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Association of hepatitis viruses with hepatocellular carcinoma in Thailand

Pisit Tangkijvanich[1], Petra Hirsch[2], Apiradee Theamboonlers[2], Issarang Nuchprayoon[2], and Yong Poovorawan[2]

[1] Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330 Thailand
[2] Viral Hepatitis Research Unit, Pediatric Department, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Abstract: Hepatocellular carcinoma (HCC) represents the most common form of malignant tumor among males in Thailand, an area endemic for hepatitis B virus (HBV) infection. Various risk factors have been associated with the development of HCC, among them exposure to certain toxins, and infection with hepatitis viruses, in particular HBV, as well as HCV in areas non-endemic for HBV infection. To examine the association of hepatitis viruses with HCC, our group investigated 101 patients who had been clinically, mainly via alpha fetoprotein level, and/or histologically diagnosed with hepatocellular carcinoma. We also examined 200 voluntary blood donors as controls. All subjects underwent serological tests for the presence of hepatitis B surface antigen (HBsAg) and anti-HCV with polymerase chain reaction (PCR) used for the detection of HBV and TT virus (TTV) DNA, and reverse transcription (RT)-PCR for the detection of HCV RNA and HGV RNA. Besides showing a clear preponderance of HCC among males, with a peak incidence the age group 51–70 years, the results obtained in the HCC patients demonstrated that the prevalence of HBV was 65%, four times that of HCV (17%), ten times that of HGV (6%), and seven times that of TTV (9%). In the controls, the prevalence of HBV was 0.5%; that of HCV, 0.5%; that of HGV, 5%; and that of TTV, 7%. These findings confirmed that hepatitis B virus was associated with the development of hepatocellular carcinoma among the Thai population, among whom case histories of chronic hepatitis and cirrhosis have also been encountered quite frequently.

Key words: hepatitis virus, hepatocellular carcinoma, Thailand

Reprint requests to: Y. Poovorawan
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Introduction

Liver cancer constitutes one of the most common forms of cancer on a global scale. Histologically, primary liver cancer can be differentiated into two major types, hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). In infants exclusively, and rather infrequently, liver cancer is found in the form of hepatoblastoma, which is definitely not associated with any of the known hepatitis viruses. HCC is commonly encountered worldwide, with a major prevalence in Asia and Africa, whereas CCA is almost exclusively concentrated in Southeast Asia and China. In Thailand, liver cancer represents the most common malignant tumor, with an incidence of HCC of 40–80 per 100000 males.[1]

Various risk factors have been associated with the etiology of liver cancer e.g., viral infection, in particular with hepatitis viruses B and C (HBV and HCV, respectively)[2] parasitic infestation, and exposure to toxins and alcohol. In most patients with HBV-associated liver cancer, HBV is found integrated in the host cellular genome, thereby providing a mechanism for the persistent expression of a virally encoded gene product in chronic hepatitis B surface antigen (HBsAg) carriers, which may represent a potential etiologic factor in the development of HCC and also have implications regarding the outcome of antiviral therapy.[3] In this context, the HBV X-protein in particular, acts as a rather non-specific transactivator, leading to mutations of tumor suppressor genes (which render them inactive), and activating proto-oncogenes.[4] In countries non-endemic for HBV, such as Japan and Europe, HCV has been identified as a major risk factor for HCC,[5,6] especially in people in high-risk groups in this particular context defined as recipients of blood or blood products, intravenous drug users (IVDUs), homosexuals, and to a lesser extent, health care professionals.

In Thailand, with its hot and humid climate, contamination of food by aflatoxin, which has been associated with the development of HCC, contributes yet another serious risk factor.[7,8] In particular, aflatoxin B1 (AFB1) exposure has been shown to correlate with a specific mutation at codon 249 in the *p53* tumor suppresser gene in liver tumors. The risk for development of HCC has been found greatest in individuals with both AFB1-DNA adducts and HBsAg, suggesting an interaction between virus and chemical toxin.[9]

The present study aimed to determine the prevalence of HBV and HCV infection in a group of patients diagnosed with hepatocellular carcinoma. The sera of these patients were also examined for the presence of TT virus (TTV) DNA, as TTV constitutes a novel parenterally transmissible agent, first described [10,11] as a single stranded, non-enveloped DNA virus highly prevalent in Japanese patients at risk of contracting blood-borne infections and named after the first patient (TT) in whom it had been discovered. To improve diagnostic efficacy and to elucidate the genetic characteristics of TTV, a Japanese group of researchers sequenced a 2.4-kb segment of the TTV genome derived from eight Japanese isolates. The region sequenced was found to contain a large open reading frame (ORF-L) coding for a protein of 768–770 amino acids highly rich in arginine at its N-terminus and harboring three or four asparagine-linked glycosylation sites clustered in its central portion.[12] Comparison of this long ORF encoded protein with those of known single-stranded DNA viruses suggested a possible phylogenetic relation of TTV with chicken anemia virus, which belongs to the family Circoviridae rather than the Parvoviridae.[12]

Further, as the literature regarding the clinical significance of hepatitis G virus (HGV) is rather scarce, we also submitted the patients' sera to reverse-transcriptase (RT) polymerase chain reaction (PCR) for tracing any HGV RNA.

Patients and methods

Population study

Hepatocellular carcinoma patients. One hundred and one patients who attended Chulalongkorn University Hospital between April 1996 and March 1998 were included in the study. They comprised 86 males (85%) and 15 females (15%); their ages ranged from 10 to 85 years (mean age, 52.9 ± 14.0 years). Regarding their geographic distribution, 44 patients were residents of Bangkok proper, another 45 resided in the surrounding central urban areas, and 12 in Thailand's northeastern rural provinces. With respect to demographic parameters potentially contributing to the development of HCC, 12 individuals had a past history of blood transfusions, 2 were intravenous drug users (IVDUs), 15 constituted known cases of chronic hepatitis and cirrhosis, 6 had a family history of HCC, and 33 admitted to serious alcohol consumption, (at least 60 g of alcohol on a daily basis for more than the past 5 years). The underlying liver disease was chronic hepatitis in 4 patients and cirrhosis, graded as Child A in 53, as Child B in 34, and as Child C in 10 patients. The HCC was diagnosed in 74 patients based on histology, and in the remaining 27 the diagnosis was based on clinical symptoms, in particular, liver mass, confirmed by alpha-fetoprotein levels above 400 IU/ml. The sera of 43 of these HCC patients had been obtained between April 1996 and July 1997 at the liver unit of Chulalongkorn Hospital and kept at −70°C for further study after the diagnosis of HCC had been confirmed by histology. The remaining 58 patients attended the liver unit at Chulalongkorn Hospital consecutively between August 1997 and March 1998. After the diagnosis of HCC, their sera were also kept at −70°C.

Voluntary blood donors. Two hundred sera collected consecutively and at random from voluntary blood donors at the National Blood Center, Thai Red Cross, between November 25 and December 2, 1996 were examined for the presence of hepatitis viruses. These 200 specimens, which served as control samples, were obtained from 150 men and 50 women, aged 19–60 years (mean, 32.7 ± 10.5 years).

All patients were informed of the objective of the study and subsequently provided their consent. Blood was obtained during examinations, and sera were separated by centrifugation and stored at −70°C until submitted to the respective tests.

Laboratory tests

Detection of HBV DNA. Aliquots of 10 µl each of each serum sample collected were transferred to 0.2-ml PCR tubes, overlaid with one drop of mineral oil and boiled in a microwave oven at maximum temperature for 4 min to inactivate any inhibitors of *Taq* polymerase inherent in human blood serum.

For DNA amplification by semi-nested PCR, a total of 40 µl of a reaction mixture containing 1 U of *Taq* polymerase (Perkin Elmer Cetus, Branchburg, NJ, USA), and each of four deoxynucleotide triphosphates, at a concentration of 200 µM, primer pairs (Biosynthesis, Lewisville, TX, USA) of Xo1, Xi3 (first round) and Xo1, Xi2 (second round) 1 µM each, 10 mM Tris, and 1.5 mM $MgCl_2$ were added directly to the heat-inactivated sera to produce a final reaction volume of 50 µl. The samples were spun in a microcentrifuge for 2 s before being placed in the thermocycler (Perkin Elmer Cetus).

The primer sequences were:

Xo1: 5′-CTC TGC CGA TCC ATA CTG C-3′ located at position 1254–1272.
Xi3: 5′-GGC ACA GCT TGG AGG CTT-3′ located at position 1883–1866.
Xi2: 5′-CAG ATG AGA AGG CAC AGA C-3′ located at position 1560–1551.

The reaction was then performed for 30 cycles, preceded by a denaturation step at 94°C for 3 min, using primer pair F1, R2, at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by an extension step at 72°C for 5 min for the first round of amplification. For the second round of amplification, the reaction was also performed for 30 cycles, preceded by a denaturation step at 94°C for 3 min, using primer pair F2, R1, at 94°C for 20 s, 51°C for 20 s, 72°C for 20 s, followed by an extension step at 72°C for 5 min.

After electrophoresis in a 2% Nusieve (FMC Bioproducts, Singapore) gel stained with ethidium bromide, the band indicating the presence of HBV DNA became visible at 279 bp after the second amplification round. Sera obtained from known HBV carriers and sera obtained from individuals vaccinated against hepatitis B were used as positive and negative controls, respectively.

Detection of HCV and HGV RNA. The reverse-transcription polymerase chain reaction (RT-PCR) was used to detect RNA from HCV and potentially that from HGV.[13] The primer sequences employed were, without exception, derived from the untranslated regions of HCV or HGV. Briefly, RNA was extracted by the guanidine method,[14] with minor modifications, prior to denaturation at 65°C for 5 min. The isolated RNA samples were reverse transcribed into cDNA using a reverse transcriptase (MuLV; Perkin Elmer), according to the manufacturer's specifications.

The sera were subsequently screened for the presence of HCV and/or HGV RNA by nested PCR, again using a commercially available preparation (Perkin Elmer) according to the manufacturer's instructions. The sequences of the four primers employed for detection of HCV RNA were: 5′-GGCGACACTCCACCATGAAT-3′ (outer sense primer, nucleotide positions −324 to −305) and 5′-CATGGTGCACGGTCTACGAG-3′ (outer anti-sense primer, nucleotide positions −17 to +3) for the first round of amplification cycles, and 5′-GGAACTACTGTCTTCACGCAG-3′ (inner sense primer, nucleotide positions −291 to −271) and 5′-TCGCAAGCACCCTATCAGGCA-3′ (inner anti-sense primer, nucleotide positions −52 to −32) for the second round. Both rounds consisted of 30 amplification cycles, each of 0.6 min at 94°C, 0.7 min at 55°C, and 1.5 min at 72°C. Any amplified product could be detected after fractionation in Tris-borate buffer by electrophoresis in 2% Nusieve gel. After staining with ethidium bromide, the product band could be visualized by ultraviolet fluorescence either at 327 bp (first round) or 259 bp (second round).

HGV RNA was detected by nested PCR in a similar manner, but using different primers (Biosynthesis): 5′-AGGTGGTGGATGGGTGAT-3′ (outer sense primer located at position 108) and 5′-TGCCACCCGCCCTCACCCGAA-3′ (outer anti-sense primer located at position 531) for the first round; and 5′-TGGTAGTCGTAAATCCCGGT-3′ (inner sense primer located at position 134) and 5′-GGRGCTGGGTGGCCYCATGCWT-3′ (where R = A or G, W = A or T, and Y = C or T; inner anti-sense primer located at position 476) for the second round.[15] After electrophoresis, the bands indicating the presence of HGV RNA in the original serum sample became visible at 421 bp (first round) and 343 bp (second round).

Sera obtained from thalassemia patients known to be positive for HCV or HGV served as the positive controls; HCV- and HGV-negative sera and sterile water served as the negative control.

Detection of TTV-DNA. DNA was isolated by the alkaline extraction method[16] Briefly, a 10-μl aliquot of plasma was pipetted into a 0.5-ml microcentrifuge tube and incubated with NaOH at a final concentration of 0.1 M at 37°C for 60 min. The solution was subsequently spun for 15 s in a microcentrifuge and neutralized with HCl at a final concentration of 0.1 M. TTV-DNA was detected by polymerase chain reaction using semi-nested primers. The amplification reaction was performed in a 50-μl reaction volume containing 1 U of Taq polymerase (Perkin Elmer Cetus), and each of four deoxynucleotide triphosphates, at a concentration of 200 μM, with primer pairs NG 059 and NG 063 for the first round, and NG061 and NG 063 for the second round, at a concentration of 1 μM each, 10 mM Tris, 1.5 mM $MgCl_2$, and 5 μl of each DNA sample. According to Okamoto et al.,[11] the nucleotide sequences of the TTV primers derived from the N-22 region, which represents the most conserved sequence of the five genotypes described to date, were: NG 059 (5′ CAG ACA GAG GAG AAG GCA ACA TG 3′), NG 061 (5′ GGC AAC ATG TTA TGG ATA GAC TGG 3′), and NG 063 (5′ CTG GCA TTT TAC CAT TTC CAA AGT T 3′). The first-round amplification reaction using primer pair NG 059 and NG 063 was performed for 30 cycles (denaturation at 94°C for 36 s, annealing at 55°C for 42 s, and extension at 72°C for 1.5 min, with final extension at 72 °C for 10 min). The second round of amplification was performed using 2 μl of the first PCR product along with primer pair NG 061 and NG 063 for 30 cycles under identical conditions, in a final reaction volume of 20 μl. Upon conclusion of the PCR, the reaction mixture was spun for 1 min at 10000 rpm, and 10 μl each of the

amplified DNAs were fractionated by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under UV light. The product band will show at 271 base pairs. The gels were photographed on a UV light box. Sera obtained from IVDUs and known to be positive for TTV-DNA were used as positive controls, and TTV-negative sera and sterile water were used as negative controls.

Serology

All sera were tested for HBsAg using a commercially available kit (Auszyme II; Abbott Laboratories, North Chicago, Ill, USA), and for anti-HCV by third generation enzyme-linked immunosorbent assay (ELISA) (Recombinant c22–3, c200, and NS5) obtained from Ortho Diagnostic Systems (Chiron, Emeryville, CA, USA).

Data analysis

The prevalences obtained for HBV, HCV, HGV, and TTV, were expressed as percentages related to the total number of patients.

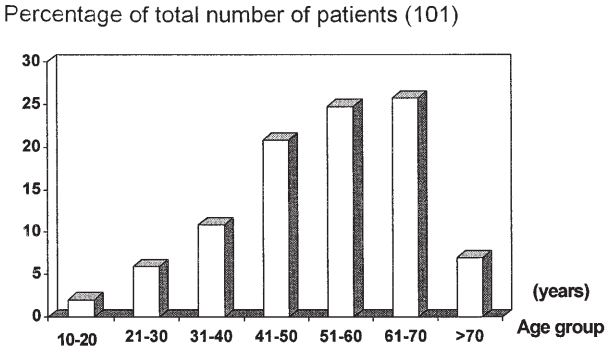

Fig. 1 Age distribution of patients with hepatocellular carcinoma

Table 1. Sex and age distribution of HCC patients in relation to viral hepatitis markers

Viral marker	Age range (years)	Mean age (years) ± SD	Male/female ratio
Hepatitis B	10–85	49.5 ± 14.2	58/8
Hepatitis C	23–72	52.7 ± 14.0	13/4
Hepatitis G	23–67	52.3 ± 25.4	4/2
Hepatitis TT	27–74	57.2 ± 15.6	8/1

HCC, Hepatocellular carcinoma

Results

In addition to the indisputable male preponderance among the 101 HCC patients (85% males versus 15% females), the prevalence of HCC in relation to age was also clearly discernible. As shown in Fig. 1, it peaked within the age group of 51–70 years, rose among those between 41 and 50 years, and fell among those over 70 years of age, the latter fall in all probability due to most HCC patients not reaching that age group. The sex and age distribution of the HCC patients in relation to viral hepatitis markers is shown in Table 1.

The results for serology, and for PCR performed for the 101 HCC patients are shown in Table 2, showing an approximately fourfold higher prevalence of hepatitis B virus over hepatitis C virus infection. By ELISA, we detected HBsAg in 66 patients, versus 12 patients who were positive for anti-HCV antibody; by semi-nested PCR, we found HBV DNA in 53 patients, versus 14 patients who were positive for HCV RNA by RT-PCR. All 53 individuals positive for HBsAg were also positive for HBV DNA whereas we found anti-HCV along with HCV RNA in only 9 patients. Thirteen patients were positive for HBsAg without the presence of HBV DNA while 3 patients were positive for anti-HCV without detectable HCV RNA. In the 101 sera tested we did not detect any positive for HBV DNA without detectable HBsAg, whereas 5 sera were positive for HCV RNA but devoid of anti-HCV antibody. Taking the number of HBsAg-positive patients as reference, the prevalence of HBV infection was about tenfold higher than that of HGV infection and about sevenfold higher than that of TTV infection.

Only one HCC patient was exclusively infected with TTV. The mean age of the nine patients in whom TTV was detected was 57.2 ± 15.6 years, compared with 52.0 ± 14.0 years in the remaining patients negative for TTV. As shown in Table 3, the prevalence of TTV infection increased with age in healthy blood donors, whereas it appeared to be equally distributed in the HCC patients.

Regarding double and/or triple infection, we found HCV RNA together with TTV DNA in two patients, HBV together with TTV DNA in two, HCV RNA along with HBV and TTV DNA in three, and HCV RNA along with HBsAg in six patients. Coinfections with HCV and HGV, both detected by RT-PCR, had occurred in two individuals. HGV RNA was found simultaneously with HBsAg in three patients, whereas HGV RNA was detected simultaneously with HBV and TTV DNA in only one of the sera tested (Table 4).

In the control group of 200 voluntary blood donors, one individual (0.5%) was HBsAg-positive, one was positive for anti-HCV and HGV RNA was detected in 10 (5%) and TTV DNA in 14 (7%).

Table 2. Association of hepatitis viruses with HCC in a total of 101 patients

	HCC (n = 101)		Blood donors (n = 200)	
Viral marker	No. +ve	(%)	No. +ve	(%)
Hepatitis B				
HBsAg (total)	66	65	1	0.5
HBsAg + HBV DNA	53	53		
HBsAg only	13	13		
Hepatitis C				
Anti-HCV (total)	12	12	1	0.5
HCV RNA (total)	14	14		
Anti-HCV + HCV RNA	9	9		
HCV RNA only	5	5		
Anti-HCV only	3	3		
Hepatitis G				
HGV RNA	6	6	10	5[a]
Hepatitis TT[b]				
TTV DNA	9	9	14	7

HB, Ag, epatitis B surface antigen; HBV, hepatitis B virus
[a] These data have been reported elsewhere[17]
[b] No. of patients with HCC tested for TTV DNA: 98

Table 3. Age distribution of subject with TTV infection in HCC patients and blood donors

	HCC		Blood donors	
Age (years)	No. of subjects	TTV +ve (%)	No. of subjects	TTV +ve (%)
≤20	2	0 (0)	25	1 (4)
21–30	6	1 (16.7)	71	2 (2.8)
31–40	11	0 (0)	55	4 (7.3)
41–50	21	3 (14.3)	35	5 (14.3)
51–60	25	0 (0)	13	2 (15.4)
>61	33	5 (15.2)	1	0 (0)
Total	98	9 (9)	200	14 (7)

Table 4. Viral hepatitis coinfections in HCC patients

Marker	No. of patients (%)
HBV + HCV	6/98 (6.1)
HBV + HGV	3/98 (3.1)
HBV + TTV	2/98 (2.0)
HCV + HGV	2/98 (2.0)
HCV + TTV	2/98 (2.0)
HBV + HCV + TTV	3/98 (3.1)
HBV + HGV + TTV	1/98 (1.0)

Note, only one patient was solely infected with TTV

In the 200 blood donors, no double or triple infections with any combination of the hepatitis viruses investigated was detected.

Of the 101 patients' samples tested, 22 (22%) were negative for all the hepatitis markers examined.

Discussion

As has been described by others, HCC frequently develops in individuals with pre-existing chronic hepatitis B virus infection, who have been shown to be at an approximately 200-fold higher risk than age-matched non-infected controls.[18] Since its discovery in 1989, hepatitis C virus has also been associated with the development of HCC, especially in areas non-endemic for HBV, for example, Europe, North America, and Japan. Therefore, our present results demonstrating an approximately fourfold higher prevalence of HBV over HCV infection in HCC patients does not come as a surprise, although a significantly higher prevalence of anti-HCV had been detected in HCC patients compared with blood donors in Thailand,[19] where HBV infection is endemic. Likewise, in the present study (in which there was a very small control group of only 200

blood donors) 12% of the HCC patients versus 0.5% of the controls were positive for anti-HCV. This percentage of anti-HCV-positive controls agrees with the data of the Thai Red Cross Transfusion Centre, which reported a prevalence of anti-HCV of 1.5% in a group of 10175 voluntary blood donors in 1991.[20] The frequency at which HBsAg was detected in our control group (0.5%), was considerably lower than the 6.45% reported in a cohort of 74530 voluntary blood donors in 1991.[21]

The prevalence of HCV-associated HCC that we identified in Thailand (12%) is comparable with that in Taiwanese (19.5%)[22] and Korean populations (28%),[23] but lower than the prevalence in several countries not endemic for HBV, where HCV is rendered more important than HBV in the pathogenesis of HCC. Antibodies to HCV were detected in 50%–70% of HCC patients in Japan,[24] and in 30%–70% of Caucasian patients with HCC.[25,26]

In our study, approximately 3% of the HCC patients had only anti-HCV antibody with no HCV RNA detectable; this may be ascribed to the patients' capacity to clear the virus. In contrast, in patients in whom anti-HCV antibody is apparently absent, yet HCV RNA can be traced by RT-PCR, the presence of active HCV infection is clearly indicated but due to genotype variations of HCV, only a limited number of serotypes are detectable with the diagnostic kits presently available,[2] whereas by the highly sensitive method of RT-PCR one single copy of viral RNA can be traced. Hence, in our present study, although we used a third-generation anti-HCV ELISA kit, in five patients devoid of anti-HCV antibody we still detected HCV RNA by RT-PCR.

The role of HGV in the etiology of HCC has been unclear, since HGV has, at least to our knowledge, not yet been implicated as a causative agent of HCC, but rather appears to represent an opportunistic virus predominantly found in recipients of blood and blood products, IVDUs, homosexuals, or in general, individuals at risk of contracting blood-borne viruses. Thus, the number of HCC patients who tested positive for HGV RNA (6%) was compatible with the number who tested positive in the control group of blood donors (5%). A study from Japan reported HGV RNA in 11 of 111 HCC patients, 10 of whom were simultaneously infected with HCV, and one with HBV.[27] We were able to detect HGV RNA in one female HCC patient who displayed no other hepatitis marker, nor did she have a history of either serious alcohol consumption or familial liver disease.

HCV-HBV coinfections were detected in 6% of the HCC patients, an incidence similar to the 6% determined for HGV infection. With regard to HCV-HBV coinfections, some authors have postulated a dominance of HCV over HBV,[6,28] while others rather argue in favor of synergism.[22,29] We found HCV-HGV coinfections in 2% of the HCC patients and HBV-HGV coinfections in 3%; in these patients HCV and HBV may well play the dominant role as the etiologic agent of HCC.

With respect to the novel hepatitis TT virus (TTV) first described by Nishizawa et al.[10] as a parenterally transmissible agent predominantly encountered in patients at risk of contracting blood-borne viruses, we detected its circulating DNA in 9 of the 98 HCC sera (9%) tested, in which the majority of patients harbored double or triple infections of TTV along with HCV (n = 2), HBV (n = 2), HCV + HBV (n = 3) and HBV + HGV (n = 1); only one patient was exclusively positive for TTV DNA. The prevalence established for TTV infection is therefore comparable with that of HGV infection and, as with HGV, there was no essential difference in the frequency of TTV infection among HCC patients compared with that in our control group of voluntary blood donors. Yet, as previously described,[30] the prevalence of HGV infection clearly decreases with increasing age, indicating clearance of the virus by the host's immune system, whereas, at least in our control group, the incidence of TTV infection was found to be increased in the higher age groups. Similar results have been reported by Takahashi's group in Japan.[31] In contrast, TTV appeared to be equally distributed among all age groups in the HCC patients; this may either constitute an artifact due to the small sample size, or it may be the consequence of the double or triple infection with other hepatitis viruses detected in most patients, a condition that could possibly endow TTV with a selective advantage. One might argue in favor of synergism between TTV and one of the other hepatitis viruses as the mechanism, which could trigger the process of malignant transformation. Yet, since TTV is found in almost equal numbers associated with HBV, HCV, HCV + HBV, and HBV + HGV, the case for synergism may be solely based on circumstantial evidence.

Considering all of the above in conjunction with the multifactorial etiology of cancer, it will require intensive and extended research efforts to even begin to understand the complex mechanisms leading to the development of HCC. To that end, the approximately 20% of HCC patients who were negative for any viral markers might prove to be the subjects who are most promising with regard to providing us with insights as to the molecular etiology of HCC. Future research may also help to shed light on the clinical significance of the novel hepatitis TT virus in connection with HCC which, due to the scarce literature at present, remains rather obscure.

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