation between TTV-DNA titers in serum and liver was found ($r = 0.28$; $P = 0.535$).

The nucleotide sequence of the TTV ORF-1 isolated from serum and liver of the seven patients with liver disease infected only by TTV, three randomly selected patients infected with TTV plus HBV or HCV, and the two TTV-positive patients without liver disease was compared. The genetic divergence among the consensus sequences of the TTV isolates in each group of patients ranged from 1% to 42%. On the other hand, the genetic divergence ranged from 3% to 43% between the consensus sequences from each group of patients and from 3% to 39% between the isolates from the patients infected only by TTV (with and without liver disease) and those with HBV or HCV coinfection. The phylogenetic analysis performed with the nucleotide sequences of the isolates from our patients and those of all of the TTV genotypes deposited in GenBank showed that the sequences of the 12 patients studied grouped together to those of the genotypes 1a, 1b, and 2, with bootstrap values of 99.7, 98.1, and 89.6, respectively (Figure 2).

TTV genome was detected by *in situ* hybridization in the liver specimens from the 17 patients with serum and liver TTV-DNA detectable by PCR. Hybridization signals

Figure 2. Neighbor-joining tree constructed with the nucleotide sequences of the TTV isolates from serum and liver of seven patients with liver disease (patients 1–7), three patients with liver disease coinfecting by HBV or HCV (patients 8–10), and two patients without liver disease (patients 11 and 12). The isolates are designated as the patient number plus S or B, indicating the origin from serum or liver, followed by the clone number. The GenBank accession number of the published TTV sequences included in the tree are: AB016942 and AB017767 (genotype 1a); AB017879 (genotype 1b); AB016952, AB017882, AB018904, AB017884, and AB060549 (genotype 2); AB017774 and AB018960 (genotype 3); AB017775 and AB017887 (genotype 4); AB017776 (genotype 5); AB017777 and AB017889 (genotype 6); AB017778 (genotype 7); AB017779 (genotype 8); AB017780 and AB017782 (genotype 9); AB017783 (genotype 10); AB017613 (genotype 11); AB021081 and AB021088 (genotype 12); AB021089 (genotype 13); AB021082 and AB021083 (genotype 14); AB021087 (genotype 15) and AB021084 (genotype 16). The bootstrap values are depicted in the nodes of the tree.

Table 2. TTV-DNA Titers in Serum and Liver of the Positive Patients Included in the Study

Patients	TTV-DNA Titers* (density units)	
	Serum	Liver
TTV alone/with liver disease $n = 7$	$3.0 \times 10^2 \pm 1.6 \times 10^2$ Range, 10^2 to 5×10^2	$2.3 \times 10^3 \pm 2.9 \times 10^3$ Range, 3×10^3 to 9×10^3
TTV alone/without liver disease $n = 2$	2×10^2 and 3×10^2	3×10^3 and 6×10^3
TTV + HBV/HCV with liver disease $n = 8$	$3.5 \times 10^2 \pm 2.8 \times 10^2$ Range, 3×10^2 to 5×10^2	$4.0 \times 10^3 \pm 2.6 \times 10^3$ Range, 3×10^3 to 7×10^3

*Expressed as the mean $\pm$ SD.

Figure 3. Detection of TTV-DNA in liver biopsies. **a:** Hybridization of a liver section from a negative control (TTV-DNA-negative in serum). **b:** *In situ* detection of TTV-DNA in the liver biopsy from a patient with TTV-DNA in serum and liver by PCR. **c, d:** Hybridization in serial sections from a liver biopsy of a TTV-DNA-positive patient predigested (**c**) and nondigested (**d**) with DNase. The **arrow** shows an example of a hepatocyte in which DNA treatment precludes TTV-DNA detection. **e–g:** Intracellular distribution of TTV-DNA in the nucleus (**e**), perinuclear (**f**), and in the cytoplasm (**g**). Counterstained with safranin. Original magnifications: **a–d**, $\times 400$; **e–g**, $\times 1000$.

were not observed in the liver biopsies from the 15 patients with liver disease without TTV-DNA in both serum and liver or in those from the six patients without evidence of liver disease that were TTV-DNA-negative in serum and liver by PCR (Figure 3, a and b).

DNase treatment before the hybridization abolished the hybridization signals (Figure 3c, 3d) although RNase pretreatment did not change the hybridization pattern. Competitive hybridization with an excess of unlabeled pCTTV plasmid diminished the signal intensity. The specificity of the *in situ* hybridization technique was further confirmed by the lack of positive signals when the slides were hybridized with the plasmid without TTV-derived sequences and when the probe was omitted in the hybridization mixture.

Regarding the liver cell types infected by TTV, *in situ* hybridization revealed positive signals only in the hepatocytes of the liver samples analyzed. The infected hepa-

toocytes were randomly distributed throughout the liver sections with no clustering of the hybridization signals in any specific area of the liver biopsies. With respect to the intracellular localization of the hybridization signals, these were mainly detected in the cytoplasm and in the nucleus of the hepatocytes although perinuclear staining was also observed in scattered hepatocytes (Figure 3, e–g). The pattern of TTV distribution and cellular localization patterns were similar in the 15 patients with liver disease and in the two cases with normal liver histology. No pathological features were observed in the hepatocytes showing hybridization signals. Furthermore, no topographical correlation was found between hepatocytes showing hybridization signals and the hepatocyte steatosis, siderosis, or the inflammatory infiltrate in the patients with cryptogenic hepatitis (Figure 4).

The mean percentage of TTV-infected hepatocytes was $11.49 \pm 8.79\%$ (range, 2.1% to 30%) in the patients

Figure 4. *In situ* detection of TTV-DNA in the liver biopsy from a patient with hepatic steatosis showing that there is no topological relationship between the presence of TTV in the hepatocytes (solid arrows) and the lipid droplets (open arrows).

studied. There were no statistical differences in the percentage of infected hepatocytes between the seven patients infected only by TTV ($6.74\% \pm 4.2\%$; range, 2.8% to 15%) and the eight patients coinfecting by HCV or HBV ($16.27\% \pm 10.5\%$; range, 2.1% to 30%) and the two cases without liver disease (8% and 10.3%, respectively).

The percentage of TTV-infected hepatocytes correlated significantly with the TTV-DNA titers in liver ($r = 0.54$; $P = 0.037$). A statistically significant correlation was observed between the percentage of TTV-infected hepatocytes in the 15 patients with liver disease infected by TTV and the ALT level ($r = 0.73$; $P = 0.002$). Interestingly, when the patients were divided into those having only TTV infection and TTV plus HCV or HBV infection, the statistically significant correlation between the percentage of infected hepatocytes and ALT levels was only maintained in the group of patients infected by TTV alone ($r = 0.8$; $P = 0.031$). Furthermore, in these patients intrahepatic TTV-DNA titers also correlated significantly with the ALT levels ($r = 0.52$; $P = 0.04$).

No correlation was observed between the percentage of TTV-infected hepatocytes and the histological activity index or its individual scores in TTV-infected patients coinfecting with HCV or HBV.

Finally, no statistical differences were observed in the histological activity index or in its scores between TTV-positive patients coinfecting with HCV or HBV and

the patients infected with HCV or HBV but without TTV (Table 3).

Discussion

Recently, Nishizawa et al⁸ reported the isolation of a novel virus named TTV from the serum of a patient with posttransfusional hepatitis without markers of any known hepatitis viruses. Since then, several authors have found an association between the presence of TTV in serum and liver disease^{9,13,22} whereas others have not.¹⁴ However, to associate TTV infection with liver damage the presence of the virus in liver cells must be demonstrated. In this sense, Okamoto et al⁹ have reported the detection of TTV-DNA by PCR in liver at titers 10- to 100-fold higher than those in serum. These data together with the fact that TTV-DNA is secreted into bile¹⁵ suggest the possible hepatotropism of the virus. In this report, we have retrospectively analyzed the presence of TTV-DNA by PCR and *in situ* hybridization in the liver biopsies of 30 patients with liver disease for whom the TTV-DNA status in serum had been previously established and prospectively in eight cases without evidence of liver damage.

TTV-DNA was detected by PCR in the liver samples from the 15 patients with circulating TTV but not in the remaining 15 patients without the viral DNA in serum. Furthermore, TTV-DNA was detected in serum and liver from two of the eight cases without liver disease. The specificity of the detection was further confirmed by the data obtained from direct sequencing of the PCR products that demonstrated that the amplified DNA corresponded to the TTV genome. The semiquantitation of the PCR products shows, in agreement with previous reports, that the TTV-DNA titers were 10 times higher in liver ($3.2 \times 10^3 \pm 1.9 \times 10^3$ density units) than in serum ($3.2 \times 10^2 \pm 2.8 \times 10^2$ density units). However, no correlation was found between the viral DNA titers in liver and in serum. There are two possible explanations for this finding. First, that TTV may infect the liver cells but does not replicate in them, and second, that the main site of TTV replication may not be the liver. In this sense, TTV-DNA has been detected in peripheral blood mononuclear cells (PBMCs).²³ The verifications of these hypotheses deserve future research.

When TTV detection was performed by *in situ* hybridization, positive signals were observed in all liver samples in which viral DNA was detected by PCR but not in the TTV-PCR-negative biopsies. The specificity of the *in situ* hybridization technique was further confirmed by the results obtained in the specificity test.

Regarding the cell types infected by TTV within the liver, hybridization signals were only observed in the hepatocytes, in both the patients with liver disease of viral or nonviral origin and the cases with normal liver histology. All considered, the data on PCR and *in situ* hybridization show that TTV can infect liver cells. However, hepatocytes with hybridization signals do not show any morphological changes, suggesting that TTV is not pathogenic for the infected cells.

Table 3. Histological Activity Index (HAI) of the Patients with Chronic Hepatitis B or C (Infected or Not by TTV) Included in this Study

	TTV-positive patients (n = 8)	TTV-negative patients (n = 26)
HAI	7.12 ± 4.05	6.50 ± 2.46
Periportal necrosis	2.25 ± 1.48	2.60 ± 0.84
Intralobular degeneration	2.00 ± 2.00	1.50 ± 1.35
Portal inflammation	2.87 ± 0.99	2.40 ± 0.84
Fibrosis	1.75 ± 1.66	1.70 ± 1.33

The hybridization signals in the hepatocytes were detected mainly in the cytoplasm and in the nucleus. However, due to the method that was used for the visualization of the *in situ* hybridization signals (light microscopy) it was not possible to discriminate if the signals observed in the nucleus were inside the nucleus or in the thin cytoplasmic layer between the cell membrane and the nucleus.

The number of TTV-infected hepatocytes was similar in the patients with liver disease (from 2.1% to 30%) and in the two cases without liver diseases (8% and 10.3%) and these percentages correlate significantly with the viral titers in the tissue. On the other hand, no differences in the number of TTV-positive hepatocytes were found between those patients positive only for TTV and those also infected by HCV or HBV. This finding suggests that HCV or HBV infection did not inhibit TTV entry into the hepatocytes or TTV replication.

A statistically significant positive correlation between the ALT levels and the number of TTV-infected hepatocytes as well as with the intrahepatic TTV-DNA titers was found in the seven patients infected only by TTV. This finding suggests that TTV may have contributed to the liver disease in these patients. As the TTV was the only etiological agent of liver disease found in two of these patients, it could be assumed that TTV is responsible for some cases of cryptogenic hepatitis, as some other authors have suggested.^{9,13,23} The fact that TTV-infected cells did not show morphological changes argues against this notion. However, TTV may be pathogenic for the infected cells by mechanisms such as alteration of the cell membrane permeability that does not imply necessarily the alteration of the cell morphology. On the other hand, the remaining five patients had a well-known cause of liver disease (liver steatosis, ductopenia, and siderosis). In addition, no topological correlation between hepatocytes showing hybridization signals and the steatosis, siderosis, or the inflammatory infiltrate was observed. Furthermore, as mentioned above, TTV was also detected in the liver of two cases with normal liver histology without differences in the infection and distribution pattern with respect to the patients with liver disease. Considered as a whole, our results indicate that TTV may not be pathogenic for the liver in all cases.

It may be argued that the TTV strain or the complexity of the viral population are different in the patients with liver disease of nonviral origin and the cases with normal liver histology. However, the sequencing data obtained from the cloned PCR products do not support this hypothesis as the TTV subtype and the complexity of the viral populations infecting both groups were similar, at least in the ORF-1 of TTV-DNA. However, changes in other regions of the viral genome that may account for a different pathogenicity of distinct viral strains cannot be discarded.

Finally, no relation between the percentage of TTV-positive hepatocytes and ALT levels and the histological activity index was found in TTV-positive patients coinfecting by HCV or HBV. Once again, this finding suggests that TTV has a minor role in causing liver disease.

In conclusion, using a specific *in situ* hybridization technique, in this study we have demonstrated that TTV can infect liver cells although our results do not support an important role for TTV in liver pathogenesis.

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