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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 pervious 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'TCAGAATTCTCACCATATTCTTGGGAACA A3' (PS1, sense nucleotides 2817-2839), 5'CACTAGTAAACTGAGCCA3' (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.

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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 phylogenic 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 phylogenic 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

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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	002-007 013,003,010 015,004 005,009
		16-24	D20G	
		26-34	N26S	
		30 -42	T40P, T40N	
		61-67	W65S	
		83-94	N87K	
		95-106	S98T, N103T, N103D	
	T cell	18-37	20G, N26S	002,-003,004 005, 007 009, 015
		83-106	N103D, N103T	
PreS2	B cell	1-45	M1I, Q2K,L12Q	002,003 005, 007, 010, 015
			Q13G, R16K, R16N,V17P	
			L20P, Y21D,F22I, F22L	
			Deletion	
			N33S, V35A,P41H	
	T cell	21-30	Deletion	002,003,005 006,007 009, 013,015
			Y21D	
			F22I	
			F22L	
		29-48	N33S	
			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 counters 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 was 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 showed 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 phylogenic 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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References

1. Lai CL, Ratziu V, Yuen MF, Poynard T. Viral hepatitis B. *Lancet* 2003; **362**: 2089-94.
2. Lupberger J, Hildt E. Hepatitis B virus-induced oncogenesis. *World J Gastroenterol* 2007; **13**: 74-81.
3. Adibi P, Ghassemian R, Alavian SM, Ranjbar M, Mohammadalizadeh AH, Nematizadeh F, *et al.* Effectiveness of hepatitis B vaccination in children of chronic hepatitis B mothers. *Saudi Med J* 2004; **25**: 1414-8.
4. Mchael K, Wolfram HG. Structure and molecular virology. In: Zuckerman A, Thomas H. Viral hepatitis. Philadelphia: Churchill Livingstone 2005; p 149-80.
5. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; **83**: 2059-73.
6. Kramvis A, Weitzmann L, Owiredu WK, Kew MC. Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. *J Gen Virol* 2002; **83**: 835-9.
7. Campos RH, Mbayed VA, Pineiro YLFG. Molecular epidemiology of hepatitis B virus in Latin America. *J Clin Virol* 2005; **34**: S8-S13.
8. Kidd-Ljunggren K, Miyakawa Y, Kidd AH. Genetic variability in hepatitis B viruses. *J Gen Virol* 2002; **83**: 1267-80.
9. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI,

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Imai M, Miyakawa Y, *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; **69**: 2575-83.

10. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; **198**: 489-503.
11. Huy TT, Ushijima H, Win KM, Luengrojanakul P, Shrestha PK, Zhong ZH, *et al.* High prevalence of hepatitis B virus pre-s mutant in countries where it is endemic and its relationship with genotype and chronicity. *J Clin Microbiol* 2003; **41**: 5449-55.
12. Chen BF, Kao JH, Liu CJ, Chen DS, Chen PJ. Genotypic dominance and novel recombinations in HBV genotype B and C co-infected intravenous drug users. *J Med Virol* 2004; **73**: 13-22.
13. Chen BF, Liu CJ, Jow GM, Chen PJ, Kao JH, Chen DS. High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases. *Gastroenterology* 2006; **130**: 1153-68.
14. Amini-Bavil-Olyaee S, Sarrami-Forooshani R, Adeli A, Sabahi F, Abachi M, Azizi M, *et al.* Complete genomic sequence and phylogenetic relatedness of hepatitis B virus isolates from Iran. *J Med Virol* 2005; **76**: 318-26.
15. Van de Peer Y, De Wachter R. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* 1994; **10**: 569-70.
16. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980; **16**: 111-20.
17. Ong HT, Duraisamy G, Kee Peng N, Wen Siang T, Seow HF. Genotyping of hepatitis B virus in Malaysia based on the nucleotide sequence of preS and S genes. *Microbes Infect* 2005; **7**: 494-500.
18. Lindh M, Gonzalez JE, Norkrans G, Horal P. Genotyping of hepatitis B virus by restriction pattern analysis of a pre-S amplicon. *J Virol Methods* 1998; **72**: 163-74.
19. Mohamadkhani A, Poustchi H, Montazeri Gh, Estakhri A, Malekzadeh R. Clinical significance of precore and core promoter mutations in genotype D hepatitis B-related chronic liver disease. *Govaresh* 2007; **12**: 36-42.
20. Amini-Bavil-Olyaee S, Sarrami-Forooshani R, Mahboudi F, Sabahi F, Adeli A, Noorinayer B, *et al.* Genotype characterization and phylogenetic analysis of hepatitis B virus isolates from Iranian patients. *J Med Virol* 2005; **75**: 227-34.
21. Bozdayi G, Turkyilmaz AR, Idilman R, Karatayli E, Rota S, Yurdaydin C, *et al.* Complete genome sequence and phylogenetic analysis of hepatitis B virus isolated from Turkish patients with chronic HBV infection. *J Med Virol* 2005; **76**: 476-81.
22. Amini-Bavil-Olyaee S, Alavian SM, Adeli A, Sarrami-Forooshani R, Sabahi F, Sabouri E, *et al.* Hepatitis B virus genotyping, core promoter, and precore/core mutations among Afghan patients infected with hepatitis B: a preliminary report. *J Med Virol* 2006; **78**: 358-64.
23. Blackberg J, Kidd-Ljunggren K. Mutations within the hepatitis B virus genome among chronic hepatitis B patients with hepatocellular carcinoma. *J Med Virol* 2003; **71**: 18-23.
24. Gunther S, Fischer L, Pult I, Sterneck M, Will H. Naturally occurring variants of hepatitis B virus. *Adv Virus Res* 1999; **52**: 25-137.

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