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Hepatitis B Virus Surface Antigen Variants Clustered Within Immune Epitopes in Chronic Hepatitis B Carriers from Hormozgan Province, South of Iran

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Abstract

Objective(s)
The aim of this study was to characterize the hepatitis B virus surface protein genotypes and sequence variations among hepatitis B virus surface antigen (HBsAg) positive chronic patients in Hormozgan province, south of Iran.

Materials and Methods
A total of 8 patients enrolled in this study. The surface gene was amplified and directly sequenced. Genotypes and nucleotide/amino acid substitutions were identified compared to the sequences obtained from the database.

Results
All strains belonged to genotype D. Overall 77 "mutations" occurred at 45 nucleotide positions, of them, 44 (57.14%) were silent (no amino acid altering) and 33 (42.86%) were missense (amino acid changing). A number of 24 (80%) out of 30 amino acid changes occurred in different immune epitopes within surface protein, of which, 9 (30%) in B cell epitopes in 7 residues (2 occurred in "a" determinant region); 8 (42.1%) in T helper epitopes in 7 residues and 7 (10%) in 4 residues inside CTL epitopes.

Conclusion
Hepatitis B virus genome containing mutated immune epitopes no longer could be recognized by specific T-cells of the host immune surveillance and did not enhance anti-HBs production. This could led to the progression of chronicity of hepatitis B virus infection.

Keywords: HBV genotypes, HBV genotype D, HBV genotype in Iran, HBV immune epitopes

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Introduction

With a 3.2 Kb genome, hepatitis B virus (HBV) is the smallest DNA virus that infects humans. The virus has a double-stranded DNA genome with four open reading frames: P, C, S and X. The S region encodes three gene products, Pre-S1, Pre-S2 and small (S) envelope proteins. These proteins are usually detected serologically as hepatitis B surface antigen (HBsAg), and they are involved in receptor binding, viral assembly and secretion. They are also important targets for immune mediated virus elimination (1) and several immune epitope specific for B, Th and CTL within the surface protein have been described (2-5). Antigenicity of HBsAg is dependent upon this complex structure. The anti-HBs (antibody to HBsAg) response following natural infection or after immunization comprises mainly antibodies that recognize the major hydrophilic region (MHR) of the protein. This comprises amino acids 99–160 that encompass the group-specific 'a' determinant, an epitope recognized by a variety of antibodies. Moreover, standard HBV subtyping and genotyping is based on the "a" determinant (6, 7).

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

In chronic carriers, the specific T cell response is significantly weaker, in contrast to acute phase, and in many patients, is undetectable (8). The T cell response ineffectiveness in the pathogenesis of chronic HBV infection has been attributed to the several factors: genetic background of the host, clonal tolerance, T cell energy (due to the high antigen load), CTL exhaustion, a Th2 type response instead of Th1, etc. In this scenario, 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 have been reported (immune-escape variants) (9-14). There have also been cases of infection that have been missed because of failure of current serological assays to detect some variant forms of HBsAg (diagnostic-escape variants) (15-18).

The aim of this study was to analyze the surface gene and protein sequences of chronic carriers in the early phase of chronicity (either before or soon after eAg seroconversion) and to allocate the pattern of variations distribution to their clinical/serologic pictures.

Materials and Methods

Sera

As a representative and eligible sample of various districts, by means of stratified multi-stage cluster sampling design, 18 HBsAg-positive chronic in-active carriers were recruited from Hormozgan province, Iran. The study population comprised sera collected during 2008. Patients were included in this study if they had HBsAg more than 6 months, with levels of ALT around the normal range. All patients were negative for antibodies against hepatitis C, hepatitis D and human immunodeficiency virus. To avoid bias on the mutational analysis, the patients needed to be HBeAg positive or at the early stage of anti-HBe seroconversion. Trained health care and field staff implemented standard questionnaires to collect data on demographic characteristics such as gender, age, educational, economic status, residency and horizontal/vertical transmitted risk factors to HBV. Aliquots (5 ml) of whole blood samples were withdrawn from each participant. Serum was, aseptically, separated in the field by centrifugation at 2000 rpm for 5 min, stored at -80°C until tested. HBV serological markers including HBsAg and anti-HBs were examined by ELISA kits manufactured by Organon Technika, Holland. Prior informed consent was obtained from all patients before

bleeding. All the procedures carried out in the Hepatitis B laboratory, Department of Virology, TUMS.

DNA extraction

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

Polymerase chain reaction

The surface gene was amplified using two pairs of primers (Table 1). A nested PCR reaction was carried out in 100 μl of a mixture containing 5 μl of DNA using HotStart Taq PCR (Qiagen, Hilden, Germany) according to manufacturer's instruction. The cycling profile for the first round PCR was one cycle of 95 °C for15 min, 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min followed by 40 cycles. For the second round of PCR, 1 μl of the first round PCR product was added to the reaction mixture with the same compositions of the first round except that S1 and S2 were replaced by S6 and S7 primers. The thermal profile was the same as first round except that cycling number was 30 cycles. Finally, 3 μl of the second round PCR products were analysed by electrophoresis in 1% agarose gel, stained by ethidium bromide, and visualized under UV light.

DNA sequencing

The HBsAg subtype of the sequences was defined by substitutions in the 'a' determinant between codons 122 and 160 inclusive. Direct sequencing of surface genes was carried out (Perkin Elmer ABI-3130XL DNA Sequencer, Fostercity, CA, USA) using 0.5 μl of appropriate primers S6 and S7 for surface gene. The results were analysed using Chromas and BioEdit softwares. Genotyping was carried out on samples using the region of surface gene specifying HBV genotypes/subtypes

Sequence analysis

After allocating a sequence to an HBV genotype by analysis of the S gene, the discovered surface gene amino acid/nucleotide variations were compared with a reference sequence obtained from Okamoto (1988, accession mumber, AB033559) and HBsAg sequences from Iranian isolates obtained from GenBank and NCBI. Comparing to 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 from GU938305 to GU938322.

Phylogenetic analysis

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Kimura 2-parameter method, and were in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 681 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

Table 1. Oligonucleotide primers used for PCR and sequencing. Base positions numbered from the EcoRI site.

Primer	Sequence 5' 3' of Oligonucleotides	Base Position	Type
S1	CCT GCT GGT GGC TCC AGT TC	56-75	Sense
S2	CCA CAA TTC (K)TT GAC ATA CTT TCC A (K=G/T)	1003-979	Anti-sense
S6	GCA CAC GGA ATT CCG AGG ACT GGG GAC CCT G	113-146	Sense
S7	GAC ACC AAG CTT GGT TAG GGT TTA AAT GTA TAC C	857-823	Anti-sense

Results

Totally, 19 HBsAg-positive patients infected with HBV were enrolled in this study, who all were native residents of Isfahan province (center of Iran). The group studied consisted of inactive hepatitis B carriers. 6 (31.6%) were female and 13 (68.4%) were male with a mean age of 39 years.

Phylogenetic analysis

The results of the phylogenetic tree revealed that Iranian HBV isolates from Isfahan were of genotype D, supported by 95% bootstrap value (1,000 replicates) (Fig. 1). Only one isolate (207) belonged to subgenotype D3, the rest contained D1. In the phylogenetic tree, a genotype E sequence (accession number AB091266) was chosen for out grouping. It is noteworthy that 3 isolates branched into 3 individual subclusters (210, 214 and 215). Samples 210 and 215

contained at least one amino acid substitution in "a" determinant. The rest of sequences branched into different lengths of clusters, maily in pairs (Figure 1).

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

The surface gene of 18 HBV isolates was amplified to identify the genotypes and other mutations located in the surface region. Overall, comparing with reference sequence (Okamoto, 1988), at the nucleotide level, a total of 162 changes occurred (Table 2). At the amino acid levels, all contained A70P compared to Okamoto reference (Table 3). We believe that this substitution was assigned as "variant" (see materials and methods). All strains belonged to genotype D (100%), subgenotype D1 (100%) and subtype ayw2 (100%).
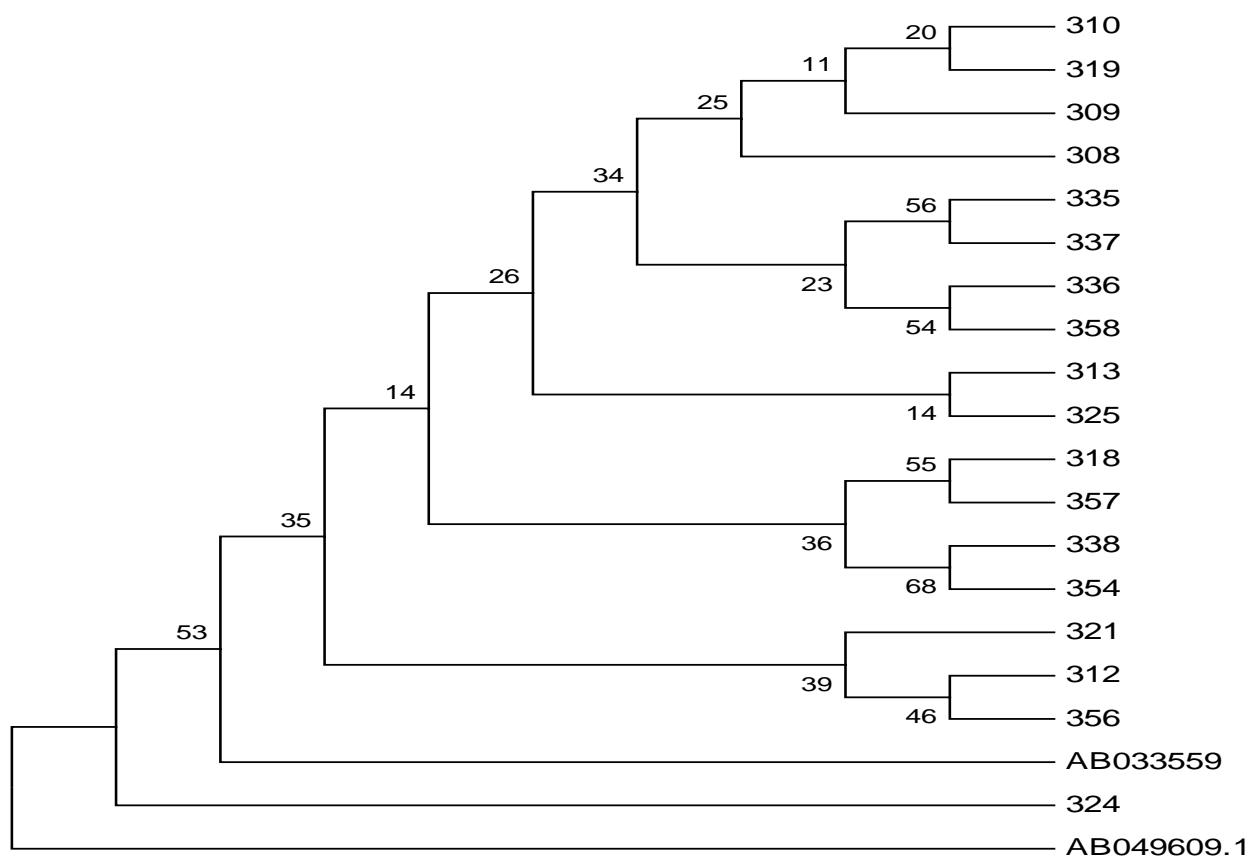
Figure 1. Neighbour joining phylogenetic trees of surface genes sequences from18 samples.

Note: S gene tree rooted with sequence AB049609 (reference genotype C). All Iranian isolates were compared to sequence AB033559 (reference genotype D, see the text). The scale denotes percentage diversity. Coding numbers indicate samples that have been analysed in the figure. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

Table 2. Alignment of complete nucleotide sequences of HBsAg from 18 sera.
Note: Nucleotides are numbered from the beginning of the HBsAg using the single letter code. Sample 324 had 3 nucleotides insertion, therefore, using Bioedit software (Clustal W alignment) 3 extra nucleotides were added at positions 337-339 for a proper alignment.

Bandar Abbas & Okamoto Nucleotides

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              10        20        30        40        50        60        70        80        90       100       110       120
     ----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
AB033559 ATGGAGAACATCACATCAGGATTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTAGACTCGTGGTGGACTTCTCTCAAT
308      ................................................................................................................................
309      ................................................................................................................................
310      ................................................................................................................................
312      ..................................................T.............................................................................
313      ................................................................................................................................
318      ..........................................................A.....................................................................
319      ...........................C.............G......................................................................................
321      ..................................................T............................................A................................
324      ................................................................................................................................
325      ................................................................................................................................
335      ................................................................................................................................
336      ................................................................................................................................
337      ................................................................................................................................
338      ................................................................................................................................
354      ..................................................T.............................................................................
356      ..................................................T.............................................................................
357      ............................................................A...................................................................
358      ................................................................................................................................

              130       140       150       160       170       180       190       200       210       220       230       240
     ----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
AB033559 TTTCTAGGGGGAACTACCGTGTGTCTTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAACTTGTGCTGGTTATCGCTGGATGTGTCTGCGGCGTTTT
308      ..............................................................................................C.................................
309      ..............................................................................................C.................................
310      ..............................................................................................C.................................
312      ..............................................................................................C.................................
313      .................A............................................................................C.................................
318      .................C..................................................................G.........C.................................
319      ..............................................................................................C.................................
321      .......................G......................................................................C.................................
324      ..............................................................................................C.................................
325      .................C............................................................................C.................................
335      ..............................................................................................C.................................
336      ..............................................................................................C.................................
337      ..............................................................................................C.................................
338      ..............................................................................................C.................................
354      ...........A...C..........G...................................................................C.................................
356      .................C............................................................................C.................................
357      ..............................................................................................C.................................
358      ..............................................................................................C.................................

              250       260       270       280       290       300       310       320       330       340       350       360
     ----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
AB033559 ATCATCTTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCA----TCAACCACCAGCACGGGA
308      ...............................................................................................TCT............................
309      ...............................................................................................TCT............................
310      ...............................................................................................TCT............................
312      ...............................................................................................TCC............................
313      .............................................................................C.................TCT............................
318      ......A........................................................................G...............TCT............................
319      ......................................................................C........................TCT............................
321      ...............................................................G...............................TCC............................
324      .............................................................G.........................AACAA.AAT.....A.......................
325      ...............................................................................................TCT............................
335      ...............................................................................................TCT............................
336      ...............................................................................................TCT............................
337      ...............................................................................................TCT............................
338      ......A........................................................................................TCC............................
354      ......A........................................................................................TCC............................
356      ...............................................................................................TCC............................
357      ......................................................................G........................TCG..................T.........
358      ...............................................................................................TCT............................
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Bandar Abbas & Okamoto Nucleotides

Table 3. Alignment of complete amino acid sequences of HBsAg which shows genotype/subtype identification and other variations of 18 sera.
Note: Amino acid residues are numbered from the beginning of the HBsAg using the single letter code. Apart from sample 324, which had one amino acid insertion, all the other samples had 226 amino acids, therefore, using Bioedit software (Clustal W alignment) 1 extra amino acid was added at positions 114 for a proper alignment.

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Bandar Abbas & Okamoto AA

                  10        20        30        40        50        60        70        80        90       100       110       120
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AB033559  MENITSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGGTTVCLGQNSQSPTSNHSPTSCPPTCAGYRWMCLRRFIIPLFILLLCLIFLLVLLDYQGMLPVCPLIPGS~STTSTG
308       ..........................................................P.......................................................XXS.....
309       ..........................................................P.......................................................XXS.....
310       ..........................................................P.......................................................XXS.....
312       ..........................................................P.......................................................XXS.....
313       ..........................................................P.......................................................XXS.....
318       ..........................................................P.......................................................XXS.....
319       ......L..V.................................................P.......................................................XXS.....
321       .......................K....................R.............P........................................A.....XXS.....
324       ..........................................................P.......................................................NKN.....
325       ..........................................................P.......................................................XXS.....
335       ..........................................................P.......................................................XXS.....
336       ..........................................................P.......................................................XXS.....
337       ..........................................................P.......................................................XXS.....
338       ..........................................................P.......................................................XXS.....
354       ...............................E...R......................P.......................................................XXS.....
356       ..........................................................P.......................................................XXS.....
357       ..........................................................P.......................................................XXS.....
358       ..........................................................P.......................................................XXS.....

                 130       140       150       160       170       180       190       200       210       220
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AB033559  PCRTCTTPAQGTSMYPSCCCTKPSDGNCTCIPIPSSWAFGKFLWEWASARFSWLSLLVPFVQWFVGLSPTVWLSVIWMMWYWGPSLYSILSPFLPLLPIFFCLWVYI*
308       .................................................................................................*
309       .................................................................................................*
310       .................................................................................................*
312       ..........................................................................N.CN...............*
313       ...................................................................................L.......*
318       ...............L...............A.............I..LA.......................*
319       .................................................................................................*
321       .................................................................................................*
324       .......................................................N.......................*
325       .................................................................................................*
335       .................................................................................................*
336       .........................................................C.......................*
337       .......T.................................A................................*
338       ...............L.................................R..................*
354       ...............L.................................R.CI.................*
356       ...........................................A.......................*
357       .................................................................................................*
358       ...........................................................C.......................*
```

Table 4. The levels of mutation rates between isolates deduced from the number and the percentage of individual sequences.

Sample Code	Mutations Pos.	Missense Mutation Pos.	Amino Acid Change	No.	Mutation Rate
308	-	-	-	0	0.00%
309	-	-	-	0	0.00%
310	-	-	-	0	0.00%
312	G36T, T339C, C420A , T426C, C432T, G611A, A617G, G620A	G611A, A617G, G620A	S204N, Y206C, S207N	8	1.17%
313	T135A, T330C, C632T,	C632T,	P211L	3	0.44%
318	G42A, T135C, A201G, C246A, T318G, C428T, G476C, C566T, C578T, T581C, T666C	C428T, C566T, C578T, T581C	S143L, G159A, T189I, S193L, V194A	11	1.61%
319	T22C, C39G, T310C, T378G	T22C, C39G	F8L, L12V	4	0.59%
321	G36T, G71A, T146G, C313G, T339C	G71A, T146G, C313G	R24K, L49R, P105A	5	0.73%
324	A303G, G334A, G335A, {335CAA336}*, T337A, C338A, C345A, C420A, G20A	[A303G, G334A], {335CAA336}*, [T337A, C338A], G20A	{112N}*, G112+1K, S113+1N, S207+1N	11	1.61%
325	T135C			1	0.15%
335	C513T			1	0.15%
336	C513T, G573C	G573C	W191C	2	0.29%
337	C379A, C513T, T530C	C379A, T530C	P127T, V177A	3	0.44%
338	C246A, T339C, C428T, C465A, T612A	C428T, T612A	S143L, S204P	5	0.73%
354	G36T, G131A, T135C, T146G, C246A, T339C, T378C, C428T, C465A, T612A, A617G, G620T	T612A, A617G, G620T, T135C, T146G, C428T	G44E, L49R, S143L, S204R, Y206C, S207I	12	1.76%
356	G36T, T339C, C432T, T551C	T551C	V184A	4	0.59%
357	G42A, T135C, T318G, A357T, T339G, C465A			6	0.88%
358	G573C			1	0.15%
Average	-	-	-	4.28	0.63%

Table 6. Amino acid mutations within HBsAg of patient groups. B cell, T helper and CTL epitopes areas with their boundaries and wild type variants indicated at top. Amino acids are described by single letter code and numbered from the beginning of HBsAg.

Bandar Abbas		Th Epitope							CTL Epitope						B Epitope				
Sample code	Amino Acid Position	24	44	49	189	191	193	194	177	184	206	207	105	112	112+1	113+1	127	143	159
	Wild Type	R	G	L	T	W	S	V	V	V	Y	S	P	-	G	K	P	S	G
	308																		
	309																		
	310																		
	312										C	N							
	313																		
	318				I		L	A										L	A
	319																		
	321	K		R									A						
	324											N		{N}*	S	N			
	325																		
	335																		
	336					C													
	337								A								T		
	338										Y	R						L	
	354		E	R														L	
	356									A									
	357																		
	358																		

Nucleotide and amino acid substitutions

In comparison with Iranian sequences obtained from the database as well as from our unpublished data, the sequences of the strains showed some variabilities over the regions sequenced (Tables 2, 3). In all, 77 "mutations" occurred at 45 nucleotide positions, of them, 44 (57.14%) were silent (no amino acid altering) and 33 (42.86%) were missense (amino acid-altering). Sample 324 contained an insertion of CAA at the nucleotide level that results to insertion of N at the amino acid level in the position 112. Table 4 shows the comparison between nucleotide and amino acid variations for the isolates. Further, it was possible to identify the level of S proteins evolution between isolates by measuring the mutation rate of individual sequences. The average mutation rate of all sequences was 4.28 (0.63%) according to the number of mutations per site (Table 4).

Mutations within immune epitopes

According to the proposed residues of immune epitopes within the HBV surface protein (Table 5), 24 (80%) out of 30 amino acid changes occurred in different immune epitopes within surface protein, of which were, 9 (30%) in B cell epitopes in 7 residues (2 occurred in "a" determinant region; 8 (42.1%) in T helper epitopes in 7 residues and 7 (10%) in 4 residues inside CTL epitopes (Table 6). Within B cell epitopes, 3 samples contained mutations in position 143. Similarly, in CTL epitopes, two samples had mutations at positions 206 and 207, 2 for each of them.

Table 5. Proposed antigenic epitopes within HBsAg.
Note: Numbers indicate amino acid residues within the surface protein. B, Th and CTL represent B-cell, T helper and CTL epitomes, respectively.

Reference	HLA restricton	Cell subsets	Sequence
Honorati	Not HLA restriction	B	100-160
Ducos-1996	ClassΠ	CD4 T	19-28
Mancini-2006	ClassΠ	CD4 T	21-65
Ducos-1996	ClassΠ	CD4 T	80-98
Mancini-2006	ClassΠ	CD4 T	186-197
Ducos-1996	ClassΠ	CD4 T	215-223
Barnab-1994	ClassI	CD8 T	171-179
Mancini-2006	ClassI	CD8 T	175-184
Mancini-2006	ClassI	CD8 T	206-215

Discussion

For a non-cytopathic virus (such as HBV) to persist, it must be able to evade immune surveillance; there must be either an ineffective antiviral immune response, or the virus must escape an otherwise efficient response. All of these might be involved in HBV persistence. The aim of this study was to characterize the mutational patterns of surface protein in chronic HBV carriers. Of 18 sequences, 11 contained different mutations within the surface protein. Of the total 77 substitutions at the nucleotide level, 46 (60%) were silent (no amino acid changing) and 32 (40%) were missense (that changed the amino acid). The ratio between silent and missense mutations in 11 sequences indicated that these proteins were under a significant selection pressure which had already been applied by both arms of cytotoxic and humoral host immune system: 27 (90%) out of 30 amino acid changes occurred in different immune epitopes, of which, 9 (30%) in B cell epitopes in 6 residues (2 occurred in "a" determinant region); 8 (26.6%) in T helper epitopes in 7 residues and 10 (33.3%) in 5 residues inside the CTL epitopes . Compared to other immune epitope mutations in this study which distributed in different residues, the occurrence of 10 CTL epitope changes in only 5 amino acid residues suggested a narrowly-focused immune selection pressure at a hotspot position for this selection. The latter finding was in consistence with the findings of other authors, especially in genotype D- infected patients (19-21).

Occurrence of genomic variation (especially in immune epitopes) is a reflection of virus-host adaptation. Appropriate reactivity of T-helper cells is a prerequisite for adequate anti-HBs production after infection with HBV, as well as after hepatitis B vaccination (22). Thus, the T-cell epitopes of HBsAg being targets for recognition by T cells should also be affected (23). In chronic HBV patients, the transition from a relative immune tolerance state to the activation of the immune system with generation of anti-HBe results in a strong selection on the viral genome, causing changes in immune targets, i.e. T and B cell epitopes that could lead to escape of the virus from

immune clearance. Some mutations are able to impair the binding of neutralizing antibodies to the viral surface (especially at "a" determinant region); viruses carrying such mutated T-cell epitopes cannot be recognized by specific T-cells of an individual, hence, will not enhance anti-HBs production (23), this could be led to the progression of chronicity of hepatitis B virus infection.

The relative importance of such mutations in different immune epitopes within HBV proteins in the pathogenesis of chronic HBV is a matter of debates. In terms of HBV proteins, some authors believe that CTL epitopes have a major role; a majority of chronic HBV carriers contained mutated residues within CTL epitopes (24-28). Others, however, showed that these mutations occurred in the Th/B cell epitopes (29-34). *In vitro*, we already showed that intracellular localization of HBcAg depended on the presence of mutations in different hepatitis B core gene B cell epitope mutations. Of 26 cloned samples, HBcAg was predominantly localized in nucleus in 13

samples in remission phase (as HBcAg is a nuclear antigen) and in cytoplasm in other 13 samples with active hepatitis. All samples with cytoplasmic localization contained B cell epitope mutations. Reversion of mutant sequences with cytoplasmic expression back to the wild type by mutagenesis led to shifting back to nuclear distribution (35).

Conclusion

In our study, the distribution of mutations within the surface protein (as an immune target for the host T cell surveillance) was not random and they were clustered in certain immune epitopes. Pattern of mutants distribution in immune epitopes deserves testing the phenotypic pictures using *in vitro* assays for further elucidation of the pathogenesis of HBV chronicity.

Acknowledgment

References

1. Wallace LA, Carman WF. Surface gene variation of HBV: Scientific and medical relevance. J Viral Hepatitis 1997; 3:5-16.
2. Honorati MC, Dolzani P, Mariani E, Piacentini A, Lisignoli G, Ferrari C, Facchini A. Epitope specificity of Th0/Th2 CD4+ T-lymphocyte clones induced by vaccination with rHBsAg vaccine. Gastroenterology 1997; 112:2017-2027.
3. Mancini-Bourgine M, Fontaine H, Bréchot C, Pol S, Michel ML. Immunogenicity of a hepatitis B DNA vaccine administered to chronic HBV carriers. Vaccine 2006; 24:4482-4489.
4. Ducos J Bianchi, Mondain AM, Pageaux G, Conge AM, Poncet R, Vendrell JP, *et al.* Hepatitis B virus (HBV)specific *in vitro* antibody production by peripheral blood mononuclear cells (PBMC) after vaccination by recombinant hepatitis B surface antigen (rHBsAg). Clin Exp Immunol 1996; 103:15-18.
5. Barnaba V, Franco A, Paroli M, Benvenuto R, De Petrillo G, Burgio VL. Selective expansion of cytotoxic T lymphocytes with a CD4+CD56+surface phenotype and a T helper type 1 profile of cytokine secretion in the liver of patients chronically infected with hepatitis B virus. J Immunol 1994; 152:3074-3087.
6. Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol 1992; 73:1201-12018.
7. Norder H, Courouce AM, Magnius LO. Molecular basis of hepatitis B virus serotype variations within the four major subtypes. J Gen Virol 1992; 73:3141-3145.
8. Chisari FV. Immunobiology and pathogenesis of viral hepatitis. Edizioni Minerva Medica, Turin. 1997.
9. Mesenas SJ, Chow WC, Zhao Y, Lim GK, Oon CJ, Ng HS. Wild-type and 'a' epitope variants in chronic hepatitis B virus carriers positive for hepatitis B surface antigen and antibody. J Gastroenterol Hepatol 2002; 17:148-152.
10. Friedt M, Gerner P, Wintermeyer P, Wirth S. Complete hepatitis B virus genome analysis in HBsAg positive mothers and their infants with fulminant hepatitis B. BMC Gastroenterol 2004; 4:11.
11. Ozaslan M, Ozaslan E, Barsgan A, Koruk MJ. Mutations in the S gene region of hepatitis B virus genotype D in Turkish patients. J Genet 2007; 86:195-201.
12. Tabor E. Infections by hepatitis B surface antigen gene mutants in Europe and North America. J Med Virol 2006; 78:S43-47.

13. Song BC, Kim SH, Kim H, Ying YH, Kim HJ, Kim YJ, *et al*. Prevalence of naturally occurring surface antigen variants of hepatitis B virus in Korean patients infected chronically. J Med Virol 2005; 76:194-202.

14. Bahramali G, Sadeghizadeh M, Amini-Bavil-Olyaee S, Alavian SM, Behzad-Behbahani A, Adeli A, *et al*. Clinical, virologic and phylogenetic features of hepatitis B infection in Iranian patients. World J Gastroenterol 2008; 14:5448-5453.

15. Ireland JH, O'Donnell B, Basuni AA, Kean JD, Wallace LA, Lau GK, *et al*. Reactivity of 13 *in vitro* expressed hepatitis B surface antigen variants in 7 commercial diagnostic assays. Hepatology 2000; 31:1176-1182.

16. Weber B. Genetic variability of the S gene of hepatitis B virus: clinical and diagnostic impact. J Clin Virol 2005; 32:102-112.

17. Weber B. Diagnostic impact of the genetic variability of the hepatitis B virus surface antigen gene. J Med Virol 2006; 78:S59-65.

18. Hollinger FB. Hepatitis B virus genetic diversity and its impact on diagnostic assays. J Viral Hepat 2007; 1:11-5.

19. Pourkarim MR, Amini-Bavil-Olyaee S, Verbeeck J, Lemey P, Zeller M, Rahman M, *et al*. Molecular evolutionary analysis and mutational pattern of full-length genomes of hepatitis B virus isolated from Belgian patients with different clinical manifestations. J Med Virol 2010; 82:379-389.

20. Datta S, Panigrahi R, Biswas A, Chandra PK, Banerjee A, Mahapatra PK, *et al*. Genetic characterization of hepatitis B virus in peripheral blood leukocytes: evidence for selection and compartmentalization of viral variants with the immune escape G145R mutation. J Virol 2009; 83:9983-9992.

21. Sloan RD, Strang AL, Ramsay ME, Teo CG. Genotyping of acute HBV isolates from England, 1997-2001. J Clin Virol 2009; 44:157-160.

22. Salazar M, Deulofeut H, Granja C, Deulofeut R, Yunis DE, Marcus-Bagley D, *et al*. Normal HBsAG presentation and T-cell defect in the immune response of nonresponders. Immunogenetics 1995; 41:366-374.

23. Bauer T, Weinberger K, Jilg W. Variants of two major T cell epitopes within the hepatitis B surface antigen are not recognized by specific T helper cells of vaccinated individuals . Hepatology 2002; 455-465.

24. Jazayeri and Carman. Virus escape, CTL or B cell epitopes Hepatitis Monthly 2005; 5:133-136.

25. Chen M, Salberg M, Thung SN, Hughes J, Jones J, Milich DR. Modelling the T helper cell response in acute and chronic hepatitis B virus infection using T-cell receptor transgenic mice. Antiviral Res 2001; 52:99-111.

26. Liu CJ, Kao JH, Shau WY, Chen PJ, Lai MY, Chen DS. Naturally occurring hepatitis B surface gene variants in chronic hepatitis B virus infection: correlation with viral serotypes and clinical stages of liver disease. J Med Virol *2002*; 68:50-59.

27. Barnaba V, Franco A, Alberti A, Balsano C, Benvenuto R, Balsano F. Recognition of hepatitis B virus envelope proteins by liver-infiltrating T lymphocytes in chronic HBV. Infection. J immunol 1989; 143:2650-2655.

28. Khakoo SI, Ling R, Scott I, Dodi AI, Harrison TJ, Dusheiko GM, *et al*. Cytotoxic T lymphocyte responses and CTL epitope escape mutation in HBsAg, anti-HBe positive individuals. Gut 2000; 4: 256.

29. Yamauchi K, Nakamura T, Yonemitsu H, Sekiya H, Katoh J, Obata H. Possible role of preS2 peptides presented by MHC class I antigen in the pathogenesis of chronic hepatitis B. J Hepatol 1993; 17:S6-9.

30. Carman WF, Thursz M, Hadziyannis S, McIntyre G, Colman K, Gioustoz A, *et al*. Hepatitis B e antigen negative chronic active hepatitis: hepatitis B virus core mutations occur predominantly in known antigenic determinants. J Viral Hepat 1995; 2:77-84.

31. Carman WF, Boner W, Fattovich G, Colman K, Dornan ES, Thursz M, *et al.* Hepatitis B virus core protein mutations are concentrated in B cell epitopes in progressive disease and in T helper cell epitopes during clinical remission. J Infect Dis 1997; 175:1093-10100.

32. Hosono S, Tai PC, Wang W, Ambrose M, Hwang DG, Yuan TT, *et al*. Core antigen mutations of human hepatitis B virus in hepatomas accumulate in MHC class II-restricted T cell epitopes. Virology 1995; 212:151-162.

33. Rehermann B, Pasquinelli C, Mosier SM, Chisari FV. Hepatitis B virus (HBV) sequence variation of cytotoxic T lymphocyte epitopes is not common in patients with chronic HBV infection. J Clin Invest 1995;

34. Maruyama T, Kuwata S, Koike K, Iino S, Yasuda K, Yotsuyanagi H, *et al*. Precore wild-type DNA and immune complexes persist in chronic hepatitis B after seroconversion: no association between genome conversion and seroconversion. Hepatology 1998; 27:245-53.

35. Jazayeri SM, Dornan E, Boner W, Waketing M, Hadziyannis S, Fattovich G, *et al*. Distribution of *in vitro* expressed HBV core protein from isolates is dependent upon sequence and correlates with disease pattern. J Infect Dis 2004; 189:1634-1645.