

RAPID COMMUNICATION

Occult hepatitis B virus infection and cryptogenic chronic hepatitis in an area with intermediate prevalence of HBV infection

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Abstract

AIM: To assess the possible role of occult HBV infection in the pathogenesis of chronic hepatitis in Iranian patients.

METHODS: After exclusion of autoimmune, metabolic and viral etiologies, 104 consecutive adult patients with histologic and biochemical features of chronic hepatitis and negative HBsAg were enrolled in the study. Qualitative PCR with a sensitivity of 150×10^3 copies/L, using two primers for Pre-S and core regions was applied to measure presence of HBV DNA in serum of the patients.

RESULTS: All 104 patients completed the study. Qualitative HBV DNA was positive in two patients (1.9%).

CONCLUSION: Occult HBV infection has negligible role in the pathogenesis of cryptogenic chronic hepatitis in Iranian patients.

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Key words: Hepatitis B; Cryptogenic chronic hepatitis; PCR

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INTRODUCTION

Several lines of evidence suggest that hepatitis B virus (HBV) may persist in the serum many years after clearance of hepatitis B surface antigen (HBsAg). These evidences include HBV transmission from liver donors even when HBsAg had been negative and the only positive serologic test was hepatitis B core antibody (HBcAb)^[1-3] and appearance of HBsAg and even overt hepatitis in previously HBsAg-negative patients after cancer chemotherapy or immunosuppression^[4,5]. It appears that low-level HBV infection may be the source of viral reactivation that is unmasked by immunosuppression. It is speculated that these occult infections may cause chronic hepatitis^[6,7], increase the rate of cirrhosis^[8,9] and eventually hepatocellular carcinoma^[10] and may transmit *via* blood transfusion.

The causes of undetectability of HBsAg in serum (in the presence of persistent HBV infection) may include mutations in the pre-S or S regions (mostly in "a" determinant region) of the virus genome^[11-14], decrease in viral load^[15] or insufficient sensitivity of the tests used to detect the HBsAg^[15]. In some regions with high endemicity (such as China), about 1/3 or more of cirrhotic patients with negative HBsAg may harbor HBV infection^[15].

The rate of HBV infection in Iran is unknown but it is the most frequent cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. It appears that Iran is in a region with intermediate or even low endemicity and the rate of positivity of HBsAg and HBcAb in the general population is 1.8%-5% and over 35% respectively^[16].

The goal of the present study was to determine whether a negative test for HBsAg in the serum is sufficient to rule out the possibility of chronic HBV hepatitis in Iranian patients with chronic liver disease and to assess the possible role of "a" determinant mutations in Iranian patients with chronic HBV hepatitis. We conducted a prospective study to determine the presence of HBV-DNA in serum of patients with chronic hepatitis and negative HBsAg.

MATERIALS AND METHODS

Patients

After completion of a questionnaire and physical examination, serum samples were collected from 104 adult (> 16 years old) patients (69 males and 35 females) with

chronic (6 mo or more) high alanine amino-transferase (ALT) and negative HBsAg who were referred for liver biopsy to endoscopy wards of Faghihi and Nemzee Hospitals, Shiraz University of Medical Sciences, Shiraz, Fars province, south of Iran, between February 1997 to December 2001.

Diagnosis of chronic hepatitis was based on a minimum of 6 mo or more persistent elevation of ALT (more than 60 or 1.5 times of upper limit of normal value) and grade of necro-inflammation of more than 3/18 of Ishak (modified Knodell) classification in liver histology. All active alcohol users (drinking of more than 20 g alcohol per day), patients with biliary obstruction, cardiac failure, active cancer or chronic infection (such as tuberculosis) and cirrhosis (shrunken liver in ultrasonography and regenerative nodules in histology and one of these features: history compatible with grade 2 of hepatic encephalopathy, endoscopically confirmed esophageal or fundal varices, ascites, hypersplenism), positive HBsAg, positive anti-smooth muscle antibody, positive anti-nuclear antibody, positive anti-mitochondrial antibody, low titers of alpha-one-anti-trypsin or ceruloplasmin, abnormal iron or copper levels, predominant steatohepatitis, biliary duct injury or congestion (Budd-Chiari syndrome) in liver histology were excluded from the study.

Demographic, biochemical and serological data, histological findings and results of PCR of our patients are shown in Table 1.

Serological assays and PCR

All serological tests including HBsAg, IgG-anti HBcAb, second generation of anti-hepatitis C virus antibody (HCVAb), hepatitis B e antigen (HBeAg) and IgG-antibody to hepatitis B e antigen (HBeAb) were performed using ELISA based on manufacturers' instructions.

PCR-assay

In order to prevent any contamination, we strictly adhered to the standard guideline of PCR procedure. Fifty microliters of serum were diluted 1:5 with 250 μ L NaOH (50 mmol/L) for DNA denaturation and RNA inactivation. Samples were then heated at 95°C for 20 min to denature proteins. Forty microliters of Tris 1 mmol/L (pH = 7.3) was then added and centrifuged at 14000 r/min for 7 min. Forty microliters of phenols-chloroform (1:1) were added and centrifuged at 14000 r/min for 7 min. Ten microliters of sodium acetate (3 mol/L) and 300 μ L absolute ethanol were added to each tube. After incubation at -70°C for 40-60 min, tubes were centrifuged at 14000 r/min for 10 min. Supernatant was removed and 50-100 μ L of TE buffer (pH = 7.8) was added to the pellets. PCR-amplification was performed using published oligonucleotide primers^[17] selected from highly conserved HBV surface gene, whereby primer 1 and primer 2 flank 597 base-pair fragment.

Five microliters of sample were added to 45 μ L of reaction mixture (2.5 U Tag polymerase, 22.5 μ mol/L of each primer, 200 μ mol/L of each deoxynucleotide triphosphate, 5 μ L of reaction buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl pH = 8.3 and 1.5 mmol/L MgCl₂]). Samples were denatured for 5 min at 94°C and then subjected to 40 cycles of 1 min at 58°C, 1 min at 72°C and 1 min at 94°C

Table 1 Demographic, biochemical and serological data and histological findings

Variable	Result
Sex (M/F)	69/35
Age (yr)	Mean: 34.8 \pm 10.3, Min: 17, Max: 71
Mean AST nkat/L (\pm SD) ¹	2312.1 (\pm 373.4)
Mean ALT nkat/L (\pm SD) ¹	2525.5 (\pm 430.1)
Mean ALK Ph ² nkat/L (\pm SD) ³	6656.3 (\pm 943.5)
HCV Ab (+/-)	68/36
Serum HBV-DNA (+/-)	2/102
Mean grade of necro-inflammation (of 18) (\pm SD)	8.2 (\pm 3.4)
Mean stage of fibrosis (of 6) (\pm SD)	3.1 (\pm 1.7)

¹Normal < 667 nkat/L, ²Alk Ph means alkaline phosphatase, ³Normal: 1084-5251 nkat/L.

in an Eppendorf thermal cycler (Master cycler 5330).

Ten microliters of reaction product was electrophoresed in a 18 g/L agarose gel made in Tris-acetate-EDTA (TAE) buffer (pH = 8-8.5) and visualized by ultra-violet illumination after ethidium bromide (10 mg/L) staining. Positive and negative controls were also treated as samples.

PCR amplification was also performed using HBV-PCR detection kit (Cinna Gen Inc., Teheran, Iran). According to manufacturer's instruction, 5 μ L of extracted DNA was added to 20 μ L of a PCR mixture, which contained primers amplifying 353 bp of the region of the core gene. Samples were denatured for 2 min at 94°C and then subjected to 40 cycles of 93°C for 40 min, 62°C for 40 min, 72°C for 40 min and finally 72°C for 10 min. The sensitivity of PCR assay was 150×10^3 copies/mL.

RESULTS

Only two patients had HBV DNA in their sera. Demographic, biochemical data, serological data, and histological findings of these patients are shown in Table 2. Both patients had major thalassemia and were on regular blood transfusion for many years.

DISCUSSION

There is much controversy and suspicion about the possible role of hepatitis B infection in pathogenesis of cryptogenic chronic parenchymal liver diseases especially in areas with intermediate or high infection rates of hepatitis B. Although the exact mechanism for active viral infection with undetectable HBsAg in the serum is not clear, both mutations in S region of the viral genome as well as low-titer infections (including possible burned-out infections) are proposed as possible explanations. It is however obvious with either of these two mechanisms being true, there should be detectable viral genome in serum of a majority of such patients using sensitive amplification techniques.

In this study, only less than 2% of our patients had HBV-DNA in their sera and it appears that HBV infection had a minimal role in causing chronic hepatitis in HBsAg negative Iranian patients with chronic hepatitis. Because of lack of a control group of normal individuals to which

Table 2 Demographic, biochemical, serological and histological data of patients with positive HBV-DNA

Variable	Patient No. 18	Patient No. 47
Age (yr)	21	27
Sex	M	F
AST nkat/L (\pm SD) ¹	1617	2917.3
ALT nkat/L (\pm SD) ¹	1667	2334
Alk Ph ² nkat/L (\pm SD) ³	2133.8	8568.4
Grade of necro-inflammation (of 18)	4	18
Stage of fibrosis (of 6)	3	5
HBsAb	-	+
HBcAb	+	-
HBsAg	+	+
HBcAb	-	-
HCV-Ab	+	+

¹Normal < 667 nkat/L; ²Alk Ph means alkaline phosphatase; ³Normal: 1084-5251 nkat/L.

the incidence of positive HBV DNA in serum can be compared, the possible role of occult HBV infection in etiology of cryptogenic liver disease in this country cannot be totally excluded, nevertheless, it can be certainly stated that this role if, exist at all will be of minimal, negligible impact.

This data is the same as that found by western researchers but in contradiction with findings in high incidence areas of the world^[15]. The possible explanation for these different results may lie in the different incidences as well as different genotypes of hepatitis B prevalence in these areas.

It should be further emphasized that two patients with positive HBV DNA in this study were both known cases of major thalassemia with a history of multiple transfusions and also had concomitant HCV infection. It is therefore can be concluded that the incidence of occult HBV infection in those with no history of high risk conditions for blood borne infections will be much lower.

Based on the findings of this study, we conclude that there is no need for HBV DNA PCR in routine work up for the chronic liver disease patients with negative HBsAg in Iran.

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