

# Clinicopathological study on TTV infection in hepatitis of unknown etiology

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## Abstract

**AIM:** To investigate the state of infection, replication site, pathogenicity and clinical significance of transfusion transmitted virus (TTV) in patients with hepatitis, especially in patients of unknown etiology.

**METHODS:** Liver tissues taken from 136 cases of non-A non-G hepatitis were tested for TT virus antigen and nucleic acid by *in situ* hybridization (ISH) and nested-polymerase chain reaction (PCR). Among them, TT virus genome and its complemental strand were also detected in 24 cases of autopsy liver and extrahepatic tissues with ISH. Meanwhile, TTV DNA was detected in the sera of 187 hepatitis patients by nested-PCR. The pathological and clinical data of the cases infected with TTV only were analyzed.

**RESULTS:** In liver, the total positive rate of TTV DNA was 32.4% and the positive signals were located in the nuclei of hepatocytes. In serus, TTV DNA was detected in 21.4% cases of hepatitis A-G, 34.4% of non-A non-G hepatitis and 15% of healthy donors. The correspondence rate of TTV DNA detection between liver tissue with ISH and sera with PCR was 63.2% and 89.3% in the same liver tissues by ISH and by PCR, respectively. Using double-strand probes and single-strand probes designed to detect TTV genome, the correspondence rate of TTV DNA detected in liver and extrahepatic tissues was 85.7%. Using single-strand probes, TTV genome could be detected in liver and extrahepatic tissues by PCR, but its complemental strands (replication strands) could be observed only in livers. The liver function of most cases infected with TTV alone was abnormal and the liver tissues had different pathological damage such as ballooning, acidophilia degeneration, formation of apoptosis bodies and focus of necrosis, but the inflammation in the lobule and portal area was mild.

**CONCLUSION:** The positive rate of TTV DNA among cases of hepatitis was higher than that of donors, especially in patients with non-A non-G hepatitis, but most of them were coinfecting with other hepatitis viruses. TTV can infect not only hepatocytes, but also extrahepatic tissues. However, the chief replication place may be liver. The infection of TTV may have some pathogenicity. Although the pathogenicity is comparatively weak, it can still damage the liver tissues.

**The lesions in acute hepatitis (AH) and chronic hepatitis (CH) are mild, but in severe hepatitis (SH), it can be very serious and cause liver function failure, therefore, we should pay more attention to TTV when studying the possible pathogens of so-called "liver hepatitis of unknown etiology".**

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## INTRODUCTION

In 1997, Nishizawa first found and reported a virus which was associated with post-transfusion hepatitis and named it transfusion transmitted virus. TTV is an unenveloped, single-stranded and circular DNA virus, which consists of 3852 nucleotides and most closely resembles the members of the Circoviridae family<sup>[1-3]</sup>. Meanwhile, different studies suggested that TTV genotypes were so much and could vary frequently<sup>[4-8]</sup>. It is believed that TTV belongs to hepadnavirus and replicates chiefly in the liver<sup>[9-11]</sup>, however, some researchers think that the replication site is in the bone marrows rather than in the livers<sup>[12]</sup>. At present, epidemiological studies suggest that TTV is mainly transmitted by blood<sup>[13-15]</sup>; blood products<sup>[16]</sup> and body fluid routes<sup>[13,17]</sup>, it can also be transmitted by mother-to-child vertical<sup>[18]</sup> and horizontal<sup>[19,20]</sup> route, fecal-oral route<sup>[9,15,21]</sup> and sexual route<sup>[22]</sup>. The infection rate of TTV DNA varies largely in healthy population and patients with liver diseases, but generally, it is higher in patients than in healthy donors<sup>[7,23-26]</sup>. The discovery of TTV once put a light on the etiological diagnoses of hepatitis cases of unknown etiology, unfortunately, the pathogenicity and clinical significance of TTV remain doubtful at this time<sup>[25,27-29]</sup>. To clarify the infection status, replication place, pathogenicity and clinical significance of TTV among hepatitis cases, especially in patients of unknown etiology, we conducted the research to test TTV DNA comprehensively among large-sample cases of hepatitis.

## MATERIALS AND METHODS

### Patients

We studied 136 hepatitis cases of unknown etiology, including 45 females and 91 males, aged 16-77 years. By serological screening, all the cases were proved to have not been infected by hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis E virus (HEV), hepatitis G virus (HGV), cytomegalovirus (CMV) or Epstein-Barr virus (EBV). Meanwhile, alcohol hepatopathy, hepatopathy caused by drugs, autoimmunity hepatopathy and liver damage caused by other systemic diseases were eliminated on the basis of the history and other clinical indexes. According to the pathological diagnostic criteria set up at the 10th National Meeting of Infection Diseases in September 2000, Xi'an, China, there were 51 cases of acute hepatitis (AH), 41 chronic hepatitis (CH), 9 acute severe hepatitis (ASH), 11 subacute severe hepatitis (SSH), 5 chronic severe hepatitis (CSH), 11 liver cirrhosis (LC) and 8

hepatocarcinoma (HCC). The liver biopsies were performed in all patients except 28 who died of liver failure and autopsies were performed. Among the 28 cases, extrahepatic tissue samples (including pancreases, kidneys, spleens and hearts) were obtained from 24 cases. The control group consists of 31 patients who had undergone abdominal operations for other diseases except liver diseases. Paired sera were also obtained from 19 of 136 cases. Sera were collected from 42 cases of non-A non-G hepatitis and from 126 cases of hepatitis A-G. Sera of 20 donors from blood bank in Beijing You'an Hospital served as controls. All the specimens of the liver tissues and the extrahepatic tissues were fixed immediately in 100mL·L<sup>-1</sup> neutral formalin and embedded in paraffin, and then sliced continuously to make 5µm thick sections and stained with hematoxylin-eosin (HE). After that, the common pathological damages of virus hepatitis were observed under microscopy and the inflammation and fibrosis of the hepatic lobules were assessed according to the Histology Activation Index (HAI, Knodell Grade) and the pathological diagnoses were made.

## Methods

**Design and synthesis of primers** The primers of PCR were contrived using the molecular biology software "Goldkey" (by the Chinese Academy of Military Medical Sciences, Beijing, China) and OLIGO 5.0 (USA), the sequence was based on the full-length TTV genome published in the Gen Bank (registry code: AF079173). The sequences were as follows: outer primers: P<sub>1</sub>: 5'-ACAGACAGAGGAGAAGGCCAAC-3'; P<sub>2</sub>: 5'-AACAGGCACATTACTACTACC-3', inner primers: P<sub>3</sub>: 5'-CCAGGAGCATATACAGAC-3', P<sub>4</sub>: 5'-TGTACTIONTCTTGCTGGTGAAAT-3'.

**Label of double-strand TTV DNA probes** Using the plasmid pGEM-ORF1 that contains the sequences of TTV genome as template (plasmid pGEM-ORF1 was provided by the Chinese Academy of Military Medical Sciences), the double-strand probes of TTV DNA were labeled with digoxigenin by PCR following the manual of Boehringer Mannheim digoxigenin kit. In the presence of template (3µL), 10×PCR buffer (6µL), labeling mixture with Dig-11-dUTP (2µL), primer P1 and P2 (1µL separately) and Taq DNA polymerase (1µL), PCR was performed for 30 cycles (94°C for 40s with an additional 3min in the first cycle, 55°C for 40s, and 72°C for 50s) in a reaction volume of 60µL. The products were separated by electrophoresis to purify the probes. The labeled probes were homologous with the 1900-2208bp region of the TTV DNA that was a part of the TTV DNA ORF1. The total length of the probe was 309bp and the titer was 1mg·L<sup>-1</sup>.

**Label of single-strand TTV DNA probes** The single-strand TTV DNA probes were labeled by asymmetric PCR, which was performed under the same conditions as the double-strand probes, except that the primer P1 and P2 were added in a rate of 1:100. The two primers were used to label the two single-strand probes. One could be used to detect the genome strand of TTV DNA (probe P37), the other could be used to detect its complementary strand (probe P38).

**In situ hybridization** Paraffin embedded sections were first parched at 60°C for 3-5h, deparaffined in xylene, digested by protease K, fixed in 40g·L<sup>-1</sup> polyformaldehyde, dehydrated through graded alcohol, and prehybridized for 10min. Then hybridization liquid was added (5-10 drops/section). After denatured for 10min (95°C in wet bin), the sections were subsequently incubated for 16-20h at 68°C in water bath bin, washed by 2×SSC, blocked by goat serum/BSA, incubated with anti-Dig-Ap. At last, chromogenic reaction was developed with NBT, BCIP lucifugously.

The confirmation assays included: (1) blank controls: the positive sections were hybridized with prehybridization solution instead of Dig-TTV DNA probes; (2) replacement assays by other probes: the positive sections were hybridized with Dig-HBV DNA probes but not TTV DNA probes; and (3) the negative sections of normal liver tissues were rehybridized, using probes with higher titer.

**Nested-PCR** After the paraffin-embedded sections had been deparaffined and treated with alcohol, the liver tissues were homogenized and incubated in proteinase K-sodium dodecyl sulfate at 52°C for 3h. And then, nucleic acids were extracted from them with phenol-chloroform and precipitated with ethanol. DNA species of chromosomal origin, which emerged as a cloudy precipitate immediately after addition of ethanol, were removed. The remaining nucleic acids were collected by centrifugation and dissolved in 200µL Tris-HCl buffer (10mmol·L<sup>-1</sup>, pH 8.0) containing 1m·L<sup>-1</sup> EDTA (TE buffer). Sera (1mL) was diluted twofold with Tris-HCl buffer (50mmol·L<sup>-1</sup>, pH 8.0) containing 150mmol·L<sup>-1</sup> NaCl and 1mmol·L<sup>-1</sup> EDTA and centrifuged in a TLA 100.3 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 190583G for 1h. The pellet was suspended in Tris-HCl buffer (10mmol·L<sup>-1</sup>, pH 8.0) containing 5mmol·L<sup>-1</sup> EDTA and supplemented with 0.5mg proteinase K per mL as well as 5g·L<sup>-1</sup> sodium dodecyl sulfate and incubated at 65°C for 2h as the direction of the STG Kit (Biotronics Tech. Corp., USA). Then, nucleic acids were extracted with phenol-chloroform, precipitated with ethanol and dissolved in 100µL of TE buffer.

Using DNA (0.1ng) extracted from the sera or livers as a template, the first-round PCR was performed with P1 and P2 (100µmol·L<sup>-1</sup> respectively) for 35 cycles (94°C for 40s with an additional 3min in the first cycle, 55°C for 40s, and 72°C for 40s with an additional 7min in the last cycle) in the presence of 10×PCR buffer (3µL), dNTPs (100µmol·L<sup>-1</sup>) and Taq DNA polymerase (1U) in 30µL of reaction volume (the reverse transcriptase, Taq DNA polymerase, dNTPs and T4 DNA Ligase were provided by Sino-American Biotech. Inc. Beijing). On the products of the first-round PCR (2µL), the second round was performed for 30 cycles under the same conditions as in the first round.

**Detection of other pathogens** The markers of hepatitis A-G viruses, CMV and EBV in sera were screened by ELISA, the steps were according to the directions of the Kits (anti-HAV IgM Kit, HBV-M Kit, anti-HCV IgM IgG Kit, HDV-M Kit and anti-HEV IgM Kit were purchased from Sino-American Biotech. Inc. Beijing, and anti-CMV IgM, anti-EBV IgM were the products of Delyea Inc., Italy). The antigens of HBV, HCV, HGV, EBV and CMV expressed in liver tissues were assayed by immunohistochemical staining using the streptavidin-peroxidase (S-P) method. The monoclonal antibody Kit against HBsAg and polyclonal antibody Kit against HBcAg were purchased from Maxim Biotech. Inc. Fuzhou, China. Purified rabbit monoclonal antibodies against HCV NS5-Ag, HGV NS5-Ag and EBV-Ag were provided by the Institute of virology of Chinese Academy of Preventive Medicine and Institute of Bacteriology & Epidemiology of Chinese Academy of Military Mmedical Sciences. Serum alanine aminotransferase (ALT) and total serum bilirubin (TSB) were assayed by automatic analyzer (COMBAS).

## Statistical analysis

Aided by statistical software SPSS 10.0, statistical analysis was performed.  $\chi^2$  test was used for unpaired enumeration data and McNemar test for paired enumeration data. If they were measurement data and normal distribution, we expressed them as  $\bar{x} \pm s$  (standard deviation) and the comparison among groups were performed by unpaired Student's *t* test. *P* values less than 0.05 was considered significant.

## RESULTS

### TTV DNA in liver tissues

In all the 136 samples of liver tissues, the total positive rate of TTV DNA was 32.4% (44/136). It was 27.5% (14/51) in acute mild hepatitis group, 36.6% (15/41) in chronic hepatitis, 45.5% (5/11) in subacute severe hepatitis, 40.0% (2/5) in chronic severe hepatitis, 36.4% (4/11) in active liver cirrhosis and 50% (4/8) in primary hepatocarcinoma. No TTV DNA could be detected in the cases of acute severe hepatitis. The positive rate in chronic liver diseases (39.5%) was significantly higher than that in acute liver diseases (23.3%,  $P=0.046$ ). However, there was no significant difference as compared with control group. After detection of other pathogen by immunohistochemical staining and *in situ* hybridization, 13 cases were confirmed to be infected by TTV alone, including 5 cases of acute hepatitis, 6 cases of chronic hepatitis and 2 cases subacute severe hepatitis (Table 1).

**Table 1** TTV DNA in liver tissues of hepatitis

Type	n	TTV(+) n(%)	TTV(-) n(%)	TTV alone n(%)
AH	51	14(27.5)	37(72.5)	5(9.8)
CH	41	15(36.6)	26(63.4)	6(14.6)
ASH	9	0(0.0)	9(100.0)	2(18.2)
SSH	11	5(45.5)	6(54.5)	0(0.0)
CSH	5	2(40.0)	3(60.0)	0(0.0)
LC	11	4(36.4)	7(63.6)	0(0.0)
HCC	8	4(50.0)	4(50.0)	0(0.0)
Total	136	44(32.4)	92(67.6)	13(9.6)
Normal	31	8(25.8)	23(74.2)	

The positive signal of TTV DNA predominantly located in the nucleoli of the hepatocytes with an appearance of blue grain. It also could be found in the cytoplasm of the hepatocytes but much weaker than that in the nucleoli. In acute hepatitis, the positive cells were distributed diffusely in the interlobular areas, and in chronic hepatitis they were aggregated in the periportal areas and in active liver cirrhosis they clustered in the pseudolobules. The positive signals could also be found in the hepatocarcinoma cells.

### TTV DNA in sera

TTV DNA could be detected in 21.4% (27/126) cases of hepatitis A-G, 34.3% (21/61) cases of non A non G hepatitis and 15% (3/20) healthy donors. There was no significant difference between the donors and the patients of hepatitis. Classified by the clinical diagnosis in AH, CH, SH and LC groups, the positive rates were 29.0% (18/

62), 23.5% (19/81), 52.9% (9/17) and 7.4% (2/27), respectively. The positive rate in SH group was significantly higher than in control group ( $P=0.014$ , Table 2).

**Table 2** TTV DNA in sera of patients

Type	n	TTV(+) n(%)	TTV(-) n(%)
NA-NG	61	21(34.4)	40(65.6)
A-G	126	27(21.4)	99(78.6)
AH	62	18(29.0)	44(71.0)
CH	81	19(23.5)	62(76.5)
SH	17	9(52.9)	8(47.1) <sup>a</sup>
LC	27	2(7.4)	25(92.6)
Total	187	48(25.7)	139(74.3)
Control	20	3(15.0)	17(85.0)

<sup>a</sup> $P<0.05$ , vs Control

In liver tissues and sera of patients of non-A non-G hepatitis, the positive rate of TTV DNA (33.0%) was significantly higher than that of hepatitis A-G (21.4%) ( $P=0.025$ ).

Among the 19 paired samples of sera and liver tissues, 12 cases had the same results when testing liver tissues by ISH and testing sera by PCR, although 5 liver tissues were positive while the paired sera were negative and 2 sera were positive but the paired liver tissues were negative. However, there was no significant difference between them ( $P=0.453$ ).

Fifty-six liver tissue specimens were assayed by PCR and at the same time by ISH. The coincidence rate of the results was 89.3% (50/56). Five cases were positive by PCR while negative by ISH and only 1 case was negative by PCR but positive by ISH. The difference was not statically significant either ( $P=0.219$ ). Fifty-six specimens of liver, pancreas, kidney, spleen and heart tissues were tested by PCR using the two probes respectively. The results suggested that the total coincidence rate was 85.7% (48/56). The rest showed positive outcomes using one probe but negative outcomes using another probe in equal proportion. The difference using the two probes was not significant ( $P=1.000$ ).

Paired specimens of liver, pancreas, kidney, spleen and heart tissues from 8 patients in whom TTV DNA had been found in the liver tissues by PCR using double-strand probes, were assayed again using P37 probes and P38 probes, respectively. The results showed that using P37 probes, 7, 7, 3, 5, 1 cases were positive in liver, pancreas, kidney, spleen and heart tissues, respectively. However, using P38 probes, only 5 specimens of liver tissues were positive and all the extrahepatic tissues were negative (Table 3).

**Table 3** TTV DNA genome and its complement chain in liver and extrahepatic tissues by PCR using single-strand probes

No.	Diagn.	Liver			Pancreas			Kidney			Spleen			Heart		
		DP	P37	P38	DP	P37	P38	DP	P37	P38	DP	P37	P38	DP	P37	P38
1	CSH	+	+	+	-	+	-	+	+	-	+	+	-	-	/	/
2	CSH	+	-	+	-	-	-	+	-	-	+	-	-	/	/	/
3	HCC	+	+	-	+	+	-	-	-	-	+	+	-	/	/	/
4	HCC	+	+	-	-	+	-	-	-	-	-	-	-	/	/	/
5	LC	+	+	+	+	+	-	+	+	-	+	+	-	+	/	/
6	LC	+	+	+	+	+	-	-	-	-	+	+	-	/	/	/
7	LC	+	+	+	+	+	-	+	+	-	-	+	-	/	/	/
8	SSH	+	+	-	+	+	-	+	-	-	-	-	-	/	/	/

DP: double-strand probe

### Liver damage of cases infected with TTV alone

In AH group, serum ALT and TSB in patients infected with TTV alone ( $325\pm 222\text{ nkat}\cdot\text{L}^{-1}$ ,  $91\pm 115\mu\text{mol}\cdot\text{L}^{-1}$ , respectively) were lower than that in patients infected with HBV only ( $931\pm 630\text{ nkat}\cdot\text{L}^{-1}$ ,  $134\pm 118\mu\text{mol}\cdot\text{L}^{-1}$ , respectively), the difference of ALT being statistically significant ( $P=0.005$ ). However, in SH group, there was no significant difference of ALT and TSB between cases infected with TTV alone ( $509\pm 361\text{ nkat}\cdot\text{L}^{-1}$ ,  $304\pm 125\mu\text{mol}\cdot\text{L}^{-1}$ , respectively) and cases infected with HBV alone ( $435\pm 239\text{ nkat}\cdot\text{L}^{-1}$ ,  $226\pm 96\mu\text{mol}\cdot\text{L}^{-1}$ , respectively) ( $P>0.05$ , Table 4).

**Table 4** Liver function of cases infected by TTV alone and by HBV alone

	Pathogen	ALT(nkat·L <sup>-1</sup> )	TSB(μmol·L <sup>-1</sup> )
AH	TTV alone	325±222 <sup>b</sup>	91±115
	HBV alone	931±630	134±118
SH	TTV alone	509±361	304±125
	HBV alone	435±239	226±96
CH	TTV alone	187±150	98±199

<sup>b</sup> $P<0.01$ , vs HBV

In the liver tissues of patients with chronic hepatitis caused by TTV alone, some histological lesions could be found in hepatic lobule, including ballooning degeneration, acidophile degeneration, formation of apoptotic bodies, focus of necrosis, amplification of portal areas and infiltration of mononuclear cells, but the inflammation and fibrosis were mild. The histological activity index ( $3.23\pm 1.86$ ) was lower than that in cases infected by HBV only ( $5.69\pm 1.34$ ), the difference being statistically significant ( $P=0.001$ ).

In cases without detectable pathogens in sera at present, after histological assay, 25.7% (35/136) were still of unknown etiology and 50.0% (68/130) could be explained to be the infection of HBV and/or HCV. Infection with TTV alone and with HGV only accounted for 9.6% (13/136) respectively, and the rate of coinfection with TTV and HGV was 5.1% (7/136) and the others were mixed infected with TTV/HGV and HBV/HCV.

### DISCUSSION

Up to now, the epidemiological survey of TTV suggested that the infection of TTV globally distributed in all kinds of species. It was reported that the positive rate of TTV in healthy donors differed largely, ranging from 1% to 93%<sup>[30-34]</sup>. Nevertheless, in patients with hepatopathy it ranged from 20% to 80%<sup>[23-25,35]</sup>. These data show that the positive rate of TTV in population varies remarkably, it may be the result of the difference of the primers used in test or the imbalance of the different regions. However, it was higher in cases of liver diseases than that in healthy donors. These conclusions were chiefly drawn on the basis of serological studies, as the investigation of the liver tissues was scarce. In our study, we detected TTV DNA directly in liver tissue specimens by ISH. The results suggested that the total positive rate of TTV DNA was 32.4% in liver tissues of cases of unknown etiology, which was coincident with that in sera (34.4%). It could be conjectured that the results from serological screening can mirror the situation of TTV DNA in liver. Moreover, the positive rate of TTV DNA in AH group (27.5%) was similar to that in the normal controls (25.8%), but it was significantly higher in chronic hepatopathy (39.5%), especially in SSH, CSH and HCC groups, than in acute hepatopathy (23.5%) and control group. It indicated that TTV was common in population, and its infection could cause acute hepatitis and fulminant hepatitis and could develop to chronic hepatitis too. It may be one of the promoters of chronicity and deterioration of acute hepatitis and can affect the development of liver

cirrhosis and hepatocarcinoma. It is worthy notice that no TTV DNA could be found in acute severe hepatitis group. We think that it might be due to the large portion of the necrosis areas.

In sera, the positive rate of TTV DNA in cases of unknown etiology was much higher than that of hepatitis A-G and the lowest in healthy donors, but the difference was not significant. We thought that it was because the samples were not enough, thus we merged the samples of liver tissues and sera to enlarge the samples and perform the statistical analysis again. The results showed the positive rate of TTV DNA in cases of non-A non-G hepatitis (33.0%) was significantly higher than that of hepatitis A-G. Therefore, when we search the pathogens of "hepatopathy with unknown etiology", we must pay more attention to the infection of TTV.

In our study, we used different methods to confirm the results. The data suggested that the positive rate in liver tissues by ISH was similar to that in sera by PCR, there were 5 cases in which TTV was positive in the liver by ISH but negative in sera by PCR, and only 2 cases were reversed. The reason might be the small number of cases or that the copies in liver tissues were higher than that in sera, and in some cases the replication of TTV DNA was so inactive that the corresponding viremia was absent. In the liver, the results of test by ISH were not significantly different from that by PCR and there was no significant difference between the results of test by PCR using the double-strand probes and single-strand probes which were complemented with the genome of TTV DNA. These methods could be used to supplement and verify each other.

TTV was once regarded as a particular hepadnavirus<sup>[36]</sup>, but some studies suggested that TTV DNA could be detected in the bone marrows and the peripheral blood mononuclear cells (PBMC), and the hepatocytes were not the only site where TTV infected and existed<sup>[11,12]</sup>. In our study, we screened the paired tissues of liver, pancreas, kidney, spleen and heart by PCR using double-strand probes and P37 probes. The results showed that TTV DNA could be detected in livers as well as in extrahepatic tissues, but in extrahepatic tissues, the positive rate was lower, and the density and intensity of positive signals were much weaker than that in liver tissues. In general, only in cases with positive TTV in liver, were there positive signals in extrahepatic tissues and there was only one case was reversed. Furthermore, there was no obvious pathological damage in the extrahepatic tissues. In conclusion, TTV can infect not only the liver, but also extrahepatic tissues. Yet, the liver is still the mainly infected site and the infection in extrahepatic tissues is an accompanying status that may act as a refuge to escape the attack of the immunity system of the host. As a result, these places can reinfect the liver as a source of infection and might be responsible for the chronicity of TTV infection and the high prevalence in all populations.

Concerning the replication site of TTV, there are much disputations at present. Okamoto *et al*<sup>[11]</sup> discovered that separated by gel electrophoresis and characterized biophysically, TTV DNA in sera migrated in sizes ranging from 2.0 to 2.5kb. However, TTV DNA in liver tissues migrated at 2.0 to 2.5kb as well as at 3.5 to 6.1kb. Both faster and slower migrating forms of TTV DNA in the liver were found to be circular and the full genomic length being 3.8kb. The further study suggested that TTV DNA migrated at 3.5 to 6.1kb represents a circular, double-strand replication intermediates of TTV. The author concluded that TTV replicated in the liver via a circular double-strand DNA. However, Kikuchi *et al*<sup>[12]</sup> found that in hepatocytes there was only TTV DNA but no mRNA by PCR and Southern hybridization, while in bone marrow tissues, especially specimens coming from biopsy, there were high titers of TTV DNA. The author suggested that TTV replicates chiefly in bone marrows but not in liver, and Okamoto *et al*<sup>[37]</sup> found replication intermediates in marrows too. Furthermore,

Maggi *et al*<sup>[38]</sup> found that TTV can replicate in activated mononuclear cells, and recent studies<sup>[39,40]</sup> suggest that viral replication takes place in multiple tissues at distinct levels in infected individuals. In order to clarify the problem, we contrived and labeled 2 single-strand probes by asymmetric PCR. Because TTV is a single-strand DNA virus, in the process of replication the replication intermediates will appear. Detection of the genomes and its complementary chains using single-strand probes could explain the existence and replication of TTV DNA in different tissues. The results suggested that in positive cases using double-strand probes, positive signals could be detected in parts of the specimens of liver, pancreas, kidney, spleen and heart tissues using single-strand probes designed to detect the TTV genome. However, using single-strand probes designed to detect the replication chain of TTV DNA, positive signals could be seen in part of liver tissues but all the extrahepatic tissues were negative. These results showed again that TTV can infect extrahepatic tissues, but the infection belongs to an accompanying state and liver is still one of the main replication places. Although we have studied the bone marrow tissues and had not compared it to the liver, it is clear at least that TTV can replicate in liver and it is the prerequisite to investigate the pathogenicity of TTV.

Discovery of TTV once triggered the expectations to explain the cases of non-A non-G hepatitis, unfortunately, the pathogenicity and clinical significance of TTV remain doubtful at this time. Most studies suggest that TTV is not responsible for hepatitis and is not one of the chief pathogens of hepatitis<sup>[23,25,30,41-43]</sup>, and in hepatitis B/C, TTV does not seem to cooperate with HBV/HCV and coinfection with TTV does not modify the clinical feature and exacerbate liver damage<sup>[44,45]</sup>. And TTV plays an insignificant role in acute fulminant and non-fulminant hepatitis<sup>[46]</sup>. But some researchers did not think so. They think that TTV may cause epidemic outbreak of hepatitis of unknown etiology by fecal-to-mouth route<sup>[28]</sup>, and be responsible for some cases of non-A non-G hepatitis<sup>[47,48]</sup>. Some studies suggest that TTV can make other systems abnormal<sup>[49,50]</sup> and may be responsible for the development of hepatocellular carcinoma in patients with type C liver disease<sup>[51]</sup>. We selected the patients of non-A non-G hepatitis who had been screened by serological and histological assays by ISH and confirmed to be infected by TTV alone, and then we observed the pathological damages, assessed the damage degree of the liver tissues by the criterion of global “Knodell Grade” and analyzed the liver function. The results show that TTV DNA is mainly located in the nuclei or cytoplasm of the hepatocytes. In the liver tissues of cases infected with TTV only the common pathological damages and inflammation would be found in the interlobular and periportal areas. The positive cells scattered diffusely in the interlobular areas in acute hepatitis and aggregated in periportal areas in chronic hepatitis or in the form of cluster in pseudolobules in the liver cirrhosis. The grade of HAI of acute or chronic hepatitis caused by TTV only was much lower than that caused by HBV. Clinically, the patients might have a raised ALT and TSB in AH group, but the elevation was mostly moderate and ALT was significantly lower than that caused by HBV. However, in SH group, the liver function change caused by TTV was similar to that caused by HBV. In CH group, the grades of inflammation activity of all the cases with TTV alone were G1-2. Therefore, TTV may have some pathogenicity, but the pathogenicity is comparatively weak and the liver damage is not serious in AH or CH. The result is identical with that of the study performed by Ge *et al*<sup>[52]</sup>. However, when it caused fulminant hepatitis, the pathogenicity is not weaker than that of HBV and Yusufu’s study<sup>[53]</sup> demonstrated that TTV may contribute to fulminant hepatic failure as a solitary infectious agent or a co-infectious agent with HCV. In addition, among these 136 cases of unknown etiology, the pathogen of 25.7% (35/136) patients was still unknown after the histological tests, 50% were caused by HBV and/or HCV,

9.6% were infected by TTV alone while 24.3% were contaminated by TTV and/or HGV, the others were coinfecting with TTV/HGV and HBV/HCV. These results suggest that we ought to pay more attention to TTV when we study the possible pathogen of so-called “liver diseases of unknown etiology”, although the conclusion should be verified by more data and more thorough studies.

On the whole, TTV is widespread in population. The positive rate of TTV DNA among hepatitis cases is higher than that of donors, especially in patients of non-A non-G hepatitis, but most of them are coinfecting with other hepatitis viruses. TTV can infect not only hepatocytes, but also extrahepatic tissues. However, the chief replication place may be the liver. The infection of TTV may have some pathogenicity. Although the pathogenicity is comparatively weak, it can still damage the liver tissues. The lesions in acute hepatitis (AH) and chronic hepatitis (CH) groups are mild, but in severe hepatitis (SH) group, it can be very serious and cause failure of liver function, therefore, we should pay more attention to TTV when studying the possible pathogens of so-called “liver hepatitis of unknown etiology”.

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