Phylogenetic Analysis of HBV Based on PreS Region in Iranian Hepatocellular Carcinoma Patients

Zahra Goodarzi 1, Reza Malekzadeh 2, Ghodratollah Montazeri 2, Seyed-Moayed Alavian 1, Mehdi Qurbanalizadgan 3, Maryam Daram 4, Seyed Mohamad Jazayeri 4*

1 Baqiyatallah Research Center for Gastroenterology and Liver Disease (BRCGL), Baqiyatallah University of Medical Sciences, Tehran, Iran
2 Digestive Disease Research Center, Shariati Hospital, Medical Sciences/University of Tehran, Tehran, Iran
3 Research Center for Molecular Biology, Baqiyatallah University of Medical Sciences, Tehran, Iran
4 Department of Virology, Faculty of Public Health, Medical Sciences/University of Tehran, Tehran, Iran

Background and Aims: There are eight genotypes (A-H) of hepatitis B virus (HBV), which show a characteristic worldwide distribution. Genotyping can be accomplished based on a partial sequence of HBV genome such as the PreS or S gene. The aim of this study was to determine the HBV genotypes in Iranian hepatocellular carcinoma (HCC) patients with chronic HBV infection.

Methods: Serum sample of 10 HCC patients with chronic HBV infection were subjected to PreS Hemi-Nested PCR. The viral genotype of each sample was determined by bi-directional sequencing of the PreS amplicon and phylogenetic analysis by comparing the nucleotide sequence with 33 reference HBV strains obtained from the GenBank.

Results: Phylogenetic analysis based on PreS region sequences disclosed that all isolated strains belonged to genotype D. Analysis of sequences revealed that all the sequences contained amino acid substitutions. In the PreS2 region of two samples, a point mutation in the start codon was found. There were some deletions with 3, 6 and 8 amino acids in PreS2 region of three samples.

Conclusions: Despite the low number of samples, these data revealed that the HBV genotype D is dominant in Iranian HCC patients. Most of the mutations are located at immunodominant epitopes involved in B or/and T cell recognition.

Keywords: HBV, Phylogenetic Analysis, PreS, Hepatocellular Carcinoma

Introduction

Hepatitis B virus (HBV) causes a variety of acute and chronic human liver diseases, including fatal fulminant hepatitis, cirrhosis and hepatocellular carcinoma (HCC). Worldwide, more than two billion people have been infected with hepatitis B and approximately 400 million people have developed chronic infection (1). Recently, it has been estimated that about 53% of HCC cases in the world are related to HBV (2). In Iran, the prevalence of HBsAg has been approximately calculated 2% and it is thought that more than 35% of Iranian population has been exposed to HBV (3).

HBV is the prototype of the Hepadnaviridae family. The HBV genome is a partial, double-stranded DNA with four open reading frames coding for the core, surface, polymerase and X proteins. The envelope gene (PreS/S) of HBV codes for three kinds of proteins which are translated from distinct mRNAs and are collectively known as the hepatitis B surface antigen (HBsAg). The major HBsAg consisting of 226 amino acids (aa) is encoded by the S gene. The middle HBsAg is coded by the PreS2 gene (55aa) and the S gene, whilst the large HBsAg is encoded by PreS1 (119 or 108 aa,
Phylogenetic Analysis of HBV in Iranian HCC patients

Ten serum samples were collected from HCC Iranian patients with chronic HBV infection. All patients had no co-infection with HCV, HDV, and HIV. All serum samples were stored at -20 °C until they were used. HBV-DNA was extracted from 200 µL of serum using DNA-QI Amp Kit (QIAGEN Inc., Valencia, CA) according to the instruction of the manufacturer. Extracted DNA was eluted in final volume of 50 µL of supplied elution buffer.

The PreS region was amplified by heme nested polymerase chain reaction (PCR) using three primers: 5′TCAGAATTCTCACCATATTCTTGGAACAA3′ (PS1, sense nucleotides 2817-2839), 5′CTACTGAACTGAGCCA3′ (PS2, antisense nucleotides 668-6875) for the outer primer pair and 'AGTAAGCTTAGAAGATGAGGCATAGCAGC3' (PS3, antisense nucleotides 415-434) (11). PCR amplification was done using Taq DNA Polymerase Kit (QIAGEN Inc., Valencia, CA). PCR reactions were done in 100 µl mixture reaction, according to the instruction of the manufacturer. First-round PCR was performed with the following parameters: preheating at 94 °C for 1 min, 5 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min; 35 cycles of 90 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min and a final 72 °C for 10 min as a final extension step. The second-round PCR was performed in the same conditions except for 25 cycles instead of 35 cycles and the annealing temperature that was at 59 °C. The PCR products were isolated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized by an ultra violate (UV) transluminator. PCR products were then subjected to bi-directional sequencing with primers PS1 and PS3 and sequencer ABI version 3130 XL.

Thirty three HBV strains obtained from the GenBank, representing each of the eight genotypes (A-H), were used in this study as references (12, 13). The wooly monkey hepatitis B virus (WMHBV), GenBank accession number AF046996, was used as out group (14). Iranian HBV PreS region sequences and whole reference sequences were aligned using BioEdit package version 7.0.5.3, and a neighbors joining phylogenetic tree was using the Treecon package (15) employing a kimura distance matrix (16).

Associations were tested by bootstrap re-sampling analysis using 1,000 replicates (14). Branches with a bootstrap value of greater than 70% were deemed well supported by the data. The nucleotide and deduced amino acid sequences were compared with reference sequence (action number AY391892) for finding the likelihood mutations in immune epitopes in this region.

Results

Phylogenetic analysis of PreS sequences disclosed that the 10 Iranian strains were classified into genotype D (Fig 1). In all strains, the length of PreS1 region was 324 bp and compared to other genotypes, there was a 33 nucleotide deletion in this region, which is characteristic for genotype D. There were some deletions with a 3, 6 and 8 amino acid in PreS2 region of three samples. Such deletions were located in the N-terminal half of PreS2 region. In addition, two strains had a point mutation at the start codon of the PreS2 region. Epitope mapping revealed that most of the mutations encompassed T cell and B cell epitopes. Table 1 demonstrates the immune epitopes and mutations within the HBV PreS region.
Discussion

Genotyping based on the PreS region alone as well as those based on the complete HBV genome are well documented. Unless whole genomic or S gene sequences were using for phylogenetic analysis of HBV, genotyping of strains based on PreS region performed in some studies and showed the same results compared with full genomic and S phylogenetic analysis (12, 13, 17, 18). In this study, the PreS region of 10 HBV strains isolated from 10 HCC patients with chronic HBV infection were amplified, sequenced and compared to known sequences from the GenBank. A phylogenetic tree based on PreS region of 10 Iranian HCC patients with chronic HBV infection compared to the known sequences from the GenBank was constructed.

Phylogenetic analysis showed that all 10 strains were clustered in HBV genotype D branch with 100% bootstrap of 1000 replicates. This was comparable with a pervious study based on full
Phylogenetic Analysis of HBV in Iranian HCC patients

Table 1. Amino acids substitutions and deletions located within immunodominant epitopes of HBV preS regions.

<table>
<thead>
<tr>
<th>HBV region</th>
<th>Epitope region</th>
<th>Epitope positions</th>
<th>Kind of mutation</th>
<th>Case No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PreS1</td>
<td>B cell</td>
<td>1-20</td>
<td>D20G</td>
<td>002-007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16-24</td>
<td>D20G</td>
<td>013,003,010</td>
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<tr>
<td></td>
<td></td>
<td>26-34</td>
<td>N26S</td>
<td>015,004</td>
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<tr>
<td></td>
<td></td>
<td>30-42</td>
<td>T40p,T40N</td>
<td>005,009</td>
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<tr>
<td></td>
<td></td>
<td>61-67</td>
<td>W65S</td>
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<td></td>
<td>83-94</td>
<td>N87K</td>
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<td>95-106</td>
<td>S98T,N103T,N103D</td>
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<td>18-37</td>
<td>N20G,N26S</td>
<td>002,-003,004</td>
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<td></td>
<td></td>
<td>83-106</td>
<td>N103D,N103T</td>
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<td>PreS2</td>
<td>1-45</td>
<td>M1I, Q2K, L12Q</td>
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<tr>
<td></td>
<td>B cell</td>
<td></td>
<td>Q13G, R16K, R16N,V17P</td>
<td>005,</td>
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<td></td>
<td></td>
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<td>L20p, Y21D,F22L,F22L</td>
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<td></td>
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<td></td>
<td>Deletion</td>
<td>007,010,015</td>
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<td></td>
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<td>N35S, V35A,P41H</td>
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<tr>
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<td>T cell</td>
<td>21-30</td>
<td>Deletion</td>
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<td></td>
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<td></td>
<td>Y21D,F22L</td>
<td>006,007</td>
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</table>

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References

9. Okamoto H, Tsuda F, Sakugawa H, Sastrosowignjo RI,


