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CTL escape mutations of core protein are more frequent in strains of HBeAg negative patients with low levels of HBV DNA

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Abstract

Background—Recent studies have suggested that Cytotoxic T lymphocytes (CTL) play a key role in eliminating hepatitis B virus (HBV).

Objectives—We aimed to investigate the role of mutations in different immune epitopes of hepatitis B core antigen (HBcAg) among Iranians with HBeAg negative chronic hepatitis B (e-CHB), and asymptomatic carriers (ASCs).

Study design—Amino acids 1-150 of HBcAg were characterized for HBV strains from 29 e-CHB patients and 48 ASCs from Iran. All patients were infected with HBV genotype D and had previously been investigated for the presence of pre-core and basic core promoter (BCP) mutants.

Results—Amino acid mutations of core protein were observed more frequently in HBV strains from ASCs than e-CHB patients (p=0.014). Asn^{67} mutation was mutually exclusive to the combination Ile^{66} and Ser^{69} (P<0.001). Substitutions for Ser^{21} and Thr12Ser were associated with lower serum levels of HBV DNA (p<0.001). None of the patients with mutations in HLA-A2 CTL epitope, 18-27, had serum HBV DNA more than 10^5 copies/mL (p<0.001). By multivariate analysis, high level (> 10^5 copies/mL) of serum HBV DNA was inversely associated with the presence of

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mutations in CTL epitopes of HBc (OR:0.11 , p=0.015), while it was directly associated with presence of promoter double $T^{1762}A^{1764}$ mutations together with G^{1757} (OR:16.87, p=0.004)

Conclusion—The inverse correlation between serum levels of HBV DNA and CTL escape mutations of the core protein in HBeAg seroconverted patients, supports the notion that selection of CTL escape mutations consolidates the persistence of HBV infection despite reducing viral fitness.

Keywords

Hepatitis B; Tc; Immune; HBcAg; Iran; viral load

BACKGROUND

More than 300 million individuals worldwide are chronically infected with hepatitis B virus (HBV). Hepatitis B e antigen (HBeAg) negative chronic hepatitis B represents the predominant form of chronic hepatitis B (CHB) in several parts of world including Iran. This form of CHB, defined as HBeAg negative CHB (e-CHB) [1], is mostly associated with mutations in the basal core promoter (BCP) and pre-core (preC) regions of HBV. The HBV genome has four open reading frames, one of which is the core gene encoding the 183 or 185 amino acid (aa) long nucleocapsid, or core (HBc) protein which is preceded by the BCP and preC regions [2].

The major B-cell epitopes of HBV are localized around the most protruding HBc region (aa 71–87 [3] involving the tip of the spike [residues 76-82]) [4, 5]. The other B-cell epitope lies around aa 129–132 [5, 6]. Another epitope, corresponding to residues 107-118, was also suggested to be a B-cell epitope, although it does not seem to be on the surface of the complete virion [7].

HLA-class-II-restricted, T helper cell epitopes of the HBc protein have been mapped to peptides corresponding to aa positions 1–20, 28–47, 50–69, 72–90, 81–105, 90–99, 108–122, 111–125, 117–131, 120–139, 126–146, and 141–165 [6, 8-11].

Special attention has been devoted to the search for HLA class I-restricted Cytotoxic T lymphocyte (CTL) epitopes within the HBc molecule. A single HLA-A2-restricted epitope HBc aa (18–27) has been identified, containing the predicted HLA-A2 binding motif with Leu at position 2 and Val at the C-terminus [8, 12]

Although, the immunological basis for HBV persistence is not fully understood, CTLs are known as the main actors of the immune system in viral clearance [13]. It is well known that the CTL response is less vigorous in chronically infected patients [11], and a robust T cell response against residues 18-27 of HBc protein [13] has been observed in patients who spontaneously clear the infection, but not in patients with CHB [8, 14, 15-16].However, the differences in immune responses among subgroups of patients with CHB patient have been less thoroughly investigated.

OBJECTIVES

In a recent study, we characterized the BCP and preC regions of HBV isolates of Iranian patients with e-CHB, and asymptomatic carriers (ASCs) [17]. The present study investigates the prevalence of mutations in different HBc immune epitopes of HBV strains in the previously studied Iranian patients and its association to serum levels of HBV DNA, aminotransferase levels, and also BCP and preC mutational patterns.

STUDY DESIGN

Patients

The study included 97 patients negative for HBeAg, and positive for anti-HBe referred to the Hepatology Clinic of Taleghani General Hospital, Tehran, Iran between March 2000 and April 2003. Based on clinical, biochemistry tests, and imaging findings, the patients were divided into two groups; Thirty-five patients were categorized as e-CHB while 62 were classified as ASCs. All patients were enrolled in the study after giving informed consent. Clinical characteristics of patients were previously described [17], and all patients were shown to be infected with HBV genotype D [17].

Methods

For core gene sequencing, HBV DNA was extracted from 200 μL of serum with QIAamp DNA mini kit (Qiagen Inc, Valencia, CA). Amplification was carried out with the primers hepA and hep66, and was nested with the primers hepA and hep68 [18]. The PCR amplicons were purified with GFX PCR DNA and gel band purification kit (Amersham, Uppsala, Sweden). Purified products were used as templates in the sequencing reaction using the dideoxynucleotide chain termination method with ABI PRISM Big dye TM terminator cycle sequencing reaction kit (Applied Biosystems, a Division of Perkin Elmer, Version 3) and the primers used in the PCR were used as sequencing primers. All amplificates were sequenced bi-directionally. The ABI PRISM 3100 Genetic Analyser (Applied Biosystems) was used for electrophoresis and data collection. The sequences obtained were edited using the SeqMan program in the LASERGENE package (DNA STAR Inc., Madison, WI). Three-dimensional predictions of the mutated HBc structures were performed on the basis of the X-ray structure of the HBc, genotype A [3], by a comparative modeling program 3D-JIGSAW [19] (http://bmm.cancerresearchuk.org/~3djigsaw/) and presented by Chimera software [20].

Statistical analysis

Statistical analysis was performed with Chi-square and Fisher's exact tests for the absence or presence of mutations within different regions. Independent t test was used to compare the average mutation numbers within the same regions (SPSS Inc. Chicago, IL). Pearson's correlation coefficient (cc) was used to calculate the correlation between each pair of regional mutations. We employed a multivariate logistic regression analysis in order to find the association between the serum HBV DNA, and a set of epitope mutations as independent variables. Correlation and regression analyses were done with the aid of Stata 11 (StataCorp, USA).

RESULTS

The core region of the infecting strain could be amplified from serum of 77 (79%) patients. Two e-CHB patients had strains with major deletions in core region and were excluded from statistical comparison. Sequencing data between amino acids 1-150 of core region in 48 ASCs and 27 e-CHB patients are presented in Fig 1. Data regarding HBV DNA and serum aminotransferase levels, preC, and core preC mutations are also available.[17]

Amino acid divergence in different regions of HBc protein

Ser^{21} and Thr^{80} were the most variable amino acid residues and could be substituted by seven and five different amino acids, respectively. Substitutions of Ser^{87} were found to be associated with substitutions of the Ser^{21} in most cases ($p<0.002$), while the latter were associated with Thr^{12} ($p<0.02$). Substitutions of Ser^{87} were also associated with Glu^{64} in most cases ($p<0.01$), while substitutions of Met^{93} were always accompanied with Asp^{64} ($p<0.001$). Asp^{64} was never

accompanied with Thr^{49} ($p<0.01$), and was more frequently associated with substitutions of Thr^{67} ($p=0.010$), Tyr^{38} ($p=0.010$), and Thr^{80} ($p=0.018$). Double amino acid mutations Ile^{66} and Ser^{69} were always accompanied with Glu64Asp and never accompanied with Asn^{67} ($p<0.001$).

Seven regions were found to be more variable within the HBV core protein and corresponded to residues 18-27, 35-45, 49-69, 76-87, 91-95, 105-116, and 130-135. It was also possible to localize each of these regions within respective immune epitope (Th, CTL, and B-cell; Table 1). Region 49-69, which is a Th epitope, was the most variable, followed by region 105-116 which is a B-cell epitope. There were significant positive correlations among the amino acid substitutions when comparing different regions. Specifically, correlations were found significant between regions18-27 and 91-95 (cc=0.43, $p=0.0001$), regions 18-27 and 76-87 (cc=0.41, $p=0.0001$). The Thr12Ser mutation was also found to be correlated with substitutions within CTL epitopes 18-27 (cc=0.43, $p=0.0001$), and 91-95 (cc=0.40, $p=0.0002$).

“Hot spot” amino acid exchanges were localized on the three-dimensional structure of HBc tetramers in the context of major CTL and B epitopes, and most variable HBc regions (Fig 2).

The overall frequency of amino acid mutations of HBc protein is higher in strains of ASCs than e-CHB patients (4.81 vs 3.22; $p=0.014$). HBV isolates of ASCs also had a higher frequency of mutation in their B-cell epitopes than isolates from e-CHB patients (2.08 vs 1.22; $p=0.026$, Table1). This difference was most significant in region aa 105-116 ($p=0.013$).

Immune epitope mutations and serum levels of HBV DNA

The overall amino acid variability of HBc protein was higher in strains from patients with lower serum levels of HBV DNA ($<10^5$ copies/mL) than in strains from patients with higher HBV DNA ($>10^5$ copies/mL) (4.49 vs 3.31; $p=0.023$, Table2), although this difference was only statistically significant in their CTL epitopes (0.93 vs 0.38, p=0.043).This difference was highly significant in region 18-27 (0.41 vs 0.00 $p<0.001$, Table 2). None of the HBV infected patients with HBV DNA greater than 10^5 copies/mL harbored amino acid mutations in region 18-27 of their isolates ($p=0.002$). Patients with HBV isolates containing Ser^{12} mutation also had lower HBV DNA than those whose isolates lacked this ($p<0.001$). The difference was also significant in region 76-87 ($p=0.018$).

HBc protein and preC/BCP mutants

Substitutions of Ser^{21} to Thr^{21} or Ala^{21} were never accompanied with $T^{1762}A^{1764}$ core promoter double mutation ($p=0.005$). Strains with BCP mutations or preC mutation had higher frequency of amino acid mutations within their immune epitopes of HBc protein compared to BCP wild type isolates, respectively, although these differences were not statistically significant (Table3). It was also found that strains containing preC mutants more frequently had a Ser^{12} ($p=0.014$).

Mutations in the context of multivariate analysis

In a multivariate analysis, with age, sex, preC mutation, core promoter mutation, and mutations within each immune epitope of HBc in the model (Table 4), the whole model was statistically significant ($p=0.006$), and high serum levels of HBV DNA ($> 10^5$ copies/mL) were inversely associated with presence of mutations in CTL epitopes of HBc (OR:0.11 , p=0.015), while they were positively associated with presence of promoter double $T^{1762}A^{1764}$ mutation together with G^{1757} (OR:16.87, p=0.004).

DISCUSSION

The HBc protein is subjected to strong folding and self-assembly requirements in order to form the highly-symmetric icosahedric HBc particle, the observed mutations were analyzed from the structural point of view. Fig. 2 represents asymmetric unit of the HBc particle consisting of two such spikes formed by two HBc dimers consisting of four HBc molecule chains: A, B, C, and D.

The observed mutations appear predominantly on the outer surface of the HBc protein, since both “hot spot” HBc epitopes: CTL 18-27 and B-cell 74-89 are externally exposed (Fig. 2, A and B). The most variable position aa 21 within the CTL epitope is one of the most exposed positions. On the other hand, the most exposed region, HBc spike, contains position 80 of the B cell epitope which is the second most variable position. Position 21 is part of a short one-turn α-helix consisting of three aa residues which is also highly exposed on the HBc surface. Correlation of definite pairs of mutations at positions 64 and 93 (Fig. 2C), and 64 and 67 (Fig. 2E) could be explained by their close contact within the four-helix bundle. Other “hot spot” positions located on outer (Fig. 2D) or inner (Fig. 2F) surfaces of the HBc particle may influence immunological or packaging HBc properties, respectively, rather than its folding and self-assembly.

In our study, mutations were found in Th/CTL epitopes of strains of both ASCs and e-CHB patients: however, contrary to our expectation; mutations were more frequent in HBV strains from ASCs. Although longitudinal studies could be more informative to correlate between HBc mutation and outcome of chronic hepatitis B, the higher frequency of these escape mutations in ASCs than in patients with liver disease emphasizes the role of these immune escape mutations for the persistence of virus rather than their role, if any, for causing liver disease.

Residues 18-27 of the HBc protein constitute an HLA-A2 restricted CTL epitope, and mutants with substitutions of amino acid 21 in the HLA-A2 restricted CTL epitope have been suggested to act as T cell receptor antagonists leading to inhibition of the CTL response to the wild-type HBV CTL epitope [22]. We found that patients having isolates with mutations within residues 18-27 never had HBV DNA exceeding 10^5 copies/mL. This level has been proposed [23] as a cut point for differentiating ASCs from e-CHB patients.

It was recently shown that replacing many positions within HLA-A2 CTL epitopes of HCV replicons severely decreased viral replication, and this might be the reason that these mutations are rarely seen in the nature [24]. In our patients acquisition of similar mutations but with a more conservative effect in lowering viral replication, like Ser21Ala might considered as a cost to viral fitness that paid by HBV to permit survival by mitigating CTL response . Another recent study also showed HBV reactivation despite reduced viral fitness due to HBsAg immune escape mutations in an HIV-coinfected patient [25]. Besides, HBV mutants that reduce viral replication rate might be compensated with other mutations that restore viral fitness like preC and BCP mutations.

In conclusion, our research corroborates the idea that selection of CTL escape mutants consolidates the persistence of HBV infection despite reducing viral fitness because these mutations are more frequently seen in the context of lower serum levels of HBV DNA. We speculate that similar mechanisms for selection of immune escape mutations occur in other chronic viral diseases as well.

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Abbrevations

aa, amino acid(s)
ASCs, asymptomatic carriers
BCP, basal core promoter
cc, correlation coefficient
CHB, chronic hepatitis B
CTL, cytotoxic T lymphocyte
e-CHB, HBeAg-negative CHB
HBc, Hepatitis B core
HBcAg, Hepatitis B core antigen
HBeAg, Hepatitis B e antigen
HBV, Hepatitis B virus
preC, pre-core
Th, T helper

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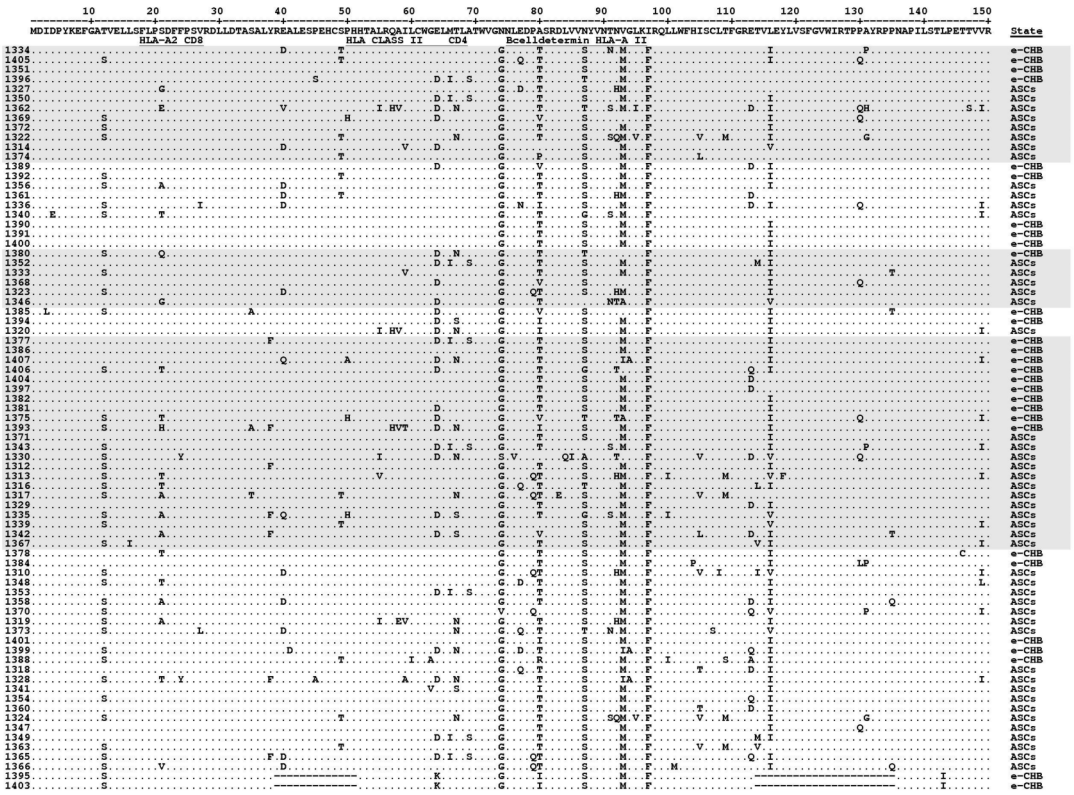

Fig 1. Amino acid sequences of the HBV core protein (aa 1-150)

Sequences of HBV core protein of patients aligned with the genotype A sequence pHBV3200 [21]. The grouping of the shaded sequences corresponds to strains with different BCP mutants previously published. [17]

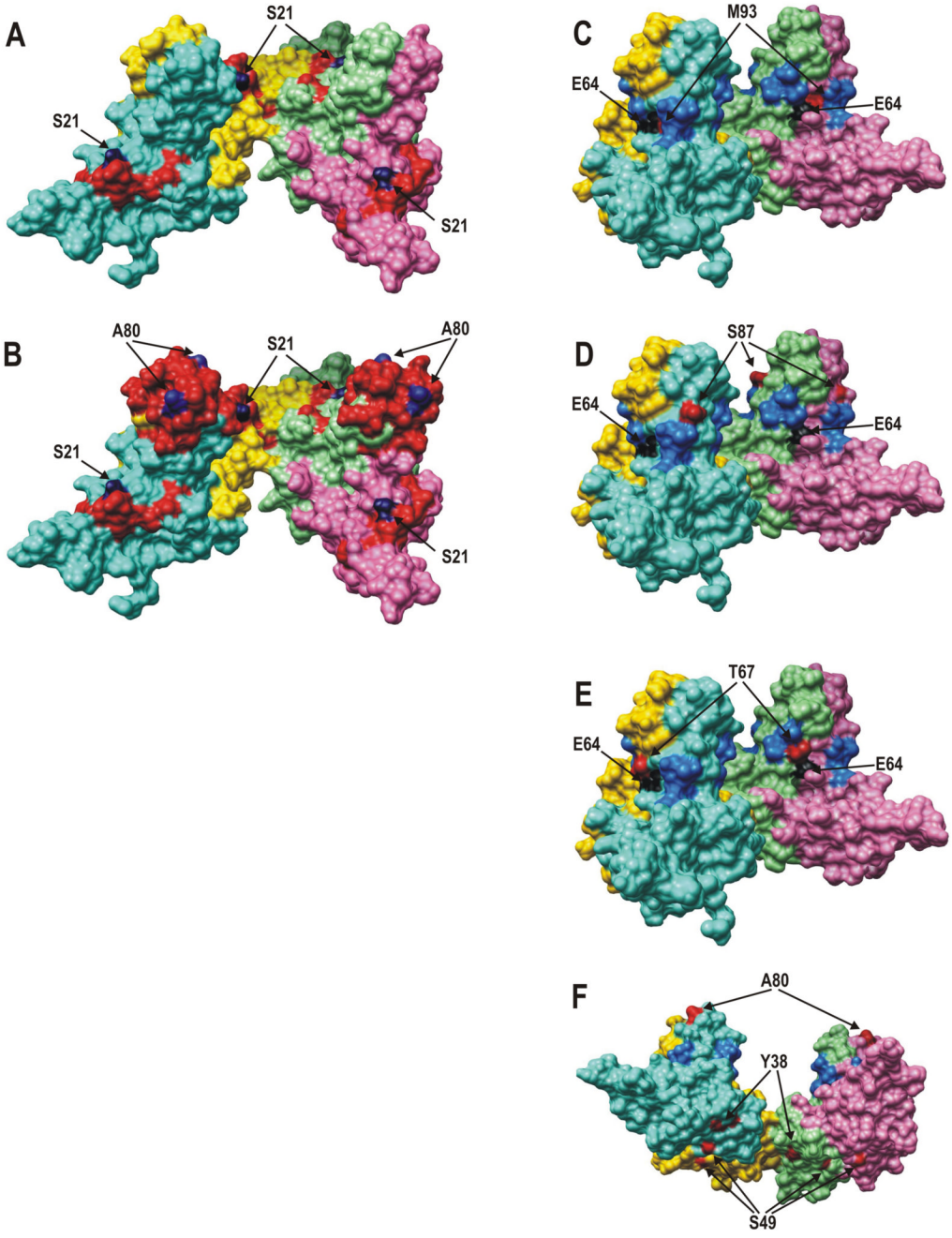

Fig 2. Appearance of major CTL and B epitopes, the most variable regions, and "hot spot" aa exchanges on the surface of the HBc tetramer

3D data are presented from the X-ray structure of the HBc, genotype A [3] by Chimera software. [20] Chains A, B, C, and D of the HBc tetramer are colored pink, light green, gold, and light blue, respectively; three different viewing angles are used for HBc drawings: (i) A and B, (ii) C, D, and E, and (iii) F. A, CTL epitope 18-FLPSDFFPSV-27 is colored red, the "hot spot" S21 is blue; B, the same as A, but the B epitope 74-NNLEDPASRDLVVNYV-89 (red) and the "hot spot" A80 (blue) are added; C, two most variable regions 64-ELMTLAT-70 and 91-TNMGL-95 are colored blue, E64 — black, and M93 — red; D, the same as C, but the S87 is colored red; E, the same as C, but the T67 is red; F — the inner part of the HBc tetramer is

exposed, “hot spots” of the inner HBc surface Y38 and S49 are red, A80 is colored red to mark the tips of the HBc spikes in the new presentation angle.

Table 1

Frequency of immune epitope mutations in HBV isolates of patients with different clinical categories

Clinical States	Th Epitopes		CTL Epitopes		B-Cell Epitopes		
Regions	**35-45**	**49-69**	**18-27**	**91-95**	**76-87**	**105-116**	**130-135**
e-CHB (n=27)							
Frequency	6	33	5	10	13	14	6
Mean[*]	0.22	1.22	0.19	0.37	0.48	0.52	0.22
Sum (Mean)	**39 (1.44)**		**15 (0.56)**			**33 (1.22)**	
ASCs (n=48)							
Frequency	18	67	19	27	34	51	15
Mean	0.38	1.40	0.40	0.56	0.71	1.06	0.31
Sum (Mean)	**85 (1.77)**		**46 (0.96)**			**100 (2.08)**	
ALL (n=75)							
Frequency	24	100	24	37	47	65	21
Mean	0.32	1.33	0.32	0.49	0.63	0.87	0.28
Sum (Mean)	**124 (1.65)**		**61 (0.81)**			**133 (1.77)**	

[*] Mean (of mutations) was calculated as frequency of mutation in each epitope divided by number of patients in each clinical group.

Table 2

Frequency of immune epitope mutations in HBV isolates of patients with different serum levels of HBV DNA

Serum HBV DNA	**Th Epitopes**		**CTL Epitopes**		**B-Cell Epitopes**		
Regions	**35-45**	**49-69**	**18-27**	**91-95**	**76-87**	**105-116**	**130-135**
>10^5 copy/ml(n=16)							
Frequency	3	24	0	6	5	10	5
Mean	0.19	1.50	0	0.38	0.31	0.63	0.31
Sum (Mean)	**27 (1.69)**		**6 (0.38)**			**20 (1.25)**	
<10^5 copy/ml(n=59)							
Frequency	21	76	24	31	42	55	16
Mean	0.36	1.29	0.41	0.53	0.71	0.93	0.27
Sum (Mean)	**97 (1.64)**		**55 (0.93)**			**113 (1.92)**	

Table 3

Frequency of immune epitope mutations in HBV isolates of patients with different categories of BCP and pre-core mutations

	Th epitopes		CTL epitpes		B-cell epitopes	
	Sum	**Mean**	**Sum**	**Mean**	**Sum**	**Mean**
BCP						
Mutants (n=58)	134	2.31	51	0.88	107	1.84
Wild-type (n=11)	6	0.55	15	1.36	14	1.27
Pre-core						
Mutants* (n=66)	113	1.71	56	0.85	121	1.83
Wild-type (n=9)	11	1.22	5	0.56	12	1.33

* These consist of isolates with A^{1896} together with two other isolates with other mutations within pre-core region, one of which abolished the pre-core start codon, and the other made another stop codon in the second aa position of pre-core polypeptide.

Table 4

Mutivariate analysis for high levels of HBV DNA (> 10^5 copy/ml) as dependant variable

Independent variable	Univariate odds ratio	Multivariate odds ratio	SEM	P-value	Confidence interval
Age	1.02	0.98	0.02	0.048	0.93-1.04
Sex	1.93	2.86	2.21	0.172	0.63-12.99
Pre-core mutation	0.45	1.74	1.67	0.562	0.27-11.40
BCP mutation ($G^{1757}T^{1762}A^{1764}$)	5.57	16.87	16.44	0.004	2.50-113.90
Mutation of Th epitopes	0.97	2.81	2.24	0.196	0.59-13.39
Mutation of CTL epitopes	0.28	0.11	0.10	0.015	0.02-0.65
Mutation of B-cell epitopes	0.43	0.40	0.32	0.245	0.08-1.90