

Association of Mutations in the Basal Core Promoter and Pre-core Regions of the Hepatitis B Viral Genome and Longitudinal Changes in HBV Level in HBeAg Negative Individuals: Results From a Cohort Study in Northern Iran

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Background: Although certain HBV mutations are known to affect the expression of Hepatitis e antigen, their association with HBV viral level or clinical outcomes is less clear.

Objectives: We evaluated associations between different mutations in the Basal Core promoter (BCP) and Pre-core (PC) regions of HBV genome and subsequent changes in HBV viral DNA level over seven years in a population of untreated HBeAg negative chronic hepatitis B (CHB) participants in Northeast of Iran.

Materials and Methods: Participants in the current study were drawn from the Golestan Hepatitis B Cohort Study (GHBCS), a cohort of approximately 2590 HBsAg positive subjects (living in Gonbad city) embedded in the Golestan Cohort Study (GCS). At baseline, HBsAg was measured in all participants and revealed 2590 HBsAg positive cases. We randomly selected 304 participants whose blood samples were taken at both baseline and seven years later in follow-up and had not been treated for HBV during this time. HBV viral load was assessed at baseline and at year 7. The BCP and PC regions of the HBV DNA, at baseline, were amplified via hemi-nested PCR and sequenced by cycle sequencing. At year 7, liver stiffness was assessed by fibroscan; also, other parameters of liver disease were assessed following standard clinical protocols. Associations were assessed via tabulation, chi-square, t-tests and logistic regression. P values < 0.05 were considered statistically significant and all tests were two-sided.

Results: Among 304 HBsAg positive participants, 99 had detectable HBV DNA at study baseline. Of these, 61.6% had PC mutations (48.5% A1896 and 25.2% G1899). In contrast to other mutations, A1896 was associated with a higher proportion of detectable HBV DNA at year 7 (39.6%) compared to patients with the wild type (13.7%) (OR: 4.36, CI95% = 1.63-11.70; P Value = 0.002). Although participants with the A1896 mutation had higher year-7 HBV viral load than participants with G1896 (2.30 ± 1.66 IU/mL vs. 1.76 ± 1 IU/mL among patients with detectable HBV; P value = 0.052), no association was observed with either serum level ALT or liver stiffness. Interestingly, mutations in the basal core promoter (BCP) region had no significant effect on virus DNA detection.

Conclusions: In this population with chronic HBeAg negative hepatitis B, an association was observed between the G1896A mutation in the Pre-core region of HBV and subsequent level of HBV DNA seven years later, which indicated that mutations in this region of HBV genome may contribute to disease progression in these patients and play an important role in HBV natural course of disease.

Keywords: Hepatitis B, Chronic; Mutation; Genome, Viral

1. Background

It is estimated that two billion people are infected with Hepatitis B virus (HBV) and 350 million people have chronic HBV infection (CHB) in the world (1). Nearly 75% of infected individuals live in Asian countries (2). Most patients infected with acute HBV undergo a three-step process: immune tolerance, immune clearance and seroconversion. However, a number of patients develop a fourth, "HBeAg negative" phase (3), in which patients have negative results for the Hepatitis e antigen (HBeAg), but have detectable HBV viral DNA in the blood. Although initially considered a late phase of infection that was manifested

in the elderly, this pattern has subsequently been reported to occur in an increasing proportion of cases worldwide. For example, HBeAg-negative CHB is the most common type of chronic hepatitis B in the European, African and Middle East countries of the Mediterranean Basin (3).

HBV genotype and HBV DNA levels are among the most important predictors of long-term clinical outcomes (4). However, specific mutations in HBV may also be important. HBV experiences evolutionary selection, with an estimated rate of nucleotide substitution of approximately 1.4 to 3.2×10^{-5} per site per year, reflecting the error rate

of the viral reverse transcriptase (4). Such mutations may alter interactions with host immune system, as well possibly potentiate viral replication, enhance virulence and promote resistance to antiviral therapies (4).

Mutations can occur throughout the HBV genome, including regions such as the pre-core, core promoter and pre-S/S genes (5), critical to the HBV function (6). Mutation of pre-core nucleotide at position 1896 from guanine (G) to adenine (A) and changes of two other nucleotides, an adenine (A) to thymine (T) transversion at nucleotide 1762 together with a guanine (G) to adenine (A) transition at nucleotide 1764 within the basal core-promoter (BCP) have been shown to prevent the synthesis of HBeAg (7). These mutations are often observed in HBeAg-negative patients with active viral replication and liver disease. Asymptomatic hepatitis B carriers sometimes have these mutations too. Double mutations in BCP T1762/A1764 have been shown to increase the risk of liver disease progression and hepatocellular carcinoma (HCC) within HBV genotypes B and C (4). Less data is available for other viral genotypes. In addition, clinical and mechanistic importance of pre-core mutations are unclear; especially G1896A, which results in a premature stop codon and termination of HBeAg translation, but does not seem to affect viral replication. Studies in population-based cohorts are particularly lacking (8,9).

Relative to other parts of the world, the prevalence of HBV in Iran varies from low to intermediate based on geographic region. Although there are an estimated 1.5 million chronically infected individuals (5), the rate of liver cancer is lower than might be expected, perhaps explained by a higher prevalence of genotype D, and high prevalence of HBeAg seronegativity or because of some mutations in core or pre-core region of the virus (10, 11). On the other hand, HBV positive individuals in Middle Eastern countries have been shown to have a high susceptibility for acquisition of pre-core or basal core promoter variants that may affect HBeAg (10), but perhaps not HBV replication or other aspects of HBV. Within Iran, there exists strong geographic variation. Whereas, the prevalence of HBV is intermediate-to-high in the Golestan province of northern Iran (12, 13), the prevalence is far lower in nearly every other province (1).

2. Objectives

In the current analysis, we conducted a longitudinal study to assess the association of mutations in the BCP and PC regions of the HBV viral genome with subsequent levels of HBV viral DNA and severity of liver disease in a cohort of untreated HBeAg negative, genotype D, chronic hepatitis B patients followed for a long time.

3. Materials and Methods

3.1. Study Population

Our study population was a subset of the Golestan Cohort Study (GCS), initiated in 2004 in Northern Iran (14-

17). As previous data suggested a high prevalence of HBV infection in this region (13), we created the Golestan Hepatitis B Cohort Study (GHBCS) from the subset of GCS participants who lived in Gonbad city (n = 9559). All 9559 of these participants were measured for hepatitis B surface antigen (HBsAg) at baseline, and 2590 participants were found to have positive results. Each participant donated a second blood sample after seven years of follow-up.

For the current analysis, we included a random sample of 394 chronically positive patients (HBsAg + longer than six months) who lacked the concomitant hepatitis C or D, human immune deficiency virus (HIV) infection, or evidence of autoimmune hepatitis, Wilson's disease or primary biliary cirrhosis. These participants were then tested for HBeAg. We excluded five HBeAg positive cases and 85 participants who lacked a second collected blood sample or who were otherwise lost to follow-up. Then, we measured HBV DNA in the remaining 304 participants, subsequently excluding 205 participants with undetectable HBV DNA at baseline. DNA samples from these 99 participants were sent for sequencing of the Basal Core Promoter (BCP) and pre-core (PC) regions of the HBV viral genome.

All included participants were HBsAg positive at both baseline and year 7. None of them received treatment during the follow-up. Informed consent was obtained and our study was approved by the ethical committee of the Digestive Disease Research Institute (DDRI) and registered in the research deputy of TUMS (No.92-02-37-19250).

Remaining in the study and follow-up were all voluntarily. Names and tests results were all confidential and just handed to the cases themselves.

3.2. Laboratory Testing

Baseline and year 7 serum samples were stored at -80°C until use. Serologic markers for HBV HBsAg, antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core (anti-HBc), HBeAg, and anti-HBe) were assessed using a commercially available ELISA kit from RADIM (Roma Rome, Italy). These tests along with HBV DNA level measurements were performed at both baseline and year 7 samples. A biochemical panel was performed on all fresh samples at year 7 consisted of Alanine transaminase (ALT), Aspartate transaminase (AST), gamma-glutamyl transferase (GGT), Complete Blood Count (CBC), Fasting Blood Sugar (FBS) and lipid profile.

3.3. HBV-DNA Quantification

HBV DNA was extracted from 200 µL of serum using QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) and then quantified in the Light-Cycler (Roche Diagnostics, Mannheim, Germany) by RealArt™ HBV LC PCR (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The linear range of this assay was 10^2 - 10^9 copies/mL. For our main analysis, we classified participants as having either detectable or undetectable HBV viral DNA in year 7 of follow-up.

3.4. Amplification of the Basal Core Promoter Region and Pre-core Motor

BCP regions were amplified using hemi-nested PCR with the sense primer PC5 5'-TCG CAT GGA GAC CAC CGT GA-3' (nt. 204-223) and the anti-sense primer PC2 5'-GGCAAAAACGAG AGTAAC TC-3' (nt. 540-559). A further hemi-nested round of amplification was performed using 2 µL of the first round product as template and the anti-sense primer 527 5'-GTA ACT CCA CAG WAG CTC C-3' (nt. 528-546). The numbering system for primer nucleotides was consistent with the genome sequence HPBADR1CG (18). The amplified products were purified using QIA PCR product purification kit (QIAGEN, Valencia, CA). PCR products were sequenced using the Big Dye Terminator Cycle sequencing Ready Reaction Kit Version 3.1 (Applied Biosystems, CA, USA).

3.5. Liver Stiffness Measurement (LSM)

Liver stiffness was measured in 2011 (end of the study) by transient elastography using the FibroScan 502 machine (EchoSense, Paris, France, 5 MHz). According to the manufacturer's guidelines, the M probe was used for subjects with thoracic perimeter less than 110 cm and the XL probe for 110 cm and above. With patient lying in the dorsal decubitus position with maximal abduction of the right arm, the probe was placed on the patient's skin, overlying the right lobe of the liver, through the intercostal spaces. At least 10 measurements were performed for each patient and the median value was recorded. Values were considered valid if the inter-quartile range (IQR) was less than 30% of the median reading.

3.6. Statistical Analysis

We used SPSS-v17 for our analysis. Categorical data were reported as percentages while continuous variables were described using means and standard deviations. Statistical analysis was conducted using Chi-square test and t-test. Odds ratio (OR) were determined with 95% confidence intervals (CI). P values < 0.05 were considered statistically significant.

4. Results

We sequenced the HBV Basal Core Promoter (BCP) and pre-core (PC) regions of 99 participants with detectable HBV DNA at baseline. Among these participants, 26 (26.3%) had detectable HBV DNA at the end of study (detectable cases); whereas, 73 (73.7%) did not (undetectable cases). Demographic, clinical and laboratory characteristics are shown in Table 1. CHB patients with a detectable HBV DNA at year 7 had a significantly higher liver stiffness, ALT, AST and GGT relative to participants with undetectable HBV DNA (Table 1). Of 99 individuals with sequenced HBV DNA, 48.5% had A1896 and 25.2% showed A1899 (Table 2).

Of these examined mutations, the only one associated with HBV DNA detection was A1896. Nineteen of 48 participants (39.6%) with A1896 had detectable HBV DNA,

whereas just 7 of 51 participants (13.7%) with G1896 had detectable HBV DNA (OR: 4.12, CI 95% = 1.54-11.03; P value = 0.003) (Table 3).

Among participants with detectable HBV DNA at year 7, the mean log₁₀ viral load was higher in those with A1896 (2.30 ± 1.66 IU/mL) than G1896 (1.76 ± 1 IU/mL); although this difference did not quite reach statistical significance (P value = 0.052), but was clinically important. In contrast, serum level of ALT and liver stiffness did not vary by this mutation.

Other examined mutations in either PC or BCP region were unrelated to ALT level, viral load or liver stiffness.

Table 1. Demographic, Clinical and Laboratory Characteristics of the Studied Chronic Hepatitis B (CHB) ^{a,b}

	All (n = 99)	Undetectable HBV DNA (n = 73)	Detectable HBV DNA (n = 26)	P Value
Sex ratio (Male/Female)	52/47	38/35	14/12	0.88
Ethnicity (Turkmen/Fars)	70/29	51/22	19/7	0.76
Age, y	59.91 ± 7.66	52.16 ± 7.82	49.92 ± 6.78	0.20
BMI, kg/m ²	27.61 ± 4.88	27.09 ± 5.12	28.25 ± 3.85	0.30
Log ₁₀ viral load, IU/mL	2.02 ± 1.31	NA	3.79 ± 1.76	< 0.01
LSM, KPa	5.93 ± 4.42	5.22 ± 2.25	7.95 ± 7.59	0.01
ALT, IU/L	26.01 ± 17.33	23.71 ± 15.70	32.46 ± 20.22	0.03
AST, IU/L	24.13 ± 13.95	22.36 ± 10.53	29.06 ± 20.22	0.03
FBS, Mg/dL	111.36 ± 45.62	109.24 ± 44.19	117.32 ± 49.85	0.44
GGT, IU/L	29.3 ± 42.62	24.00 ± 13.98	44.17 ± 79.03	0.04
HDL, mg/dL	59.11 ± 15.77	59.21 ± 16.79	58.84 ± 12.78	0.92
LDL, mg/dL	114.82 ± 30.75	114.99 ± 30.52	114.35 ± 31.98	0.93
TG, mg/dL	125.30 ± 89.05	115.51 ± 74.72	152.78 ± 118.07	0.07
Plt, × 10 ³ /m ³	223.22 ± 57.58	224.79 ± 52.44	218.80 ± 71.06	0.65

^a Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; GGT, gamma glutamyl transferase; HDL, high density lipoprotein; LDL, low density lipoprotein; LSM, liver stiffness measurement; NA, not applicable; TG, triglycerides; Plt, Platelets.

^b Data are presented as mean ± SD.

Table 2. Different Mutations in Basal Core Promoter (BCP) and Pre-core (PC) Regions at Baseline Phase in 99 CHB Cases

Mutation Site	Numbers (%)
Pre-core motor region	
G1896 (A)	36 (36.4)
G1899 (A)	13 (13.1)
G1896A+G1899A	12 (12.1)
Basal core promoter region	
T1753 (C, A, G)	4 (4)
G 1764 (A)	5 (5)
Double mutation A1762/G1764	6 (6.1)
Double mutation + C1766 (T, G, A)	5 (5.1)
Double mutation + T1753	6 (6.1)
Double mutation +C1766+T1753	3 (3.1)

Table 3. Association Between Baseline G1896A Mutation and Viral Level at the End of Study ^{a,b}

Hepatitis B Virus replication status at the end of study	Undetectable	Detectable	Total	OR	CI 95%	P Value
G1896A mutation						
G1896 (wild type)	44 (86.3)	7 (13.7)	51 (100)	4.12	1.54-11.03	0.003
A1896 (mutant)	29 (60.4)	19 (39.6)	48 (100)			
Total	73	26	99			

^a Abbreviation: CI, Confidence Interval; OR, Odd Ratio.

^b Data are presented as No. (%).

5. Discussion

Mutations in the HBV viral genome may affect interactions with host immune system, alter HBV natural history and affect clinical outcomes. In the current longitudinal study, we found that participants infected with Hepatitis B virus with the A1896 mutation had higher HBV DNA levels after seven years of follow-up than those infected with the wild type variant. Interestingly, the only HCC case in this population showed A1896 variant which emphasizes the importance of this mutation and consequent higher viral load.

5.1. A1896 Variant, HBV DNA Level and Viral Load

Previous associations between A1896 mutation and HBV DNA level have typically been null, although with mostly cross-sectional designs not directly compatible with our study. For example, a recent study of 186 HBeAg negative chronic HBV patients from Morocco found no association, although associations were found with other HBV mutations (8). Similar null findings were found in a study of 123 HBV genotype D patients in Tunisia (19), 67 patients in Turkey (20) and 150 patients in Korea (21). Nevertheless, other studies observed an association, such as a Korean study of 475 patients (22). Heterogeneity across studies could reflect chance as well as differences in study design, patient populations, viral genotypes and HBeAg status.

There are multiple ways by which HBV mutations could affect HBV viral levels over time. Thus, despite in vitro data indicating that HBV pre-core mutations (A1896 and A1899) have no appreciable effect on HBV replication capacity (23), altered interactions with host immune system or perhaps other mechanisms could lead to changes in HBV levels over time.

5.2. Associations Between the A1896 Mutation and Liver Disease Severity

We found no association between the A1896 mutation and disease severity, consistent with most but not all previous studies. For example, Yoo et al. reported frequent A1896 and BCP mutations in a cross-sectional study of 413 HBeAg negative chronic hepatitis B (genotype C) patients in Korea, however found no association with disease severity (24). Similarly, no association was observed

in a cross-sectional study of 109 chronic HBV patients in Pakistan (25). In contrast, a Chinese case-control study observed evidence for a positive association between the pre-core A1896 mutation and HCC in HBeAg negative untreated patients (genotype C) (26).

Other studies, however, observed inverse associations for A1896 with liver disease severity and HCC (8, 27). Yang et al. found that individuals with the A1896 mutation (genotype C) had lower risk of HCC over 13 years of follow-up than those with wild type G1896 in a large Taiwanese study (27). Similar results were found in a Moroccan study for liver disease progression (8). Inconsistencies across studies may reflect differences in HBV genotype and HBeAg status or chance. As for studies of HBV DNA level, large future studies are needed to distinguish the effects of HBV genotype, HBeAg and HBV viral mutations.

5.3. Association Between other Examined Mutations (pre-core or BCP) and HBV Infection Markers

In the present study, we found no association for other examined mutations with either HBV DNA level or markers of liver disease (ALT level and liver stiffness). In contrast to these findings, cross-sectional studies of HBV viral level and liver disease severity have generally suggested associations with A1762T/G1764A double mutation (6, 26, 28, 29), although these studies were mostly conducted among participants with other HBV genotypes.

For example, Zheng et al. in their cross-sectional study in China on 341 untreated older HBV patients reported that BCP T1762/A1764 double mutations and pre-core A1899 mutation were associated with HCC development in HBeAg positive patients (26). In a cross-sectional hospital-based study in Taiwan, Liu et al. observed an association between T1762/A1764 and HCC (6). In addition, Parekh observed an association between core promoter mutations, although other than T1762/A1764, with HBV DNA replication and HBeAg expression (28) in a French study. Study specific differences may reflect differences in HBV viral genotype.

Unfortunately, we lacked sufficient participants with cirrhosis or HCC to investigate these important end-

points. Whether low rates of HCC in our area (16) reflect the preponderance of genotype D in our population, lack of HBeAg among chronically infected HBV patients, or particular HBV mutations, such as those in the pre-core or basal core promoter, requires further studies. Although, there are some studies in Iran that hypothesized a negative selection imposed on HBV genome, low frequency of mutations and unique characteristics of genotype D could be involved in the low prevalence of poor prognosis complications like HCC and cirrhosis in our country compared to others (30, 31).

Finally, it may be important to examine HBV mutations in concert as opposed to individually. Some studies indicated that the A1762T/G1764A mutation typically appears earlier than G1896A during the course of HBV infection (28). It is possible that the effects of particular mutations may vary by the presence of other mutations. As our study was too small to conduct such an analysis systematically, future such studies are needed.

5.4. Strengths and Limitations

Key strengths of our study include its longitudinal design and comprehensive characterization of HBV and liver disease. Our study also benefited a population-based setting and enrolled participants of a homogenous ethnic group who were all HBeAg negative and infected by HBV genotype D, allowing us to disentangle associations of viral mutation from viral genotype and other important aspects of HBV.

The most important limitation was a relatively small size of cases with progressive liver disease, which limited our ability to assess disease progression and possible associations with combinations of HBV mutations. We also lacked data on liver stiffness and other markers of liver disease at baseline, which caused limitation in assessing the possible association between the baseline and follow-up markers. Our results may also not apply to other populations, particularly among patients infected with other genotypes of HBV or HBeAg positive ones.

5.5. Conclusions

In a longitudinal study of individuals infected with genotype D HBeAg negative HBV, participants with the pre-core A1896 mutation were more likely to have detectable HBV DNA after seven years of follow-up than those with the wild-type G1896.

5.6. Key Message

Mutations in the HBV genome may contribute to high levels of detectable virus in some HBeAg negative patients.

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Authors' Contributions

Sima Besharat and Ashraf Mohamadkhani contributed in the study design, performing tests and writing the paper. Hossein Poustchi and Abdolvahab Moradi were the scientific managers and contributed in the final editing of the paper. Gholamreza Roshandel contributed in statistical analysis of data. Aezam Katoonizadeh and Neal Freedman edited the paper for grammatical and writing errors.

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