

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

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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,

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depending on subtypes), PreS2 and S genes⁽⁴⁾. Thus far, HBV has been classified into eight genotypes designated A to H, which show typical geographical distribution throughout the world.

Genotype A is mainly detected in Northwestern Europe, North America, and Africa; whereas Genotype B and C are found in Southeastern Asian populations. Genotype D is the commonest of genotype in the world and the predominant one in Mediterranean basin. Genotype E is found in Africans and genotype F in the aboriginal populations of South America. Genotype G has been limited to HBV carriers in France and Georgia. Genotype H is confined to the Amerindian populations of Central America⁽⁵⁻⁷⁾. It seems that the diversity of HBV genotypes could also be related to different clinical patterns of infections⁽⁸⁾. At the first, genotyping of HBV was based on nucleotide (nt) diversity of 8% or more in the whole genome⁽⁹⁾. However, genotyping can be accomplished based on a partial sequence of the HBV genome such as the PreS or S genes⁽¹⁰⁾.

There are some reports of genotyping of HBV from Iran based on complete genome. In this study, we sequenced the PreS region of HBV-DNA extracted from serum sample to determine the genotypes and mutations in the immunodominant epitopes present in Iranian HBV carriers with HCC. This study is important, as it determines the distribution of genotypes and compares it with findings of previous studies.

Materials and Methods

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-QIAamp 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 hemi nested polymerase chain reaction (PCR) using three primers: 5'TCAGAATTCTCACCATTCTTGGGAACA A3' (PS1, sense nucleotides 2817-2839), 5'CACTAGTAACTGAGCCA3' (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 violet (UV) transilluminator. 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 woolly 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 (accession 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.

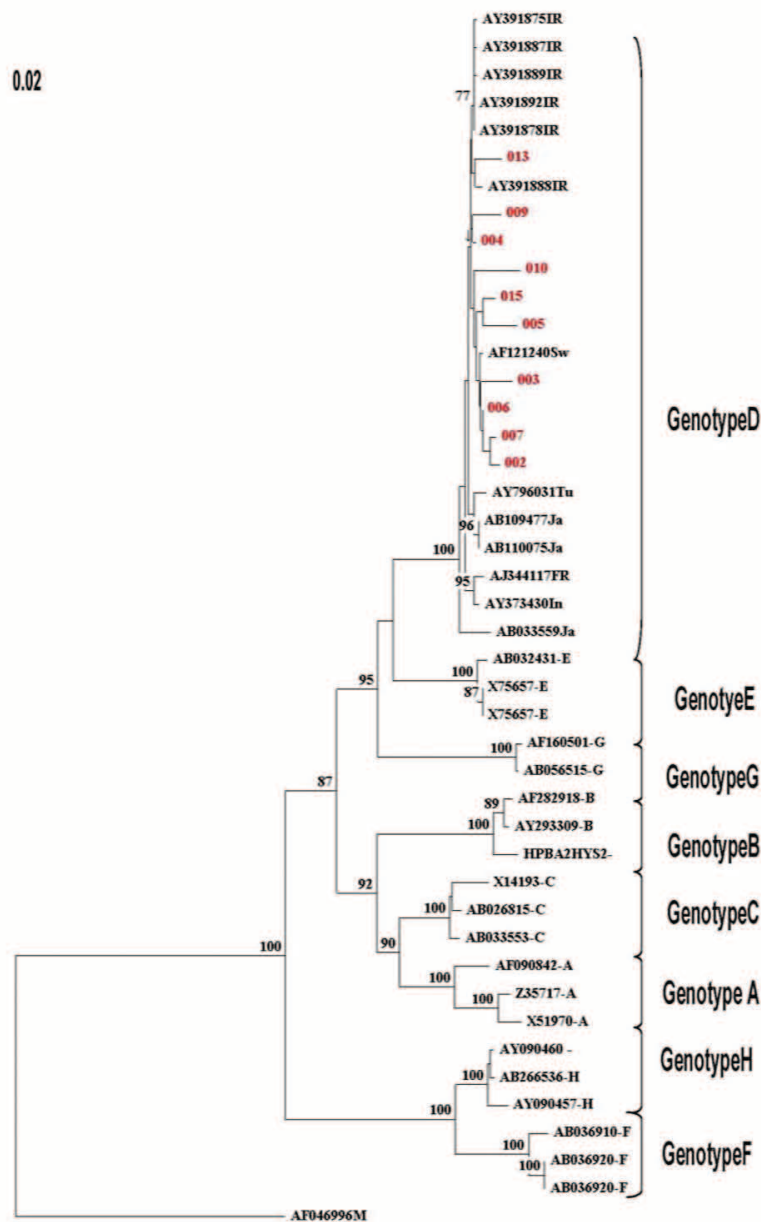


Figure 1. Phylogenetic analysis based on the PreS region of the HBV strains from this study and 33 human HBV strains from GenBank. The scale bar indicates the percentage of nucleotides divergence. Iranian sequences determined in this study, are indicated number (002, 003...). The letters A to H designate HBV genotypes. Bootstrap values based on 1000 replicates are shown at each main branch.

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

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

HBV region	Epitope	Epitope positions	Kind of mutation	Case No.
PreS1	B cell	1-20	D20G	002-007
		16-24	D20G	
		26-34	N26S	013,003,010
		30-42	T40P, T40N	
		61-67	W65S	015,004
		83-94	N87K	005,009
	95-106	S98T, N103T, N103D		
	T cell	18-37	20G, N26S	002,-003,004
83-106		N103D, N103T	005, 007 009, 015	
PreS2	B cell	1-45	M1I, Q2K,L12Q	002,003
			Q13G, R16K, R16N,V17P	
			L20P, Y21D,F22I, F22L	005, 007, 010, 015
			Deletion	
			N33S, V35A,P41H	
	T cell	21-30	Deletion	002,003,005
			Y21D	
			F22I	
			F22L	
		29-48	N33S	006,007 009, 013,015
			V35A	
			P41H	
S47T				

genomic sequences (14, 19). In addition, a previous study among Iranian HBV chronic patients based on the S and C ORFs sequences showed that HBV genotype D was predominant among the Iranian population (20). All these data together indicate that Iranian patients with chronic HBV do not show genotyping diversity. The HBV genotype D is also prevalent in neighboring countries such as Turkey and Afghanistan (21, 22). Moreover, Phylogenetic analysis showed that a Swedish strain (AF121240Sw) is located in the same cluster as Iranian strains. We suggested that this strain may have been isolated from an Iranian person.

In this study, investigation of PreS2 sequence region of HCC patients revealed that there were some deletions in two isolates and there were point mutations that abolished the start codon. These PreS2 variants have been described earlier in other genotypes (11, 13, 23). All of these investigations have shown that internal deletions and point mutation at PreS2 start codon are common among HBV strains in sera from patients with HBV infection and

HCC. Another aspect of our study was the occurrence of those mutations in the immunodominant epitopes of the PreS region. Epitope mapping in this region showed that most amino acid substitutions and all deletions in PreS region occurred in T cell and B cell epitopes. These mutations may emerge during the course of infection under the antiviral pressure of the host immunity (24). There are some reports that showed PreS2 mutants harbored a deleted immune epitope escaped from the host immune surveillance and contributed to more progressive liver damage and finally hepatocarcinogenesis.

In conclusion, this study has shown that all 10 HCC patients with chronic HBV infection were in cluster of genotype D. We also found that the genotype assignment can be performed based on phylogenetic analysis of PreS region. Another finding is that PreS2 mutants may be common in HCC Iranian patients with chronic HBV infection.

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