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Original Article

HBV Surface Ag mutations in chronic carriers are rare in Baloochstan provinces of Iran: a community with a low rate of HBV-related cirrhosis and HCC

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Abstract

Background and Aims: The aim of this study was to determine the correlation between the hepatitis B virus surface Ag (HBsAg) genotypes and variations in the clinical/serological pictures among HBsAg positive chronic patients from South Khorasan province of Iran.

Methods: Twenty-five patients were enrolled in this study. The HBs Ag gene was amplified and was directly sequenced. Genotypes and nucleotide/amino acid substitutions were characterized comparing with sequences obtained from the database.

Results: All strains belonged to genotype D, subgenotype D1 and subtype ayw2. Eight samples (group I) contained at least one mutation at the single amino acid level. Five out of 8 samples showed ALT levels above the normal range of which only one sample was anti-HBe positive. Group II (17 samples) did not contain any mutation, 4 were anti-HBe positive and 9 had increased ALT levels. In both groups, in anti-HBe positive patients who showed high levels of ALT, only one sample had amino acid mutations. Conversely, 7 of 20 samples with HBeAg positivity had mutations. In both groups, from a total of 18 amino acid mutations, 5 (27.5%) and 13 (72.5%) occurred in anti-HBe and HBeAg positive groups respectively. In general, there was no correlation between the occurrence of mutations and HBeAg status/ALT levels of the patients.

Conclusion: The relatively small number of nucleotide/amino acid mutations might belong to either the initial phase (tolerant phase) of chronicity in our patients, regardless of being anti-HBe positive; or that even in anti-HBe positive phase in Iranian genotype D-infected patients, a somehow tolerant pattern due to the host genetic factors may be responsible.

Keywords: HBsAg variants; HBV genotype D; HBV genotypes Iran; HBV chronic carriers

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Introduction

Hepatitis B virus (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. Using HBV genotype sequencing,

HBV genome variability can usefully be classified into at least eight families (genotypes) based on sequence divergence in the entire genome of >8-<17, with a characteristic geographic distribution (1-4). The different genotypes of HBV are stable forms of the virus, which are the results of random changes selected over years of population pressure (5). Based on the variability of the S-gene, a worldwide molecular epidemiology of HBV has been established (6). Thus, genotype A is common in North-Western Europe, North America, and Africa. Genotypes B and C are confined to Asia. Genotype D is the most widely distributed genotype and occurs all over the world with highest prevalence in the Mediterranean, the Middle East, and India, while E is the predominant genotype in West Africa. G is a genotype described from chronic HBV carriers in North America and France (3). Genotype F occurring in Central and South America and genotype H was reported in Central America, Mexico, and California which are considered to be the original HBV genotypes of Amerindians (4).

Recent publications from different parts of Iran indicated that genotype D is the only detected HBV genotype (7-14). This unique pattern may indicate a recent isolation of people in the absence of intermixing with other genotypes led to a homologous pattern (15).

The clinical manifestations of persistent HBV infection correlate somehow with distinct genotypes. Prospective, case-control and cross-sectional studies predominantly but not entirely indicate that the severity and outcome of chronic hepatitis B are more serious in patients infected with genotype C and D compared with B and A respectively (16). Clinical differences between genotype A and D infections have been reported from Europe, where these genotypes are frequent and differences between genotypes A and D prevailing in Western countries, India and the United States, are increasingly coming to the fore (16-23).

In this study, we analyzed the relationship between the HBV surface genetic and protein variations, liver function (assessed by alanine aminotransferase [ALT] levels), and anti-HBe status in a group of patients who were residents of north-eastern part of Iran.

The aims of the present study were to elucidate the prevalence of naturally occurring HBsAg variants and the correlation between those mutations according to the clinical features and HBeAg serostatus in chronic HBV patients from eastern part of Iran.

Methods

Patients

To cover the whole province of South Khorasan, we studied seven regions based on population and geographical zones. A total of 25 sera samples were collected. All cases had positive test for HBsAg detected by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Enzygenost® HBS Ag, USA). The patients had not previously received any antiviral drugs or hepatitis B vaccines, and were not co-infected with HCV, HDV, or HIV. Informed consent was taken from all patients and the study protocol was approved by the local ethics committee. Five mL of venous blood sample was taken from each patient and then was centrifuged immediately. Sera were stored at -80°C.

DNA extraction

HBV DNA was extracted from 200 µl of sera using Qiagen Mini Blood Kit (Qiagen , Hilden, Germany) according to manufacturer's instruction. In brief, 20 µl of protease was added to the serum in a 1.5 ml tube. Then, 200 µl of Al buffer was added to each tube, was vortexed and was incubated for 10 min in 56°C. For DNA precipitation, 200 µl of ethanol was added to the mixture, and the mixture was centrifuged for 1 min. Components were transferred to a collection tube contained filter tube. Trapped DNA was washed in two steps by AW1 and AW2 buffers to eliminate impurities together with centrifugation after each step. Finally, DNA was eluted using 100 µl of elution buffer, stored in -20°C.

Polymerase Chain Reaction

Surface gene including 'a' determinant antigenic domain sequences was chosen for

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amplification in order to determine HBV genotype/subtype and other mutations. First round HBsAg amplification was performed by S1 5′- CCT GCT GGT GGC TCC AGT TC -3′ (position: 56-75) as sense primer and S2 5′- CCA CAA TTC (K)TT GAC ATA CTT TCC A (K=G/T)-3′ (position: 1003-979) as anti-sense primer. HBsAg second round PCR was performed by S6 5′- GCA CAC GGA ATT CCG AGG ACT GGG GAC CCT G -3′ (position: 113-146) as sense primer and S7 5′- GAC ACC AAG CTT GGT TAG GGT TTA AAT GTA TAC C -3′ (position: 857-823) as anti-sense primer. PCR program for first round consisted of 94C 4 min, followed by 35 cycles 94C 30 sec, 62C 35 sec, 72C 30 sec, followed by 72 C 10 min, and a similar program was applied for the second round PCR, but with 10 cycles decreasing in program. PCR elements in all reactions 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 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.

DNA sequencing

The HBsAg genotypes and subtypes of the sequences were 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 analysed using Chromas software.

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) and HBsAg sequences from Iranian isolates obtained from GenBank and NCBI using Bioedit version 7.0.9. Comparing with the former, any amino acid changes were defined as "variant" (host HLA-determined). With regard to the latter (Iranian database sequences), amino acid differences were defined as "mutation". Sequences have been submitted to GenBank, numbered from GU938342 to GU938364 and from HQ008866 to HQ008868.

Phylogenetic analysis

Phylogenetic analysis was done and a neighbor-joining phylogenetic tree was constructed using the MEGA 4 employing a Kimura distance matrix (24). Associations were tested by bootstrap re-sampling analysis using 1000 replicates. Associations with a bootstrap value of greater than 70% were deemed significant.

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 exact test.

Results

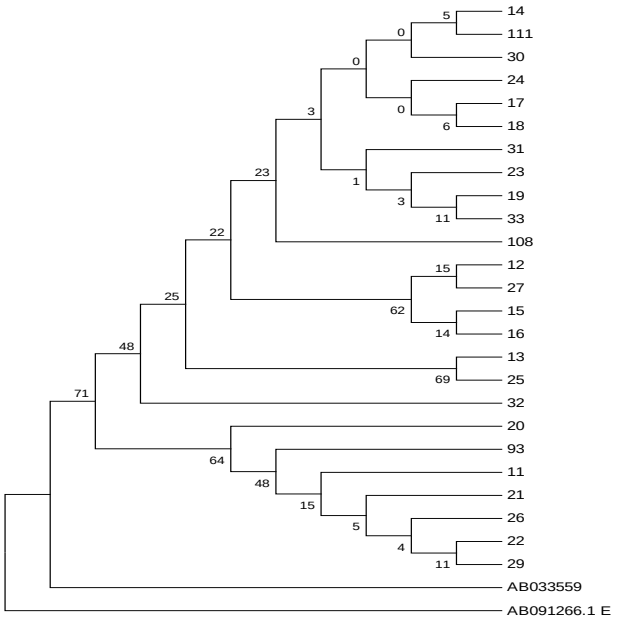

Fig. 1. Neighbour joining phylogenetic trees of surface genes sequences from 25 samples. Note: S gene tree rooted with sample AB091266. Coding numbers indicate samples that have been analysed in the figure. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary 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.

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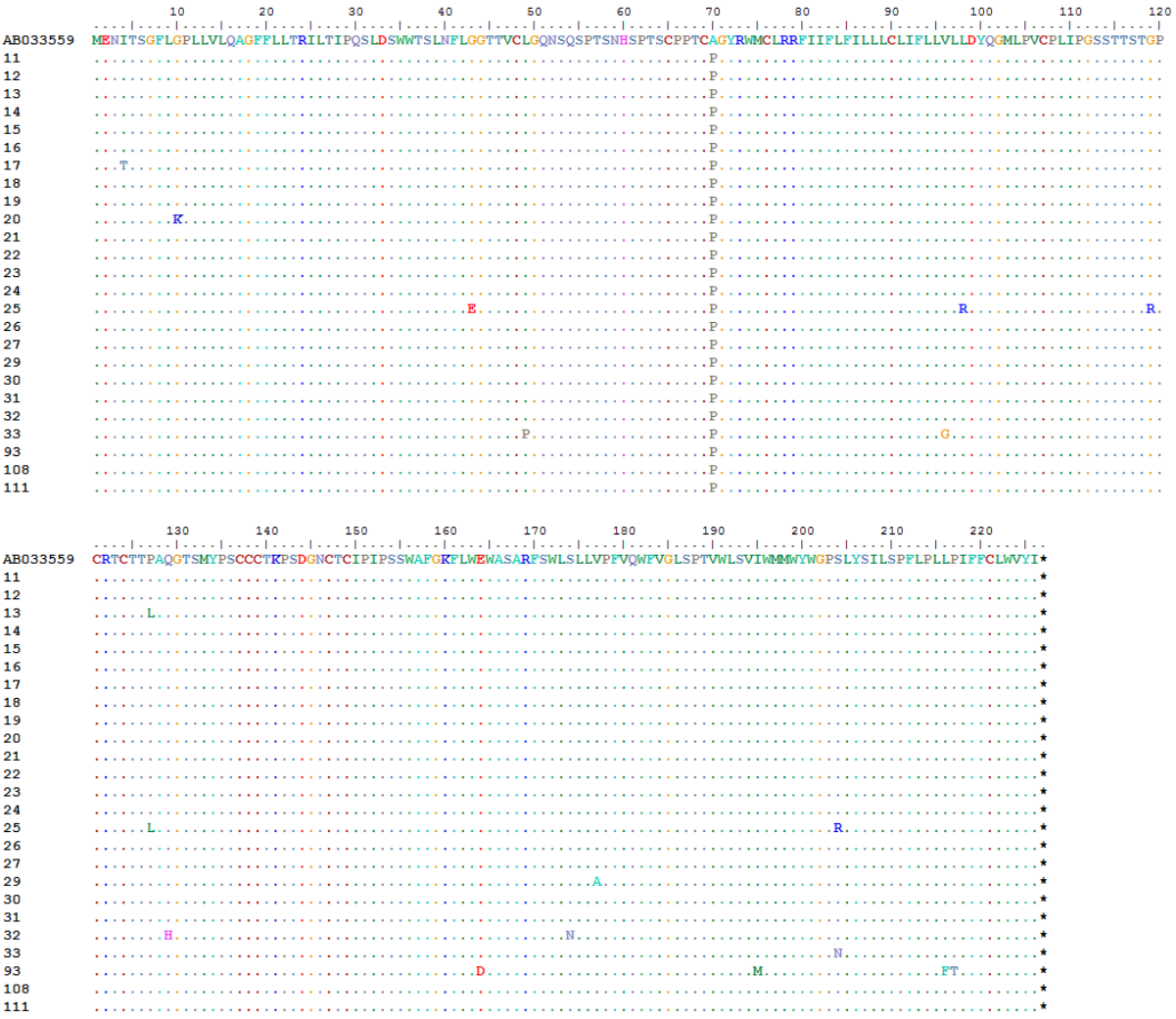

Fig. 2. Alignment of complete amino acid sequences of HBsAg which shows genotype/subtype identification and other variations of 25 sera using Bioedit software
Note: Amino acid residues are numbered from the beginning of the HBsAg using the single letter code.

Twenty-five HBsAg-positive patients infected with HBV were enrolled in this study, all of whom were native residents of Southern Khorasan Province (North-East of Iran). Four of them (15/4%) were female and 21 (84/6%) were male, with a mean age of 28/5 years. 20 (80%) of samples were HBeAg positive, whereas 5 (20%) were anti-HBe positive. The mean ALT and AST were 103 and 76 IU/L respectively. The studied patients were divided into two groups: group I (8 samples) who contained at least one amino acid change and group II (17 samples) who did not contain any amino acid mutation.

Phylogenetic analysis

The distribution of positive cases by age which is A phylogenetic tree (Fig. 1) was designed for the isolates studied by rooting with a genotype E sequence (accession number: AB091266). All isolates belonged to genotype D and subgenotype D1. It is noteworthy that all Iranian isolates clustered in a distinct branch that separated them from the reference Okamoto genotype D. There were seven pairs of clusters (14 isolates). However, only 2 pairs (12, 15, 16 and 27) contained similar patterns of either nucleotide or amino acid variations specifying pairing homology. The rest of

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Table 1. 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
11	C345T, C465T			-
12	C444A			-
13	[T11C, C12A], C380T	[T11C, C12A], C380T	P127L	0/33
14	[T11C, C12A], T288A	[T11C, C12A],T288A		-
15	C444A			1
16	C444A			1
17	T11C	T11C	I4T	-
18				0
19				0
20	C345T, C465T,G28A,G29A		G10K	3
21	C345T, C465T			-
22	C345T, C465T			-
23				0
24				0
25	G128A, T293G, G355A, C380T, T612A	G128A, T293G, G355A, C380T, T612A	G43E, L98R, G119R, P127L, S204R	-
26	C345T, C465T			2
27	C444A			-
29	T318A, C345T, C465T, T530C	T318A, C345T, C465T, T530C	V177A	0/75
30				0
31				0
32	A387C,G521A	A387C	Q129H- S174N	0/5
33	T146C,C246A,T287G,A366G, T384A,G611A,		L49P-V96G-S204N	1
93	C345T, C465T, G492T, C513A, A585G, A648T, C649A, G664C	G492T, A585G, A648T, C649A	E164D, I195M, L216F, P217T	1
108				0
111				0
Average	-	-	-	1.26

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Table 2. The correlation between HBeAg/Anti-HBe status, ALT levels and the details of amino acid substitutions.

Group	Sample code	Amino Acid Change	Gender	HBeAg/ HBeAb	ALT
Group 1 (n=8)	13	P127L	M	+/-	57
	17	I4T	M	+/-	25
	20	G10K	M	+/-	37
	25	G45E-L98R-G119R-P127L-S204R	M	-/+	181
	29	V177A	M	+/-	85
	32	Q129H-S174N	M	+/-	31
	33	L49P-V96G-S204N	F	+/-	21
	93	E164D-I195M-L216F-P217T	M	+/-	47
Group 2 (n=17)	11	-	M	+/-	40
	12	-	M	+/-	30
	14	-	M	+/-	42
	15	-	F	+/-	21
	16	-	M	-/+	183
	18	-	M	+/-	34
	19	-	M	+/-	9
	21	-	M	-/+	136
	22	-	M	+/-	55
	23	-	M	+/-	165
	24	-	M	+/-	18
	26	-	M	+/-	45
	27	-	M	+/-	60
	30	-	M	-/-	59
	31	-	F	-/+	116
	108	-	M	+/-	82
	111	-	F	+/-	24

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Table 3. The nucleotide and amino acid substitution patterns in correlation with the HBeAg status as well as the levels of ALT of patients.

Mutational Patterns	**Number of mutations HBeAg (*n=20*)/ Anti-HBe (*n=5*)**	**Number of mutations Normal ALT (n=6)/Increased ALT(n=19)**
Surface Gene Mutations (Nucleotide level) (*n=48*)	43/5	5/43
Surface Protein Mutations (Amino Acid level) (*n=18*)	13/5	3/15
B cell epitope Mutations (*n=4*)	2/2	0/4
T helper epitope Mutations (*n=5*)	4/1	1/4
CTL epitope Mutations (*n=6*)	4/2	1/5

strains branched into individual subclusters with different genetic distances. Nonetheless, they were not characterized by specific nucleotide and/or amino acid substitutions.

Substitutions in comparison with reference genotype D

Overall, comparing with reference sequence (Okamoto, 1988), at the nucleotide level, of a total of 192 changes in 33 positions, 69 (36%) and 123 (64%) were missense and silent, respectively (results are not shown). At the amino acid level, 43 substitutions were found. All strains contained A70P compared with Okamoto reference (fig. 2). We believe that this substitution was assigned as "variant" (see material and methods). According to the above mentioned description, 25 out of 43 amino acid changes were variants and 18 changes were mutations (see below). All strains belonged to genotype D (100%) and the only detected subtype was ayw2 (100%) (fig. 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 genotype differences described above, the sequences of the strains showed a little variability over the sequenced regions. In fact, at the nucleotide levels, 48 "mutations" occurred at 23 nucleotide positions, 21 (43.7%) of which were missense (amino acid altering) and 27 (56.3%) were silent (no amino acid changing). Table 1 shows the comparison between nucleotide and amino acid variations for the isolates. Group II (17) samples did not contain any mutations. 8 samples (group I) contained at least one mutation at the amino acid level; 18 amino acid mutations were found in 16 residues, of which 15 (83.3%) occurred within surface protein immune epitopes in 13 residues (Table 1). Three mutations were found in "a" determinant (Q129H and two in P127L). However, they were not specified as a specific

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subtype. Furthermore, it was possible to identify the level of S proteins evolution between isolates by measuring the mutation frequency of individual sequences in both groups (Table 1). The average mutation frequency of all sequences was 1.26 according to the number of mutations per site.

Surface protein variations and clinical status

There was no significant association between the age and gender of patients and the patterns of amino acid substitutions (results are not shown). The correlations of HBeAg/Anti-HBe status and ALT levels with nucleotide/amino acid variations in both groups have been shown in Table 3 and Table 4. In group I, 6 out of 8 samples (76.5%) showed ALT levels above the normal range, only one sample of which was anti-HBe positive. Similarly, 13 (75%) out of 17 samples in group II had increased ALT levels. Statistically, there was no significant association between two groups in terms of increased ALT and patterns of amino acid variations (*P* value: 0.65). Besides, the association between occurrence of amino acid substitutions and HBe Ag/Anti-HBe positivity was not significant (*P* value: 1.0). However, in spite of the fact that all patients with anti-HBe positivity contained high levels of ALT (ranged between 59 and 183), only one sample (20%) had amino acid mutations (Table 1). From a total of 20 samples with HBeAg posititvity, 7 samples (35%) had mutations. In both groups, from a total of 18 amino acid mutations, 5 (27.5%) and 13 (72.5%) occurred in anti-HBe and HBeAg groups, respectively.

Discussion

The patient samples included in this study represented selected material from an infectious diseases outpatient clinic located in north-eastern part of Iran. The results showed the presence of HBV genotype D, sub-genotype D1 and subtype ayw2 in all HBV infected patients. The results of genotyping were in agreement with previous reports on genotype/subtype distribution in different regions of Iran (7-14). Unpublished data from our lab indicates that genotype D has been the only genotype of hepatitis B circulating in this country, despite the presence of legal and illegal immigrants from the neighbor countries with potential importation of other HBV genotypes which would result in a high rate of intermixing between populations and consequently, mixed-genotype patterns (15).

Majority of patients (80%) were HBeAg positive in the present study. Although 5 (20%) patients contained anti-HBe, clinical and molecular data indicated that they were in tolerant phase of chronic disease. Besides, from a total of 18 amino acid mutations, 5 (27.5%) and 13 (72.5%) amino acid mutations occurred in anti-HBe and HBeAg groups, respectively. However, previous data (25-27) indicated that HBeAg seroconversion led to the higher number of amino acid substitutions. Occurrence of 18 amino acid mutations in 11 positions indicated that there were not any hotspot residues for these substitutions.

Furthermore, the ratio between silent and missense substitutions in our study (S/M= 1.26), indicating that the surface proteins were under the constraints for substitutions, led to a low mutation frequency at the protein level. It seems that in the HBeAg phase of chronic disease, because of toleragenic effect of HBeAg, a few mutations occur in the surface protein. Transition from HBeAg positivity to anti-HBe positivity is somehow a prolonged process, during which a number of amino acid changes are proportional to the interaction between virus and host immune status. Thus, in the presence of host immune surveillance, the occurrence of such mutations is inevitable. However, the number of amino acid substitutions was higher in patients who were HBeAg positive than anti-HBe-positive patients. Therefore, we believe that the relatively small number of nucleotide/amino acid mutations in the latter group might belong to the initial phase (tolerant phase) of chronicity in our patients, regardless of being anti-HBe positive. More likely, even in anti-HBe positive phase in Iranian genotype D-infected patients, a somehow tolerant pattern due to host genetic factors is observed (see below). Analyzing sequential samples during HBeAg seroconversion and comparing the

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serial sequences needs the above hypothesis to be confirmed.

All selected patients were chronic carriers, with the mean period of 5 years of chronicity. All had been infected by genotype D. Based on current investigations on genotype D of HBV, new findings show that HBV genotype D is able to cause a severe disease and a higher rate of drug resistance in comparison with other studied genotypes. Thakur et al. (28) reported that genotype D of HBV correlates with more severe liver disease than HBV genotype A in India, especially in young HBV infected patients, in whom it might lead to HCC. Likewise, in a survey, genotype D of HBV was associated with more active disease in comparison with HBV genotypes A to F (29). We were not able to compare our group of patients with genotype D with the other groups of HBV genotypes. However, according to the epidemiologic studies, prevalence of cirrhosis and HCC, the major complications of chronic HBV infection are relatively low in Iran (30-31) and HCC is not included in the list of top ten cancers in Iranian population (regardless of role of HBV as an etiology) (32-35).

In conclusion, in the spectrum of HBV chronicity, as time goes by (and especially after HBeAg seroconversion), the accumulation of mutations in different HBV proteins occurs. Although the impact of such mutations on the pathogenesis of cirrhosis and HCC is not clear, the rough conclusion is that due to the negative selection in Iranian HBV genome, these complications are lower than other published studies. A definite conclusion needs mutational analysis of sequential samples from different stages of chronically-infected individuals, ranging from inactive carriers to HCC cases in a cohort study.

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