HEPATITIS VIRUSES AND CHRONIC LIVER DISEASE

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Abstract. We have investigated several groups of Thai patients diagnosed with chronic liver disease including chronic hepatitis, cirrhosis and hepatocellular carcinoma, as well as cholangiocarcinoma, for the prevalence of infection with either one of the hepatitis viruses B, C, G and the novel hepatitis virus TT (TTV). The 168 patients tested comprised 120 men and 48 women with their median age ranging from 42.3 to 62.3 years. Screening for antibodies to HBV and HCV was performed by a commercially available serological test kit, for the presence of HBV and TTV DNA by PCR, and of HCV and HGV RNA by RT-PCR, respectively. There was a clear two-fold higher prevalence of HBV (49%) over HCV (27%) infection and a four-fold higher frequency compared to HGV (13%) and TTV (11%) infection, respectively, in those individuals with chronic hepatitis, cirrhosis, and hepatocellular carcinoma, whereas all but one patient with cholangiocarcinoma the etiology of which has been ascribed to parasitic infestation, were free of all viral markers. In Thailand chronic HBV, and to a lesser extent, chronic HCV infection represent the two most common causes of hepatitis potentially proceeding to chronic liver disease, whereas the clinical significance pertinent to HGV and TTV remains to be elucidated.

INTRODUCTION

Viral hepatitis represents a major cause of chronic liver disease, namely, chronic hepatitis, cirrhosis and its fatal complication, hepatocellular carcinoma (HCC). Hepatitis B (HBV) has been a significant health problem worldwide with a particularly high incidence in South-East Asia, China and sub-Saharan Africa (Lee, 1997). In these areas, hepatitis B virus infection accounts for the majority of cases of chronic hepatitis, cirrhosis and HCC. The relative risk for chronic HBV carriers to develop HCC is approximately 100 times greater than that of the uninfected population (Beasley, 1988). The time in an individual's life when infection occurs appears crucial as, for example, infections during adulthood carry a far lower risk than infections in early life or in the course of delivery by a carrier mother, which often progress to chronic infection and HCC (Poper *et al*, 1987).

In countries with a low prevalence of HBV, for example, Southern Europe and Japan, hepatitis C virus (HCV) constitutes the most frequent cause of chronic liver disease and HCC (Colombo *et al*, 1989; Saito *et al*, 1990). HCV infections are usually contracted by adults as a consequence of either blood transfusion or intravenous drug use with nonsterile equipment. Unfortunately, a tendency towards chronicity is the most distinguishing characteristic of HCV infection, occurring in more than 85% of the cases with a history of acute infection. Chronic hepatitis C usually develops into HCC in the course of approximately two to three decades (Tong *et al*, 1995) with most cases preceded as well as accompanied by cirrhosis.

Hepatitis G virus (HGV) is a newly identified flavivirus discovered almost simultaneously in 1995 by investigators from Abbott Laboratories and Genelabs Technologies, Inc (Simons *et al*, 1995; Linnen *et al* 1996). Current data suggest HGV infection to only rarely cause acute liver disease and the majority of patients not to develop chronic hepatitis (Alter *et al*, 1997). Due to common modes of transmission, HGV frequently occurs as a coinfection with HCV and, to a lesser extent, with HBV. The course of chronic hepatitis in patients with HCV-HGV coinfection invariably correlates with HCV infection (Di Bisceglie, 1996; Wang *et al*, 1996) A causative role of HGV in the etiology of HCC has as yet not been established.

Hepatitis TT virus represents a novel hepatitis virus first isolated by representational difference analysis (RDA) (Lisitsyn *et al*, 1993) as a clone of 500 nucleotides from the serum of a patient with post-transfusion hepatitis of unknown etiology

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Table 1
Characteristics of patients included in the study.

Group	No. of subjects			Age (years)	Mean age (years)
	Male	Female	All		
1. Chronic hepatitis	41	12	53	17-78	42.3
2. Cirrhosis	37	28	65	26-87	53.1
3. HCC	37	6	43	10-85	56.8
4. Cholangiocarcinoma	5	2	7	45-67	62.3
Total	120	48	168		

(Nishizawa *et al*, 1997). Subsequently, the agent in question has been molecularly cloned and characterized as a single-stranded DNA virus. To date, approximately 3.7 kb of its genome have been sequenced and subjected to homology search which did not yield any nucleotide sequences showing a significantly high sequence homology (Okamoto *et al*, 1998). With this virus having been shown to employ a parenteral route of transmission as hepatitis viruses B, C and G, it might well turn out to be the causative agent in those approximately 30 % of chronic liver disease and hepatocellular carcinoma patients in whom none of the above hepatitis viruses could be traced (Songsivilai *et al*, 1996; Linnen *et al*, 1996; Alter *et al*, 1997)

The aim of the present study was to determine the prevalence of infection with HBV, HCV, as well as HGV and TTV among various groups of Thai patients diagnosed with chronic liver disease, including chronic hepatitis, cirrhosis and HCC. Furthermore, we investigated patients with cholangiocarcinoma (CCA), the second most common primary liver cancer in the Thai population, as to previous infection with either one of those four hepatitis viruses.

MATERIALS AND METHODS

Population study

Four groups of patients, having attended Chulalongkorn University Hospital between August 1997 and January 1998, were included in the study. The first group consisted of 53 patients with chronic hepatitis. The second group included 65 patients with cirrhosis graded as Child A in 34, as Child B in 17, and Child C in 14 cases, respectively. The third group comprised 43 patients with HCC, the diagnosis based on histology in 28 cases, and in the remaining 15 on serum alpha-fetoprotein levels above 400 IU/ml with liver tumor features on scintigraphy. The fourth group encompassed 7 patients with cholangiocarcinoma diagnosed by liver biopsy. Table 1 summarizes the main characteristics of the subjects in these four groups.

All patients were informed as to the objective of the study performed to elucidate the viral etiology of their respective liver disease and subsequently provided their consent. Blood was obtained during examinations, sera were separated by centrifugation and stored at -70 °C until subjected to the respective test.

Laboratory tests

Detection of HBV DNA: Aliquots of 10 µl each of every 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 minutes in order to inactivate any inhibitors of *Taq* polymerase inherent in human blood serum.

For DNA amplification by semi-nested polymerase chain reaction (PCR), a total of 40 µl of a reaction mixture containing 1 U of *Taq* polymerase (Perkin Elmer Cetus), and each of four deoxynucleotide triphosphates at a concentration of 200 µM, primer pairs (Biosynthesis, Lewisville, Texas) of Xo1, Xi3 (first round) and Xo1, Xi2 (second round) 1 µM each, 10 mM Tris, 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 seconds 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 1569-1551.

The reaction was then performed for 30 cycles, preceded by a denaturation step at 94°C for 3 minutes, using primer pair Xo1, Xi3 at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by an extension step at 72°C for 5 minutes for the first round of amplification, and for 30 cycles, preceded by a denaturation step at 94°C for 3 minutes, using primer pair Xo1, Xi2 at 94°C for 20 seconds, 51°C for 20 seconds, 72°C for 20 seconds, followed by an extension step at 72°C for 5 minutes for the second round, respectively.

After electrophoresis in a 2 % Nusieve 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: Reverse-transcription polymerase chain reaction (RT-PCR) was used to detect RNA from HCV and potentially that from HGV (Kwok and Higuchi, 1989). The primer sequences employed were, without exception, derived from the untranslated regions of HCV or HGV. Briefly, RNA was extracted by the guanidine method (Cha *et al*, 1991), with minor alterations, prior to denaturation at 65°C for 5 minutes. The isolated RNA samples were reverse transcribed into cDNA using a reverse transcriptase kit (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 applying commercially available kits (Perkin Elmer) according to the manufacturer's instructions. The sequences of the four primers employed for detection of HCV RNA were 5´-GGCGACACT CCACCATGAAT-3´ (outer sense primer, nucleotide positions -324 to -305) and 5´-CATGGTGCA CGGTCTACGAG-3´ (outer anti-sense primer, nucleotide positions -17 to +3) for the first round of amplification cycles, and 5´-GGAACTACTGT CTTCACGCAG-3´ (inner sense primer, nucleotide positions -291 to -271) and 5´-TCGCAAGCACCC TATCAGGCA-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 minutes at 94°C, 0.7 minutes at 55°C, and 1.5 minutes at 72°C. Any amplified product could be detected after fractionation in Tris-borate buffer by electrophoresis (120 V for 50 minutes) 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, Lewisville, Texas): 5´-AGGTGGTGGAT GGGTGAT-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´-TGGTAGTCGTAAA TCCCGGT-3´ (inner sense primer located at position 134) and 5´-GGRGCTGGGTGGCCYCAT GCWT-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 (Jarvis *et al*, 1996). 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 thalassemic patients known to be positive for HCV or HGV served as the positive controls and sterile water served as the negative control.

TTV-DNA extraction: DNA was isolated employing the alkaline extraction method (Kaneko *et al*, 1989). 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 minutes. The solution was subsequently spun for 15 seconds in a microcentrifuge and neutralized with HCl at a final concentration of 0.1 M.

TTV-DNA detection: 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, primer pairs NG 059 and NG 063 for the first round, and NG061 and NG 063 for the second round, respectively, 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* (1998) the nucleotide sequences of the TTV primers derived from the N-22 region, which represents the most conserved sequence of the 5 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 seconds, annealing at 55°C for 42 seconds, and extension at 72°C for 1.5 minutes, final extension at 72°C for 10 minutes). The second round of amplification was performed using 2 μl of the 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 centrifuged for 1 minute at 10,000 rpm, and 10 μl each of the amplified DNA 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 IVDU and known to be positive for TTV-DNA and sterile water were used as positive and negative controls, respectively.

Serology: All sera were tested for hepatitis B surface antigen (HBsAg) using a commercially available kit (Auszyme II, Abbott Laboratories, North Chicago, Ill), as well as for anti-HCV by ELISA (Ortho Diagnostic Systems, Chiron Corp, Emeryville, CA).

Data analysis: The prevalence obtained for HBV, HCV, HGV and TTV, respectively, was expressed in per cent related to the total number of patients in each group (chronic hepatitis, cirrhosis, hepatocellular carcinoma and cholangiocarcinoma), as well as in per cent related to the total number of patients in the study.

RESULTS

The results of serology, as well as PCR performed on the 4 study groups are shown in Table 2. The prevalence of hepatitis B infection in all patients is about two-fold higher than that of hepatitis C and four-fold higher than that of hepatitis G as well as TTV infection, respectively. We detected HBsAg in 82 patients, whereas 46 patients were positive for anti-HCV antibody. By semi-nested PCR, we found HBV DNA in 52 patients, while 31 patients were positive for HCV RNA, 22 for HGV RNA, and 19 for TTV single stranded DNA, respectively. Contrasting the 52 cases positive for both HBsAg and HBV DNA, we determined anti-HCV along with HCV RNA in only 22 patients. Furthermore, the number of cases positive for HBsAg without the presence of HBV DNA amounted to 30 whereas 15 patients were positive for anti-HCV without detectable HCV RNA. Among the 168 sera tested we did not detect any positive for HBV DNA without detectable HBsAg, but found 9 cases positive for HCV RNA devoid of anti-HCV antibody. Finally, 31 cases (19%) of chronic liver disease, including HCC, and 6 cases (86%) of CCA turned out to be negative for all the hepatitis markers.

DISCUSSION

Our data clearly demonstrate hepatitis B virus to be the major cause of chronic hepatitis, cirrhosis and HCC. The prevalence of HBV in these three groups has been shown to be approximately twice that of HCV and four-times that of HGV as well as of TTV, respectively. However, HBV markers, including HBV DNA and HBsAg, are found in less than 40% of cirrhotic patients, a level much lower than that detected in chronic hepatitis and HCC cases. This result could be explained by the fact that approximately 20% of the cirrhotic patients selected for the study were serious alcohol consumers which appears to be the single underlying cause of cirrhosis found in these patients. As to the prevalence of HGV infection, we detected HGV RNA in approximately 10% of the patients, a frequency amounting to twice that previously observed among blood donors in Thailand (Poovorawan *et al*, 1998) and in most countries around the world (Cheung *et al*, 1997).

Among HCC patients in Thailand, an area endemic for HBV infection, the prevalence of HCV-related cases is comparable to that in Taiwanese (19.5 %) (Chuang *et al*, 1992) and Korean populations (28 %) (Kim and Park, 1993), but it is definitely lower than the frequency reported from several countries not endemic for hepatitis B, where HCV is rendered more crucial regarding the etiology of hepatocellular carcinoma. In Japan, for example, the prevalence of antibodies to HCV amounts to between 50 and 70 % of HCC cases (Suga *et al*, 1994), and among Caucasian populations to between 30 and 70 % (Ruiz *et al*, 1992; Di Bisceglie *et al*, 1994) The average age of patients with HCV-related HCC tends to be higher than that of HBV-related hepatoma (60.4 *vs* 49.2 years) possibly reflecting the timing of hepatitis viral infection (Di Bisceglie *et al*, 1994).

Studies of patients serologically negative for HBsAg but harboring HBV DNA in serum or liver tissue have been documented (Brechot *et al*, 1985; Thiers *et al*, 1988). These results would suggest a level of viral replication too low for serological detection resulting from hepatitis B infection in the

Table 2
Association of hepatitis viruses with liver disease in 168 cases.

Viral marker	Chronic hepatitis (53)	Cirrhosis (65)	HCC (43)	CCA (7)	Total (168)
Hepatitis B	36 (67%)	22 (34%)	24 (56%)	0	82 (49%)
HBsAg (total)	36	22	24	0	
HBV DNA (total)	25	11	16	0	
HBsAg + HBV DNA	25	11	16	0	
HBsAg (only)	11	11	8	0	
HBV DNA (only)	0	0	0	0	
Hepatitis C	18 (34%)	18 (28%)	9 (21%)	1	46 (27%)
anti-HCV (total)	14	15	7	1	
HCV-RNA (total)	11	14	6	0	
anti-HCV + HCV-RNA	7	11	4	0	
anti-HCV (only)	7	4	3	1	
HCV-RNA (only)	4	3	2	0	
Hepatitis G	6 (11%)	11 (17%)	5 (12%)	0	22 (13%)
Hepatitis TT	11 (21%)	5 (8%)	3 (7%)	0	19 (11%)
Hepatitis B + C	3 (5.5%)	1 (1.5%)	3 (7%)	0	7 (4%)
Hepatitis B + G	4 (7.5%)	1 (1.5%)	2 (4.5%)	0	7 (4%)
Hepatitis B +TT	6 (11%)	3 (4.5%)	0	0	9 (5%)
Hepatitis C+ G	1 (2%)	3 (4.5%)	2 (4.5%)	0	6 (3.5%)
Hepatitis C+TT	4 (7.5%)	0	2 (4.5%)	0	6 (3.5%)
Hepatitis B + C +G	0	1 (1.5%)	0	0	1 (0.5%)
Hepatitis B+C+TT	1 (2%)	0	0	0	1 (0.5%)
No viral marker	2 (4%)	18 (28%)	11 (25.5%)	6 (86%)	37 (22%)

The percentages given are relative to the respective patient group indicated on top.

distant past, yet with the mostly fractionated viral DNA left integrated in the hepatocyte DNA (Liang *et al*, 1993). Alternatively, they could be due to changes in the viral nucleotide sequence leading to an altered immunological profile (Kremsdorf *et al*, 1993).

In contrast, cases with only HBsAg, but no HBV DNA detectable - approximately 35% of the hepatitis B patients in our study - clearly indicate the respective individuals' ability to eliminate the virus. Regarding HCV infection, there were approximately 30% of cases only positive for anti-HCV, yet negative for HCV RNA. On the contrary, about 20% of the patients were serologically negative for anti-HCV but had HCV RNA detectable in their sera, which indicates active viral replication, but due to the genotype variations of HCV and the limited number of serotypes traceable with the diagnostic kits presently available some serotypes escape detection (Songsivilai *et al*, 1996).

Considering HBV-HCV coinfections, other studies have shown that patients with concurrent infections have more severe liver disease than those infected with only one virus (Fong *et al*, 1991). Furthermore, dual infections with HBV-HCV may imply an increased risk for the development of HCC (Kew *et al*, 1997). Coinfection with HBV and HCV usually results in the suppression of one of the two viruses with HCV, however, exhibiting a tendency towards dominance over HBV (Fattovich *et al*, 1991; Liaw, 1995). In our study, HBV-HCV coinfections were detected in approximately 4% of the patients, a similar prevalence to that reported from other Asian countries (Panigrahi *et al*, 1997). Of those seven patients, four were positive for HCV RNA, while three harbored HBV DNA.

In contrast to HBV-HCV coinfection, hepatitis virus G has little or no impact on the course of hepatitis C infection. The clinical picture of liver disease in the patients with HCV-HGV coinfection was not different from that observed in those infected only with HCV (Tanaka *et al*, 1996). In addition, recent data have demonstrated HGV coinfection to have no impact on the clinical course of acute hepatitis A, B, or C (Alter *et al*, 1997). Thus the clinical significance of hepatitis virus G

remains unclear, as it rather appears to represent an opportunistic virus predominantly detected in individuals at risk for blood-borne transmission. Due to the very recent discovery of hepatitis TT virus, presently there are neither data available regarding its impact on HBV and/or HCV infections, nor with respect to its overall clinical significance.

In the present study, HCV-HGV and HCV-TTV coinfections both amounted to 3.5%, HBV-HGV and HBV-TTV coinfections to 4% and 5%, respectively, cases in which HCV or HBV may well play the dominant role as the etiologic agent of chronic liver disease and HCC. Interestingly, one case of our cirrhosis patients harbored triple hepatitis virus infections with HBV, HCV and HGV, and in one of our chronic hepatitis patients triple infections with HBV, HCV and TTV were detected.

Contrasting HCC, cholangiocarcinoma (CCA) is usually not associated with underlying cirrhosis, but occurs predominantly in males at an average age at presentation of approximately 60 years, while it is rarely found in individuals below the age of 40 years (Okuda *et al*, 1997). As CCA is endemic in northeastern Thailand, where infestation with the liver fluke, *Opisthorchis viverrini*, is common (Kurathong *et al*, 1985), and as no association with viral hepatitis or aflatoxin exposure has been reported (Srivatanakul *et al*, 1991), the predominantly negative results for all the viral markers obtained with our CCA group are not surprising. In any event, among chronic hepatitis, cirrhosis and HCC patients, after careful exclusion of other potential causes of chronic liver disease, such as alcohol, drugs and autoimmune disorders, there remains a small percentage of patients (5-10%) negative for all the known viral hepatitis markers. It seems probable that, in these cases, as yet unidentified viral agents may trigger the processes leading to chronicity.

In conclusion, in Thailand, chronic HBV infection and, to a lesser extent, chronic HCV infection are the two most common causes of hepatitis which can proceed towards chronic hepatitis, cirrhosis and hepatocellular carcinoma. The clinical significance of the rather novel hepatitis G virus, as well as that of the very recently characterized hepatitis TT virus with respect to actual liver disease is still uncertain. In the present study, coinfections with distinct hepatitis viruses were not as rare as previously assumed, but their respective impact on the clinical outcome remains to be further elucidated. Finally, the search for novel hepatitis viruses harboring the potential to trigger the development of chronic liver disease should continue.

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