

Occult Hepatitis B Virus Infection in a Cohort of Liver Disease Patients with Chronic Hepatitis C: A significant Association of HCV Genotype 1b in South India

Shanmugam Saravanan, Vidya Madhavan, Kailapuri G Murugavel, Pachamuthu Balakrishnan, Sunil Suhas Solomon, Dhakshinamoorthy Subashini, Greer Waldrop, Venkataraman Jayanthi, Suniti Solomon, Sadras P Thyagarajan

Shanmugam Saravanan, Vidya Madhavan, Kailapuri G Murugavel, Pachamuthu Balakrishnan, Sunil Suhas Solomon, Dhakshinamoorthy Subashini, Greer Waldrop, Suniti Solomon, Sadras P Thyagarajan, Y.R.Gaitonde Centre for AIDS Research and Education, Chennai, India

Sadras P Thyagarajan, Sri Ramachandra University, Chennai, India
Venkataraman Jayanthi, Stanley Medical College and Hospital, Chennai, India

Sadras P Thyagarajan, Dr. ALM. Post Graduate Institute for Basic Medical Sciences, University of Madras, Chennai, India

Correspondence to: Shanmugam Saravanan, PhD, Assistant Professor, Y.R.Gaitonde Centre for AIDS Research and Education (YRG CARE), Voluntary Health Services (VHS) Hospital, Taramani, Chennai-600 113, India. saravanan@yrgcare.org

Telephone: +91-44-22542929 Fax: +91-44-22542939

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ABSTRACT

AIM: Occult hepatitis B virus infection (OHBI) is characterized by low viral replication in the absence of Hepatitis B surface antigen (HBsAg), and although this has been described among people with chronic hepatitis C liver disease in the western world, its prevalence and clinical significance remains unclear in the Indian subcontinent. Hence the present study was carried out to analyse the frequency and clinical outcomes of occult HBV infections among patients with Hepatitis C Virus (HCV) -related chronic liver disease (CLD) and its associations with HCV genotype.

METHODS: Hepatitis B Virus DNA by polymerase chain reaction (PCR) in serum samples of 260 HBsAg negative subjects with chronic HCV related liver disease, and in 70 apparently healthy volunteers who were negative for both HBsAg and HCV antibody was investigated. Serum samples reactive by at least two independent PCR assays were considered HBV DNA positive.

RESULTS: HBV-DNA was detected among 19 of the subjects with HCV-related chronic liver disease (CLD) (7.3%), which was significantly higher ($p=0.2$) than the rate found among the controls (4.3%). HBV occult infection was more frequent (37.5%) in 24 anti-HBs negative/anti-Hepatitis B core Antigen (anti-HBc) positive

patients than in 180 anti-HBs/anti-HBc positive (5 %, $p<0.05$). HCV RNA positivity by qualitative PCR was significantly ($p<0.001$) higher among subjects with occult HBV infection compared to those without occult HBV infection. Further, HCV genotype 1b was predominantly associated with occult HBV (73%), especially among subjects with hepatocellular carcinoma (HCC) ($p<0.05$) as compared to non-occult HBV cases.

CONCLUSION: This study suggests that in areas endemic for HBV infection, in patients with HCV, especially of genotype 1b, occult HBV should be considered and HBV DNA testing should be performed.

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Key words: Occult hepatitis B; Anti-HBc; Hepatitis B and C virus; CLD in India; HCV genotype

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INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections account for a substantial proportion of cases of chronic liver disease (CLD) including chronic hepatitis, cirrhosis, and liver cancer. It is widely believed that almost 400 million HBV carriers and 170 million HCV carriers exist worldwide^[1,2]. HBV and HCV share same routes of transmission; thus, coinfection with both viruses is likely, particularly in areas where the two viruses are endemic^[3,4]. The diagnosis of active HBV infection is usually based on the detection of hepatitis B surface antigen (HBsAg), and the disappearance of this antigen indicates clearance of HBV^[1]. However, HBV genomes may also be present among people who are negative for HBsAg. This "occult" HBV infection has been associated with HCV-infection, particularly in western countries^[5,6,9-11]. These occult HBV infections in the setting of HCV infection have been associated with more severe liver damage and the development of hepatocellular

carcinoma (HCC)^[5,12,13]. Though a prevalence rate of 5.12% of occult HBV infection has been reported in northern India^[14], the prevalence and clinical associations of HBV “occult” infection on HCV infection are not known in southern part of India. Hence the current study was undertaken to study the frequency and clinical outcomes of occult HBV infections in patients with HCV-related chronic liver disease (CLD) and the associated HCV genotype.

METHODS

Study design

A total of 260 consecutive patients with CLD who had a diagnosis of chronic active hepatitis C, referred from various gastroenterology departments of hospitals from southern India to the National Referral Centre for Viral Hepatitis, Department of Microbiology, Faculty of Medicine, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, and Chennai, India were enrolled. We also enrolled 70 apparently healthy volunteers who were negative for both HBsAg and HCV antibody as controls. The study was approved by the Institutional Ethics Committee of the University of Madras, Taramani, Chennai, South India.

Patients

The study group consisted of 330 subjects and their demographics were given in table 1. Available biochemical, clinical, and liver

Table 1 Demographic characteristics of patients with chronic HCV infection, depending on the presence or absence of occult HBV infection (*n*=260).

| Variables | ¹ Occult HBV positive | Non-Occult HBV |
|---|----------------------------------|----------------|
| <i>n</i> =260 | 19 (7.3%) | 241(92.7%) |
| Age | 44±12 | 46±15 |
| Sex | | |
| Male (<i>n</i> =162) | 11 (58%) | 151 (63%) |
| Female (<i>n</i> =98) | 8 (42%) | 90 (37%) |
| Risk Factors | | |
| Sexual Promiscuity | 0 (0%) | 15 (6%) |
| IDU's | 0 (0%) | 1 (0.4%) |
| BT/hospitalization/surgery | 8 (42%) | 90 (37%) |
| Tattoo/ear piercing | 5 (26%) | 75 (31%) |
| Exposure to unsterilized syringes & needles | 5 (26%) | 42 (17%) |
| Unknown | 1 (5%) | 18 (7.5%) |
| Laboratory Investigations | | |
| ALT | 158±12 | 140±18 |
| AST | 138±19 | 128±12 |
| Liver diseased patients | | |
| Chronic hepatitis (<i>n</i> =183) | 5 (26%) | 178 (74%) |
| Liver cirrhosis (<i>n</i> =48) | 4 (21%) | 44 (18%) |
| Hepatocellular carcinoma (<i>n</i> =29) | 10 (53%) | 19 (8%) |
| HCV RNA by qualitative PCR | 17 (89%) | 136 (56%) |

¹ Serum samples reactive by at least two independent qualitative diagnostic PCR assays were considered HBV DNA positive.

histopathological information were used to correlate with the virological profile. The diagnosis of CLD including mild chronic hepatitis, moderate or severe chronic hepatitis, liver cirrhosis, and HCC were assessed based on histological activity index (HAI), per standard guidelines^[15]. HCC was assessed by pathologic examination of needle aspiration cytology/biopsy collected from the liver lesions of the study participants by experienced gastroenterologists. All subjects were interviewed on the basis of a pre-designed questionnaire regarding occupation, history of jaundice (its nature, etiology and outcome), blood transfusion, surgery/dental/gynecological procedures, exposure to syringes and needles, ear piercing, sharing of razors, blades, mother being HBsAg and/or anti-HCV positive, information on high risk behavior such as intravenous drug use, number of lifetime sexual partners,

tattooing, etc. None of the patients had a history of haemodialysis or organ transplantation.

Specimens and methods

Five milliliters of blood sample was collected aseptically, serum was separated, aliquoted and stored at -70°C until tested. The following HBV and HCV markers were detected using commercial ELISA kits; IgG anti HBc, HBsAg, anti HBs (Biorad Laboratories, USA) and anti-HCV (Murex Diagnostics, UK), using known positive and negative controls. Serum was assayed for HBV-DNA by PCR and HCV-RNA by RT-PCR employing methods described by Cheung and Cha^[16,17]. The sensitivities of the PCR assays were ten and 50 copies of HBV-DNA and HCV-RNA per specimen respectively, as determined by testing of serial 10-fold dilutions of HBV-DNA/HCV-RNA with known concentrations. Serum samples that were reactive by at least two independent PCR assays were considered to be positive for HBV-DNA/ HCV-RNA. To avoid false-positive results, the methods described by Kwok and Higuchi^[18] were adapted. HCV genotyping was carried out using a type-specific detection system of the PCR-amplified 5' non-coding region (5' NCR) (Inno-LiPA, Innogenetics, Belgium) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences software (SPSS for Windows, version 13.0, Chicago, IL). The median values of ALT levels accordingly, with or/ without occult HBV, and liver profile were analyzed using the Mann-Whitney U test (non-parametric). All *p* values were 2-sided hypotheses.

RESULTS

Of the total 260 patients with chronic HCV infection and negative HBsAg tests in the cohort, the sera of 19 (7.3%) were positive for HBV-DNA by PCR revealing an occult HBV infection. The prevalence of occult HBV infection in HCV carriers was comparable to the healthy adult controls (4.3%) (Exact *p*=0.25) (Table 2). In addition,

Table 2 Frequency of HBV DNA in sera of 70 healthy controls and 260 HCV carriers by serological patterns and liver status (*n*=330).

| Group and serological pattern or liver status | No. of individuals | No.s (%) positive for HBV-DNA ¹ | <i>p</i> value |
|---|--------------------|--|----------------|
| Healthy controls | 70 | 3 (4.3%) | 0.2 |
| Negative for all HBV markers | 12 | 0 (0%) | |
| Positive for anti-HBc alone | 4 | 2 (50%) | |
| Positive for anti-HBc and anti-HBs | 54 | 1 (1.8%) | |
| HCV infected patients | 260 | 19 (7.3%) | 0.2 |
| Negative for all HBV markers | 46 | 0 (0%) | |
| Positive for anti-HBc alone | 24 | 9 (37.5%) | |
| Positive for anti-HBc and anti-HBs | 190 | 10 (5.2%) | |
| HCV infected patients | 260 | 19 (7.3%) | 0.2 |
| Chronic hepatitis | 183 | 5 (2.7%) | |
| Liver cirrhosis | 48 | 4 (8.3%) | |
| Hepatocellular carcinoma | 29 | 10 (34%) | |

¹ Serum samples reactive by at least two independent qualitative diagnostic PCR assays were considered HBV DNA positive.

none of the 12 controls and 46 chronic HCV patients negative for all three serological markers of HBV infection were positive for HBV-DNA by PCR amplification.

Among the 70 healthy adult controls and 260 patients infected with HCV, the prevalence of occult HBV infection ranged from 0 to 50% among subjects, those positive for anti-HBc alone, and those positive for both anti-HBs and anti-HBc (Table 2). Occult HBV was not seen among those who were negative for all markers. The mean age

of patients with HCC, HCV infection and occult HBV infection was similar to those who had HCV infection alone (51 ± 4 vs. 51 ± 5 years). Most of the occult HBV infection was seen among aged patients (>40 years) and though ALT was elevated its not significant compared to those patients with non-occult HBV infection (Table 1).

Among 65 patients with chronic HCV and HBV co-infection (15 occult and 50 non-occult) and who were tested for HCV genotype, we found that 1a, 1b, 3 and mixed infection genotypes were found in 9 (14%), 38 (58%), 12 (18%), and 6 (9%) patients, respectively (Table 3). Genotype 1b predominance was observed among both occult (73%; 11/19) and (54%; 27/54) non-occult HBV (Table 3) cases. When these patients were stratified by the presence or absence of occult HBV infection, the frequency of HCC was significantly higher among patients with HCV and occult HBV coinfection. Although more HCV viremic patients were observed in occult HBV infections compared to non-occult HBV, the difference was not markedly significant.

Table 3 Distribution of HCV genotypes among Chronic HCV Patients.

| Variables | Occult HBV ¹ (n=19) | Non-occult HBV (n=241) |
|-------------------------------------|-----------------------------------|---------------------------|
| HCV RNA by qualitative PCR | 17 (89%) | 136 (56%) ² |
| Number genotyped | 15 | 50 |
| HCV Genotypes: | | |
| 1a | 3 (20%) | 6 (12%) |
| 1b | 11 (73.3%) | 27 (54%) |
| 3 | 1 (6.6%) | 11 (22%) |
| 3 and 4 | 0 (0%) | 6 (12%) |
| Distribution of 1b genotype: | | |
| Chronic hepatitis | 2 (18%) | 10 (37%) |
| Liver cirrhosis | 1 (9%) | 5 (19%) |
| Hepatocellular carcinoma | 8 (73%) | 12 (44%) ³ |

¹ Serum samples reactive by at least two independent qualitative diagnostic PCR assays were considered HBV DNA positive; ² $p < 0.001$ and; ³ $p < 0.05$ for the comparison with occult and non-occult.

DISCUSSION

This study found that 7.3% of the patients with HCV-related chronic hepatitis had detectable HBV DNA levels, despite the absence of circulating HBsAg. This prevalence of occult HBV infection in the setting of HCV infection was higher than among healthy controls in southern India, which is a HBV intermediate region. Though the reasons for the lack of HBsAg and, in some cases, of all HBV markers despite the persistence of HBV infection are not known OHBI is considered to represent the window period of acute or chronic infection with reduced viral replication or an escape mutant in pre S region resulting in undetectable HBsAg in the peripheral axis^[19]. It is assumed that, due to low HBV loads in many of the cases, HBV may not be identifiable by detection of HBsAg and that the infection is often asymptomatic or has non-specific symptoms. This serologically unapparent infection makes the true global incidence of HBV hard to estimate.

In line with earlier reports^[5, 9-11, 13] we have also seen that the prevalence of occult HBV infection was increased in patients with chronic HCV infection (7.3%) as compared to healthy adults (4.3%), implying that the risk of occult HBV infection among HCV carriers is comparatively high in India and this appears to be found more frequently among older patients (>40 years) with evidence of liver disease, suggesting that liver disease progresses insidiously. Recent epidemiological and molecular studies have indicated that persistent HBV infection may have a critical role in the development of HCC in especially HBsAg-negative patients^[7], since HBV has potential oncogenicity as a consequence of the capacity of the viral DNA to get integrated into the host genome^[8].

It is increasingly recognized that after a person is exposed to HBV, infection persists in the liver for prolonged periods^[20-22] in which case, the HBV DNA could be detectable despite the absence of serum HBsAg. There is also growing evidence to support an increase in the risk of HCC among patients with HCV and are co-infected with occult HBV. Japan, an endemic area for HBV and HCV, has $>75\%$ of HCC cases and are believed to be attributed to HCV-related CLD, and $\sim 15\%$ due to HBV-related liver disease^[23]. On the contrary, the incidence of HCC in India is reportedly very low and varies from 0.2 and 1.9%^[24]. Currently, HBV and HCV are increasingly recognized as the major risk factors for the development of HCC in India^[25-27]. Many published reports support our observation that the presence of anti-HBc in the serum of patients with HCV-related HCC is a common clinical finding, which suggests that previous exposure to HBV may be a risk factor for HCC in patients with HCV-related CLD^[28]. However, all these findings were obtained from retrospective studies and were not confirmed prospectively.

Our findings also demonstrate that occult HBV infection is significantly higher among the HCV genotype 1b infected CLD patients, suggesting that genotype 1b may either enhance occult HBV infection or the two infections are likely to be transmitted together. However, this will require careful analysis in prospective settings. Though consequences of occult HBV infection are not clear, a number of studies suggest that occult HBV is associated with an increased risk of HCC in chronic hepatitis C and persistent low level viremia can cause liver damage independent from other disease factors, particularly in India. We conclude that, the importance of tests to detect HBV DNA permitted the identification of HBV infections in HBsAg-negative HCV patients. Hence, we suggest that PCR-based HBV screening is necessary in patients with HCV associated liver diseases, although its etiological importance and benefit of HBV treatment have not been established.

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Authors' contributions: SSa, and SPT conceived and designed the study; SSa enrolled and interviewed the patients; VM, SSS and SSa managed and followed-up the cases; SSa used the diagnostic kits to detect HCV genotype and also performed the HBV and HCV qualitative PCR; KGM, PB, VM and DS acquired, analysed and interpreted the data. VJ, SS and SPT critically revised the manuscript. All authors read and approved the final manuscript. SSa and SPT are guarantors of the paper.

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