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HBsAg Mutants Clustered Mainly Outside of "a" Determinant in Chronic Carriers From Azarbayjan Province, Iran

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Background: Hepatitis B virus (HBV) genetic and protein variations have been observed in chronic patients frequently. However, their role in the pathogenesis of chronicity has not been explored yet.
Objectives: The aims of this study were to determine the genotypes as well as the patterns of variations distribution in chronically infected patients from the north western part of Iran.
Materials and Methods: The surface genes from 17 chronic carriers were amplified, sequenced and subsequently aligned using international and national Iranian database.
Results: All strains belonged to genotype D, subgenotype D1 and subtype ayw2. Of all 56 "mutations" that occurred at 37 nucleotide positions, 25 (44.6%) were missense (amino acid altering) and 31 (55.4%) were silent (no amino acid changing) (S/M ratio: 1.2). At the amino acid level, 21 (91.3%) out of 23 amino acid mutations occurred in different immune epitopes within the surface proteins, of which 3 (14.3%) occurred in B cell, 8 (38%) in T helper and 10 (47.7%) inside CTL epitopes. In general, the association between amino acid mutations and especially, immune epitope substitutions was more significant in terms of HBeAg status than ALT levels in patients.
Conclusions: The distribution of amino acid mutations as well as the ratio between missense and silent nucleotide mutations (dN/dS) showed that a narrowly focused immune pressure had already been on the surface protein (especially CTL epitopes) which led to the emergence of escape mutants in these patients who were in tolerance phase of chronicity.

Keywords: HBV Genotype D; HBsAg variants; HBsAg Epitopes Mutations

1. Background

HBV infection is one of the most significant global health problems. In spite of vaccination against HBV, this infection is responsible for more than 1 million deaths annually worldwide. 5-10% of infected adolescents or adults progress to a chronic carrier state, whereas up to 90% of infected neonates develop chronicity (1-3). Hepatitis B surface (HBsAg) protein is an important target for immune mediated virus elimination. Several immune B, Th and CTL specific epitopes have been described within the surface protein (4-7). Several "escape mutants" have been described so far, all showing changes in the so called "a" determinant of HBsAg within the MHR, of which, "vaccine-selected escape mutants" have been identified in children who acquired HBV infection despite the presence of surface antibody (anti-HBs) (8-11). Besides vaccination-associated HBsAg mutants, mutations in the HBV surface gene have been reported in chronic carriers who did not receive hepatitis B immunoglobulin or vaccine (12-14). Moreover, a study suggested a 10.8% frequency of HBsAg variants among Asian–Indian chronic HBV carriers (15).

The antibody against the HBs surface antigen (anti-HBs) is important in elimination of HBV (16, 17). Therefore, various mutations arising as a result of natural selection to evade the immune surveillance of the infected host would be expected. It seems that the consequence of selection pressure posed by anti-S antibodies would be the emergence of immune escape mutations in this protein which no longer could be recognized by the host immune system. Results would be the presence of virus (and sometimes a high level of viral load) in a chronically

Implication for health policy/practice/research/medical education:
HBV mutational studies have confirmed their implications on the route of transmission, potential pathogenesis and treatment decision.

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infected patient.

2. Objectives

This study was carried out in order to investigate the surface gene and protein variations implications in the chronic state of the disease and their correlation with the clinical features of chronic HBV carriers from Turkish province of Azarbayjan, Iran.

3. Materials and Methods

3.1. Sera

Patients with HBV infection attending the outpatient clinic at Azarbayjan research center for gastroenterology between 2005 and 2007 were included in the study. The inclusion criteria comprised of a serologically proven HBV infection (HBsAg positivity) with concomitant tests for HBeAg, anti-HBe and ALT. 17 chronic carriers who did not contain HDV, HIV and HCV co-infection were selected. Informed consents were taken from all patients and the study protocol was approved by the local ethics committee. The serological markers for Hepatitis B were identified using enzyme linked immune sorbent assay (ELISA) technique (Diaplus, Inc. USA).

3.2. DNA Extraction

Viral nucleic acid was extracted from serum using the Qiagen Mini Blood Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. 50 μl of elution buffer was added and the recovered nucleic acid was either tested for HBV DNA the same day or stored at −20 °C for analysis within 1 week.

3.3. Polymerase Chain Reaction

The whole HBsAg gene including 'a' determinant antigenic domain sequences was chosen for amplification in order to determine HBV genotype/subtype and other mutations. In an in-house methodology, first round HBsAg amplification (365 bp) was done by S1 50- CCT GCT GGT GGC TCC AGT TC -30 (position: 56-75) as sense primer and S2 50- CCA CAA TTC (K)TT GAC ATA CTT TCC A (K = G/T)-30 (position: 1003-979) as anti-sense primer. HBsAg second round PCR (270 bp) was performed by S6 50- GCA CAC GGA ATT CCG AGG ACT GGG GAC CCT G -30 (position: 113-146) as sense primer and S7 50- GAC ACC AAG CTT GGT TAG GGT TTA AAT GTA TAC C -30 (position: 857-823) as anti-sense primer. PCR program for first round consisted of 94°C 4 min, followed by 35 cycles 94°C 30 sec, 62°C 35 sec, 72°C 30 sec, followed by 72°C 10 min, and a similar program was applied for the second round PCR, but with 10 cycles decreasing. PCR elements in all reactions were identical and consisted of lx PCR buffer, 1.5 mM MgCl2, 0.2 mMdNTPs, 1.5 U Taq DNA polymerase HotStartTaq PCR (Qiagen, Hilden, Germany), 0.25 mM first and 0.5 mM the second round primers, 5 μl extracted HBV DNA for the first round, and 1 μl of the first round amplicon for the second round PCR reaction as template. To keep away from the risk of false-positive consequences, all PCR assays were performed with precautions against cross-contamination.

3.4. DNA Sequencing

The HBsAg subtype of the sequences was defined by substitutions in the 'a' determinant between codons 122 and 160 inclusive. Direct sequencing of surface gene was carried out (Genetic Analyzer ABI- 3130 DNA Sequencer, Fostercity, CA, USA) using 2 pmol of appropriate primers; S6 and S7 for surface gene. The results were analyzed using Chromas program. Sequences of surface gene were aligned using the BioEdit Package, version 7.0.9.

3.4.1. Sequence Analysis

After allocating a sequence to an HBV genotype by analysis of the S gene, the surface gene amino acid/nucleotide variations found were compared with a reference sequence obtained from Okamoto (1988, accession number AB033559), HBsAg sequences from Iranian isolates obtained from GenBank, NCBI and the ones from our own laboratory reports. Sequences have been submitted to GenBank, numbered from HM348619 to HM348635.

3.4.2. 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. There were a total of 681 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (18). Three of each 9 common HBV genotypes (A to I) were chosen for comparison. A genotype E (X75657) was chosen for out grouping between isolates.

3.4.3. Statistical Analysis

Data were represented in contingency tables and the association between categorical variables were analyzed using Chi-Square tests and Fisher exact test. The P value less than 0.05 was set for significant results.

4. Results

Seventeen HBsAg-positive patients were enrolled in this study, all of whom were native residents of Azarbayjan province (north-west of Iran). The group studied consisted of chronic hepatitis B carriers. Seven (41.2%) were female and 10 (58.8%) were male, with a mean age of 44.5 years. 5 and 12 of patients were HBeAg and anti-HBe positive respectively. The mean ALT value of patients was 60.4 IU/L (Table 1).

Table 1. Baseline Characteristics of the Patients Included in the Study

Sample Code	Age	Gender	HBeAg	Anti-HBe	ALT
34	28	Female	+		60
35	45	Male		+	50
39	37	Female		+	38
40	48	Male		+	32
44	50	Male	+		48
45	15	Female	+		47
46	29	Male	+		122
47	54	Male		+	32
48	75	Male		+	98
56	42	Male		+	70
59	66	Female		+	20
147	47	Female		+	65
148	51	Female		+	46
149	13	Female	+		14
150	51	Male		+	79
151	48	Male		+	156
345	57	Male		+	50

4.1. Phylogenetic Analysis

The results of the phylogenetic tree revealed that Iranian HBV isolates from Azarbayjan were of genotype D (Figure 1). All sequences were clustered within D1 subgenotype. Five sequences with a higher genetic distance from others (40, 56, 59, 151 and 345) showed individual clusters within the tree. These individual clusters contained at least two amino acid substitutions (Table 2). Eight sequences clustered in 4 pairs. Each pair showed a somehow similar (but not rather identical) pattern in terms of sequence variability. For instance, they contained at least either one nucleotide or amino acid substitution in comparison with reference genotype D (Okamoto, AB033559). Overall, comparing with reference sequence (Okamoto, 1988), at the nucleotide level, of a total of 160 changes, 113 (70.6%) and 47 (29.4%) were silent and missense, respectively (results are not shown). At the amino acid levels, all contained A70P compared to Okamoto reference. We believe that these substitutions were intragenotypic differences. Thus, 23 out of 40 amino acid changes were true mutations (see below).

4.2. 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 little variability over the sequenced regions. In general, 56 “point mutations” occurred at 37 nucleotide positions, of them, 25 (44.6%) were missense (amino acid altering) and 31 (55.4%) were silent (no amino acid changing) (Table 2). At amino acid level, 23 substitutions occurred, 3 were in positions 120 and 129 (Table 2), assigning for “a” determinant region. Furthermore, it was possible to identify the level of S proteins evolution between isolates by measuring the mutation frequency of individual sequences. The average mutation frequency of all sequences was 1.03 according to the number of mutations per site (Table 2).

4.3. Amino Acid Mutations within the Surface Protein Immune Epitopes

Twenty-one (91.3%) out of 23 amino acid mutations occurred in different immune epitopes within the surface protein, of which 3 (14.3%) occurred in B cell epitopes in 3 residues; 8 (38%) in T-helper epitopes in 6 positions and 10 (47.7%) inside CTL epitopes in 4 residues (Table 3). Three mutations were found in “a” determinant (two and one in residues 120 and 129, respectively).

4.4. Surface Gene and Protein Variations and Clinical/Demographic Status

There was no significant association between the sex and age of patients and the frequency of surface gene and protein variations (P value for each item was > 0.05). Table 4 shows the correlation between ALT levels and HBeAg status of patients with the number of nucleotide/amino acid variations. Five (29.5%) of samples were HBeAg positive, whereas 12 (70.5%) were anti-HBe positive. Eleven (19.6%) and 45 (80.4%) of nucleotide substitutions (regardless of being silent or missense) occurred in the former and latter, respectively. Similarly, 3 (13%) and 20 (87%)

of amino acid mutations occurred in HBeAg and anti-HBe groups, respectively.

When the correlation between the patient's ALT levels and the surface protein variations were analyzed (Table 4), the results showed that in patients with increased levels of ALT, 34 and 14 nucleotide and amino acid changes occurred, respectively (P value = 0.13 and 0.08, respectively). In patients with normal ALT levels 22 and 9 of such substitutions were found. Surface proteins immune epitopes showed a higher number of mutations in the group of patients with positive anti HBe (19 vs 2). In total, the association between amino acid mutations and especially, immune epitope substitutions were more significant in terms of HBeAg status than ALT levels (Table 4).

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	Nucleotide Mutation	Misssense Mutation	Amino Acid Change	Mutation Frequency
34	A342G, C566T	C566T	T189I	1
35	A342G, C566T	C566T	T189I	1
39	C345T, G529A, T600C	G529A	V177M	0.5
40	C369T, C420A, G492A, A617G, G620A	A617G, G620A	Y206C, S207N	0.6
44	C465T			-
45	C465T			-
46	C465T			-
47	C465T			-
48	C465T			-
56	A201G, C246A, T321C, C444T, G620C, T623C	G620C, T623C	S207T, I208T	0.5
59	C246A, C345T, C358A, A387C, C566T, C618T, A619C	C358A, A387C, C566T, A619C	P120T, Q129H, T189I, S207R	1.3
147				-
148	C345T			-
149	A303G, [A491G, G492T], A585G, C513A, A585G	[A491G, G492T], A585G	E164G, I195M	1
150	A534C			-
151	A327G, C358T, C578T, A366G, A610C, A617G	C358T, C578T, A610C, A617G	P120S, S193L, S204R, Y206C	2
345	T22C, G71A, T135A, A136C, C246A, C345T, A366G, C465A, [A617G, C618T], A619C, T623C	T22C, G71A, A136C, [A617G, C618T], A619C, T623C	F8L, R24K, T46P, Y206C, S207R, I208T	1.4
Average	-	-	-	1.03

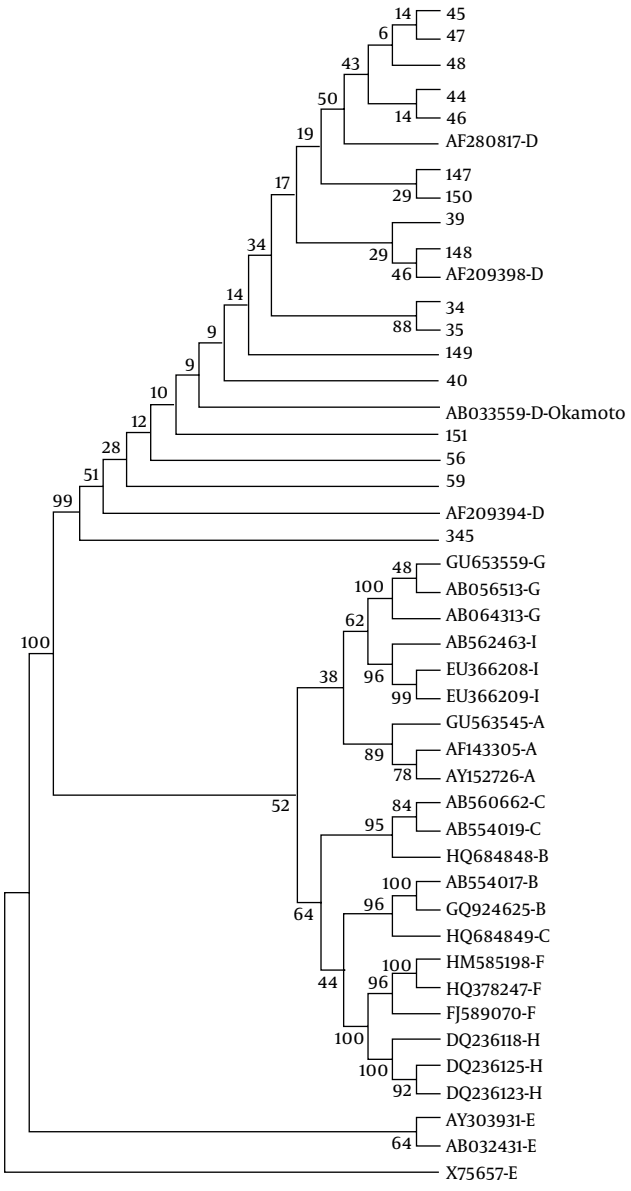
Table 3. Amino Acid Mutations Within HBsAg of Patient Groups: B cell, T helper and CTL Epitopes. Samples are Arranged in Accordance to the Arrangement of Immune Epitopes. Amino Acids are Described by Single Letter Code and Numbered From the Beginning of HBsAg.

Azarbayjan		Th Epitope						CTL Epitope				B Epitope	
Sample code	**Amino Acid Position**	**24**	**46**	**129**	**189**	**193**	**195**	**177**	**206**	**207**	**208**	**120**	**129**
Wild Type	R	R	T	Q	T	S	I	V	Y	S	I	P	Q
34					I								
35					I								
39								M					
40									C	N			
44													
45													
46													
47													
48													
56										T	T		
59				H	I					R		T	H
147													
148													
149							M						
150													
151						L			C			S	
345	K	K	P	Q	T	S	I	V	C	R	T	P	Q

Table 4. Correlation Between the Number and Distribution of Surface Variations and the Serologic/Biochemistry Pictures of the Isolates

Mutational Patterns	HBeAg (n = 5) / Anti-HBe (n = 12)	Normal ALT (n = 4) / Increased ALT (n = 13)	P Value	P Value	P Value	P Value
Surface Gene Mutations (Nucleotide level) (n = 56)	11/45	18/38	0.13			
Surface Protein Mutations (Amino Acid level) (n = 23)	3/20	8/15	0.08			
B cell epitope Mutations (n = 3)	0/3	2/1	0.4	0.03		0.17
T helper epitope Mutations (n = 8)	2/6	3/5	0.59	0.03	0.11	0.17
CTL epitope Mutations (n = 10)	0/10	3/7	0.2	0.03	0.11	

Figure 1. Evolutionary Relationships of 17 Taxa



The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

5. Discussion

An intermediate to a low endemicity has been reported for HBV infection in Iran (19). The main route of HBV transmission is vertical and most cases have a positive history of HBV infection in their family (20). The patients included in this study represent selected patients from a north-western Iranian infectious diseases outpatient clinic. It was possible to sequence the S genes for 17 HBV strains from Azarbayjan province. Genotype D and subtype ayw2 accounted for 100% of isolates. Published and unpublished data from Iran and from our laboratory indicated that there has been an obvious uniqueness for this virus genetic pattern in Iran (21-24). We already hypothesized that this unique pattern of homology is related to the relatively recent distribution of HBV in Iran compared to other countries in the region (21). When the patients were analyzed for variations, it was observed that anti HBe positive patients were more likely than HBeAg positive patients to have nucleotide and amino acid mutations (45 and 20 vs 11 and 3, positive respectively). Furthermore, anti HBe positive patients were more likely than HBeAg patients to have mutations within the surface protein immune epitopes (19 vs 2, respectively). However, no difference in ALT levels was found when mutated and nonmutated strains were compared.

These findings are interesting in the context of the immunotolerance that is thought to exist in the studied patients. This indicated that the proportion of deduced amino acid changes in these chronically infected patients was high. Indeed, the emergence of frequent mutations in patients with prolonged period of chronicity is inevitable (see below). The ratio between missence and silent mutations (dN/dS > 1) in 10 (who did contain amino acid substitutions) sequences indicated that these proteins were under a slightly positive selection pressure which had already been applied by both arms of cytitoxic and humoral host immune systems; overall, 18 (85.7%) out of 21 amino acid substitutions occurred at T cell levels. Compared to other immune epitope mutations, the occurrence of 10 CTL epitope changes in only 4 amino acid residues suggested a narrowly focused immune selection pressure at a hotspot position for this selection.

The latter finding was consistent with the findings of other authors, especially in genotype Dinfected patients (25-27).The distribution of the mutations within known surface protein immune epitopes reflects the virus host interaction with a prolonged infection period. Being a structural protein, HBsAg is an immune target. The consequence of selection pressure posed by anti S antibodies would be the emergence of immune escape mutations in this protein which no longer could be recognized by the host immune system. The results would be the presence of virus (and sometimes a high level of viral load) in a chronically infected patient. However, the presence of HBsAg mutants has been reported in some patients with chronic HBV infection who have not received either active immunization or HBIG. This observation can be justified by the fact that the host immune pressure alone is able to drive the selection of HBV mutants in such cases (14, 28, 29). The occurrence of Th and CTL epiotpe mutations in these patients indicates an ineffective T cell response. It has already been shown that these responses are weak and sometimes undetectable during the chronic state of

the infection (30).

In conclusion, mutation in the HBsAg in chronic HBV patients seems to be a common phenomenon which might have some implications that could finally lead to progression of chronicity of hepatitis B virus infection.

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Authors' Contribution:

For preparing this manuscript, all authors contributed equally.

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