Surface gene mutations of hepatitis B virus among high-risk patients with occult hepatitis B virus infection

Rasool Hamkar\textsuperscript{a}, Arezoo Aghakhani\textsuperscript{b}, Safyeh Soufian\textsuperscript{c}, Mohammad Banifazl\textsuperscript{d}, Nastaran Ghavami\textsuperscript{a}, Mahsa Nadri\textsuperscript{a}, Masoomeh Sofian\textsuperscript{e}, Farrokhlagha Ahmadi\textsuperscript{a}, Effat Razeghi\textsuperscript{a}, Ali Eslamifar\textsuperscript{b}, Amitis Ramezani\textsuperscript{b,*}

\textsuperscript{a}Tehran University of Medical Sciences, Tehran 14155, Iran
\textsuperscript{b}Clinical Research Department, Pasteur Institute of Iran, Tehran 13164, Iran
\textsuperscript{c}Arak Payame Noor University, Arak 38135, Iran
\textsuperscript{d}Iranian Society for Support Patients with Infectious Diseases, Tehran 19568, Iran
\textsuperscript{e}Arak University of Medical Sciences, Arak 38149, Iran

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Abstract

Surface gene mutants of hepatitis B virus (HBV) have been reported in a variety of patient groups. Because of limited data regarding these mutations in patients with occult HBV infections; we aimed to determine these mutations among high-risk patients with occult HBV infection. The presence of HBV-DNA was determined in patients with isolated anti-HBc by real-time polymerase chain reaction (PCR). Then, surface gene region was amplified by nested PCR and mutations were analyzed after sequencing. The mutations that resulted in nonfunctional hepatitis B surface antigen (HBsAg) were insertion of single nucleotide in 2 cases, which causes frameshift and single-nucleotide replacement, and premature stop codons at Leu15 and Gly10 in the other 2 cases. Amino acid substitution at amino acid position 207(S207N) was found in the other isolates. Our study suggested that “a” region mutations did not play a major role in HBsAg detection, and other genetic and nongenetic factors may be responsible for failure to detect HBsAg by routine laboratory tests.

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1. Introduction

Occult hepatitis B virus (HBV) infection harbors potential risk of HBV transmission through blood transfusion, hemodialysis (HD), and organ transplantation (Hu, 2002). Occult HBV infection is characterized by presence of HBV infection with undetectable hepatitis B surface antigen (HBsAg). A number of explanations for the persistence of HBV-DNA in HBsAg-negative samples have been proposed, including integration of HBV-DNA into the host’s chromosomes (Zuckerman, 2000), genetic variations in the S gene (Ackerman et al., 1994; Liang et al., 1990), and the presence of immune complexes in which HBsAg may be hidden (Chemin et al., 2001; Grob et al., 2000). In addition, occult hepatitis B may be due to the window period after acute HBV infection (Brechot et al., 2001), poor laboratory detection of HBsAg due to low level of HBs antigenemia, underlying hepatitis C virus (HCV) coinfection (Zuckerman, 2000), immunosuppression, or other host factors (Carman, 1997; Jilg et al., 2001). Recently, there has been concern about isolated hepatitis B core antibody (anti-HBc) individuals (Groeb et al., 2000; Jilg et al., 2001; Weber et al., 2001) in which anti-HBc is the only detectable HBV marker in the absence of HBsAg or antibody to HBsAg (anti-HBs). Of these cases, 10% to 40% have persistent HBV-DNA (Alhababi et al., 2003; Weinberget al., 2000).
Mutations in the HBV surface (S) genes have been reported in a variety of patient groups, with variable rates of occurrence. However, the biologic significance of this mutation is still unclear (Ozaslan et al., 2007). In some patients, these mutants are characterized by a glycine to arginine mutation at residue 145 (G145R) within the “a” determinant (amino acids 124–147), due to a G to A transition at nucleotide position 587. Changes outside the “a” determinant, such as a double amino acid substitution at codons 116 and 118 (Tang et al., 1998) or the presence of a translational stop codon at position 199 (Pichoud et al., 1999), have also led to failure of HBsAg detection. Thus, the “a” determinant epitope cluster may extend both upstream and downstream of amino acid positions 124 to 147 and involve the entire major hydrophilic region of the surface proteins (Carman, 1997; Chen et al., 1996).

Some studies reported that “a” determinant mutations did not play a major role in HBsAg detection (Grethe et al., 1998; Weinberger et al., 2000) and mutations in the S gene, which lead to premature stop codons that may affect the HBsAg secretion and detection (Wakil et al., 2002).

Because of the apparent increase in prevalence of S mutants and limited data regarding these mutations in patients with occult HBV infections, we aimed to determine the surface gene mutations of HBV among high-risk patients with occult HBV infection.

2. Materials and methods

2.1. Patients

In this cross-sectional study, 395 patients including 289 patients on chronic HD and 106 HIV-infected subjects were enrolled. A questionnaire that gathered epidemiologic and clinical data was completed by clinicians. This project was approved by the Iranian Society for Support Patients With Infectious Diseases ethics committee, and informed consent was obtained from patients before their enrollment.

2.1.1. Blood samples collected from all patients were stored at −80 °C

All samples were tested for HBsAg, anti-HBs, hepatitis C antibody (anti-HCV), and total anti-HBc by enzyme immunoassay (EIA). The commercial EIA kits used were as follows: HBsAg and anti-HBs (Hepanosticka Biomerieux, Boxtel, the Netherlands), anti-HBc (Dia.Pro Diagnostic BioProbes, Milan, Italy), anti-HCV (Biorad, Segrate, Italy). Recombinant immunoblot assay (RIBA Innogenetics, Ghent, Belgium) was employed to confirm anti-HCV reactivity. Alanine aminotransferase (ALT) was also determined in all of the patients.

Human immunodeficiency virus antibody (anti-HIV) was determined by EIA (MP Biomedicals, Illkirch, France), with positive tests confirmed by Western blot assay (DiaPlus, San Francisco, CA). All assay protocols, cutoffs, and result interpretations were carried out according to the manufacturers’ instructions.

In patients with isolated anti-HBc (HBsAg negative, anti-HBs negative, and anti-HBc positive), HBV-DNA was extracted using High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s instructions. HBV-DNA was determined quantitatively by real-time polymerase chain reaction (PCR) using the artus HBV RG PCR kit (QIAGEN, Hamburg, Germany) on the Rotor-Gene 3000 real-time thermal cycler (Corbett Research, Sydney, Australia). The analytic detection limit of the kit is 20 IU/mL according to the user manual. HBV-DNA below 50 IU/mL is reported as less than 50 IU/mL.

2.2. Amplification of HBV S-ORF

The most conserved regions of S gene sequences were amplified by nested PCR, using primers to amplify the sequence between nt 155 and nt 787, yielding an amplicon of 633 bp. The outer primers were PrS2 (forward, nt 2820–2837, 5′-GGGACACCATATTCTTGG) and S1R (reverse, nt 842–821, 5′-TTAGGGTTTAAATGTATACCCA). The inner primers were HBS1 (forward, nt 155–173, 5′-ATGGGACTACATCACATCAG-3′) and YS2 (reverse, nt 767–787, 5′-GGGACTCAAAGATGTGTATCAG) (Zeng et al., 2004). A 10-μL extracted DNA was added to an amplification mixture containing 5 μL of 10× PCR buffer, 1.5 μL of MgCl2 (50 mmol/L), 1 μL of dNTP mix (100 mmol/L each), 1 μL (2.5 U) of Taq polymerase (CinnaGen, Tehran, Iran), and 2 μL of each of outer primers (10 pmol) in a total volume of 50 μL. The PCR profile was an initial 3-min denaturation at 94, followed by 35 cycles of amplification including denaturation for 45 s at 94, annealing for 60 s at 53, and extension for 90 s at 72. Strand synthesis was completed at 72 for 6 min. One microliter of the first-round PCR products was then subjected for the second-round PCR under the same conditions but with the inner primers.

2.3. Sequencing assay

Second-round PCR products were subjected for bidirectional automated sequencing using both forward and reverse inner primers (Kawasar Biotech, Tehran, Iran). Sequencing was performed twice more on 2 samples harboring single-nucleotide insertion and 2 other samples that showed premature stop codon. Nucleotide sequences were aligned with CLUSTALW program using BioEdit software (BioEdit Sequence Alignment Editor Software, Department of Microbiology, North California State University). Genetic distance was estimated using the Kimura 2-parameter matrix (Kimura, 1980). A phylogenetic tree was constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987). Bootstrap resampling and reconstruction were carried out 1000 times to confirm the reliability of the phylogenetic tree (Felsenstein, 1985). The nucleotide sequences of HBV isolates reported in this article can be found in the GenBank.
2.4. Statistical analysis

The Mann–Whitney U, χ², and r² tests were used with the SPSS 16 Package program for statistical analysis (SPSS, Chicago, IL). Data are presented as mean ± SD or, when indicated, as an absolute number and percentage. The 95% confidence interval (95% CI) was calculated.

3. Results

A total of 395 patients, including 289 patients on chronic HD and 106 HIV-infected subjects, were enrolled in this study.

The mean age of HD patients was 55 ± 16 (range, 15–89) years. Sixty percent of them were male. The duration of HD was 5.2 ± 5.1 years. HBsAg, anti-HBs, anti-HCV, and anti-HIV were found in 2.8%, 77.5%, 3.1%, and 940) cells/mm³. The mean log₁₀ HIV viral load was 1.97 ± 2.03. Coinfection with HCV and HBV occurred in 67% and 3.8% of patients, respectively. The mean ALT level was 16.4 ± 9.4 IU/L.

The mean age of HIV-positive patients was 36.6 ± 9.6 (range, 23–60) years. Of the patients, 74.5% were male and 25.5% were female. The mean CD4 count was 349.08 ± 181.07 (2–940) cells/mm³. The mean log₁₀ HIV viral load was 1.97 ± 2.03. Coinfection with HCV and HBV occurred in 67% and 3.8% of patients, respectively. The mean ALT levels were 32.4 ± 20.1 IU/L. The possible routes of HIV transmission were intravenous drug use (52.8%), infected husband (24.5%), heterosexual contact (3.8%), and heterosexual contact and intravenous drug use (5.6%), and in 3.7%, the route of HIV acquisition was not identified.

Of the 395 patients, 40 (18 HD and 22 HIV-infected patients) had isolated anti-HBc (10.13%; 95% CI, 7.1–13.1%). HBV-DNA was detectable in 12 of 40 patients (30%; 95% CI, 15.8–44.2%) who had isolated anti-HBc. Of these 12 patients, 9 of them were HD patients and 3 of them were HIV-infected subjects. Plasma HBV-DNA load was less than 50 IU/mL in all of these patients.

In 8 patients (5 HD and 3 HIV-infected patients), the amount of DNA was enough for analysis. In these patients, surface gene mutations were analyzed after direct sequencing. The characteristics of these patients were summarized in Table 1.

The phylogenetic analysis results showed that our isolates were clustered in the genotype D branch with other genotype D HBV reference genes (Fig. 1).

Table 1. Characteristics of patients with occult HBV infection

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex (M/F)</th>
<th>Age (year)</th>
<th>Genotype</th>
<th>S mutations</th>
<th>Clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>30</td>
<td>D</td>
<td>Frameshift</td>
<td>HIV</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>44</td>
<td>D</td>
<td>S207N</td>
<td>HIV</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>30</td>
<td>D</td>
<td>S207N</td>
<td>HIV</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>62</td>
<td>D</td>
<td>Stop codon</td>
<td>HD</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>82</td>
<td>D</td>
<td>Stop codon</td>
<td>HD</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>68</td>
<td>D</td>
<td>Frameshift</td>
<td>HD</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>60</td>
<td>D</td>
<td>S207N</td>
<td>HD</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>48</td>
<td>D</td>
<td>S207N</td>
<td>HD</td>
</tr>
</tbody>
</table>

The development of HBV mutations interfering with viral replication or antibody recognition of HBsAg in serologic assays has been known for many years. However, when viral DNA is detected and HBsAg is not, several types of mutations can explain this discrepancy. Many of these mutations affect the amino acid sequence of the antigenic “a” region of the S gene. The most frequent mutation is G145R, and the most disrupting were substitution of the cysteines in positions 121 124, 137, or 147 (Grethe et al., 1998; Kao et al., 2002; Sakugawa et al., 2001; Weinberger et al., 2000). Unique insertion variants have been described of T by A at position 44 and G by T at position 28, respectively (Fig. 2). The TTA→TAA stop mutation led to a premature stop codon at position Leu15, and the GGA→TGA led to a premature stop codon at position Gly10 in the S gene.

Serine to asparagine substitution at residue 207 (S207N), due to a G to A transition at nucleotide position 620, was found in the other 4 sequences (Fig. 3A and B).

4. Discussion

In this study, the genotype and surface gene mutations of HBV among high-risk patients with occult HBV infection were investigated. There is little information on HBV genotypes in occult infections in Iran. We showed that all HBV isolates belonged to genotype D, which was in accordance with other studies reporting that genotype D is the only detectable genotype in different clinical forms of HBV infections in Iran (Aghakhani et al., 2009).

The most important mutations that resulted in nonfunctional HBsAg were insertion of single nucleotide in 2 cases, which causes frameshift and single-nucleotide replacement, and premature stop codons at Leu15 and Gly10 in the other 2 cases, which cause failure in detection of HBsAg with routine diagnostic tests.

S207N mutations were also identified in the other 4 cases. As the viral load was low in our samples, it is possible that failure to detect HBsAg may be related to a low level of antigenemia besides to the mutation in our cases. No “a” determinant mutations were found in any of the HBV isolates.

The development of HBV mutations interfering with viral replication or antibody recognition of HBsAg in serologic assays has been known for many years. However, when viral DNA is detected and HBsAg is not, several types of mutations can explain this discrepancy. Many of these mutations affect the amino acid sequence of the antigenic “a” region of the S gene. The most frequent mutation is G145R, and the most disrupting were substitution of the cysteines in positions 121 124, 137, or 147 (Grethe et al., 1998; Kao et al., 2002; Sakugawa et al., 2001; Weinberger et al., 2000). Unique insertion variants have been described
in patients with occult HBV infection from the Far East (Carman et al., 1995; Hou et al., 1995; Yamamoto et al., 1994). A 2- to 8-amino acid insertion was noted between codon positions 121 and 124 in such patients. However, most studies have revealed missense mutations occurring within the protective "a" determinant of HBsAg in patients from Europe, Asia, and Africa (Cooreman et al., 1999; Ireland et al., 2000; Seddigh-Tonekaboni et al., 2000; Waters et al., 1992).

However, recently some studies suggest that the “a” region mutations did not play a major role in HBsAg detection and the diagnosis of occult infection (Grethe et al., 1998; Weinberger et al., 2000). Novel mutations in the S-ORF, which lead to premature surface gene termination, might affect the production of HBsAg and need further study (Wakil et al., 2002).

A study on HBV surface protein mutations in HIV-infected Brazilian patients with occult HBV infection

Fig. 1. Phylogenetic tree constructed using a Kimura 2-parameter matrix and the NJ method. The numbers represent the percentages of bootstrap replicates (of 1000 totals) for the node. Iranian sequences determined in this study are indicated by FJ prefix.

Fig. 2. Position of stop codons and single-nucleotide insertions that resulted in nonfunctional HBsAg. ¹Stop codons. ²Single-nucleotide insertions.
showed a stop codon mutation at position 216 in the HBsAg in one case and E164D and I195M substitutions in HBsAg in another patient (Araujo et al., 2008). In Datta et al. (2007) study on a blood donor with occult HBV infection, a point mutation (T→A) at the 207th nucleotide of the HBsAg ORF, which resulted in a premature stop codon at position 69, was found. Yang et al. (2003) reported an HBsAg gene stop codon caused by a point mutation upstream of the “a” determinant. Their finding was very similar to the deletion mutation of HBsAg gene located at the nucleotide 31 of the HBsAg gene, which led to a frameshift and introduced a stop codon after 21 amino acids of HBsAg described by Weinberger et al. (2000).

Because not all patients with occult HBV infection had “a” determinant mutations, other factors may be responsible for the failure to detect HBsAg and multiple mechanisms may be responsible for occult HBV infection (Fang et al., 2009). Such factors may include mutations outside the “a” determinant (Grethe et al., 1998; Pichoud et al., 1999), a low level of replication and genotype of the viral strain (Weinberger et al., 2000). However, more likely, failure to detect HBsAg in patients with occult HBV infection may be due to low levels of viral replication, which may allow escape from conventional immunoassays (Hou et al., 2001).

In a study of 249 sera from HBV-infected patients living in 25 provinces of Iran, the most frequent mutations in Iranian isolates were Ser143Leu, Ile10Leu, Gln101Arg, Pro120Ser, Thr118Ala, Ser136Tyr, and Thr118Lys. One isolate had an insertion (15 nucleotides) between nucleotides 222 and 223, which resulted in an LLPGS amino acid insertion. The TGG →TGA stop mutation was detected in 2 isolates and led to a premature stop codon at position Trp172 in the S gene (Mohebbi et al., 2008). In our study, the analysis of the S region of HBV isolates of the occult HBV-infected patients did not show any mutations, which were found in overt Iranian HBV cases.

In conclusion, HBV genotype D was the only detectable genotype in Iranian patients with occult HBV infection. The most important mutations that resulted in nonfunctional HBsAg were insertions of single nucleotide that causes frameshift and single-nucleotide replacement, and premature stop codons. No “a” determinant mutations were detected. Thus, the existence of occult HBV infection among individuals with isolated anti-HBc could not be explained fully by mutations in the “a” determinant region of surface gene in our population. This survey suggested that the “a” region mutations did not play a major role in HBsAg detection, and other genetic and nongenetic factors may be responsible for the existence of occult HBV infection and failure to detect HBsAg by routine laboratory tests.

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References


