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Mutation Hot Spots in Hepatitis B Surface Antigen in Chronic Carriers from Khoozestan Province, Southern of Iran

Fatemeh Ramezani[1], Mehdi Norouzi[1], Gholam Reza Sarizade[2], Vahdat Poortahmasebi[1], Ebrahim Kalantar[3], Lars Magnius[4], Helen Norder[4], Esteban Domingo[5], and Seyed Mohammad Jazayeri[1]

[1] Hepatitis B Molecular Laboratory, Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2] Khoozestan Province Blood Trasfusion, Ahvaz, Iran
[3] Gholhak Medical Laboratory, Tehran, Iran
[4] Virological Department, Swedish Institute for Infectious Disease Control, Solna, Sweden
[5] Centro de Biología Molecular, Severo Ochoa, (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain

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ABSTRACT

Mutations in the human hepatitis B virus (HBV) genome contribute to its escape from host immune surveillance and result in persistent infections. The aim of this study was to characterize the molecular variations of the surface gene and protein in chronically-infected patients from the southern part of Iran.

The surface genes from 12 HBV chronic carriers were amplified, sequenced and subsequently aligned using international and national Iranian database.

All strains belonged to genotype D, subgenotype D1 and subtype ayw2. Of all 30 mutations occurred at 22 nucleotide positions, 18 (60%) were missense (amino acid altering) and 12 (40%) were silent (no amino acid changing). The mean mutation frequency (missense to silent nucleotide ratio), was 1.5, indicating application of a high positive selection pressure on the surface proteins. At the amino acid level, of 17 substitutions, 15 (88%) occurred in different immune epitopes within surface protein, of which 7 (46.6%) in B cell epitopes in 5 residues; 7 (46.6%) in T helper epitopes in 6 positions; 1 (7%) in inside CTL epitopes in 1 residue.

We therefore conclude that the distribution of 93.2% of amino acid mutations inside B and T helper immune epitopes as well as the ratio between silent and missense nucleotide mutations showed a positive, focused immune selection pressure on the surface protein, which led to the evolution and emergence of escape mutants in these patients.

Keywords: Chronic HBV; HBV escape mutations; HBV immune epitope; HBsAg mutation.

Corresponding Author: Seyed Mohammad Jazayeri, MD, PhD; Department of Virology, Hepatitis B Molecular Laboratory, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Tel/Fax: (+98 21) 8899 2660, E-mail: jazayerism@tums.ac.ir

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INTRODUCTION

Hepatitis B virus (HBV) infection is among the top ten causes of death worldwide due to chronic liver disease[1] and accounts for an estimated 370 million chronic infections.[2]

The morbidity and mortality of persistent HBV infection are a major public health concern. More than one million deaths every year are due to end-stage HBV liver disease, such as decompensated liver cirrhosis and hepatocellular carcinoma (HCC).

Hepatitis B virus surface protein (HBsAg) contains two loops, formed by disulfide bridges between cysteines 124/137 and 139/147.[3] This region, particularly the second loop (between amino acid 139 and 147, ''a'' determinant) is commonly described as a major B-cell epitope which is highly conserved and the anti-HBs (antibody to HBsAg) response comprises mainly antibodies that recognize this epitope cluster.[4] Being a structural protein, HBsAg is an immune target. Moreover, the humoral response to HBsAg is T-cell-dependent. At least 4 regions within HBsAg contain epitopes for major histocompatibility complex (MHC) class II restricted CD4+ T cells.[5,6] Therefore, during the HBV chronicity, the T-cell epitopes of HBsAg which are targets for T-cell recognition might be involved.[7] The distribution of the mutations within known surface protein immune epitopes reflects the virus-host interaction with a prolonged infection period.

Mutants within immune epitopes of HBV represent a significant role and isolated cases of infection with HBV variants bearing substitutions in these regions, are predicted to escape from host's immune surveillance (immune-escape variants). There have been increasing reports on the emergence of different escape mutations in chronic HBV carriers who did not receive hepatitis B immunoglobulin or vaccine[8,9] distributed either within or outside the "a" determinant, including T cell epitopes.[10]

The aim of this study was to characterize the molecular variations of the hepatitis B surface protein in chronic active patients from Southern region of Iran.

MATERIALS AND METHODS

Sera

This cross-sectional study has been carried out on 12 blood donors who were diagnosed to be HBsAg positive and subsequently, were referred from the Khoozestan Blood Organization. All patients were interviewed and examined by gastroenterologists to evaluate the clinical findings and the results of the investigative workup (liver histology, ultra sonography and laboratory tests such as serologic, biochemical and virological tests) in order to determine the clinical status of the patients. They did not contain HDV, HIV and HCV co-infection. Informed consent was obtained from each patient and ethical approval for the study of human subjects was granted by local ethics committee.

Samples were tested by (ELISA) with commercial kits for serologic detection (Diapro, Milan, Italy). Two ml of sera were taken from each patient and stored at -80° C for further investigations.

DNA Extraction

HBV DNA was extracted from 200µl of each patient's serum, using a QIAmp DNA Blood minikit (Qiagen, Hilden, Germany), according to manufacturer's instructions. The eluted DNA was stored in -20°C.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was carried out in 100 µl of a mixture containing 5µl of the extracted DNA, using standard methodology. The complete surface gene was amplified using approperiate primers (Table 1). First round PCR was performed using 1 U of *Taq*DNA polymerase (HotStartTaqPCR, Qiagen, Hilden, Germany), 0.25 mM of each dNTP, 10x reaction buffer, 12.5 pmol of S1 and S2 primer. For the second round PCR, 1 µl of first round PCR product was added to 99 µl of the reaction mixture with the same composition as the first round except that S1/S2, were replaced by S6/S7.

Table 1. Oligonucleotide primers used for PCR and sequencing. Base positions numbered from the EcoRI site.

Primer	Sequence 5'… 3' of Oligonucleotides	Base Position	Type
S1	CCTGCTGGTGGCTCCAGTTC	56-75	Sense
S2	CCACAATTC (K)TTGAC ATA CTTTCC A (K=G/T)	1003-979	Anti-sense
S6	GCACACGGA ATT CCGAGG ACT GGGGACCCT G	113-146	Sense
S7	GAC ACC AAG CTT GGT TAG GGT TTA AAT GTA TAC C	857-823	Anti-sense

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***DNA* Sequencing**

The nucleotide sequence of the S gene was determined with a Genetic Analyzer sequencing ready reaction kit and run on an ABI- 3130 DNA Sequencer (Fostercity, CA, USA). The primers used for sequencing the S ORF are summarized in Table 1. Sequence analysis was performed with Chromas software and BioEdit Package version 7.0.9. Briefly, genomic sequences obtained for the HBV S gene were compared with all HBV genotype D references sequences used on the NCBI website as well as from the published and unpublished Iranian sequences from the Genbank.

Sequence Analysis

After allocating a sequence to an HBV genotype by analysis of the S gene, the surface gene amino acid/nucleotide variations that were found were compared with a reference sequence obtained from Okamoto (1988, accession number, AB033559)[11] and HBsAg sequences from Iranian isolates obtained from GenBank and NCBI and from our own laboratory reports. Comparing to the former, any amino acid changes defined as "variant" (host HLA-determined). With regards to the latter (Iranian database sequences), amino acid differences were defined as "mutation". Sequences have been submitted to GenBank, numbered from HM348682-HM348693.

Phylogenetic Analysis

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Kimura 2-parameter method and were in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 681 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.[12]

RESULTS

Twelve HBsAg-positive patients infected with HBV were enrolled in this study. All patients were native residents of Khoozestan province (south of Iran). The group studied consisted of in-active chronic hepatitis B carriers, all but one were anti-HBe positive with various levels of low to moderate HBV viral load and normal to slightly high levels of ALT (>30 in male and >19 in female). All were positive for anti-HBc, however, negative for anti-HBs (results not shown).

Phylogenetic Analysis

The results of the phylogenetic tree revealed that Iranian HBV isolates from Khoozestan were of genotype D and subgenotype D1 supported by 95% and 97% bootstrap value (1,000 replicates), respectively (Figure 1). In the phylogenetic tree, a genotype E sequence (accession number AB091266) was chosen for out grouping. Of two pairs of clusters (168, 177 and 171, 174), the former contained a mutation, L109Q, and the latter did not contain any nucleotide or amino acid mutations. The rest of sequences which showed separate individual clusters with different genetic distances within the tree were not characterized by amino acid substitution specifying that clustering patterns. Isolate 167 which showed the highest genetic distance was separated from all other strains, which contained the largest number of nucleotide and amino acid variations, including one stop codon at residue 69 of surface protein.

Substitutions in Comparison with Reference Genotype D (Okamoto, AB033559)

Overall, comparing with reference sequence (Okamoto, 1988), at the nucleotide level, of a total of 116 changes in 28 positions, 86 (74%) and 30 (26%) were silent and missense, respectively (results not shown).

At the amino acid levels, all contained A70P compared to Okamoto reference. We believe that this substitution was assigned as "variant" (see material and methods). According to the above mentioned description, 19 out of 49 amino acid changes were variants and the other 30 changes were mutations (see below).

Genotyping

Analysis of variation within the S gene of 12 patients with chronic HBV infection demonstrated that the only detected subtype was D (100%), subgenotype D1 (100%) and subtype ayw2 (100%) (Figure 1).

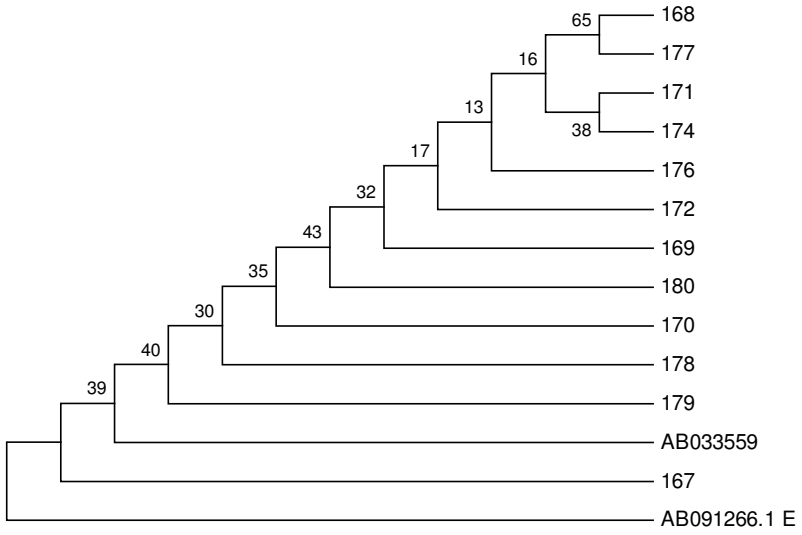

Figure 1. Neighbour joining phylogenetic trees of surface genes sequences from 12 samples. Note: S gene tree rooted with sequence AB049609 (reference genotype C). All Iranian isolates were compared to sequence AB033559 (reference genotype D, see the text). The figure shows bootstrap values of ≥70% and scale denotes percent diversity. Coding numbers indicate samples that have been analyzed in the figure. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

Table 2. Nucleotide (total and missense) and amino acid substitutions as well as the levels of mutation frequencies between isolates deduced from the number and the percentage of individual sequences.

Sample Code	Total Nucleotide Mutations	Nucleotide Misssense Mutation	Amino Acid Change	Mutation Frequency
167	G131A, A201G, T207A, A327G, A343G, A360C, T427C, T465C	G131A, T207A, A343G, T427C	G44E, C69Stop, T115A, S143P	1.00
168	T326A	T326A	L109Q	1.00
169	C345T			0.00
170	[C134A, T135G], C414T, G620C	[C134A, T135G], G620C	T45K, S207T	3.00
171				0.00
172	G71A	G71A	R24K	1.00
174				0.00
176	G131A	G131A	G44E	1.00
177	T326A	T326A	L109Q	1.00
178	C88A, A201G, T239C, T465C	C88A, T239C	Q30K, F80S	1.00
179	T135C, C167A, T310C, T337A, C345T, T465C	C167A, T337A	P56Q, S113T	0.50
180	G40A, T326A, T400G	G40A, T326A, T400G	V14M, L109Q, Y134D	3.00
Average	-	-	1.8	1.04

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Table 3. Amino acid mutations within B cell, T helper (Th) and CTL epitopes of HBs proteins. Amino acids are described by single letter code and numbered from the beginning of HBsAg. The wild type residues aligned according to genotype D consensus sequence.

		Th Epitope						CTL Epitope	B cell Epitope				
	Amino acid position	**24**	**30**	**44**	**45**	**56**	**80**	**207**	**109**	**113**	**115**	**134**	**143**
	Wild type	**R**	**Q**	**G**	**T**	**P**	**F**	**S**	**L**	**S**	**T**	**Y**	**S**
Sample code	167			E							A		P
	168								Q				
	169												
	170				K			T					
	171												
	172	K											
	174												
	176			E									
	177								Q				
	178		K				S						
	179					Q				T			
	180								Q			D	

Nucleotide and Amino Acid Substitutions

In comparison with Iranian sequences obtained from the database as well as from our unpublished data, in addition to the genotypic characterization described above, the sequences of the strains showed a few variability over the regions sequenced. In all, 30 "mutations" occurred at 22 nucleotide positions, of them, 18 (60 %) were missense (amino acid altering) and 12 (40%) were silent (no amino acid changing) (Table 2). At amino acid level, 17 substitutions occurred. Furthermore, it was possible to identify the level of S protein evolution between isolates by measuring the mutation frequency of individual sequences (Table 2). The average mutation frequency of all sequences was 1.5 according to dN/dS) missense to silent mutations per site. The average number of amino acid mutations for all isolates was 1.8.

Amino Acid Mutations within the Surface Protein Immune Epitopes

15 (88%) out of 17 amino acid mutations occurred in different immune epitopes within surface protein, of which 7 (46.6%) in B cell epitopes in 5 residues; 7 (46.6%) in T helper epitopes in 6 positions; 1 (7%) in inside CTL epitopes in 1 residue (Table 3). Two mutations occurred in "a" determinant: Y134D and S143P.

There were no significant correlations between the occurrence and distribution of mutations with age, gender, ALT levels and HBeAg status of the patients (results not shown).

DISCUSSION

It was possible to sequence the S gene for 12 HBV strains from Khoozestan province. Genotype D, subgenotype D1 and subtype ayw2 accounted for 100% of isolates. Published and unpublished data from our laboratory indicated that in Iran, there has been an obvious predominance of these virus genetic patterns.[13,14] We already hypothesized that this unique pattern of homology is related to the relative recent distribution and circulation of HBV in Iran compared to other countries in the region.[14]

The ratio between silent and missense nucleotide mutations in our patients was about 0.66. This indicated that the proportion of deduced amino acid changes in these chronically infected patients was tremendously high, and that these proteins were under a significant positive selection pressure which had already been applied by humoral host immune system: 15 (88%) out of 17 amino acid mutations occurred in different immune epitopes within surface protein. It is not unexpected, as with prolonged period of chronicity, especially in the presence of anti-HBe, the emergence of frequent mutations in these patients is inevitable.

However, the relative importance of such mutations in different immune epitopes within HBV proteins in the hierarchy of protective immunity is a matter of debates. In terms of HBV proteins, some authors believe that CTL epitopes have a major role; a majority of chronic HBV carriers contained mutated residues within CTL epitopes.[15-17] Others, however, showed that these mutations occurred in the Th/B cell epitopes.[18-21] In our group-studied, nearly, half of the of mutations (46.6%) were occurred in B cell epitope within major hydrophilic region (MHR), encompassing amino acid residues 100-160. This was in concordance to our previous data. *In vitro*, we already showed that intracellular localization of HBcAg depended on the presence of mutations in different hepatitis B core gene B cell epitope mutations.[22] Of 26 cloned samples, HBcAg was predominantly localized in nucleus in 13 samples in remission phase (as HBcAg is a nuclear antigen) and in cytoplasm in other 13 samples with active hepatitis. All samples with cytoplasmic localization contained B cell epitope mutations. Reversion of mutant sequences with cytoplasmic expression back to the wild type by mutagenesis led to shifting back to nuclear distribution.[22] Furthermore, the similar numbers of mutations occurred in T helper immune epitopes within the surface proteins. The main role of T helper subsets in the immunosurveillance system is to synthesize cytokines and growth factors that stimulate (help) specific lymphocytes, including B cells.

Taken together, substitutions of 93.2% of amino acids in B and T helper residues highlights the importance of the humoral arm of immune system in the pathogenesis of HBV chronicity. The consequence of selection pressure posed by anti-S antibodies would be the emergence of immune escape mutations in this protein which no longer can be recognized by the host immune system. Some mutations are able to impair the binding of neutralizing antibodies to the viral surface (especially at “a” determinant region); viruses carrying such mutated T-cell epitopes cannot be recognized by specific T-cells of an individual, hence, will not enhance anti-HBs production,[23] and this can lead to the progression of chronicity of hepatitis B virus infection. Also, the occurrence of Th and CTL epiotpe mutations indicates an ineffective T cell response, as already shown that these responses are weak and sometimes undetectable during the chronic state of the infection.[24] The results presented in this paper are not defined by comparing HBV sequences at different time points for the same individuals. Rather, it is defined by its occurrence in highly conserved positions.

In conclusion, it appears from this work that there are variations in the structural protein of HBV in chronic patients. For a better interpretation, the allocation of such molecular variations to the clinical, serological and biochemical pictures needs to be explored. In this scenario, even an individual variation must be taken into account.

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