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Original Article

Nucleotide Substitutions in Hepatitis B Viruses Derived from Chronic HBV Patients

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Abstract. ***Background:*** **Mutations in the S gene (HBsAg), pre-core (PC), and basic core promoter (BCP) of the hepatitis B virus (HBV) infection are correlated with disease progression. This study assessed the frequency of mutations in the S gene, PC, and BCP regions in chronic hepatitis B (CHB) patients.**
Methods: **104 formerly known CHB patients who visited Tehran Hepatitis centers, were included. The viral load of samples was determined based on the TaqMan method. Regions of the S gene, PC and BCP were amplified by the nested PCR. Positive PCR products were sequenced and analyzed.**
Results: **33 successfully sequenced S gene region revealed all the derived strains were genotype D, with the majority (90.9%) belonging to the ayw2 subtype, and the rest (9.1%) to the ayw1 subtype. The prevalence of mutations was found to be 51.0% and 18.0% in the HBsAg and the Major Hydrophilic Region, respectively. 70.0% of amino acid changes within HBsAg occurred in different immune epitopes, of which 27.0% and 72.0% were located in B cell and Th epitopes, respectively. 26 successfully sequenced PC and BCP regions showed at least one mutation in 84.6% of the HBV strains. The PC and BCP mutations were G1896A (61.0%), G1899A (23.0%), A1762T/G1764A (23.0%) and G1764T/C1766G (26.0%). None of the strains with A1762T/G1764A mutation carried the G1764T/C1766G mutant.**
Conclusions: **Our results showed common mutations within HBsAg, occurring in immune epitopes, a high rate of G1896A mutations in the PC region, and a negative correlation between the emergence of A1762T/G1764A mutation and the G1764T/C1766G mutant in the BCP region.**

Keywords: Chronic hepatitis B; Mutation; Hepatitis B virus; Hepatitis B surface antigen; Hepatitis B e antigen.

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Introduction. Hepatitis B virus infection is a global epidemic health problem, which leads to cirrhosis, hepatocellular carcinoma, and severe liver disease.[1] Over 257 million people are chronic carriers of this virus.[1,2] The disease progression and the treatment response depend on the virus genotype in infected individuals.[3] HBV consists of four overlapping open reading frames (ORFs) and encodes seven viral proteins, including three envelop proteins (pre-S1, pre-S2, S), core protein, polymerase, HBx protein (as a transcriptional transactivator) and HBe protein.[4] HBV genome is classified into ten genotypes (from A-J) by

an intergroup difference > 8%.[5] The subtypes are associated with Hepatitis B surface antigen (HBsAg) epitopes, located in a region that comprises the two external loops of the molecule. HBsAg subtypes are differentiated based on two sets of determinants (d/y and w/r) and a determinant named (q).[4] As a result of the reverse transcriptase activity of HBV polymerase, this virus displays a wide genetic diversity. The immune system of the host and antiviral therapy are involved in the evolution of the HBV genome.[6]

HBsAg is a significant target for immune-mediated virus elimination.[7] This protein bears an antigenic structure, termed the Major Hydrophilic Region (MHR, aa 99-169). The MHR being the main target for neutralizing antibodies, encompasses the major B cell epitopes, the "a determinant" domain (aa 124-147).[8] The humoral response against HBsAg, is furthermore, T cell dependent. As a result, the appropriate reactivity of T cell, since it was a prerequisite for adequate anti-HBs products should affect the T cell epitopes within HBsAg which are a target for the recognition by T cells.[7] Mutations occurring within these immune epitopes play a significant role, and viruses carrying such mutations are predicted to evade host immune surveillance.[9]

Hepatitis B e antigen (HBeAg) is a secreted protein and a marker of active viral replication. In the natural course of infection with HBV, seroconversion from HBeAg to anti-HBe usually indicates the end of the active viral replication. However, mutations in the pre-core (PC) and basic core promoter (BCP) cause HBeAg to turn negative in patients with chronic HBV, even though replication continues and HBV-DNA is detectable.[2,10] The most common mutations involve G1896A in the PC region and the simultaneous presence of G1764A/A1762T in the BCP region,[11,12] which results to the premature termination of HBeAg expression and decreased level of HBeAg, respectively.[10-12]

Mutations in the core/pre-core and surface regions are correlated with disease progression, ranging from asymptomatic HBV carriers to fulminant hepatitis. Extensive studies have been done to associate these mutations with enhanced virulence. Nevertheless, it is still difficult to analyze the role of viral versus host factors in the progression of the disease.[11] In spite of extensive studies of HBV in Iran, there is little data on hepatitis B genome characterization. The aim of the present study was to investigate the frequency of mutations in the core/pre-core and the surface region of the hepatitis B virus derived from Iranian patients.

Patients and Methods.

Samples. The present study involved a total of 104 formerly known chronic hepatitis B patients (Positive for HBsAg for at least six months) who visited Tehran Hepatitis centers between 2014 and 2015. The ELISA method (Biokit, Spain) was used to test HBsAg, HBsAb, HBeAg, and HBeAb of all the serum samples. The HBsAg positive samples were subjected to DNA extraction using a commercial kit (High pure viral nucleic acid kit, Roche, Germany). Extracted DNA was stored at -70°C for PCR.

HBV viral load determination. The viral load of serum samples was determined by COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0 (Roche, USA).

DNA amplification of the PC and BCP regions. The region harboring the pre-core and basal core promoters of HBV was amplified by nested PCR using appropriate primers.[13] First-round PCR was carried out with 5 µl of extracted DNA in a total amplification mixture of 50 µl containing Taq polymerase, dNTP, primers, and PCR buffer. The PCR profile was preheated at 95°C for 15 min, followed by 35 cycles of amplification (95°C for 45 s, 53°C for 45 s, 72°C for 1 min), with a final extension at 72°C for 7 min. For the second round PCR, 5 µl of the first round PCR products were used as a template with the same condition as the first round.

DNA amplification of surface gene. The surface region was amplified by nested PCR using suitable primers.[14] The PCR condition was an initial 5 min preheating at 94°C, then 35 cycle amplification (94° 30 s, 56° 30 s, 72° 60 s) with a final extension at 72°C for 10 min. For the second round PCR, 5 µl of the first round PCR products were used as a template with the same condition as the first round except for the slightly altered annealing temperature (62°C instead of 56°C).

Sequencing and phylogenetic analysis. The positive PCR products were subjected to purification and sequencing (ABI 3730XL DNA Analyzer, Bioneer, Korea). Nucleotide sequences were aligned and analyzed using Bio Edit software version 7.0.0.

Statistical analysis. Statistical analysis was performed with Chi-square or Fisher exact test for categorical variables and with independent samples t-test for continuous variables using the SPSS version 21.0 software package. P values (two-tailed) less than 0.05 were considered statistically significant. The logarithms of HBV DNA levels were used for analysis.

This study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences, Infectious Diseases, and Tropical Medicine Research Center (approval number 2013/6/2), and was in accordance with the Helsinki Declaration of 1964.

Results. Overall, 104 serum samples from patients with chronic HBV infection (75 males, 29 females;

mean age 41.9+14.7 years) were obtained. A total of 20 (19.2%) patients had inactive HBV, 74 (71.2%) had chronic active HBV, and 10 (9.6%) had cirrhosis. Patients with chronic active HBV 74/104 (71.2%) and patients with cirrhosis 10/104 (9.6%) received antiviral therapy. Patients with a history of Lamivudine treatment were administered with Lamivudine again, and patients with a history of Tenofovir treatment or naïve treatment experience received Tenofovir. 55.8% (58/104) of them had a record of HBV infection among their family. The mean AST and ALT values of patients were 107± 69.3 and 137±93.7, respectively (**Table 1**).

All the samples were positive and negative for HBsAg and HBsAb, respectively. A total of 32.7% (34/104) and 66.3% (69/104) of patients had HBeAg and HBeAb, respectively. Based on the nested PCR results, 43 (41.0%) samples were positive for HBVDNA (limit of detection; LOD = 15 IU/mL). The surface region could be sequenced for 33 (76.7%) HBV infecting strain, and the sequence of the PC and BCP regions could be determined in 26 (60.5%) cases. All the derived strains were genotype D, and the S gene sequences revealed that the majority of isolates (30/33, 90.9%) were found belonging to ayw2, and the rest (3/33, 9.1%) to ayw1.

Amino acid mutations within the surface gene. Five (5) mutations were detected within the surface gene in the patients (F8L, T118M, E164D, T189I, and W196L). The prevalence of strains with S region mutation (single or multiple) found in all cases was 51.0% (17/33). Eight (8) of the cases had a single mutation, 3 cases 2 mutations, 6 cases 3 mutations. The frequency of mutations in the MHR was seen in 18.0% (6/33) of isolates (T118M, E164D). The most common amino acid change found within HBsAg was W196L in 13 (39.0%) isolates. A total of 32 amino acid changes, 22 (70.0%) occurred in different immune epitopes within the surface protein, of which 6 (27.0%) and 16 (72.0%) were located in B cell and Th epitopes, respectively (**Figure 1**). Also, AST was significantly higher among patients with F8L HBV mutants (P=0.001) (**Table 3**).

Mutations in the PC and BCP regions. At least one mutation was detected in the PC region in 60.0% (13/22) and 75.0% (3/4) of the HBeAg negative and HBeAg positive patients, respectively. In the BCP region, at least one mutation was observed in 54.5% (12/22) and 75.0% (3/4) of the HBeAg negative and HBeAg positive patients, respectively.

A high proportion (61.0%, 16/26) of G1896A mutation occurred in the PC region. The G1899A mutation was found in 6 (23.0%) isolates and had concomitant G1896A change. In the BCP region, the most common mutations were A1762T (30.0%, 8/26) and G1764T (30.0%, 8/26), followed by G1764A (26.0%, 7/26), C1766G (26.0%, 7/26), C1766T (11.5%, 3/26). A1762T and G1764A were frequently detected together in 23.0% (6/26) of the isolates. Similarly, G1764T and C1766G were frequently seen together in 26.0% (7/26) of cases. However, none of the patients with A1762T/G1764A mutation carried the G1764T/C1766G mutant (**Table 2**). There was no significant relationship between BCP, pre-core and surface mutations with HBV viral load, HBeAg and

Table 1. Demographic, biochemical, and virological data of the patients.

Characteristics	Inactive carriers (n=4)	Chronic active (n=23)	Cirrhosis (n=6)	Total
Age (years)	38.5 ± 13.4	32.5 ± 10.9	54.4 ± 10.3	47 ± 16.2
Sex M/F	1/3	5/18	1/5	7/26
HBeAg/ HBeAb	0/4	6/17	0/6	6/27
AST (IU l–1)	34.2± 5.4	58± 33	85± 97	60± 50
ALT (IU l–1)	41± 10	100± 75	69± 75	87± 72
HBV DNA (IU ml–1)	$2\times10^3 \pm 10^3$	$1.6\times10^6 \pm 1.5\times10^6$	$2\times10^5 \pm 5\times10^5$	$1.2\times10^6 \pm 1.4\times10^6$

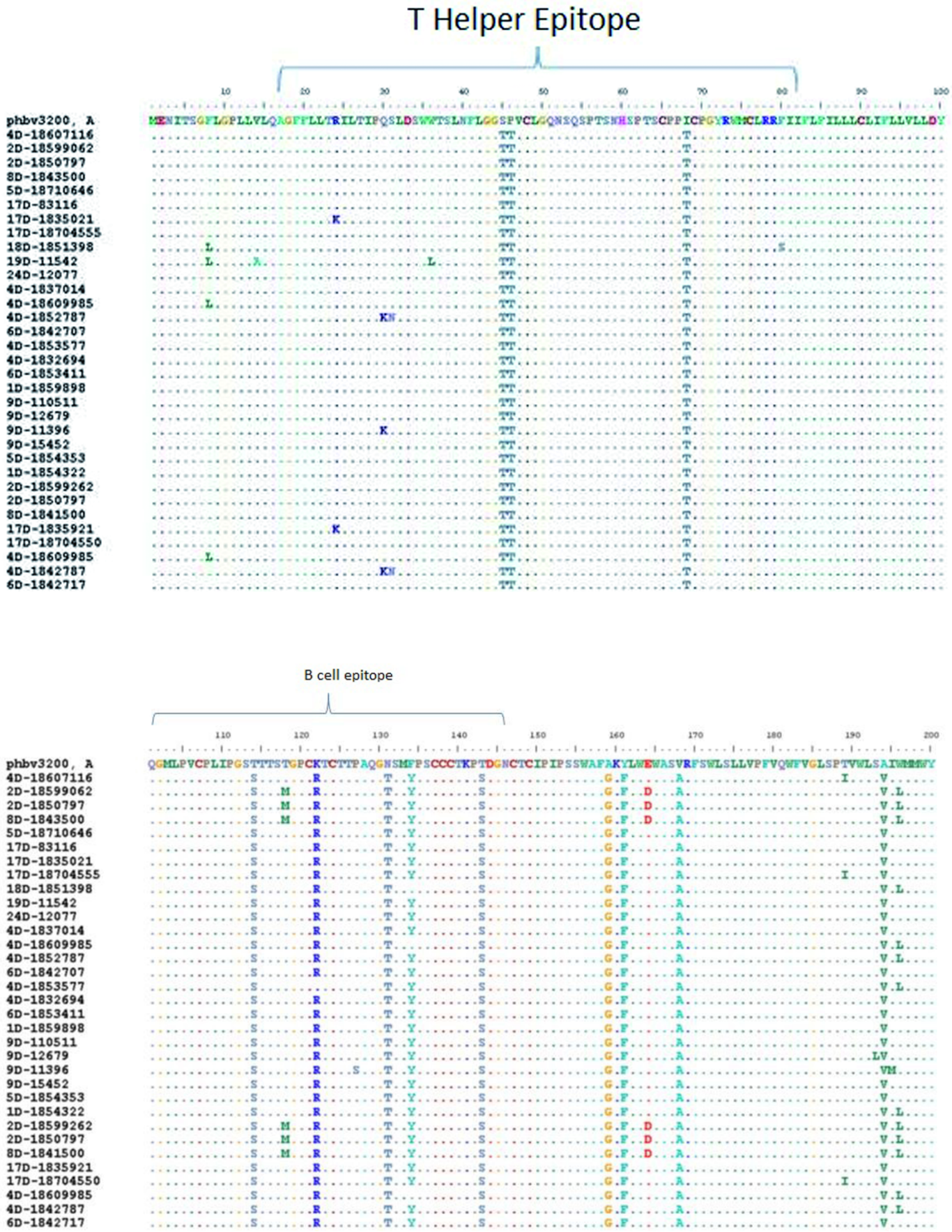
Data are presented as mean ± SD or N/N

Table 2. Frequencies of mutations in precore and core promoter region in strains from patients.

Clinical stage	A1762T	G1764T	G1764A	C1766T	C1766G	A1896T	G1899A
Inactive carriers (n=4)	1(0.03)	1 (0.03)	1 (0.03)	-	1(0.03)	3 (0.11)	1(0.03)
Chronic active HBV (n=16)	4(0.15)	6 (0.23)	3 (0.11)	1 (0.03)	6(0.23)	12(0.46)	4(0.15)
Cirrhosis (n=6)	3 (0.11)	1 (0.03)	3 (0.11)	2 (0.07)	-	1 (0.03)	1(0.03)
Total (n=26)	8 (0.3)	8(0.3)	7 (0.26)	3(0.11)	7(0.26)	16 (0.61)	6(0.23)

Data are presented as N (%)

Figure 1. Amino acid mutations within B cell, T helper (Th) and CTL epitopes of HBs proteins. Amino acids are designated by single letter code and numbered from the beginning of HBsAg.



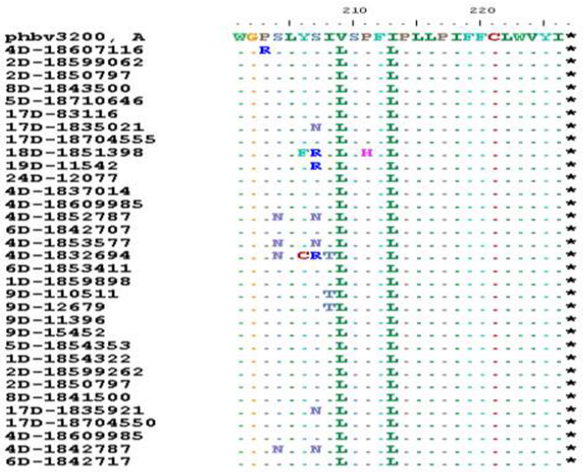


Table 3. Frequencies of mutations in surface region in strains from patients.

Clinical stage	F8L	T118M	E164DD	T189I	W196L
Inactive carriers (n=4)	1(0.03)	-	-	-	-
Chronic active HBV (n=23)	2(0.06)	4(0.12)	4(0.12)	3 (0.09)	9(0.27)
Cirrhosis (n=6)	1 (0.03)	2 (0.06)	2 (0.06)	-	3(0.09)
Total (n=33)	4 (0.12)	6(0.18)	6(0.18)	3(0.09)	12(0.36)

Data are presented as N (%)

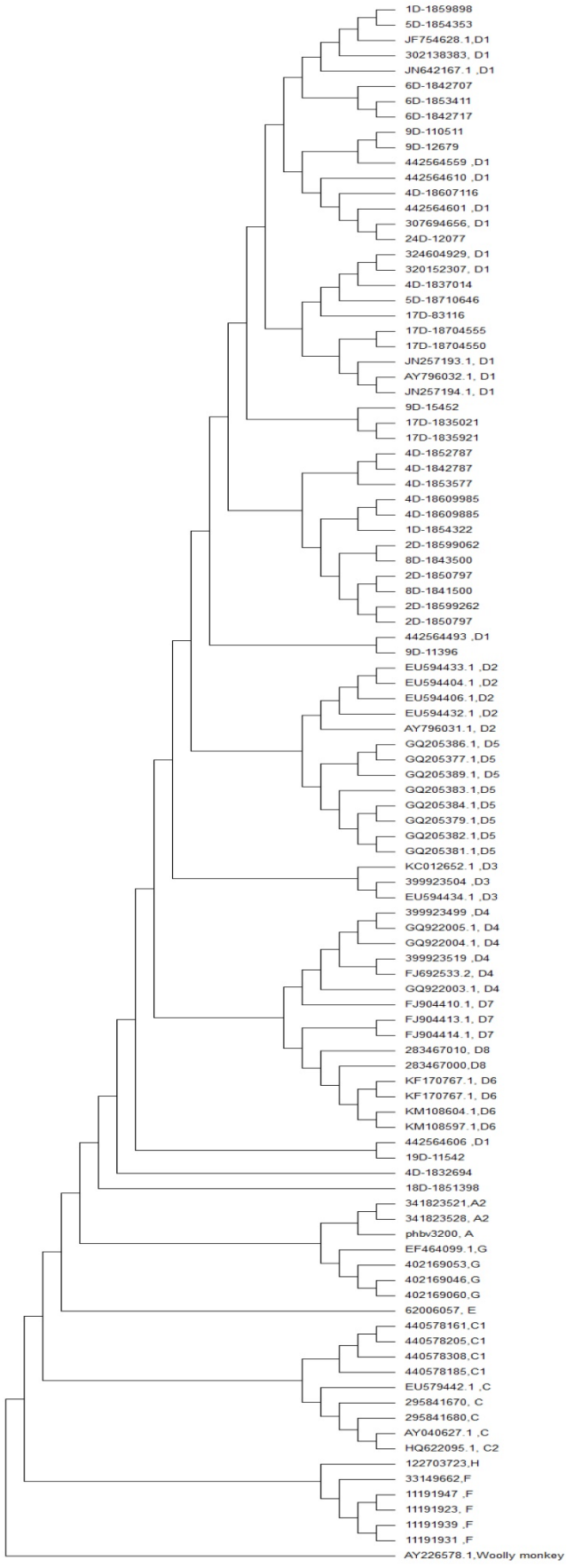
Table 4. P value of HBV genome mutations association with HBV viral load, HBeAg statue and liver function in this study.

		HBV viral load	p-value	AST	p-value	ALT	p-value	HBeAg	p-value
A1762T	Positive	$3.5\times10^5\pm50\times10^5$	0.1	59±46	0.8	96±119	0.7	0/8	0.2
	Negative	$1.2\times10^6\pm1.5\times10^6$		66 ±61		82±51		4/14	
G1764T	Positive	$1.6\times10^6\pm2\times10^6$	0.09	66±34	0.9	82±42	0.8	3/5	0.07
	Negative	$6.5\times10^5\pm7.3\times10^5$		63±62		88±81		1/17	
G1764A	Positive	$2.1\times10^5\pm3\times10^5$	0.08	61±50	0.8	96±129	0.7	0/7	0.5
	Negative	$1.2\times10^6\pm1.4\times10^6$		65±59		83±49		4/15	
C1766T	Positive	$2.1\times10^5\pm3\times10^5$	0.3	69±48	0.8	74±56	0.7	3/4	1.0
	Negative	$1\times10^6\pm1.4\times10^6$		63±57		88±80		1/18	
C1766G	Positive	$1.8\times10^6\pm2.1\times10^6$	0.1	57±28	0.7	78±50	0.7	3/4	0.04
	Negative	$6.2\times10^6\pm7.2\times10^6$		66±62		89±86		1/18	
A1896T	Positive	$1.1\times10^6\pm1.5\times10^6$	0.3	56±38	0.4	89±88	0.8	3/13	1.0
	Negative	$6.5\times10^5\pm8.5\times10^5$		74±76		83±61		1/9	
G1899A	Positive	$4.6\times10^5\pm8.7\times10^5$	0.3	78±48	0.4	123±133	0.1	1/5	1.0
	Negative	$1\times10^6\pm1.4\times10^6$		59±58		75±49		3/17	
F8L	Positive	$3.7\times10^5\pm4.7\times10^5$	0.2	104±113	0.07	130±80	0.2	0/4	0.5
	Negative	$2.1\times10^5\pm3\times10^5$		54±34		79±72		6/21	
T118M	Positive	$5.9\times10^5\pm6.5\times10^5$	0.3	52±33	0.6	68±27	0.5	0/6	0.3
	Negative	$1.2\times10^6\pm1.5\times10^6$		64±55		90±81		6/19	
E164D	Positive	$5.9\times10^5\pm6.5\times10^5$	0.3	52±33	0.6	68±27	0.5	0/6	0.3
	Negative	$1.2\times10^6\pm1.5\times10^6$		64±55		90±81		6/19	
T189I	Positive	$2.2\times10^6\pm1.4\times10^6$	0.1	49±50	0.6	79±45	0.5	1/2	0.4
	Negative	$1\times10^6\pm1.4\times10^6$		63±52		74± 14		5/23	
W196L	Positive	$8.3\times10^4\pm8.4\times10^4$	0.9	70± 68	0.4	88± 57	0.8	0/12	0.06
	Negative	$1.4\times10^6\pm1.7\times10^5$		54± 36		83±83		6/14	

Data are presented as N (%)

liver enzymes (**Table 4**), except an association between C1766G and HBeAg negativity which was significant (P=0.04).

Figure 2. UPGMA phylogenetic tree of surface genes sequences from 33 HBV strains. The tree rooted with HBV Woolly Monkey (AY226578) sequence. Genetic distances were estimated using the Kimura 2-parameter matrix. Clustering of sequences was supported by 1000 resamplings of the data sets.



Discussion. Analysis of the S gene sequence revealed that the majority of isolates (30 out of 33, 90.9%) belonged to the awy2 subtype while the rest (3 out of 33, 9.1%) were of the awy1 subtype. These findings are in agreement with other Iranian studies[6,15] reporting that the awy2 subtype was predominant. However, our results showed a higher rate of the awy1 subtype than previously reported.[6,15]

Hepatitis B surface antigen encompasses several B, Th, and CTL epitopes. The significance of substitution within these immune epitopes in the pathogenesis of chronic HBV is controversial.[7] Mutations occurring within the MHR, especially the "a determinant" domain, may alter the antigenicity; thus, this conformational change may contribute to false-negative serological tests, the presence of occult hepatitis, escaping vaccine-induced immunity and failure of the HBV immunoglobulin (HBIg) therapy.[16-18] Viruses harboring mutated T-cell epitopes may not be recognized by the T-cell of an individual; thus, it will not increase anti-HBs production and, it can result in the progression of chronicity.[9]

The result of this study indicated that most of the amino acid changes (22 out of 32, 70.0%) appeared in different immune epitopes, of which 6 (27.0%) and 16 (72.0%) were located in the B cell and Th epitopes, respectively. These results are in line with the finding by Moradi et al.,[19] showing the most occurrence of mutations (42.5%) in the Th immune epitope. However, it is in contrast to observations by Norouzi et al.[20] and Khedive et al.[7] stating that most of the mutations were clustered in the CTL immune epitope.

A prevalent mutation in the PC region of the HBV genome is a substitution at position G1896A (codon 28), resulting in the disappearance of HBeAg.[21] The rate of mutation at residue 1896 correlates with the HBV genotype and varies geographically. The pre-core mutations are more frequently seen in the D genotype and are more often observed in the Mediterranean region.[22]

In our study, G1896A mutation was detected in 16 (61.0%) out of 26 isolates (12 CHB patients, 1 cirrhosis, 3 inactive carriers). This finding is in line with other studies in France in 252 HBsAg positive carriers[23] and Korea in 472 patients with chronic HBV infection ,[24] which reported the substitution of G1896A in 54.9% and 55.0% of subjects, respectively. However, the present study showed a higher rate of G1896A than other studies in Iran by Ghabeshi et al. in 50 CHB patients,[25] Moradi et al. in 120 CHB patients[26] and Soleimani in 69 CHB patients[27] which demonstrated the presence of G1896A in 46.0%, 36.66% and 17.3% of patients, respectively. It was speculated that this higher frequency might be affected by the host immune system.

In this study, another common pre-core mutation at position G1899A was detected in 6 (23.0%) patients (4

CHB patients, 1 cirrhosis, 1 inactive carrier). It was observed that all subjects with the G1899A variant carried G1896A. Some studies revealed that G1899A is found to be associated with the severity of liver diseases.[28,29] Our finding is comparable with a study which showed G1899A in 29.3% of patients.[23] Another study performed in Korea indicated that all isolates with a G to A change at position 1899, had a concomitant G1896A change.[24]

A predominant double mutation in the basic core promoter region involves a G to A change at nucleotide 1764 and an A to T change at nucleotide 1762. This mutation may cause a decrease in the HBeAg level-up to 70.0% and increase viral genome replication.[30] The mechanism by which the G1764A/A1762T dual mutation enhances the virulence of HBV is not fully understood. It is thought that this double mutation forms a new binding site for the hepatocyte nuclear factor 1 (HNF1), leading to a reduced pre-core RNA expression and enhanced pre-genomic RNA transcription.[31,32]

The result of the present study revealed the presence of A1762T and G1764A mutations in 30% (4 CHB patients, 3 cirrhosis, 1 inactive carrier) and 26% (3 CHB patients, 3 cirrhosis, 1 inactive carrier) of subjects, respectively. The proportion of patients with A1762T/G1764A dual mutant in our study (23.0%,6/26) (3 CHB patients, 3 cirrhosis) is consistent with recent findings from Iran (19.6%),[26] Malaysia (26.9%) in 93 HBV carriers(26.9%)[33] and Morocco in 221 chronic carriers(22.9%).[34]

Another frequent dual mutation detected in the present study was a G to T change at nucleotide 1764 and a C to G change at nucleotide 1766. It has been suggested that the G1764T/C1766G mutant creates a new binding site for the hepatocyte nuclear factor 3 (HNF3), and increases core promoter activity.[35]

In our data, the G1764T/C1766G mutant was seen in 26.0% (7/26) of patients (6 CHB patients, 1 inactive carrier). None of the patients with G1764T/C1766G mutation carried A1762T/G1764A substitution. This finding is similar to the study by Sendi et al. in 97 CHB patients with HBeAg negative reporting that 30.0% of subjects had G1764T/C1766G double mutation, and the combined mutational patterns T1762/A1764/ G1766 or T1762/T1764/G1766 which would not generate binding sites for HNF1 or HNF3 were not seen.[35] Some studies revealed that the A1762/G1764A mutant accompanied by G1757A is associated with lower viral load and ALT level; hence, G1757A acts as an inhibitor to the A1762/G1764A mutant.[31] Nevertheless, the simultaneous presence of the A1762T/G1764A in conjunction with G at position 1757 is more efficient. When there is G1757A, the C1766G/G1764T double mutant is more efficient than the A1762T/G1764A mutation.[35] More extensive research work is needed to explore the tendency to either A1762T/G1764A or C1766G/G1764T.

Conclusions. Our results showed that most mutations within the S region were clustered in the Th immune epitope. Furthermore, the present data indicate a high rate of G1896A mutant in the PC region among Iranian CHB patients and a negative correlation between the emergence of A1762T/G1764A mutation and G1764T/C1766G mutant in the BCP region.

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