Virus Research 242 (2017) 166–172

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Prevalence of occult hepatitis B virus infection and Torque teno virus infection and their association with hepatocellular carcinoma in chronic hepatitis C patients

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ARTICLE INFO

Keywords:
Hepatocellular carcinoma
Occult HBV
TTV
Hepatitis C
Hepatocarcinogenesis

ABSTRACT

Background: The role of occult hepatitis B virus (HBV) infection and Torque teno virus (TTV) infection in the development of hepatocellular carcinoma (HCC) in chronic hepatitis C patients is still uncertain.
Aim: The aim of the present study was to investigate the prevalence and significance of OBI and TTV infection, and to examine the genetic diversity of these viruses, in chronic hepatitis C patients with and without HCC.
Methods: Sera from 151 hepatitis C virus (HCV)-infected patients (49 patients with HCC and 102 without HCC) negative for HBV surface antigen (HBsAg) were tested for the presence of OBI and TTV infection by semi-nested and group-specific multiplex PCR assays, respectively. Nucleotide sequencing of HBV S region was further performed.
Results: OBI and TTV infection were detected in 5 (3.3%) and 68 (45%) patients, respectively. HBV isolates were classified into genotypes A (4/5, 80%) and D (1/5, 20%), and no HBsAg escape mutation was observed. TTV phylogenetic group 3 was the most prevalent among both HCC and non-HCC patients. OBI and TTV infection were significantly more frequent in patients with HCC than patients without HCC ($p = 0.003$, and $p = 0.009$, respectively). Moreover, TTV infection was associated with HCC (OR = 2.23, 95% CI = 1.04–4.80, $p = 0.040$), independently of liver cirrhosis.
Conclusions: A low prevalence of OBI was observed in patients with HCV-related chronic liver disease, and TTV infection was an independent factor associated with the occurrence of HCC. Whether TTV influences the progression of liver disease in chronic hepatitis C patients remains to be elucidated.

1. Background

Liver cancer is the second leading cause of cancer-related mortality, estimated to be responsible for over 700,000 deaths each year worldwide (WHO/IARC, 2012). Hepatocellular carcinoma (HCC) is by far the most common type of primary liver cancer with approximately 80% of all cases associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections (Perz et al., 2006). These risk factors induce chronic liver damage often leading to cirrhosis, which is present in 80–90% of patients with HCC (El-Serag, 2011; Llovet et al., 2003).

HBV infection is a major cause of chronic liver disease, affecting over 240 million people worldwide (Schweitzer et al., 2015). HBV contains a partially double-stranded DNA genome of approximately 3200 nucleotides (nt) in length. At present, HBV is classified into 10 genotypes (A-J) and a growing number of subgenotypes (Tong and Revill, 2016). Occult HBV infection is defined by the presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing negative for HBV surface antigen (HBsAg) (Raimondo et al., 2008). Several reports showed a significantly higher prevalence of OBI in HCV-positive patients with HCC compared with non-HCC chronic hepatitis C patients, supporting the hypothesis of a synergistic interaction between OBI and HCV in promoting HCC (Huang and Hollinger, 2014; Pollicino and Saitta, 2014; Shi et al., 2012; Squadrito et al., 2013; Tanaka et al., 2004). However, findings from other studies indicate no influence of OBI on the natural history of chronic hepatitis C, particularly regarding the risk of developing HCC

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http://dx.doi.org/10.1016/j.virusres.2017.09.022
Received 8 June 2017; Received in revised form 27 September 2017; Accepted 28 September 2017
Available online 29 September 2017
0168-1702/

(reviewed in Coppola et al. (Coppola et al., 2015)).

Torque teno virus (TTV) was first identified in 1997 in the serum of a Japanese patient with post-transfusion hepatitis of unknown etiology (Nishizawa et al., 1997). The virus has a single-stranded circular DNA genome of negative polarity, approximately 3800 nt in length (Miyata et al., 1999; Mushahwar et al., 1999). TTV has a wide genetic diversity, with multiple genotypes classified into five major phylogenetic groups (1–5) (Peng et al., 2002). Recently, two new phylogenetic groups (6 and 7) were identified in eastern Taiwan indigenes (Hsiao et al., 2016). Co-infection with TTVs of distinct genotypes/phylogenetic groups in the same individual is a common event (Niel et al., 2000). Due to its high prevalence in the general population, a causative role of TTV in human diseases remains uncertain. However, a higher prevalence of TTV was found to be associated with several pathological conditions such as liver and respiratory diseases, hematological disorders, and cancer (reviewed in Spandole et al. (Spandole et al., 2015)). Particularly, it was suggested that TTV infection may influence the development of cirrhosis and HCC in patients with chronic hepatitis C (Moriyama et al., 2001; Tokita et al., 2002). In addition, certain TTV genotypes/phylogenetic groups were suggested to be more pathogenic than others in patients with liver disease (Okamura et al., 2000; Tanaka et al., 2000; Tokita et al., 2001).

The objective of the present study was to investigate the prevalence and significance of OBI and TTV infection, and to examine the genetic diversity of these viruses, in HCC and non-HCC chronic hepatitis C patients.

2. Methods

2.1. Patients

This study included 151 Brazilian patients with HCV-related chronic liver disease [68 males and 83 females; age 60 ± 10 years (mean ± standard deviation, SD; range 19–86 years)] referred to the Clementino Fraga Filho University Hospital (HUCFF), Rio de Janeiro, Brazil, during the period 2010–2015. Among them, 49 patients had HCC (47 with cirrhosis and 2 without cirrhosis), and 102 patients had chronic hepatitis without HCC (55 with cirrhosis and 47 without cirrhosis). Patients with autoimmune liver disease, alcohol ingestion > 20 g/day, metabolic disorders, hereditary hemochromatosis, and hemoglobinopathies were excluded from the study. Data on the immune status of the patients were not collected; however, no patient was under immunosuppressive drugs. All patients were negative for HBsAg. The diagnosis of HCV infection was based on the simultaneous positivity for anti-HCV and HCV-RNA in the serum. The diagnosis of liver cirrhosis was made by liver biopsy or by the presence of clinical and laboratory features of portal hypertension at ultrasound or upper endoscopy. HCC diagnosis was based on liver imaging (angiography, ultrasonography, and/or computed tomography). The study protocol was approved by the Research Ethics Committee of the Clementino Fraga Filho University Hospital (approval number 139/10) and written informed consent was provided by all study participants.

2.2. Nucleic acids extraction and amplification

Viral DNA was extracted from 0.2 mL of serum by using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Nucleic acids were re-suspended in 50 µL of TE buffer.

Amplification of the HBV S region was attempted by a semi-nested PCR assay. The first round of amplification was performed with 2 µL of extracted DNA, 2 units of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.2 µM forward and reverse primers each. While primer pairs PS1 (5′-CCATATTCTTGGGAACAAGA-3′, nt 2826 to 2845) and SR (5′-CGAACCACTGAACAAATGGC-3′, nt 704–685) were applied in the first PCR round to generate a product of 1099 nt, PS1 was replaced by S1 (5′-CTTCTCGAGGACTGGGGACC-3′, nt 124–143) in the second round, amplifying a product of 580 nt. Thermal conditions for PCR amplifications included an initial denaturation step at 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 57 °C for 40 s and 72 °C for 1 min 30 s followed by a final extension step, 72 °C for 7 min. PCR products were analyzed on 1% agarose gels. Negative and positive controls were added in both extraction and PCR procedures, and a sample was considered positive when HBV DNA was repeatedly found after amplification of newly extracted material. A multiplex PCR assay able to simultaneously detect TTV isolates from phylogenetic groups 1–5 was performed as described previously (Devalle and Niel, 2005). Briefly, a large segment (> 3000 nt) of the TTV genome was first amplified using the 'universal' oligonucleotide primers T1S and T1A. One microliter of reaction product was used for nested multiplex PCR experiments, which were performed in the presence of six primers in a single tube. One of the primers (T2S) was a 'universal' sense primer, while the other five (T2G1A to T2G5A) were antisense primers, each of which specific for a particular TTV phylogenetic group. After electrophoresis, PCR bands of expected sizes (393, 678, 884, 111, and 485 nt for groups 1–5, respectively) were visualized on 3% agarose gel.

Table 1
Characteristics of the patients with chronic hepatitis C according to the occurrence of occult HBV infection.

	Occult HBV positive (n = 5)	Occult HBV negative (n = 146)	*p* value
Age[a]			
< 60	1 (20%)	64 (46.4%)	NS (0.377)
≥60	4 (80%)	74 (53.6%)	
Gender			
Male	3 (60%)	65 (44.5%)	NS (0.658)
Female	2 (40%)	81 (55.5%)	
HCC diagnosis			
Positive	5 (100%)	44 (30.1%)	0.003
Negative	0	102 (69.9%)	
Liver cirrhosis			
Positive	5 (100%)	97 (66.4%)	NS (0.175)
Negative	0	49 (33.6%)	
TTV DNA			
Positive	3 (60%)	65 (44.5%)	NS (0.658)
Negative	2 (40%)	81 (55.5%)	
Anti-HBc			
Reactive	3 (60%)	30 (20.5%)	NS (0.070)
Non-reactive	2 (40%)	116 (79.5%)	
Anti-HBs			
Reactive	2 (40%)	59 (40.4%)	NS (1.000)
Non-reactive	3 (60%)	87 (59.6%)	

NS, not significant.
[a] Age not informed in eight patients.

2.3. HBV direct nucleotide sequencing and genotyping

PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and bidirectionally sequenced using BigDye Terminator Kit v3.1 (Applied Biosystems, Foster City, CA). Sequencing reactions were analyzed on an ABI3730xl automated sequencer (Applied Biosystems). The HBV genotype was determined by phylogenetic analysis using the MEGA program version 6.0 (Tamura et al., 2013) and reference sequences available in GenBank. Nucleotide sequences of the S gene were aligned using ClustalW algorithm implemented in MEGA. Maximum-likelihood phylogenetic analysis was performed using the Kimura 2-parameter model (1000 bootstrap replicates) of MEGA. The HBV serological subtype was predicted based on the deduced HBsAg amino acid sequence using the HBV Serotyper Tool (Bell and Kramvis, 2015). Previously published mutations in the S region associated with "escape" or with diminished antibody binding were predicted by using the Geno2pheno[HBV] online tool available at http://hbv.geno2pheno.org/index.php.

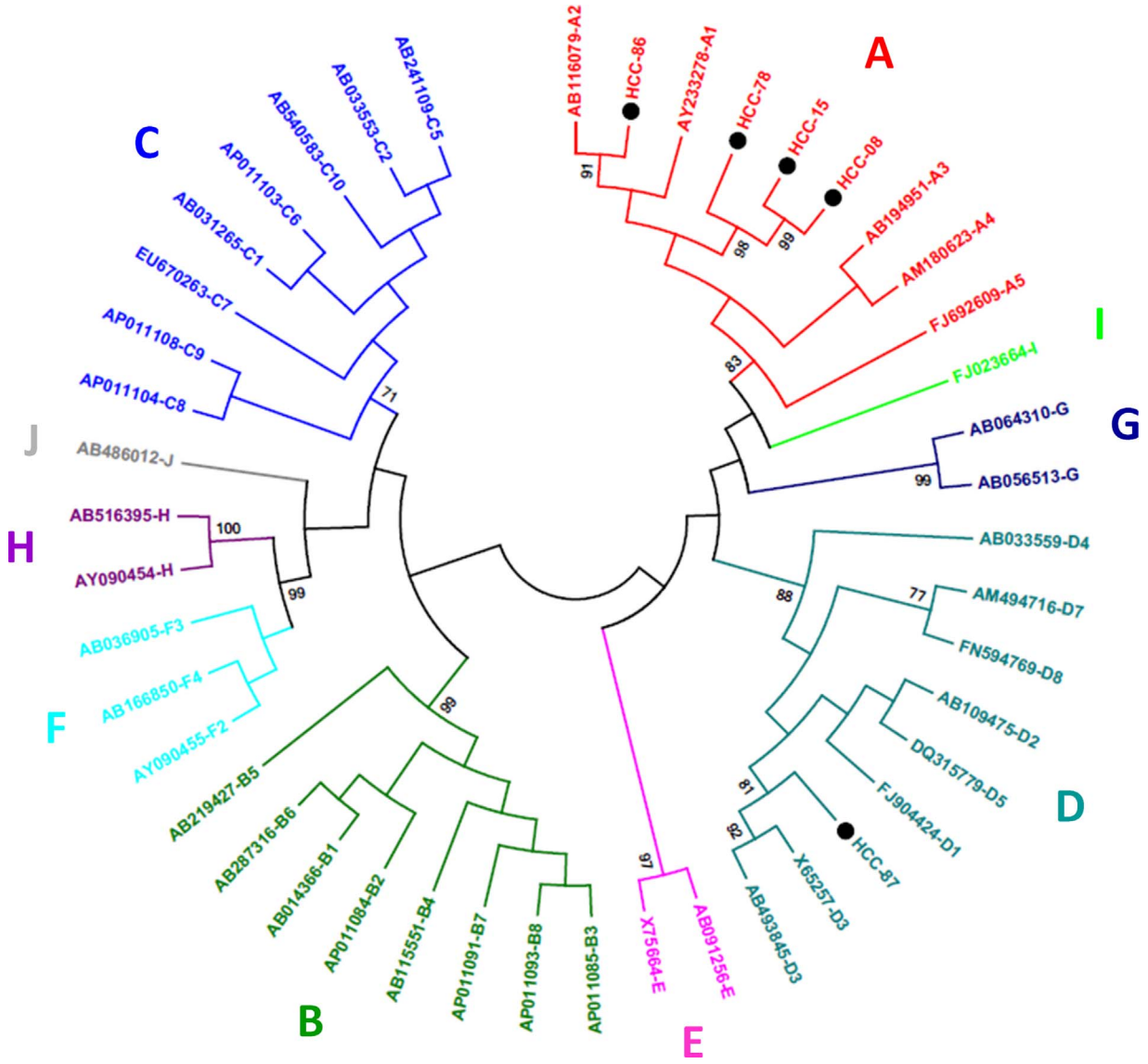

Fig. 1. Phylogenetic analysis of HBV S gene sequences using the Maximum Likelihood method. Reference sequences are indicated by their GenBank accession numbers followed by the subgenotype. Letters A–J represent the HBV genotypes. The sequences generated in this study are denoted HCC, followed by the sample number and are identified with a black circle. Values at internal nodes indicate percentages of 1000 bootstrap replicates that support the branch.

Table 2
HBV genetic and serological data in HCV-infected patients with hepatocellular carcinoma and occult HBV infection.

Patient	Genotype	Serological subtype	HBsAg escape mutation	Serological markers
HCC-08	A	*adw2*	none	anti-HBc; anti-HBs
HCC-15	A	*adw2*	none	anti-HBc; anti-HBs
HCC-78	A	*adw2*	none	none
HCC-86	A	*adw2*	none	anti-HBc
HCC-87	D	*ayw2*	none	none

2.4. Statistical analysis

The associations between categorical variables were assessed using Pearson's Chi square test or Fisher's exact test. A stepwise multivariate logistic regression modeling, defining HCC (with/without) as the dependent variable was performed. The selected covariates included in the model were those with a p value < 0.20 in the univariate analysis. Results were presented as odds ratios (OR) with their 95% confidence intervals (CI). All statistics were performed with SPSS statistical package version 21.0 (SPSS Inc., Chicago, Il, USA) and a 2-tailed p value < 0.05 was regarded as significant.

3. Results

3.1. Comparison between OBI positive and OBI negative patients

Five (3.3%) of the 151 patients with HCV-related chronic liver disease were positive for OBI. All OBI-positive patients were diagnosed with HCC. The comparison of several features between the OBI-positive and-negative patients is shown in Table 1. OBI was significantly associated with occurrence of HCC (p = 0.003). Other variables, such as age, gender, liver cirrhosis, TTV DNA, anti-HBc, and anti-HBs did not differ significantly between the two groups (Table 1).

3.2. HBV genetic and serological characterization of the OBI cases

By phylogenetic analysis, it was demonstrated that four (80%) and

Table 3
Characteristics of the patients with chronic hepatitis C according to the occurrence of TTV infection.

	TTV DNA positive (n = 68)	TTV DNA negative (n = 83)	*p* value
Age[a]			
< 60	20 (30.8%)	45 (57.7%)	0.001
≥60	45 (69.2%)	33 (42.3%)	
Gender			
Male	34 (50%)	34 (41%)	NS (0.324)
Female	34 (50%)	49 (59%)	
HCC diagnosis			
Positive	30 (44.1%)	19 (22.9%)	0.009
Negative	38 (55.9%)	64 (77.1%)	
Liver cirrhosis			
Positive	52 (76.5%)	50 (60.2%)	0.038
Negative	16 (23.5%)	33 (39.8%)	
Occult HBV			
Positive	3 (4.4%)	2 (2.4%)	NS (0.658)
Negative	65 (95.6%)	81 (97.6%)	
Anti-HBc			
Reactive	16 (23.5%)	17 (20.5%)	NS (0.695)
Non-reactive	52 (76.5%)	66 (79.5%)	
Anti-HBs			
Reactive	24 (35.3%)	37 (44.6%)	NS (0.317)
Non-reactive	44 (64.7%)	46 (55.4%)	

NS, not significant.
[a] Age not informed in eight patients.

one (20%) of the five HBV isolates clustered with genotypes A and D reference sequences, respectively (Fig. 1). All genotype A isolates displayed determinants of the serological subtype *adw2*, while the genotype D isolate, *ayw2*. Analysis of the deduced amino acid sequence of HBsAg did not show any escape mutation. Among the five patients with OBI, three had a seropositive-OBI (two were reactive for both anti-HBc and anti-HBs, and one for anti-HBc alone) and two had a seronegative-OBI (anti-HBc and anti-HBs negative) (Table 2).

3.3. Comparison between TTV DNA positive and TTV DNA negative patients

Sixty-eight (45%) of the 151 patients with HCV-related chronic liver disease were positive for TTV DNA. The comparison of several features between the TTV DNA-positive and-negative patients is shown in Table 3. TTV infection was significantly associated with older age ($p = 0.001$), occurrence of HCC ($p = 0.009$) and liver cirrhosis ($p = 0.038$). Other variables, such as gender, OBI, anti-HBc, and anti-HBs did not differ significantly between the two groups (Table 3).

3.4. TTV genetic characterization and co-infection with several TTV phylogenetic groups

Among the HCV infected patients, the TTV phylogenetic group 3 was the most prevalent (75%), followed by group 1 (27.9%), group 5 (25%), group 4 (20.6%), and group 2 (1.5%). Comparison of the prevalence of the phylogenetic groups between patients with and without HCC did not show a significant difference (group 1, 30% and 26.3%; group 2, 0 and 2.6%; group 3, 73.3% and 76.3%; group 4, 26.7% and 15.8%; group 5, 30% and 21.1%, respectively, $p > 0.05$) (Fig. 2A).

Co-infection of different TTV phylogenetic groups in a single patient was also assessed. The mean number of groups in patients with and without HCC was 1.6 and 1.4, respectively. No significant difference was observed in the degree of co-infection between patients with and without HCC (one group, 60% and 65.8%; two groups, 23.3% and 26.3%; three groups, 13.3% and 7.9%; four groups, 3.3% and 0; five groups, 0 and 0, respectively, $p > 0.05$) (Fig. 2B).

3.5. Comparison between patients with and without HCC

Forty-nine (32.5%) of the 151 patients with HCV-related chronic liver disease were diagnosed with HCC. Table 4 shows the comparison of several features between patients with and without HCC. OBI and TTV infection were significantly more frequent in patients with HCC than in those without HCC (10.2% vs. 0, $p = 0.003$; 61.2% vs. 37.3%, $p = 0.009$, respectively). Furthermore, HCC was significantly associated with higher age ($p < 0.001$), and presence of liver cirrhosis ($p < 0.001$). Other variables, such as gender, anti-HBc, and anti-HBs did not differ significantly between the two groups (Table 4).

Table 5 shows the final model of variables independently related to HCC in the multivariate logistic regression analysis. TTV infection and presence of liver cirrhosis were found to be independent factors associated with the occurrence of HCC in patients with HCV chronic infection (OR = 2.23, 95% CI = 1.04–4.80, $p = 0.040$, and OR = 18.45, 95% CI = 4.22–80.60, $p < 0.001$, respectively).

4. Discussion

The occurrence of HCC is a major problem in chronic hepatitis C, with annual rates ranging from 1% to 4% in HCV-related cirrhotic patients (El-Serag, 2012). In Brazil, almost 9000 deaths were attributed to HCC in 2013(INCA, 2017), and chronic HCV infection is the major risk factor, accounting for more than 50% of the cases (Carrilho et al., 2010).

In the present study, the prevalence and significance of OBI and TTV infection, as well as the genetic diversity of these viruses, were investigated in chronic hepatitis C patients with and without HCC. Herein, OBI was detected in 5/151 (3.3%) HCV infected patients. Several studies assessing the prevalence of OBI in patients with chronic HCV infection have reported dissimilar rates of HBV DNA positivity, which may be explained by differences in the prevalence of HBV infection in the different geographic regions, the detection limits of HBV DNA assays, and/or the biological material analyzed (liver tissue or serum). In particular, previous studies analyzing serum samples of Brazilian HCV infected patients found frequencies of OBI ranging from 0 to 24% (Alencar et al., 2008; Branco et al., 2007; Fontenele et al., 2015; Motta et al., 2010; Pereira et al., 2006; Peres et al., 2005).

The majority (4/5, 80%) of the HBV isolates analyzed here belonged to genotype A, and one (20%) isolate to genotype D, which is in accordance with previous studies reporting genotype A as the most frequently found genotype in Brazil, followed by genotype D (Araujo et al., 2004; Barros et al., 2014; Mello et al., 2007; Oliveira et al., 2016).

A limitation of this study was the inability of including OBI in the multivariate logistic regression modeling due to the low number of OBI positive patients (n = 5). However, all five patients positive for OBI were diagnosed with HCC and a significant association between OBI and HCC was observed in univariate analysis ($p = 0.003$). These results corroborate previous reports suggesting a possible pro-oncogenic role played by OBI in patients with HCV-related chronic liver disease (Huang and Hollinger, 2014; Pollicino and Saitta, 2014; Shi et al., 2012; Squadrito et al., 2013; Tanaka et al., 2004). Of note, a longitudinal study, evaluating for a median time of 11 years the clinical outcome of 326 HCV infected patients, demonstrated that those with OBI were at a significantly higher risk of progression toward cirrhosis, HCC development, and lower survival than OBI negative patients (Squadrito et al., 2013). Similarly, a meta-analysis conducted by Shi et al. (Shi et al., 2012) demonstrated that OBI increased the risk for HCC in HCV-infected populations (relative risk = 2.83, 95% CI = 1.56–4.10) and appeared to be a cofactor or precipitating event in the development of HCC in prospectively followed HCV-coinfected patients.

In the present study, TTV infection was detected in 68/151 (45%) patients with HCV-related chronic liver disease. Similar frequencies of TTV infection have been observed among dialysis patients (36%) (Takemoto et al., 2015), renal-transplant patients (54%) (Takemoto

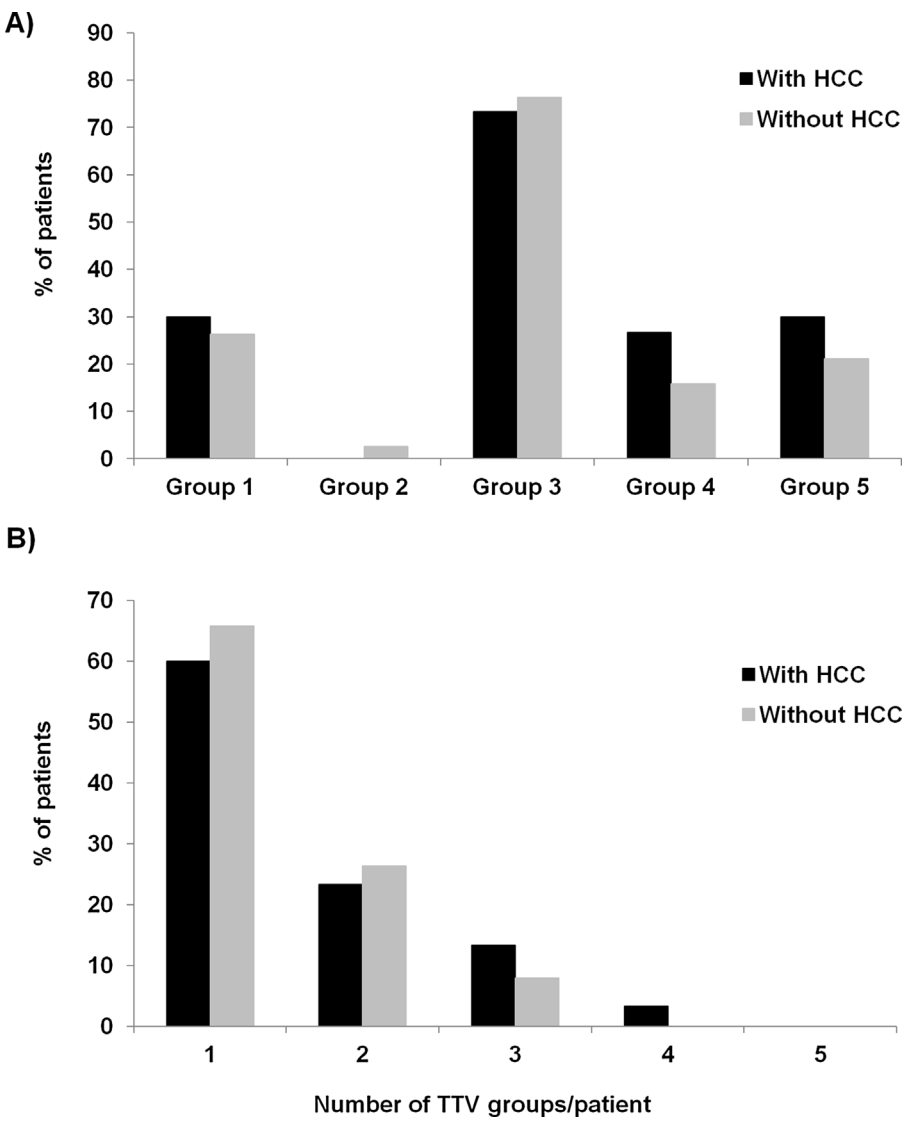

Fig. 2. TTV infection in chronic HCV patients with and without HCC. A) Frequencies of TTV phylogenetic groups. B) Degree of co-infection with different TTV groups. Columns represent the percentage of patients infected with 1, 2, 3, 4, or 5 TTV groups.

et al., 2015), blood donors (46%) (Devalle and Niel, 2004), and HBV carriers (54%) (Devalle and Niel, 2004), by using the same set of PCR primers. Overall, TTV phylogenetic group 3 was the most prevalent group observed here, while 2 and 4 were the less frequent ones, corroborating previous findings in different populations (Biagini et al., 2006; Cancela et al., 2016; Devalle and Niel, 2004; Maggi et al., 2003; Pinho-Nascimento et al., 2011). Here, no significant difference was observed concerning the prevalence of the phylogenetic groups between HCC and non-HCC patients. In addition, it has been suggested that immunosuppressed patients are more likely to be co-infected with several TTV isolates than other individuals (Devalle and Niel, 2004; Devalle and Niel, 2005; Shibayama et al., 2001). A slight increase in the mean number of co-infections with different TTV phylogenetic groups was observed in HCC patients compared with non-HCC patients (1.6 and 1.4, respectively), although without statistical significance ($p > 0.05$).

Among the HCV infected patients analyzed here, TTV prevalence was significantly higher in older patients ($p = 0.001$), which is consistent with previous reports showing a gradual increase of TTV infection rates with age (Hsieh et al., 1999; Saback et al., 1999; Spandole et al., 2015). In addition, TTV infection was associated with the occurrence of HCC (OR = 2.23, 95% CI = 1.04–4.80, $p = 0.040$), independently of liver cirrhosis, which is a well-known risk factor for HCC. These findings are in accordance with a previous study showing that high TTV loads were a significant independent factor associated with HCC among Japanese patients with chronic hepatitis C (Tokita et al., 2002). Likewise, Zein et al. (Zein et al., 1999) demonstrated that TTV was significantly more prevalent among HCV patients with advanced liver disease (decompensated cirrhosis and HCC) than those with stable disease (chronic hepatitis and compensated cirrhosis). Moreover, it was demonstrated that TTV-infected cirrhotic patients with chronic hepatitis C had higher levels of irregular regeneration of hepatocytes (a main risk factor for the development of HCC) than TTV-non-infected patients (Moriyama et al., 2001), supporting the hypothesis that TTV plays a role in hepatocarcinogenesis. However, Tokita et al. (Tokita et al., 2002) provided an alternative explanation for the significant association between high TTV viremia and occurrence of HCC observed in their study. Based on previous findings suggesting that an impaired immune system or suppression of the immune system is involved in elevated TTV viremia (Christensen et al., 2000; Shibayama et al., 2001; Touinssi et al., 2001), the authors did not exclude the possibility of high TTV viremia reflecting the immune status of the HCC patients. In the same way, this hypothesis cannot be ruled out to explain the results observed here.

In conclusion, OBI was infrequent in patients with HCV-related chronic liver disease. This is the first study that describes an association between TTV infection and HCC in Brazilian patients chronically infected with HCV. Whether TTV infection mediates the progression of

Table 4
Characteristics of the patients with chronic hepatitis C according to the occurrence of HCC.

	With HCC (n = 49)	Without HCC (n = 102)	*p* value
Age[a]			
< 60	12 (25%)	53 (55.8%)	< 0.001
≥60	36 (75%)	42 (44.2%)	
Gender			
Male	25 (51%)	43 (42.2%)	NS (0.383)
Female	24 (49%)	59 (57.8%)	
Liver cirrhosis			
Positive	47 (95.9%)	55 (53.9%)	< 0.001
Negative	2 (4.1%)	47 (46.1%)	
Occult HBV			
Positive	5 (10.2%)	0	0.003
Negative	44 (89.8%)	102 (100%)	
TTV DNA			
Positive	30 (61.2%)	38 (37.3%)	0.009
Negative	19 (38.8%)	64 (62.7%)	
Anti-HBc			
Reactive	14 (28.6%)	19 (18.6%)	NS (0.207)
Non-reactive	35 (71.4%)	83 (81.4%)	
Anti-HBs			
Reactive	18 (36.7%)	43 (42.2%)	NS (0.597)
Non-reactive	31 (63.3%)	59 (57.8%)	

NS, not significant.
[a] Age not informed in eight patients.

Table 5
Final model of the variables independently related to HCC in chronic hepatitis C patients.

Variables	Odds ratio (95% CI)[a]	*p* value
TTV-DNA		
Negative	1	
Positive	2.23 (1.04–4.80)	0.040
Liver cirrhosis		
Negative	1	
Positive	18.45 (4.22–80.60)	< 0.001

[a] 95% confidence interval.

liver disease or whether it is an indicator of the HCC patient's compromised immune system still needs to be elucidated.

Competing interests

The authors have no conflicts of interest to disclose.

Funding

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant number 444071/2014-8].

Acknowledgements

The authors thank the DNA Sequencing Platform (RPT01A) of Oswaldo Cruz Foundation for technical support.

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