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Original Article

Incidence of Hepatitis B virus Surface Antigen (HBsAg) Mutations in Naïve Treated of Chronic Carriers from Tehran Metropolis, Iran

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Abstract

Background and Aims: Naturally occuring hepatitis B virus (HBV) with surface mutations in a variety of chronic hepatitis B (CHB) patients who have received no vaccine or HBIG bearing substitutions in surface protein, have been reported. Current knowledge concerning the prevalence of these naturally occurring surface antigen mutations among Iranian carriers is limited.

Materials and Methods: In a retrospective cross-sectional study, 119 HBV inactive chronic carriers were enrolled. The surface gene was amplified, sequenced and subsequently aligned using international and national sequence database.

Results: All strains belonged to genotype D, subgenotype D1 and subtype ayw2. In 74 (62.18%) of patients, 146 (68.8%) out of 212 amino acid mutations occurred in different immune epitopes within surface protein, of which 28 (19.17%) in B cell, 37 (25.34%) in T helper and 81 (55.47%) inside CTL epitopes. 13 (8.9%) and 15 (10.27%) of amino acid substitutions occurred outside and within the "a" determinant in Major Hydrophilic Region (MHR). While 11 (9.24%) and 77 (64.7%) patients who harbored amino acid mutations, were HBeAg and anti-HBe positive, respectively (p=0.004). 9 and 63 amino acid mutations occurred in different HBsAg epitopes in HBeAg and anti-HBe positive patients, respectively (P=0.04).

Conclusion: HBV mutants within the immune epitopes of surface Ag seem to be extremely common among chronic carriers from Tehran, especially those who are anti-HBe positive, indicating that after HBeAg seroconversion, due to the selection pressure of Ag e antibody, the occurrence of mutation is an inevitable effect of the evolutionary process.

Keywords: HBsAg mutations, HBV genotype D, HBV Iran, HBsAg immune epitopes

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Introduction

Human hepatitis B virus (HBV) is a non-cytopathic DNA virus that causes various manifestations of liver disease. Chronic HBV infection frequently leads to the development of cirrhosis and hepatocellular carcinoma (HCC). However, the mechanisms by which HBV escapes immunological surveillance and persists chronic carriers, remain obscure.

In chronic carriers, the specific T cell response is significantly weaker and in many patients is undetectable, in contrast to acute phase (1-4). The T cell response ineffectiveness in the pathogenesis of chronic HBV infection has been attributed to the several factors of which the escape mutants within immune epitopes of HBV constitute a significant role and isolated cases of infection with HBV variants bearing substitutions in these regions, are predicted to escape from immune surveillance (immune-escape variants). The consequence of selected 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.

Naturally occurring HBV with surface mutations in a variety of chronic hepatitis B (CHB) patients without receiving any vaccine or HBIG bearing substitutions in these regions, with variable rates of occurrence regardless of being within(5-7) or outside of "a" determinant in particular T cell epitopes, have been reported (8-11) and several immune epitope specific for B, Th and CTL within the surface protein have been described.(١٦-١٢)

However, current knowledge concerning the prevalence of these naturally occurring surface antigen mutations is limited. Furthermore, the prevalence of these variants according to the clinical state and HBeAg serostatus has been investigated recently by authors.

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The aim of this study was (i) to determine the genotypes of HBV in patients with in the metropolitan city of Tehran, and (ii) to characterize the molecular variations and to compare those variations to the serologic/clinical data of the chronic patients.

Methods

Sera

This retrospective cross-sectional study were used 119 serum samples which were previously collected from patients referred to Tehran Hepatitis Network and Digestive Disease center for GI, and Tehran had been kept frozen during (2006-2008). All the index patients had chronic HBV infection; and all were interviewed and examined by gastroenterologists 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) were considered 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 a low to moderate HBV DNA levels, persistent or intermittent elevation in the serum ALT levels, and compatible liver biopsy. The study was approved by the Ethics Committee of Tehran Hepatitis Network (code 16-86-10-12) and an informed consent was obtained from each participant. All authors respected their patients' privacy and assured that patients' confidentiality was regarded. With no evident of co-infection with other hepatitis viruses, and human immunodeficiency virus (HIV), they were treatment-naive. The diagnosis of chronic liver disease was made by clinical, biochemical, radiological and endoscopic criteria. The clinical data of all patients were reviewed retrospectively in order to collect information about previous laboratory tests (aspartate aminotransferase (AST); alanine aminotransferase (ALT) and demographic (gender and age) data).

Commercially available enzyme-linked immune assay kits were used according to the manufacturer's instructions to test serological

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markers (HBsAg, HBeAg and anti-HBe) (Organon, Technika, and Holland).

DNA extraction and Polymerase chain reaction

Viral nucleic acid was extracted from serum using the Qiagen Mini Blood Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations, and the recovered nucleic acid was either tested for HBV DNA the same day or stored at −20°C to be analyzed within 1 week. To analyze mutation patterns and their frequencies in the MHR of the surface gene, a nested PCR protocol was used as described previousl (17) .

Direct sequencing

Direct sequencing of surface gene was carried out (Genetic Analyzer ABI- 3130 DNA Sequencer, Foster City, CA, USA) using 2 pmol of appropriate primers: S6 and S7 for surface gene (17). The results were analyzed using Chromas program. Sequences of surface gene were aligned using the BioEdit Package, version 7.0.9.

Sequence analysis

The reference sequences were available in the GenBank database with the corresponding accession number AB033559 (18) among various HBV genotypes. The sequences were compared with this original sequence for identifying variants. After allocating a sequence to an HBV genotype, the surface gene amino acid/nucleotide variations that were found were compared with HBsAg sequences from Iranian isolates obtained from GenBank and NCBI; and from our own laboratory reports. Comparing with the former, any amino acid changes defined as "variant" (host HLA-determined). With regards to the latter (Iranian database sequences), amino acid differences defined as "mutation". Sequences have been submitted to GenBank, numbered according to the time of submission: HM358277-329, HM358335-39 and KC176076-136.

Phylogenetic analysis

Phylogenetic analysis was performed and a neighbor-joining phylogenetic tree constructed using the MEGA 5 employing a Kimura distance matrix (19). One isolate from each genotype A to H as well as one from each subgenotypes D (D1 to D8) were chosen from NCBI. For outgrouping, a genotype A (accession numbers: AY161161 from India) and a genotype G (accession numbers: AB056513 from USA) were chosen. Samples were tested by bootstrap resampling analysis using 1000 replicates. Associations with a bootstrap value of greater than 70% were presumed to be significant.

Statistical analysi:

Descriptive statistics including mean±SD, percentage and contingency table were used to present the data. Chi-square test (with Yates correction), fisher exact test and Mann-Whitney U test were employed to find the significant variation in distribution across the HBeAg and anti-HBe positive. P-value less than 0.05 considered as the significance level. All analysis carried out, using SPSS version 18.

Results

119 serum samples from HBsAg-positive patients infected with HBV which had enrolled in this study were used. The main characteristics of patients are presented in table 1. As it is shown 21 (17.6%) and 94 (78.9%) of patients were HBeAg and Anti-HBe positive, respectively. The mean age was 34.8±13.1 (mean±SD) years (range: 1.5-71 years, median: 34 years). In HBeAg positive, the mean age was 25±12.7 and in Anti-HBe positive the mean was 36±12.3. In HBeAg positive, the mean age was 25±12.7 and in Anti-HBe positive it was 36±12.3. The study population was consisted of 92 males (77.3%) and 27 females (22.7%). In Anti-HBe positive, 76.2% was male, and in HBeAg positive, 76.6% was male, respectively. The mean ALT level was 84.1±66.7±sd (results not shown). The mean HBV DNA levels was 1.5x103 (results not shown).

Phylogenetic Analysis

In the phylogenetic tree deduced from 30 randomly-selected isolates along with reference sequences belonged to different HBV genotypes and subgenotypes; all sequences clustered within genotype D (100%), subgenotype D1 supported by 95% and 97% bootstrap value (1,000 replicates) (fig 1).

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Table 1: The main characteristics and evolutionary status of surface genes and proteins between 119 isolates

Number of samples	119
Male/female	92 (77.3%)/27(22.7%)
HBeAg/Anti-HBe	21 (17.64%)/94 (78.99%)
Number of nucleotide mutations in comparison with the Iranian reference sequence	473
Mean and ratio of nucleotide mutations in comparison with the Iranian reference sequence (per site)	0.58-3.97%
Number of nucleotide silent mutations	227
Ratio of silent mutations (silent/total)	0.47
Number of missense mutations	246
Ratio of missense mutations (missense/total)	0.52
Nucleotide mutations rate (silent/missense)	0.92
Number and percentage of stop codons	14 (6.6%)
Number of amino acid changes	212
Mean and ratio of amino acid change (per site)	0.44-1.02
Number of amino acid changes in immune epitopes	146
Number of amino acid changes in non-immune epitopes	66
Immune/Non-immune ratio	2.21
Number and percentage of amino acid changes in B cell immune epitopes	28 (19.17%)
Number and percentage of mutations within “a” determinant	14 (9.5%)
Number and percentage of mutations outside “a” determinant	11 (7.53%)
ratio of “a” determinant to non-a determinant mutations (within MHR)	1.27
Number and percentage of amino acid changes in Th cell immune epitopes	37 (25.34%)
Number of amino acid changes in CTL cell immune epitopes	81 (55.47%)

Substitutions in comparison with reference genotype D (Okamoto, AB033559:

Analysis of variation within the S gene of 119 patients with chronic HBV infection demonstrated that the only detected subtype was D (100%) and subtype ayw2 (100%) (Results not shown).

Overall, comparing with this reference sequence, at the nucleotide level, six changes occurred (G208C, A339T, C360A, A420C, C438T and T513C), of which G208C was a non-synonymous substitution that at the amino acid levels altered were alanine to proline (A70P) (Results not shown). We believe that this substitution was assigned as “variant” (see material and methods). According to the above mentioned description, 473 and 212 changes at

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Table 2: The main characteristics and evolutionary status of surface genes and proteins between 119 isolates

HBsAg Substitution levels (no of patients)	HBeAg Positive *(N= 21) 17.64%*	Anti-HBe Positive *(N= 94) 78.99%*	P-Value
Nucleotide mutation (N= 113)	19	90	0.30
Amino Acid mutation (N=90)	11	77	0.004
"a" determinant Mutations (N= 12)	2	9	0.99
Total No-mutations (N=29)	10	17	0.004
Immune Epitope Mutations (N=74)	9	63	0.04
T helper epitope Mutations (N= 29)	1	28	0.017
CTL Epitope Mutations (N= 52)	5	46	0.036
B cell Epitope Mutations (N= 20)	4	14	0.63
Mutations within 'a' determinant (N= 15)	2 (0.94%)	12 (5.66%)	0.63
Mutations outside 'a' determinant (N= 13)	2 (0.94%)	9 (4.24%)	0.29
Non-Immune Epitope Mutations (N= 53)	6	45	0.11

the nucleotide and amino acid levels were mutations, respectively (Table 1).

Nucleotide and amino acid substitutions

In comparison with Iranian sequences obtained from the database as well as from our data, in addition to the genotypic characterization described above, the sequences of the strains showed a few variability over the regions sequenced. In all, 473 "muta¬tions" occurred, of which 246 (52%) were missense (amino acid altering) and 227 (48%) were silent (no amino acid changing) (Table 1). At amino acid level, 212 substitutions occurred in 90 (75.63%) of patients (Table 1). Table1 shows the comparison between nucleotide and amino acid variations from the isolates. 13 isolates had stopped codon either in positions 69 or 216. Further, it was possible to identify the level of protein surface evolution between isolates by measuring the mutation rate of individual sequences (Table 1). The average mutation rate of all sequences (non-synonymous to synonymous substitutions, dN/dS) was 0.92 according to the number of mutations per site. The average nucleotide mutation frequency was 3.97 per sample. There was a probability of 0.58% for substitution per nucleotide position. The

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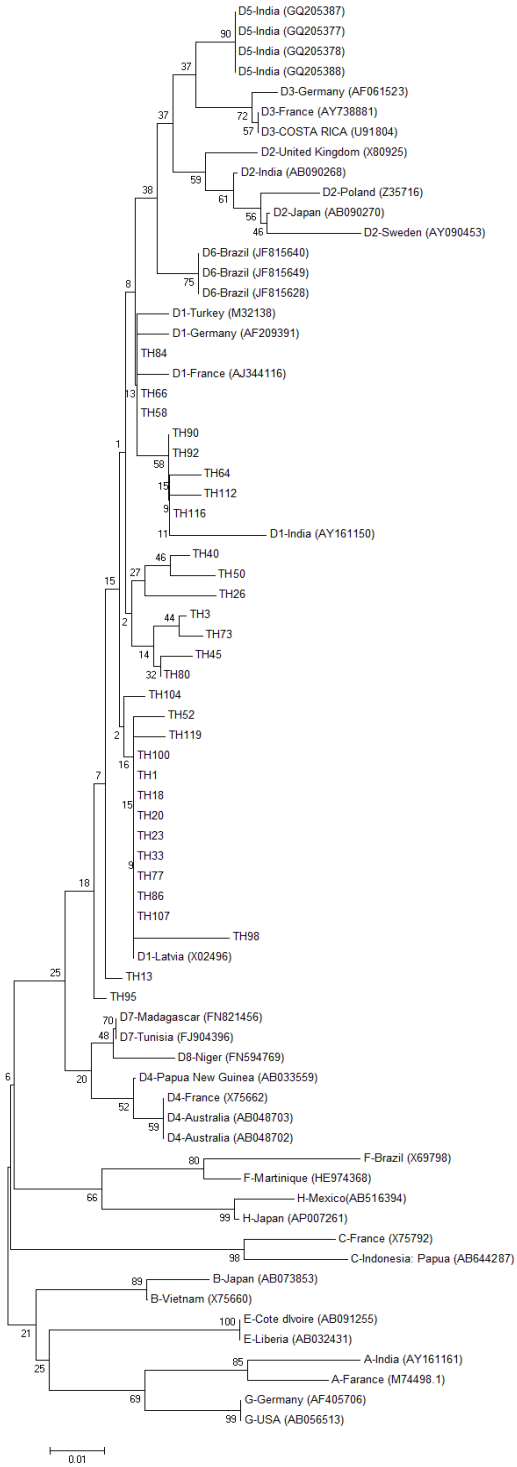

Fig. 1. Evolutionary relationships of 119 taxa: The evolutionary history was inferred using the Neighbor Joining method. The bootstrap consensus tree, inferred from 1000 replicates, is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in 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.

average amino acid mutation frequency was 1.89 mutations per sample. There was a probability of 0.84% for substitution per amino acid position (Table1).

Amino acid mutations within the surface protein immune epitopes

In 74 (62.18%) of patients, 146 (68.86%) out of 212 amino acid mutations occurred in

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different immune epitopes within surface protein, of which 28 (19.17%) in B cell; 37 (25.34%) in T helper and 81(55.47%) inside CTL epitopes (Table1). 14 (11.7%) and 11 (9.2%) of patients had amino acid substitutions within and outside the "a" determinant region of surface protein, respectively (results not shown). Mutations in the former residue did not allocate for a certain subtype and/or genotype, thus they were mutation, not variants (results not shown).

Surface protein variations and clinical status

Table 2 shows the correlation between HBeAg/anti-HBe status and the number of nucleotide/amino acid variations. The total number of nucleotide (regardless of being silent or missense) substitutions was not significantly different in both groups. However, 11 (9.24%) and 77 (64.7%) of patients who harbored amino acid mutations were HBeAg and anti-HBe positive, respectively (P=0.004) (Table 2). Likewise, comparing both groups in terms of distribution of amino acid mutations, showed that 9 and 63 amino acid mutations occurred in different HBsAg epitopes in HBeAg and anti-HBe positive patients, respectively (P=0.04). Furthermore, the number of mutations within T helper and CTL mutations were found to be more in anti-HBe positive than HBeAg positive patients with significant correlations: P values: 0.017 (T helper epitopes) and 0.036 (CTL epitopes), respectively (Table 2). Despite occurrence of a majority of mutations within and outside the "a" determinant in anti-HBe positive versus HBeAg positive groups, however, they did not reach significant correlation (Table 2). Interestingly, 27 patients (22.68%) did not contain any mutations at amino acid levels (17 anti-HBe positive vs 10 HBeAg positive, P=0.004). There was no correlation between the amino acid substitutions and the ALT/AST and HBV DNA levels as well as biopsy results (Results not shown).

Discussion

The prevalence and the types of variants of the S gene should be recorded; because this will affect policy decisions related to managing the HBV infected chronic carriers. Therefore, in the present study, mutations in the whole HBsAg were analyzed comprehensively in 119 chronic patients from capital Tehran.

Genotype D, subgenotype D1 and subtype ayw2 accounted for 100% of isolates. Published data from our laboratory indicated that in Iran, there has been an obvious uniqueness of this virus genetic pattern (20-23). We have already hypothesized that this unique pattern of homology is related to the recent distribution and circulation of HBV in Iran comparing to other countries in the region (20, 24).

HBV mutation has a complicated scenario. In fact, this infectious phenomenon seemed to be the potential mechanism for the pathogenesis basis of chronicity and the clinical complications. Recent studies have shown that HBV surface protein (HBsAg) is more variable than it was initially thought to be, and amino acid exchanges are scattered over the whole molecule. The overall ratio between silent and missense nucleotide mutations in all patients was 0.92. This indicated that the proportion of deduced amino acid changes in these chronically infected patients was high and a positive selection pattern has been exerted on the sequences, as more than 75.63% of the patients including the present study, were found to be infected with one or more of these variants.

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 selective 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 prevalence of T cell and 'a' determinant epitope mutants according to the presence of HBeAg was compared. The transition from an immune tolerant state to an

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immune clearance state with the generation of antibodies against HBeAg results in a strong selective pressure on the viral genome. Since HBeAg, is highly immunogenic at the B and T cell levels, the prevalence of these variants in HBeAg-negative patients in an immune clearance state would be expected to be higher than in HBeAg positive patients in an immune tolerant state. Despite the majority of anti-HBs antibodies that appear after natural infection are directed against the 'a' determinant epitope cluster in several studies (16-18, 25); it is interesting that most of the amino acid changes observed in our survey were clustered in 2 regions; residues 193 and residues 207 of the small surface protein. These residues have been shown to stimulate the host T helper and CTL epitopes, respectively (15). Occurrence of 212 amino acid mutations in 70 positions indicated that there were hotspot residues for these substitutions. In total, a majority of patients who harbored the mutations were anti-HBe positive, indicating that after seroconversion, due to the selective pressure of e antibody, the occurrence of mutation is an inevitable effect of evolutionary process. This was in accordance to the previous data carried out on the core protein elsewhere (26-29). On the other hand, there were no significant associations between biochemistry as well as hepatitis activities (according to biopsy results) of patients with the patterns of amino acid substitutions in the surface proteins of the patients .

Our results shows that the frequencies of mutations within the major hydrophilic region (regardless being either within or outside the 'a' determinant) are comparable for anti-HBe and HBeAg positive patients (Table 2), which supports the presence of selective pressure for mutations in the MHR region. 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 (outside the "a" determinant) as mutations in those residues have been reported previously (5, 7-9, 11).

In conclusion, HBV surface mutants seem to be extremely common among chronic carriers from Tehran, and mutants with a potential impact on relevant aspects of the HBV infection should be expected in a significant proportion of carriers. For a better interpretation, the allocation of such molecular variations to the clinical, serological and biochemical pictures needs to be explored. In this scenario, even an individual variation must be taken into account.

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