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Mutations in the S gene region of hepatitis B virus genotype D in Golestan Province-Iran

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Abstract Mutations of HBsAg especially within the "a" determinant could alter the antigenicity of the protein causing failure of HBsAg neutralization and escaping from the host's immune system, resulting in active viral replication and liver disease. This project aimed to investigate mutation in the S gene region of HBV infected patients in Golestan Province-Iran. HBV-DNA extractions from plasma and PCR of 100 patients were performed. Direct sequencing and alignment of S gene were applied using reference sequence from Gene Bank database. All isolates were belonged to genotype D, subgenotype D1, subtype *ayw*2. Overall 92 point mutations occurred. Of them, 40 (43.47%) were missense and 52 (56.52%) were silent. Mutations were detected in 95 cases (95%). Five of 40 mutations (12.5%) occurred in "a" determinant and 13 (32.5%), 17 (42.5%), and 2 (5%) were seen in antigenic epitope regions of B cell, CD_4^+ and CTL, respectively. Frame shift mutations were seen in 22 cases (22%). 14% of mutations occurred at Major Hydrophilic Region(MHR) area which P120T/S and R122K/T substitutions were the most frequent ones (4%). Mutation in G145R of the S gene was observed in one case. A large number of MHR mutants are in association with failure of HBsAg detection, vaccine, and immunotherapy escape. This study showed "a" determinant S gene mutations in HBV infected people with HBsAg positivity in Golestan Province-Iran. The rate of mutation in our study was 95%. Collectively, the results of this project exhibited that most of mutations were clustered in CD_4^+ antigenic epitopes.

Keywords Hepatitis B virus · S gene · Mutation · Golestan · Iran

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Introduction

Hepatitis B (HBV) is one of the most common infectious diseases, which may lead to chronic liver disease, cirrhosis, and hepatocellular carcinoma [1]. HBV S gene has three open reading frames (ORFs), including pre-S_1, pre-S_2, and S gene; its product is the small HBsAg protein SHB [2]. Full length genome analysis of HBV subclassified them into eight genotypes, named A to H [3]. The subtypes of HBV were originally defined by antibodies raised against the small HBsAg (SHBs) antigenic determinants are called as *ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adw4*, *adrq*− and *adrq*+ serotypes. With the emergence of complete HBV genome, eight genotypes from A to H were identified based on more than 8% of differences [4]. There is a certain correlation between serotype and genotype, but it is far from perfect [3]. HBV replicates to high titers in infected individuals, the replication of HBV DNA proceeds through a RNA reverse transcriptase intermediary step, since the reverse transcriptase activity of the HBV polymerase protein lacks a proof-reading function, random mis-incorporation of bases into the replicating DNA strand

occurs [5]. Mutation in the HBsAg, have now been documented worldwide [6]. The HBsAg "a" determinant (amino acids 124–147) is located within the major hydrophilic region (MHR amino acids 99–169) of this surface protein [7]. The potent B-cell epitope of HBsAg is borne by aa 124–147 of the S gene product, making two loop structures maintained by a disulfide bond between Cys-124 and Cys-137 as well as Cys-139 and Cys-147 [4]. Subtyping and genotyping of HBV is based on rasing of variety of antibodies against the "a" determinant [8]. Mutation of HBsAg particularly within the "a" determinant could alter the antigenicity of the protein, leading to fail neutralization of HBsAg by anti-HBs antibody and may cause escaping from the host's immune system [2]. Amino acid substitutions within the "a" determinant of HBsAg can lead to conformational alterations that may affect the binding of neutralizing antibodies. The first described escape mutation is G145R, which is related to a Guanine to Adenine mutation at nucleotide position 587. Many other substitutions described within the "a" determinant in the HBsAg, were outside of "a" determinant sequence and in the pre-S region [9]. A molecular model of HBV polymerase indicated that substitution in residues that located beneath the template strand of HBV nucleic acid near the active site of the reverse transcriptase may enhance polymerization either by repositioning the template strand of nucleic acid or by affecting other residues involved in the polymerization reaction [10]. Iran is considered as a country with low endemicity of chronic HBV infection [11] however, comparing to higher prevalence of HBV infection in Golestan Province with about 8.9% in comparison with other studies published in Iran to date (3%) [12]. Recent study in our research group has showed 85% efficiency of HBV vaccination in children aged under 1 year old, that 85% of them were Anti-HBs positive and Anti-HBc negative, and among 15% of them 4 were Anti-HBs and Anti-HBc positive and one of them was HBsAg positive,were which is lower than universal predicted rate of 95% [13]. This study aimed to investigate the rate of mutation in the S gene region of HBV in patients infected by this virus in Golestan Province-Iran.

Materials and methods

Sample selection

In this study, one hundred patients with proved HBV infection by Real-time PCR who have been referred by specialist for HBV infection diagnosis to virology diagnostic laboratory of Golestan University of Medical Sciences were entered in this study. None of the patients were vaccinated for HBV or had immunoglobulin therapy; all patients were negative for antibodies against hepatitis C, hepatitis D and human immunodeficiency virus. In addition to these samples, HBV-DNA positive and HBV-DNA negative control groups were also used. Blood samples were collected in anti-coagulant EDTA 5% and plasma was separated and used for further examination. All patients has been tested for serological marker of HBV (HBsAg) using commercial ELISA kit from DIALAB GmbH. All the samples were HBsAg positive.

DNA extraction

HBV-DNA was extracted from 200 µl plasma for each sample using QIAamp DNA Mini Kit (QIAGEN, Hamburg, Germany) following the manufacturer's instruction. Extracted DNA was stored at −20°C for PCR.

Primer selection

Suitable primers were selected to achieve maximum sensitivity for PCR to amplify the S gene region providing common regions for three types of surface antigens and first loop of "a" determinant. Following primers were yielded a 482 bp amplification product corresponding to nucleotide position 250–732 [6].

F-5′-CTA GAC TCG TGG TGG ACT TCT C-3′.
R-5′-AAG CCA NAC ART GGG GGA AAG C-3′.

PCR and DNA amplification

5 µl of extracted DNA was added to an amplification mixture containing 5 µl of 10X PCR buffer, 5 µl of $MgCl_2$ (25 mmol/L), 0.5 µl of dNTP (10 Mm), 0.5 µl of Taq DNA polymerase (QIAGEN, Hamburg, Germany) (5 U/µl) and 1 µl of each primers (10 pmol) in a total volume of 50 µl with distilled water. The PCR profile was an initial 5 min denaturation at 94°C, followed by 30 cycles of amplification including denaturation for 20 s at 94°C, primer annealing for 30 s at 60°C and extension for 40 s at 72°C, with a final extension at 72°C for 5 min. The 482 bp amplification products were analyzed by gel electrophoresis on 1.5% agarose gel stained with ethidium bromide to determine HBV-DNA positive and negative samples.

DNA sequencing and mutation analysis

The positive PCR products were subjected for automated sequencing (Metabion Company). For detection of mutation and analysis, nucleotide sequences were aligned with standard hepatitis B sequence [14, Accession number: AB033559] from Gene Bank database, And also with standard hepatitis B sequence of Iran in Gene Bank [8, Accession number: GU938305].

Results

The mean age of patients was 35.46 years. Of them 61% were male and 39% were female. Comparison of our nucleotide sequences with reference sequence showed all isolates were belonged to genotype D based on previous study [15], subgenotype D_1, subtype ayw_2, according to information that summarized in Table 1 based on amino acid residues specifying determinants of HBsAg [3]. Only three cases showing A70P mutations were HBeAg positive.

In the case of Amino acid mutation level, our findings with using Gene Runner software revealed the occurrence of 92 point mutations with 40 mutations (43.47%) as missense (amino acid altering) mutation and 52 mutations (56.52%) as silent (no amino acid altering) mutation. Of 100 patients, 73 (73%) of them showed point mutations in the region from nucleotide 143–582 that codes amino acids 47–194. Frame shift mutations were also showed in 22 patients (22%). Five of them (5%) did not show any mutations in their S region.

Most of the frame shift mutation (68.18%) was happened at amino acid positions 150 and 152.

In the case of point mutation distribution and frequency in the patients, our results were demonstrated 43 (58.90%) with single mutation, 13 (17.80%) with double mutations, 7 (9.58%) with triple mutations, 7 (9.58%) with four mutations, and 2 (2.72%) with five mutations in the sequenced region of them (Table 2).

Data from amino acid mutation level were described substitution of A70P in 58 (58%) cases and substitution of A70T in 2 (2%) cases with no significant effect on immune response. HBsAg MHR region possessed 14% of whole mutations and the positions that amino acid changes occurred are as following: Y100F, L109R, I110L, G112R, S117I, P120S/T, R122K/T, M133I, Y134H, T140I, S143L, G145R, G159R, E164V 0.5 of 40 mutations (12.5%) was occurred in "a" determinant. Distribution of these mutations was at M133I, Y134H in the first loop of "a" determinant and the rest of substitutions including T140I, S143L and G145R were occurred in the second loop of "a" determinant.

MHR variants with known functional effects are shown in Table 3. The variants involved in failure of HBsAg detection were found in samples of 11 patients (11%). The variants involved in immunotherapy escape were detected in samples of 5 patients (5%) whereas variants associated with vaccine escape were detected in samples of 4 patients (4%). The amino acid substitution G145R, which is associated with vaccine escape, was identified in one isolate (1%).

According to the proposed residues of antigenic epitopes within the HBV surface protein, our results demonstrated that of 40 amino acid changes, 13 (32.5%), 17 (42.5%), 2(5%) were seen in B cell epitopes, CD_4^+ and CTL

Table 1 HBsAg amino acid determinants and distribution of HBV genotypes and subtypes [18]

Position	Amino acid	Specify	Genomic group	Subtype	Areas of high prevalence
122	Lys	*d*			
	Arg	*y*			
127	Pro	w_1^a/w_2	D	ayw_2	Mediterranean area
	Thr	w_3			
160	Leu/Ileu	w_4		ayw_3	India
	Lys	*w*			
	Arg	*r*			

[a] w_1 reactivity also requires Arg 122, Phe 134, and/or Ala 159

Table 2 Distribution of frequency and position of point mutations in HBV patients in Golestan Province-Iran

No (%)	Frequency of point mutation	Position
43 (58.90)	1	A70P, G71X, R122T/K, L87M, P120S, I82L
13 (17.80)	2	A70P, V177L, P62L, L87M, M133I, L175F, I82L, P120S, S55C, A70T, S143L, T140I , Y134F, G145R, R79H
7 (9.58)	3	A70P, P62A, C65S, L186R, T189L, E164V, V190L, P62L, S64F, T140I
7 (9.58)	4	R79F, F80L, F85C, L89P, I110L, P120T, V190L, L89I, L109R, S117I, R122T, Y100F, L109R
2 (2.72)	5	G112R, P120S, R122T, W74G, M75R, L77M, A70T, S193 N, V194Y, P62L
Total	73 (100%)	

Table 3 Amino acid substitutions in the MHR of HBsAg with known functional effects according to Avellon and Echevarria [26]

Substitution in MHR	Number of cases	Description		
		Vaccine escape	Immunotherapy escape	Failure of HBsAg detection
P120S	$n = 3$	+	−	+
P120T	$n = 1$	−	+	+
R122K	$n = 4$	−	−	+
M133I	$n = 1$	−	+	+
T140I	$n = 2$	−	+	−
S143L	$n = 1$	−	−	+
G145R	$n = 1$	+	+	+

Table 4 Distribution and position of S region mutations in proposed antigenic epitopes [8]

Patients sequence	Cell subsets	Amino acid	Reference
Y100F, L109R, I110L, G112R, S117I, P120S/T, R122K/T, M133I, Y134H/F, T140I, S143L, G145R, G159R	B cell	100–160	Honirati
–	Th (CD4)	19–28	(Ducos 1996)
P62L, C65S, S55C, S64F	Th (CD4)	21–65	(Mancini 2006)
F80L, I82L, F83C, F85C, L87M, L89I	Th (CD4)	80–98	(Ducos 1996)
L186R, T189L, V190G, W191A, L192Y, S193N, V194Y	Th (CD4)	186–197	(Mancini 2006)
–	Th (CD4)	215–223	(Ducos 1996)
L175F, V177L	CTL (CD8)	171–179	(Barnab 1994)
–	CTL (CD8)	175–184	Mancini-2006
–	CTL (CD8)	206–215	Mancini-2006

epitopes, respectively and 8 mutations (20%) were out of these regions (Table 4).

Discussion

We have aligned our identified hepatitis B surface protein genotype and subtype with gene bank standard [14, Accession number: AB033559] and Iranian introduced strain [8, Accession number: GU938305]. All isolates were belonged to genotype D, subgenotype D_1 and subtype ayw_2; these are comparable with other cases studied in Iran by Daram et al. [16] Norouzi et al. [8], and Hamkar et al. [17]. Our previous study as well shown that all isolates of HBV in Golestan Province were belonged to genotype D [15]. This was in accordance with other studies reporting subgenotype D_1 and subtype ayw_2 are prevalent in Mediterranean area [3].

In comparison with previous reports from different part of Iran, showing 100% mutations in S gene region of isolated HBV [8, 16, 17], our findings showed some cases (5%) of HBV without any mutation in their S region. We have demonstrated mutations in 95% of patients showing lower rate of mutation than reports from Iran.

The most common mutation in this study was occurred at position A70P in 58 (58%) patients. However, it is not located in the antigenic epitopes within the HBV surface protein; compared to results in Daram et al. [16] and Norouzi et al. [8] studies. Also we have not seen this mutation (A70P) in 13 (13%) cases. For the first time, we also have find amino acid substitution at position A70T in 2 (2%) patients.

Mutations especially at "a" determinant region are able to impair the binding of neutralizing antibodies to the viral surface; viruses carrying such mutated T-cell epitopes cannot be recognized by specific T-cells of an individual, hence, will not enhance anti-HBs production, this could be led to the progression of chronicity of HBV infection. The importance of such mutations in different antigenic epitopes within HBV proteins in the pathogenesis of chronic HBV is a matter of debates. Some authors believe that CTL epitopes have a major role; a majority of chronic HBV carriers contained mutated residues within CTL epitopes. Others, however, showed that these mutations occurred in the CD_4^+/B cell epitopes [16].

Our study showed that, most of the amino acid substitutions (32 of 40 amino acid changes) 80% occurred in different antigenic epitopes of S gene, with distribution of 13 (32.5%) in B cell epitopes, that 5 of them were located in "a" determinant, 17 (42.5%) in CD_4^+ and 2(5%) in CTL epitope, whereas 8 mutations (20%) were positioned out of this region. It is comparable with data reported by Norouzi et al. [8] describing occurrence of 42.1% mutations in CD_4^+ epitope. But is different from findings of Daram et al. [16] in Iran showing most occurrence of mutations in CTL epitope (27.2%).

According to the study on Moroccan HBV infected patients, the global prevalence of MHR variants was 15% with the most frequent substitution at position P120T/S (3.7%) [18]. In Serbia mutation in MHR of HBV patients has been reported by overall frequency of 22.6% and substitution at position 120 (9.1%) was the most frequent [19]. This frequency has been reported in Argentina with 14.8% MHR mutation [20]. These are comparable with our results showing overall frequency of 14% MHR mutations with the most frequent ones of P120T/S and R122K/T (4%).

Interestingly, in comparison with a study of 249 sera from HBV-infected patients living in 25 Provinces of Iran [8], which were reported most frequent mutations of isolates was located at positions S143L, I10L, G101A, T118A, S136T, T118L, and P120S, our findings showed different feature of mutations with just two similar mutation at positions S143L and P120S/T. This difference could be due to high variability of sampling from different area of Iran that showing different pattern of above mutations in different Provinces.

Substitutions in numerous residues of S gene including positions 116, 118, and 120 could alter the "a" determinant conformation [21]. Substations that lead to conformational change of the 'a' determinant may cause a considerable decrease of properly folded surface antigen, which may render the virus particles less immunogenic in inducing an effective neutralizing antibody to clear the virus [6]. Nucleotide substitutions that lead to amino acid changes within the second loop of "a" determinant may result in reduced binding or failure to detect serum HBsAg in diagnostic assays with polyclonal and/or monoclonal antibodies [7]. However, some recent studies suggested that the "a" determinant region mutations did not play a major role in HBsAg laboratory detection and paraclincal diagnosis of occult HBV infection [8]. This is comparable with our results that reveal all 100 patients were HBsAg positive by DIALAB GmbH and shows following distribution in the first and second loop of "a" determinant. M133I and Y134H were occurred in the first loop of "a" determinant and the rest of substitutions including T140I, S143L, and G145R were occurred in the second loop of "a" determinant. It may result in an increased likelihood of HBV escaping identification by the immune system [6]. Our findings showed most of mutations were happened in the second loop of "a" determinant and is different with results of a study in Turkey that reveals higher rate of mutations at the first loop of "a" determinant [6].

In addition, De Maddalena et al. [22] found that genotype D strains carry more mutations in the "a" determinant of HBsAg with potential escape mutants in non-vaccinated subjects. Therefore, they indicate the need for careful surveillance of these variants in areas in which genotype D predominates.

The first description of an HBsAg escape mutant was made in a child born to an HBsAg-positive mother who developed acute hepatitis B despite being vaccinated and passively immunized against HBV [23]. The variant G145R seems to be the hottest spot for an escape mutation to occur and usually selected under immune pressures after administration of the HBV vaccine with or without concurrent HBIg, or following treatment with polyclonal or monoclonal HBIg, and caused to reduce affinity of HBsAg to antibodies induced with vaccination [24]. Naturally occurring surface gene variants have also been reported around the world in persons who have not been immunized [7]. Identified viral mutants are naturally circulating, because none of these patients were vaccinated with HBV or had immunoglobulin therapy. Escape mutants with mutations in the S gene would pose a substantial risk to the community, because current hepatitis B immunoglobulin and vaccines are not effective in preventing infection with them [25]. This substitution reported as a vaccine-escape

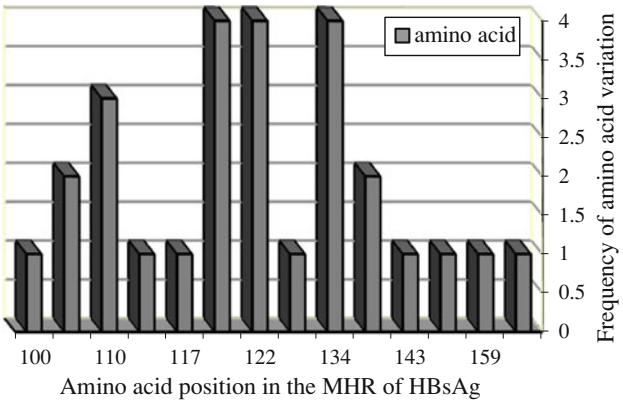

Fig. 1 Frequency and distribution of amino acid substitutions in the MHR of HBsAg in 19 patients

mutation [4]. In our study, we found one patient (1%) that displayed G145R substitution for the first time in Iran [8, 16, 17]. This frequency was similar to results reported in Spain and Serbia, 1.5 and 1.8%, respectively [19, 26]. Therefore, the presence of immune-escape mutants in our population should be considered in the immunization program (Fig. 1).

According to the study on Moroccan HBV infected patients, the global prevalence of MHR variants was 15% with the most frequent substitution at position P120T/S (3.7%) [27]. In Serbia mutation in MHR of HBV patients has been reported by overall frequency of 22.6% and substitution at position 120 (9.1%) was the most frequent [19]. This frequency has been reported in Argentina with 14.8% MHR mutation [20]. These are comparable with our results showing overall frequency of 14% MHR mutations with the most frequent ones of P120T/S and R122 K/T (4%).

The clinical characteristics of patients with MHR variants indicate a significant association between the occurrence of MHR variants and advancing age of the patients (>40 years). This result was consistent with that obtained by study in morocco [27] and could be explained that older age is more associated with longer history of HBV infection.

It has been demonstrated that the M133 V substitution led to the antigenic alteration [18]. Mutation at amino acid position M133I were already reported by Oon et al. [28], who has been shown that this mutation increase ability of vertical transmission of HBV to fetus. Further study in the case of babies who were born from HBV infected mothers should be done to confirm above idea. In our study, M133I substitution was found in 1(1%) case, and this case was female that indicates more consideration should be done about this group of patients to avoid prevalence of disease in the whole society.

Conclusion

A large number of MHR mutants are in association with failure of HBsAg detection, vaccine, and immunotherapy escape. This study showed S gene "a" determinant mutation in HBV infected people in Golestan Province-Iran. Our results demonstrate that the rate of mutations in S gene residues was 95%, while 5% of our samples were without any mutations. Mutation in 145th amino acid of S gene in genotype D of HBV is reported for the first time in Iran. Mutations in second loop of "a" determinant may result in an increased likelihood of HBV escaping identification by the immune system. Collectively, the results of this project exhibited that most of the mutations were clustered in CD_4^+ antigenic epitopes.

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