Original Article

Prevalence and Clinical Feature of Occult Hepatitis B Infection in Thai Patients with Chronic Hepatitis C

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ABSTRACT

Background: Occult hepatitis B is defined by the presence of the hepatitis B virus (HBV)-DNA in a serum or in the liver tissue in the absence of hepatitis B surface antigen (HBsAg). The prevalence and clinical correlates of occult hepatitis B remain incompletely defined. This cross-sectional study was carried out to determine the prevalence of occult hepatitis B with chronic hepatitis C disease in Thailand.

Method: Using the polymerase chain reaction, we searched for HBV DNA in formalin-fixed liver samples from 49 HBsAg-negative patients with hepatitis C virus (HCV)-related chronic hepatitis. Twenty-three patients had detectable antibodies to the HBV core antigen (anti-HBc) while another fourteen patients had no detectable antibodies. We also studied five HBsAg-positive patients as a control group.

Results: Intrahepatic HBV DNA was not detected in 50 specimens examined by nested PCR to identify HBV DNA and HBV genotype. In HBsAg-positive control groups, HBV DNA was detected in three of five specimens.

Conclusions: A very low occult HBV infection rate was found in HCV Thai patients, but this may be not a true prevalence of occult HBV. Fresh frozen liver tissue or peripheral mononuclear cell may be a better sample for HBV testing. For future study, a new nested PCR technique to detect HBV DNA for other organs should be developed to evaluate the "true prevalence" of occult HBV infection.

Key words : Occult hepatitis B, chronic hepatitis C, hepatitis B virus

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INTRODUCTION

Hepatitis C infection is a common chronic liver disease worldwide that can lead to liver cirrhosis and liver failure. Between 180 and 200 million people all over the world are infected with this virus. The prevalence of chronic hepatitis C in Thailand is estimated at about 0.7-4%, depending on the geographic area, with about 800,000-1,000,000 patients approximately.

Hepatitis B infection is also another common disease with local prevalence of about 3-5%. Both hepatitis B and hepatitis C share similar transmission routes, such as needle-sharing practice among intravenous drugs addicts, or exposure to blood or blood products of infected patients. As a result, a significant number of patients are co-infected with the hepatitis B and hepatitis C virus. In Thailand, the prevalence of co-infection is about 3-9%.

Over the past two decades, many investigators have probed into occult hepatitis B infection. Although virology testing and genome amplification method have improved, the most definitive diagnosis requires demonstration of hepatitis B viral DNA in the liver tissue. However, a low level of viral DNA can also be found in the blood stream of a small number of patients[1]. Such so-called occult hepatitis B infection can nowadays be detected not only in patients with positive anti-HBs or anti-HBc but also in hepatitis B seronegative patients due to the advance in diagnostic technology.[2-5]

Clinical observation has shown that occult hepatitis B infection plays an important role in the reactivation of hepatitis B infection in patients with haematological malignancy, HIV-infection, chemotherapy and organ transplants.[6] Among chronic hepatitis patients, those with occult hepatitis B infection trend to have a more severe liver disease than those without.[7] Based on such observation, several studies on occult hepatitis B infection in chronic hepatitis C patients have been made. Some studies found that occult hepatitis B could cause more severe liver disease[8-16] and could reduce the response rate to IFN treatment[17,18], which would adversely affect the current standard treatment of peg-interferon plus ribavirin.

According to medical literature, the prevalence of occult hepatitis B in chronic hepatitis C patients is rather high.[19,20] There are no reports as yet on the prevalence of occult hepatitis B infection in Thai patients with chronic hepatitis C. This study was aimed at determining the prevalence of occult hepatitis B infection in Thai patients with chronic hepatitis C, employing a combination of PCR on formalin-fixed embedded tissues, clinical characteristics, and liver histology. Information gained from this research would provide preliminary data for further study on occult hepatitis B infection in chronic hepatitis C patients, as well as a better understanding of the natural history and treatment of occult hepatitis B and help provide a more effective treatment.

METHODS

Patients

A review was made of medical charts of patients with chronic HCV-related liver disease with undetectable HBsAg, who underwent diagnostic liver biopsy at Prince Songkla University Hospital between 2003 and 2008. All patients were HIV-negative and did not consume excess alcohol.

HCV infection was defined by the presence of anti-HCV antibodies and HCV RNA in serum. Forty-nine specimens of formalin-fixed, paraffin-embedded liver tissue were collected for this preliminary study. The collection and use of liver tissue for this study were approved by the appropriate Institutional Ethics Committees.

Liver Tissue

The liver tissues were stored in formaldehyde for histopathological analysis of the lesions and for further analysis of HBV DNA by PCR. A pathologist (S.K) who was blinded to the clinical data assessed histopathology findings, using modified Knodell score as grade (0-18 points) and stage (0-6 points) in all cases.

HBV DNA Analysis

DNA extraction from FFPE Tissue

DNA was extracted using the "MagneSil® genomic fixed tissue system". A section was placed in a 1.5 ml microcentrifuge tube. One-hundred microlitres of freshly made "Incubation buffer/proteinase K" solution was added and incubated at 56˚C overnight. There was no need to deparaffinize thin tissue sections. After removing the tube and adding two volumes of lysis buffer, 7 μl of resin slurry was added to the proteinase K-treated sample. Sample lysis buffer/resin mix was mixed by vortex for three seconds at high speed, then incubated at room temperature for five minutes. The tube was placed in a magnetic stand for separa-

tion. All the lysis buffer was discarded, and 100 µl of prepared 1× wash buffer was added. All wash buffer was discarded, and the washing step was repeated for a total of three washes. The resin was air-dried in the magnetic stand for five minutes.

Twenty-five microlitres of elution buffer was added and mixed by vortex for two seconds, and then placed in a temperature of 65˚C for five minutes, before immediately placed on the magnetic stand. The DNA-containing solution was carefully transferred to a container of choice.

Amplification

The first-round PCR primers (outer primer pairs) and the second-round PCR primers (inner primer pairs) were designed on the basis of the conserved nature of the nucleotide sequences in regions of the pre-S1 through S genes, irrespective of the six HBV genotypes.

P1 (sense) and S1-2 (antisense) were universal outer primers (1,063 bases). B2 was used as the inner primer (sense) with a combination called mix A for genotypes A, B, and C. Mix A consisted of antisense primers BA1R (type A specific), BB1R (type B specific), and BC1R (type C specific). B2R was used as the inner primer (antisense) with a combination called mix B for genotypes D, E, and F. Mix B consists of sense primers BD1 (type D specific), BE1 (type E specific), and BF1 (type F specific). After optimizing the process, the first PCR was carried out in a tube containing 40 µL of the reaction mixture, made up of 0.5 µM of each of the outer primers, 200 µM of each of the four deoxynucleotides, 2.5 units of Taq DNA polymerase, 5 µL of reaction buffer and 1.5 mM $MgCl_2$. Then, the sample was added. The samples were denatured for two minutes at 94˚C, followed by 40 cycles each consisting of 40 s at 94˚C, one minute at 57˚C, and one minute at 72˚C in an PCT100 (MJ Research) thermal cycle. One microliter aliquot of the first-round PCR product was added to two tubes containing the second sets of each of the inner primer pairs, each of the deoxynucleotides, Ampli Taq Gold DNA polymerase, and PCR buffer as in the first reaction. The samples were amplified for 35 cycles, consisting of preheating at 95˚C for two minutes, 20 cycles of amplification at 94˚C for 20 seconds, and 68˚C for 50 seconds.

Detection of PCR Product

Ten micro-litres of the reaction product was electrophoresed on a 3% agarose gel, stained by ethidium bromide, and evaluated under ultraviolet light. Negative and positive controls were also treated as samples. We used DNA samples of HBV with different genotypes, which were confirmed by genotype sequencing, as controls. Beta-globin was used as an internal control (house-keeping gene).

Statistical Analysis

Student's *t*-test and the Mann-Whitney test were used to analyse quantitative data. Fisher's exact test was used to analyse qualitative data and for comparing proportions. All *p*-values are two-tailed and a *p*-value of <0.05 was considered to be indicative of statistical significance.

RESULTS

All liver specimens were from biopsies taken in 2008. The primer sequences for HBV genotyping by nested PCR was shown in Table 1. The baseline characteristics of patients were shown in Table 2. Intrahepatic HBV DNA was not detected in the 50 specimens examined by nested PCR for iHBV DNA and HBV genotype. In the control group with HBsAg positive specimens, HBV DNA was detected in 3 of the 5 specimens. All positive specimens had high levels of HBV DNA, with between 10^4-10^5 and genotype C being found in all 3 specimens, but 2 of the 5 showed negative results. These negative specimens had low levels of HBV DNA, about 10^3-10^4. (Table 3)

DISCUSSION

The prevalence of HBV differs in varying geographic areas and in different populations.[31] Cacciola I *et al*., reported the prevalence of occult HBV infection in HCV infected patients in Italy at 33%[8] while In Japan the prevalence of occult HBV infection was 70% in the non-B, non-C chronic hepatitis group,[32] and occult HBV in HCV liver disease was up to 60%.[33]

The prevalence of occult HBV in Thailand has not been established, but the prevalence of HBV and HCV co-infection was in the region of 3-9%. We assumed a similar prevalence of occult hepatitis B infection to the co-infection. The number of liver tissue samples in this study was considered adequate for evaluation, but there turned out to be no HBV DNA positively in any of the 49 specimens.

Table 1. Primer sequences used for HBV genotyping by nested PCR.

Primer	Sequence A (position, specificity, and polarity) 5'- 3'	Position	Type	Size
First PCR				
P1b_F	TCA CCA TAT TCT TGG GAA CAA GA	2823-2845		
S1-2_R	CGA ACC ACT GAA CAA ATG GC	685-704		
Second PCR				
Mix A				
B2_F	GGC TCM AGT TCM GGA ACA GT'	67-86	A to E	
BA1R	CTC GCG GAG ATT GAC GAG ATG T	113-134	A	67
BB1R	CAG GTT GGT GAG TGA CTG GAG A		B	281
BC1R	CAG CCT AGG AAT CCT GAT GTT G	165-186	C	122
Mix B				
BD1_F	GCC AAC AAG GTA GGA GCT	2979-2996	D	119
BE1_F	CAC CAG AAA TCC AGA TTG GGA CCA	2955-2978	E	171
BF1_F	GYT ACG GTC CAG GGT TAC CA	3032-3051	F	97
B2R	GGA GGC GGA TYT GCT GGC AA	3078-3097	D to F	

An "M" represents a nucleotide that could be either an A or a C; a "Y " represents a nucleotide that could be either a C or aT. "nt" stands for nucleotide.

Table 2. Baseline characteristics of patients.

	HBsAg -ve with chronic HCV patient (N = 49)	Anti HBc positive (N = 23)	Anti HBc negative (N = 14)
Characteristics			
Age	48.18 ± 11.02	48.6522 ± 11.68	46.93 ± 9.00
Sex (M/F)	27/22	15/8	4/10
Liver Function Test			
AST (U/L)	82.36 ± 49.62	73.26 ± 45.65	85.21 ± 39.44
ALT (U/L)	117.91 ± 92.92	106.91 ± 78.65	103.92 ± 57.08
ALP (U/L)	81.51 ± 27.89	81.48 ± 25.32	75.07 ± 27.98
Total bilirubin (mg/dL)	0.75 ± 0.42	0.80 ± 0.51	0.67 ± 0.37
Albumin (g/dL)	4.34 ± 0.42	4.42 ± 0.36	4.39 ± 0.27
PT	11.98 ± 1.94	11.80 ± 2.55	11.77 ± 0.88
Liver histology (HAI)	7.59 ± 3.91	8.26 ± 3.80	6.29 ± 3.24

AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; PT = prothrombin time

Table 3. HBsAg negative with HCV patients.

HBV DNA	HBsAg -ve with HCV patient (N = 49)	Anti HBc positive (N = 23)	Anti HBc negative (N = 14)
Liver tissue	0/49	0/23	0/14

The prevalence of occult HBV infection in hepatocellular carcinoma in the study by Alencar et al, was shown to be as in our study. HBV DNA was detected in none of the formalin-fixed liver tissues but in only one of 50 serum specimens.[29] Ding X, *et al*. in a 6-country multicenter study to investigate the relationship between HBV and p53 mutation in hepatocellular carcinoma, reported that intrahepatic HBV DNA was detected in about 10% (44 of 449) of all formalin-fixed liver tissues.[30]

Such finding was probably related to the fact that the small liver biopsy fragments analysed did not con-

Table 4. HBs Ag positive specimen for control group.

No.	Paraffin Block	Results			Conclusion/Result	HBV DNA
		B-globin	Mix-A	Mix-B		
1	51-4108	+	+	-	HBV genotype C122 bp	67,5000 IU/ml
2	51-4876	+	-	-	Negative	17,000 IU/ml
3	51-6812	+	+	-	HBV genotype C122 bp	>1000000 IU/ml
4	51-6813	+	+	-	HBV genotype C122 bp	>1000000 IU/ml
5	51-4623	+	-	-	Negative	3,330 IU/ml

+ = Positive, - = Negative

tain any viral particles that might have been present in other liver fragments. Another possible explanation was that only formalin-fixed embedded paraffin tissues (in which some of the DNA might have been degraded during the process) were analysed, and perhaps a higher sensitivity could have been achieved had we analysed freshly obtained liver fragments that would be immediately transferred to a DNA-conserving storage solution or frozen at -70˚C.

In conclusion, occult HBV infection was not demonstrated in HCV patients in our study, but this may not reflect the true prevalence of occult HBV infection as the PCR system did not detect HBV DNA in some of the HBsAg-positive control group (HBsAg-positive formalin-fixed liver specimen). Using the alternative PCR method as previously described[30] may well increase the sensitivity of the test. For future study to evaluate the "true prevalence" of occult HBV infection, fresh-frozen liver tissue or peripheral mononuclear cells may be a better sample, and a new nested PCR technique at other regions should also be developed to detect HBV DNA.

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