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Hepatitis B virus surface protein mutations clustered mainly in CTL immune epitopes in chronic carriers: results of an Iranian nationwide study

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SUMMARY. Mutations within the coding region of hepatitis B surface antigen (HBsAg) have been found naturally in chronic carriers. To characterize the mutations of HBsAg from Iranian chronic carriers who were vaccine and/or medication naive. The surface genes from 360 patients were amplified and directly sequenced. The distribution of amino acid substitutions was classified according to different immune epitopes of the surface protein. All isolates belonged to genotype D. 222 (61.6%) of 360 patients contained at least one amino acid substitution. 404 (74.5%) of 542 amino acid changes occurred in different immune epitopes of HBsAg, of which 112 (27.7%) in 32 residues of B-cell epitopes (62 in the 'a' determinant); 111 (27.4%) in 32 residues of T helper; and 197 (48.7%) in 32 residues inside cytotoxic T lymphocyte (CTL) epitopes. One Th (186–197) and two CTL (28–51 and 206–215) epitopes were found to be hotspot motifs for the occurrence of 213 (52.7%) substitutions. 20 stop codons were identified in different epitopes. There was a significant association between amino acid substitutions and anti-HBe seropositivity; however, the correlation between such changes with viral load and ALT levels was not significant. In chronic hepatitis B virus(HBV) carriers, positive selection in particular outside the 'a' determinant appeared to exert influence on the surface proteins. These changes could be immune escape mutations naturally occurring due to the host immune surveillance especially at the T-cell level.

Keywords: Chronic HBV carriers, HBV escape mutations, HBV immune epitopes, HBV T-cell response.

INTRODUCTION

The morbidity and mortality associated with hepatitis B virus (HBV) infection manifest themselves in conditions such as cirrhosis and primary liver cancer that develop slowly during chronic disease. Globally, 30% of cirrhosis and 53% of primary liver cancer are attributed to HBV infection [1,2]. The diversity of clinical syndromes and

Abbreviations: HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MHR, major hydrophilic region.

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disease manifestations associated with HBV strongly suggests that the outcome of this infection is determined by the quality and vigour of the antiviral immune response produced by the infected individual. The pathogenic mechanisms responsible for liver cell injury in HBV infection are not well understood, although it appears that the virus is not directly cytopathic for the infected liver, and a strong immune reaction kills a large number of hepatocytes to clear the virus [3,4]. This leads to the pathologic consequences of acute and chronic hepatitis failure as well as cirrhosis.

In chronic carriers, the specific T-cell response is significantly weaker, in contrast to the acute phase, and in many patients, it is undetectable [5,6]. The T-cell response ineffectiveness in the pathogenesis of chronic HBV infection has been attributed to several factors: genetic background of the host [7,8], clonal tolerance [9], T-cell anergy (due to the high antigen load) or ignorance [10], cytotoxic T lymphocyte (CTL) exhaustion [6], a Th2-type response instead of Th1 [11,12], etc. In this scenario, escape mutants within immune epitopes of HBV constitute a significant problem. The HBsAg protein is an important target for immune-mediated virus elimination, and several B, Th and CTL immune epitopes within the protein have been described [13–17]. In recent years, HBsAg mutants have attracted great academic interest, and research work has concentrated on the emergence of HBV mutants especially with mutations in the 'a' determinant of HBsAg, in the following settings: (i) isolated cases of infection with HBV variants bearing substitutions in the 'a' determinant of HBsAg, predicted to escape from immune surveillance (immune escape variants) [18–20]; (ii) following vaccine and/or hepatitis B immunoglobulin (HBIG) administration, with amino acid exchanges in HBsAg, which lead to reduced or abolish completely binding of neutralizing antibodies (vaccine-/HBIG-escape variants) [21–25]; (iii) cases of infection that have been missed because of failure of current serological assays to detect some variant forms of HBsAg (diagnostic escape variants) [26–30].

In this study, we aimed to explore the surface protein variations between different ethnic groups of Iranian HBV treatment-naïve chronic carriers. We also wanted to establish whether there were any correlations between the frequency and the pattern of mutations within known immune epitopes of surface proteins.

MATERIALS AND METHODS

Study design

Three hundred and sixty HBsAg-positive chronic carriers who were referred to the Iranian Hepatitis Network (2004–2010) were enrolled in a cross-sectional study. To cover the whole ethnic regions of the country, we studied seven regions based on different ethnic populations and geographical zones. All patients were interviewed and examined by a gastroenterologist to evaluate the clinical findings and the results of the investigative workup (liver histology, ultrasonography and laboratory tests such as serologic, biochemical and virological tests) in order to determine the clinical status of the patient. Chronic hepatitis was defined as HBsAg positivity with or without the presence of HBeAg and low-to-moderate HBV DNA levels, persistent or intermittent elevation in serum ALT levels and compatible liver biopsy. Patients with HDV, HIV and HCV co-infection were excluded, as were those on antiviral treatment or who were vaccinated. All patients gave their informed consent, and the study protocol was approved by the local ethics committees. 5-mL aliquots of whole blood samples were withdrawn from each participant. Serum was aseptically separated in the field by centrifugation at 2000 rpm for 5 min and stored at −20 °C until tested. HBV serological markers including HBsAg and anti-HBs were examined by ELISA kits manufactured by Organon Technika, Holland.

DNA extraction

Hepatitis B virus DNA was extracted from 200 μL of serum using the Qiagen Mini Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. In brief, 20 μL of proteinase *K* was added to the serum in a 1.5-mL tube. Then, 200 μL of Al buffer was added to each tube, vortexed and then incubated for 10 min at 56 °C. For DNA precipitation, 200 μL of ethanol was added to the mixture followed by the centrifugation for 1 min. The contents of each tube were then transferred to collection tubes containing filter columns. Trapped DNA was washed in two steps by AW1 and AW2 buffers to eliminate impurities together with centrifugation after each step. Finally, the DNA was eluted using 100 μL of elution buffer and stored at −20 °C.

Polymerase chain reaction

Specific primers suitable for nested PCR were used as described previously [31]. First-round HBsAg amplification was performed by S1 (5′- **CCT GCT GGT GGC TCC AGT TC**-3′, nt positions: **56-75**) as sense primer and S2 [5′- **CCA CAA TTC (K) TT GAC ATA CTT TCC A (K=G/T)**-3′, nt positions: **1003-979**] as antisense primer. Second-round PCR was performed using S6 (5′- **GCA CAC GGA ATT CCG AGG ACT GGG GAC CCT G** -3′, nt positions: **113-146**) as sense primer and S7 (5- **GAC ACC AAG CTT GGT TAG GGT TTA AAT GTA TAC C** -3′, nt positions: **857-823**) as antisense primer. Cycling conditions for first-round PCR consisted of 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 62 °C for 35 s, 72 °C for 30 s and then one cycle at 72 °C for 10 min. A similar programme was applied for the second-round PCR, but for 25 cycles instead. PCR elements

were identical and consisted of lx PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 U Taq DNA polymerase (HotStart Taq, Qiagen, Hilden, Germany), 0.25 mM first and 0.5 mM 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 as template.

All PCR assays were performed with precautions against cross-contamination. To prevent carry-over contamination during PCR, each step of the procedure was performed in a separate room with dedicated equipment and directional flow from the beginning of the procedure to the end. Negative controls containing serum or water were also included in each extraction run, and an extra negative control containing water was included.

DNA sequencing

The whole surface antigen region obtained from the second round of PCR was sequenced with a DNA sequence analyzer (Perkin Elmer ABI-3130XL DNA Sequencer, Foster City, CA, USA) using 0.5 μL of appropriate primers S6 and S7. Electropherograms were analysed with the Chromas program and checked manually to confirm base assignment.

Sequence analysis

For the purposes of sequencing alignment, 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) and the consensus HBsAg sequences from Iranian isolates obtained from GenBank, NCBI, and from our laboratory using the BioEdit package version 7.0.9. Comparisons with the reference sequence identified amino acid changes that were defined as 'variant' (host HLA-determined). With regards to comparison with Iranian database sequences, any amino acid differences were defined as 'mutations'. Sequences have been submitted to the GenBank under accession numbers HM348619-35, GU938342-61, GU938363-64, HQ008867-68, KC176161-72, KC176137-41, HM348636-81, KC176142-60, GU938305-12, GU938314-22, HM348694-714, HM348682-93, GU938323-41, KC176076-99, KC176100-30, HM358277-99, HM358300-29, HM358335-39, KC176131-36.

Allocation of mutations to surface protein immune epitopes

The pattern of mutated amino acid distribution was established using the published data on the different HBV surface protein immune epitopes (Table 1) In total, 1, 4 and 4 regions have been proposed for B-cell, T helper and CTL immune epitopes across the surface protein, respectively.

Table 1 Proposed antigenic epitopes within hepatitis B surface antigen (HBsAg)

Sequence	R Cell subsets	HLA restriction	Reference
100–160	B	Not HLA restriction	[17]
19–28	Th	Class II	[14]
28–51	CTL	Class I HLA-A2	[16]
80–98	Th	Class II	[14]
171–179	CTL	Class I	[13]
175–184	CTL	Class I HLA-A2	[15]
186–197	Th	Class II	[15]
206–215	CTL	Class I	[15]
215–223	Th	Class II	[14]

Statistical analysis

Descriptive statistics were used such as frequency; mean and standard deviation comparisons between groups were made using the chi-squared test and Fisher's exact test.

RESULTS

Three hundred and sixty HBsAg-positive chronic patients were enrolled in this study, which were all native residents of different regions of Iran. All were chronic carriers, HBV DNA-positive and treatment-naïve. 247 (68.61%) were male, and 113 (31.38%) were female with a mean age of 36.34 ± 12.42 years (Mean ± SD) (Table 2). The mean ALT levels were 81.95 ± 64.85 IU/L (Mean ± SD). 74 (20.5%) and 248 (68.8%) were HBeAg- and anti-HBe-positive, respectively (Table 2). 3 (0.83%) were positive, and 35 (9.72%) were negative for both markers (results not shown).The mean viral load of the patients was 15000 copies/mL (results not shown).

Genotyping

Analysis of variation within the *S* gene of the 360 patients with chronic HBV infection demonstrated that the only detected subtype was *D* (100%); subgenotypes were D1: 97.7% (n = 352), D2: 0.55% (n = 2), D3: 0.27% (n = 1), D5: 0.83%(n = 3) and D8: 0.55% (n = 2). Finally, all isolates belonged to subtype ayw2 (100%) (results not shown).

Evolutionary changes within hepatitis B surface antigen

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 variability over the regions sequenced. In total, 222 (61.6%) of 360 patients had at least one mutated amino acid. 72 (20%) patients did not

Table 2 Details of evolutionary changes within the surface proteins studied

Characteristic variables	Number (%)
Number of samples	360
Male/female (%)	247 (68.61)/113 (31.38)
HBeAg-positive/ anti-HBe-positive (%)	74 (20.55)/248 (68.88)
Nucleotide mutations	1104
Silent nucleotide mutations (%)	562 (50.09)
Missense nucleotide mutations (%)	542 (49.09)
Silent/missense ratio	1.03
Amino acid mutations	542
Stop codons (%)	32 (5.9)
Immune epitope amino acid changes (%)	404 (74.5)
Nonimmune epitope amino acid changes (%)	138 (25.5)
B-cell epitope amino acid changes (%)	112 (27.72)
Th-cell epitope amino acid changes (%)	111 (27.47)
CTL epitope amino acid changes (%)	197 (48.76)

have any nucleotide and amino acid substitutions at all. In total, 1104 nucleotide substitutions occurred, of which 542 (49%) were missense (amino acid altering), and 562 (50.9%) were silent (no amino acid change) (Table 2). At the amino acid level, 542 substitutions were noted affecting 164 positions. The mean ratio between silent to missense substitutions (*dS* to *dN*) in each ethnic group was 1.03. The nucleotide mutation frequency (indicating the probability of nucleotide substitution per individual site) was 3.61 (0.53%). 32 (5.6%) nucleotide mutations were of nonsense substitutions, which led to stop codons in different positions. The amino acid mutation frequency (indicating the probability of amino acid mutation per individual site) was 1.68 (0.74%) (Table 2).

Amino acid mutations within surface protein immune epitopes

According to the proposed residues of immune epitopes within the HBV surface protein (Table 1), 404 (74.5%) of 542 amino acid changes occurred in different immune epitopes within the surface protein, of which 112 (27.7%) occurred in 32 residues of B-cell epitopes; 111 (27.4%), in 32 residues of T helper; and 197 (48.7%), in 32 residues inside CTL epitopes (Table 2, Fig. 1).

Mutations within B-cell epitopes

Analysis of the major hydrophilic region (100–160) showed that of the total amino acid substitutions (542), 62 (11.4%) occurred within the 'a' determinant region (Fig. 1). Especially, there were four hotspot residues within this domain, which accounted for 31 substitutions: 7, 7, 10 and 7 substitutions in amino acid residues 127, 129, 134 and 143, respectively. Only one patient had a G145E substitution. Of 50 mutation events within the major

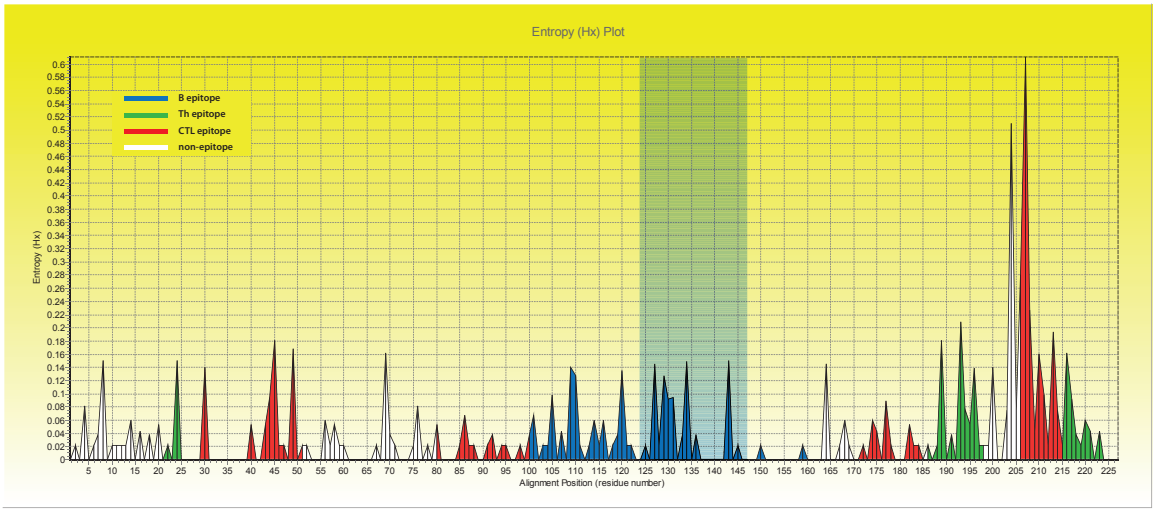

Fig. 1 Positions of amino acid substitutions within the different surface protein immune epitopes highlighted in different colors. The major hydrophilic region (MHR) including the "a" determinant (amino acid 125–147) is highlighted in cyan. Amino acids are numbered from the beginning of HBsAg. Only positions at which changes occurred are shown, so relative proportion of areas is skewed in favour of regions where substitutions occurred, respectively.

Table 3 Basic characteristics of the different ethnic groups studied in relation to the pattern of amino acid distributions within the surface protein immune epitopes

	Number of patients		
HBsAg substitution levels	HBeAg-positive (N = 74) 20.5% (P Value)	Anti-HBe-positive (N = 248) 68.8% (P Value)	Abnormal ALT (N = 330) 91.6% (P Value)
Nucleotide mutation	56 (0.18)	206 (0.02)	265 (0.88)
Amino acid mutation	36 (0.008)	166 (0.002)	206 (0.18)
Immune mutation	28 (0.002)	144 (0.005)	177 (0.89)
Th epitopes	11 (0.03)	69 (0.002)	78 (0.88)
CTL epitopes	16 (0.006)	102 (0.00)	113 (0.86)
B-cell epitopes	13 (0.17)	49 (0.06)	74 (0.17)
NonImmune mutations	21 (0.38)	86 (0.007)	103 (0.63)

hydrophilic region (however outside the 'a' determinant), 39 (78%) occurred predominantly between residues 105 and 120 (Fig. 1, blue colour).

Mutations within Th epitopes

Hundred and eleven mutations in 32 residues were found among four Th epitope domains (totalling 50 residues altogether) within the proteins studied (Table 1): 15 (13.5%), 17 (15.3%), 51 (45.9%) and 28 (25.2%) in domains 19–28, 80–98, 186–197 and 215–223, respectively (Fig. 1, green colour). Five positions 24, 189, 193, 196 and 216 contained 11, 11, 16, 8 and 11 amino acid changes, respectively (results not shown). 10 of 11 changes at position 216 were stop codon mutations.

Mutations within CTL epitopes

Of 197 mutations in 32 positions within the CTL epitopes, 48 (24.3%), 17 (8.6%), 18 (9.1%) and 114 (57.8%) substitutions occurred within epitopes 28–51, 171–184, 183–191 and 206–215, respectively (Fig. 1, red colour). Substitutions at positions 45, 49, 206, 207, 208, 210 and 213 accounted for 12, 11, 17, 46, 16, 9 and 13 amino acid changes (results not shown).

Surface protein variations and clinical status

When the correlation between the patterns of distribution of surface protein amino acid variations within the immune epitopes and HBeAg status of the ethnic groups was analysed, the results showed that 28 and 144 of the patients had amino acid substitutions in the HBeAg and anti-HBe groups, respectively (P value: 0.002 and 0.005, respectively) (Table 3). The most significant association was found between the occurrence of amino acid mutations within B, Th and CTL epitopes in anti-HBe-positive individuals. In those patients who were both HBeAg- and anti-HBe-negative, the results indicated that the majority of amino acid mutations occurred in nonimmune epitopes (14.8% *vs* 3.6%, P = 001). The comparison between amino acid substitutions with mean ALT showed that 206 of amino acid changes occurred in groups with abnormal ALT (P value = 0.18). However, the association between abnormal ALT levels and amino acid substitutions in epitopic and non-epitopic locations in patients who harboured the mutations was not significant (P values: 0.89 *vs* 0.63) (Table 3). Similarly, in total, there was no significant correlation between HBV DNA levels ($<10^4$ *vs* $>10^4$ copies/mL) and the occurrence of amino acid substitutions at all (results not shown).

DISCUSSION

The presence of HBsAg mutants has been reported in patients with chronic HBV infection who have not received either active immunization or HBIG, and it is thought that in such cases, the host immune pressure alone is able to drive the selection of HBV mutants [18–20].

We aimed to investigate HBsAg mutations in patients with chronic HBV disease. All patients had been infected by genotype D. It was interesting that most of the amino acid changes observed in immune epitopes were clustered in five regions: first, the 'a' determinant; second, positions 186–197 and 215–223, specifying Th domains; and third, positions 28–51 and 206–215 specifying CTL domains. Of a total of 404 mutations within HBsAg immune epitopes, 308 (76.2%) occurred within T-cell domains, suggesting a narrowly focused immune selection pressure at a hotspot position. The latter finding was in agreement with the findings of other authors, especially in genotype *D*-infected patients [32–34].

What is the impact of single amino acid substitutions on the antigenicity of HBsAg, and is the immunogenicity of HBsAg necessarily impaired if such mutations occur? The humoral immune response to HBsAg is T-cell-dependent. Thus, appropriate reactivity of T cells is a prerequisite for adequate anti-HBs production after infection with HBV.

Therefore, the T-cell epitopes of HBsAg being targets for the recognition by T cells should also be affected. Although we did not carry out *in vitro* experiments to determine T-cell reactivity of those epitopes, multiple amino acid changes (alone and/or in combination) in surface-exposed regions of HBsAg outside the 'a' determinant have been found to reduce and/or abolish the reactivity of monoclonal and polyclonal anti-HBs diagnostic antibodies [26,35–38]. These mutations especially include amino acid residues between the positions 19–28, 80–98, 110–120, 148–162 and 206–215 (Table 1). It seems that, as an alternative, virus-neutralizing activity may reside in antibodies related to distantly located amino acid residues in other parts of the protein rendering the viral particle less immunogenic in producing an effective neutralizing anti-HBs response to clear the virus [36]. Also, changes located outside the 'a' determinant region were reported from immunized infants born to HBV carrier mothers [39–41]. Furthermore, some of these mutant proteins had reduced binding to monoclonal antibodies against the 'a' determinant. These amino acid substitutions may occur often in one isolate and may contribute collectively to the reduction of antibody binding [42] leading to the progression of chronicity. Interestingly, none of such substitutions in our study led to the nonreactivity of HBsAg in the patients studied.

In the present study, two characteristic features concerning the prevalence and mutation patterns of HBsAg were observed. First, an unexpectedly higher prevalence of mutations was observed within HBsAg immune epitopes versus nonepitope domains, 74.5% *vs* 25.5%, respectively. Second, a relatively higher mutation frequency was observed in positions specifying for T-cell immune epitopes (76.2%), especially CTL domains (48.7%). The two above issues indicated that these substitutions in chronic carriers do not occur randomly; instead, a significant selection pressure that had already been applied by both arms of the cytotoxic and humoral host immune system (a positive selection) led to an ineffective T-cell response, as it has already been shown that these responses are weak and sometimes undetectable during the chronic state of the infection [5,6].

The relative importance of such mutations in different immune epitopes within HBV proteins in the hierarchy of protective immunity is a matter of debate [43]. The finding that 48.7% of the mutations occurred within CTL epitopes were in disagreement with the finding obtained by some other authors. In several studies on chronic HBV-infected patients, investigators found that in anti-HBe-positive patients, who went into remission, putative escape mutations appeared in the T helper epitopes. Conversely, in those with ongoing disease, they occurred in B-cell epitopes. One can thus speculate that after HBeAg is lost by the selection of a precore stop codon variant, probably driven by the immune response against HBeAg, either an effective CTL response occurs against the core, which clears the virus, or there is a poor CTL response [10]. In the absence of an adequate CTL response, anti-HBc kills hepatocytes via complement or natural killer cells; selection of numerous mutations in humoral epitopes is then inevitable. Thus, a significant role of the humoral immune response in terms of T helper cell epitope nonrecognition on the pathogenesis of HB chronic infection may exist [10–12,18,20,44,45]. These findings are consistent with the results obtained by Ferarri *et al.* [46], who found a higher level of anti-HBc antibodies in chronic HBV patients than in acute patients. They hypothesized that chronic exposure of hepatocytes to HBcAg could lead to T-cell-independent B-cell immunogenicity. The end result would be the presence of virus (and sometimes with a high level of viral load) in a chronically infected patient. On the other hand, Peng *et al.* [47] showed that the persistent stimulation by HBeAg in serum probably imposed more pressure upon the CD8+ T cells, which resulted in high expression of PD-1 and CTLA-4 specifically on HBV-specific CD+ T cells. The high surface expressions of PD-1 and CTLA-4 may then downregulate the CD8+ T cellular responses and thus lead to the promotion and maintenance of viral persistence. Nevertheless, it is possible that mutations, for example, in CTL epitopes of some patients cannot be tolerated because of lethal effects on HBV replication and *vice versa* for either Th or B epitopes. Weiland *et al.* showed in mice that immunization with a DNA vaccine containing an immunodominant HLA-A*02012-restricted epitope of HBsAg (amino acid residue 20–28) that mutagenized from L to V at position 39 did not alter the HBsAg-specific expression and its immunogenicity to B cells, but had a strong impact on priming multispecific CD8+ T-cell responses [48].

The results of the present study showed that mutant HBV strains with changes in epitopic regions of HBsAg should be expected in almost 61.9% of the Iranian carriers, a significant frequency. There were no strong associations between viral load and biochemistry as well as hepatitis activity (according to biopsy results) of patients with various patterns of amino acid substitutions in the surface proteins (results not shown). This frequency does not seem to depend on the patient's gender or age. All the selected patients were chronic carriers. A majority of patients who harboured the mutations were anti-HBe-positive, indicating that the HBeAg seroconversion (due to the selection pressure of e antibody) led to a number of amino acid substitutions within the surface protein. Thus, the occurrence of mutation is an inevitable effect of the evolutionary process.

In conclusion, an unexpectedly high prevalence of naturally occurring HBsAg variants was observed in Iranian chronic treatment- and vaccine-naïve patients that were associated with mutations outside the 'a' determinant. Some novel variants and mutation patterns were also detected in the present study. These variants or mutants

arise as a result of natural selection to evade the immune surveillance of the infected host. These results may suggest a role of these mutations in T-cell epitopes in the establishment of chronic HBV infection. Therefore, the high prevalence of HBsAg variants and unique mutation patterns in Iranian patients may be associated with the extraordinary predominance of genotype D, which could influence biological aspects of HBV isolates in this region.

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