Clinical, virologic and phylogenetic features of hepatitis B infection in Iranian patients

Golnaz Bahramali, Majid Sadeghizadeh, Samad Amini-Bavil-Olyaee, Seyed-Moayed Alavian, Abbas Behzad-Behbahani, Ahmad Adeli, Mohammad-Reza Aghasadeghi, Safieh Amini, Fereidoun Mahboudi

AIM: To characterize the clinical, serologic and virologic features of hepatitis B virus (HBV) infection in Iranian patients with different stages of liver disease.

METHODS: Sixty two patients comprising of 12 inactive carriers, 30 chronic hepatitis patients, 13 patients with liver cirrhosis and 7 patients with hepatocellular carcinoma (HCC) were enrolled in the study. The HBV S, C and basal core promoter (BCP) regions were amplified and sequenced, and the clinical, serologic, phylogenetic and virologic characteristics were investigated.

RESULTS: The study group consisted of 16 HBeAg-positive and 46 HBeAg-negative patients. Anti-HBe-positive patients were older and had higher levels of ALT, ASL and bilirubin compared to HBeAg-positive patients. Phylogenetic analysis revealed that all patients were infected with genotype D (mostly ayw2). The G1896A precore (PC) mutant was detected in 58.1% patients. HBeAg-negative patients showed a higher rate of PC mutant compared to HBeAg-positive patients ($\chi^2 = 9.682, P = 0.003$). The majority of patients with HCC were HBeAg-negative and were infected with PC mutant variants. There was no significant difference in the occurrence of BCP mutation between the two groups, while the rate of BCP plus PC mutants was higher in HBeAg-negative patients ($\chi^2 = 4.308, P = 0.04$). In the HBV S region, the genetic variability was low, and the marked substitution was P120T/S, with a rate of 9.7% ($n = 6$).

CONCLUSION: In conclusion, HBV/D is the predominant genotype in Iran, and the nucleotide variability in the BCP and PC regions may play a role in HBV disease outcome in HBeAg-negative patients.

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Key words: Hepatitis B virus; Clinical and virologic features; Genetic variability; Phylogenetic analysis

Peer reviewer: George V Papatheodoridis, PhD, 2nd Academic Department of Internal Medicine, Hippokration General Hospital, 114 Vas. Sofias ave., Athens 1527, Greece

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INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most important infectious diseases worldwide and is a major global health problem. Approximately one million people die annually because of acute and chronic HBV infection despite the availability of effective vaccines and effective antiviral medications[1]. HBV replicates via the reverse transcriptase enzyme system which lacks proofreading ability; therefore, new virions

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possess diverse genetic variability. Different selection pressures such as host immunity (endogenous pressure), and vaccine or antiviral agents (exogenous pressure) influence the production of HBV quasispecies in infected individuals. It has been demonstrated that mutations in the HBV genome not only impact the replication fitness of the virus (phenotypical effect) but can also influence the disease outcome, as well as the response to treatment (clinical effect). Mutations in the HBV surface (S), precore (PC) and basal core promoter (BCP) genes are observed frequently in HBV infected patients, and studies show that these mutations are associated with the clinical outcomes of HBV disease. The most clinically relevant mutations in the S region arise in the immunologic “a determinant” domain, and neutralizing antibodies (anti-HBs) are targeted against this epitope. The most frequent and clinically important mutations in the PC and BCP regions are G1896A and A1762T/G1764A, respectively; which are often detected in HBeAg-negative chronic HBV infected patients. Moreover, it has recently been documented that HBV genotypes may also contribute to the clinical features, disease outcome, and response to antiviral therapy.

Iran is located in the Middle East, and has an intermediate-to-low prevalence of the HBV infection. The prevalence of HBV infection in Iran is around 2% and it appears that after the implementation of the HBV National Vaccination Programme (started in 1993), the HBV infection rate in young children has diminished significantly. There are very few reports on the molecular epidemiology of HBV in Iran. Recently, a study on the clinical and serological findings of HBV infection in Iran was published, however, there are no reports on the association of the clinical, serological, virologic (HBV genetic variability) and phylogenetic features of HBV infection. In the present study, we have attempted to determine the HBV genetic variability, and its association with clinical outcome in HBV infected patients at different stages of liver disease.

**MATERIALS AND METHODS**

**Patients**

Sixty two HBsAg-positive patients who were referred to the Tehran Hepatitis Centre (2004-2006), were enrolled in a cross-sectional study. The study population consisted of 79% males (n = 49) and 21% (n = 13) females. The mean ± SD age was 37.3 ± 12.3 years (range: 15-64 years, median 36 years). All patients 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) in order to determine the clinical status of the patient. We followed the American Association for the Study of Liver Disease (AASLD) practice guidelines with regard to the diagnostic criteria. Briefly, inactive carriers had persistent HBV infection without significant necro-inflammatory disease. Chronic hepatitis was defined as HBsAg positivity with or without the presence of HBeAg and a high HBV DNA (> 100 000 copies/mL) level determined by the Amplicor HBV monitor, persistent or intermittent elevation in the serum ALT levels, and compatible liver biopsy. Liver cirrhosis and hepatocellular carcinoma (HCC) were confirmed by liver biopsy. Informed consent was obtained from the patients before collecting blood samples. Sera from the patients was frozen at -20°C in aliquots, until used for virological examination.

**Serologic, virologic and biochemical parameters**

All patients were tested for HBV serological markers (HBsAg, anti-HBs, total anti-HBc, HBeAg, and anti-HBc), hepatitis D virus (HDV), hepatitis C virus (anti-HCV) and human immunodeficiency virus (anti-HIV) using commercial kits (DIA PRO Diagnostic Bioprobes, Srl, Italy). Coinfected patients with HIV, HDV and HCV were excluded from the study. Liver function tests such as serum albumin, total bilirubin, ALT, AST and ALP were measured by an auto-analyzer. HBV DNA viral load was determined using the Cobas Amplicor HBV Monitor test (Roche Applied Science, Mannheim, Germany).

**Detection of S and BCP/C mutations**

HBV DNA was extracted using a nano-particle magnetic beads kit (BILATEC AG, Viernheim, Germany) according to the manufacturer’s instructions. The HBV S/pol and BCP/C regions were amplified as previously described. Negative serum samples from subjects with no HBV markers served periodically as a negative control. The PCR amplicons were purified using the AccuPrep gel purification kit (Bioneer Inc, Alameda, CA), sequenced bi-directionally with inner primers using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the data were collected by an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems).

**Phylogenetic and sequence analysis**

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1, as well as BioEdit, version 7.0.4.1 as described previously. Briefly, sequences of BCP/C plus S regions (approximately 1000-bp) were block aligned by the CLUSTAL X program and corrected visually; the Kimura two-parameter algorithm was used for genetic distance calculation. A phylogenetic tree was generated by the neighbour-joining method, and bootstrap re-sampling and reconstruction was carried out 1000 × to confirm the reliability of the phylogenetic tree.

**Statistical analysis**

The data were statistically analyzed using the SPSS software, version 11.0 (SPSS, Inc., Chicago, IL). P < 0.05 was considered significant.

**RESULTS**

**Clinical and demographic data**

Based on the clinical and laboratory findings the
patients were divided into four categories: 19.4% patients (n = 12) were inactive HBsAg carriers, 48.4% (n = 30) had chronic hepatitis B infection, 21.0% (n = 13) were diagnosed with cirrhosis, and 11.3% (n = 7) had HCC. The clinical and laboratory findings (serologic, biochemical, and virologic) are summarized in Table 1. The clinical features of HBsAg-positive patients and anti-HBs-positive patients are shown in Table 2. Based on the HBsAg serology status, 16 patients were HBsAg-positive and 46 patients were anti-HBs-positive. There was no significant difference in age, ALT, AST, and bilirubin levels (biochemical parameters), and HBV viral load between HBsAg-positive and HBsAg-negative groups; however, a significant difference in the gender distribution was observed (χ² = 10.96, P = 0.003) (Table 2). All chronic hepatitis B patients with HCC were HBeAg-negative. The rate of HBeAg-positive isolates was significantly higher (χ² = 0.003) in HBeAg-negative patients (P = 0.003) (Table 2). One patient had both positive HBeAg and anti-HBe status.

**HBV genotype and subtype**

The phylogenetic tree was constructed using the block alignment of HBV S plus BCP/C gene sequences (62 HBV isolates from this study) along with different HBV genotype (A to H) sequences retrieved from the GenBank[20] as reference genes. The phylogenetic tree revealed that all Iranian isolates were branched with other genotype D of HBV reference isolates with a high bootstrap value, 99%, 1000 × replicates (Figure 1). Thus, all Iranian patients were infected with only genotype D. To test the HBV subtype, the amino acid mapping on the HBV S gene protein was performed. Based on the presence of Arg¹²², Thr¹²⁵, Pro¹²⁷, and Lys¹⁴⁰ residues, HBV were subtyped as ayw2 and ayw3, respectively.

**Characteristics of nucleotide substitution in the S and BCP/C regions**

Amino acid sequences of a portion of the S region of all 62 isolates of the study patients were compared with the amino acid sequences of the reference genes. Amino acid mapping revealed that the S region was relatively conserved; however, an important substitution of P120T/S was observed. P120T/S substitutions were detected in 9.7% of chronic hepatitis and cirrhotic patients (n = 6). No G145R substitution was identified in the isolates; whereas, some substitutions such as P127T, T131I, Y134H, D144N and I152T were observed in the immunologic domain of the “a determinant” region. With regard to the mutations in the BCP and C regions (Table 3), a high rate of G1896A PC mutant variants (58.1%, 36/62) was detected in the isolates. The rate of precore mutant isolates was significantly higher (χ² = 9.682, P = 0.003) in HBsAg-negative patients (69.5%, n = 32) compared to HBsAg-positive patients (25%, n = 4). In the HBV precore region, mutation of G1899A (Gly-to-Asp, at codon 29) was found in 37.1% isolates (n = 23), and was mostly detected in patients with cirrhosis (61.5%), and HCC (42.8%). All isolates had T1585 which is specific for genotype D.

### Table 1  Clinical, serological, virological and biochemical features of patients infected with HBV (mean ± SD)

| Clinica-status                  | Number (%) | Sex | Age (yr) | HBsAg+ | Anti HBs+ | Anti HBe+ | HBsAg+ | Anti HBe+ | ALT (IU/L) | AST (IU/L) | Alk (mg/dL) | T-Bil (mg/dL) | Albumin (g/dL) | HBV DNA (log copies/mL) |
|--------------------------------|------------|-----|----------|--------|----------|----------|--------|----------|------------|------------|-------------|--------------|---------------------|----------------------|---------------------|
| Inactive HBsAg carriers        | 12 (19.4)  | M   | 30.6 ± 12| 9      | 3        | 3        | 9      | 3        | 38.3 ± 1   | 31.4 ±     | 282.2 ±     | 0.7 ±        | 4.77 ±            | 4.3 ±              |
| Chronic hepatitis              | 30 (48.4)  |     | 34.5 ± 30| 5      | 4        | 5        | 5      | 5        | 25.8 ±     | 12.1 ±     | 199.9 ±     | 0.241 ±      | 1.25 ±            | 1.57 ±             |
| Liver cirrhosis                | 13 (21.0)  | M   | 44.5 ± 13| 4      | 4        | 4        | 4      | 4        | 11.6 ±     | 11.6 ±     | 120.7 ±     | 74.7 ±       | 125.4 ±           | 0.49 ±            |
| HCC                            | 7 (11.3)   |     | 11 (9)   | 1      | 1        | 1        | 1      | 1        | 18.8 ±     | 22.2 ±     | 229.3 ±     | 0.63 ±       | 0.67 ±            | 2.03 ±            |
| Total                          | 62 (100)   | M   | 37.3 ± 62| 13     | 13       | 13       | 13     | 13       | 123.5 ±    | 285.1 ±    | 402 ±       | 3.1 ±        | 7.22 ±            | 5.2 ±             |

NS: Not significant.

### Table 2  Comparison of demographic and para-clinical features between HBsAg-positive and anti-HBs-positive individuals (mean ± SD)

| HBsAg-positive | Anti-HBs-positive | P   |
|----------------|-------------------|-----|
| Age (yr)       | 35.0 ± 12.8       | 38 ± 12.2 | NS  |
| Genotype       | D                 | D    | NS  |
| ALT (IU/L)     | 70.7 ± 63.5       | 101.7 ± 204.3 | NS  |
| AST (IU/L)     | 69.3 ± 67.6       | 98.6 ± 134.07 | NS  |
| T-Bil (mg/dL)  | 1.18 ± 0.58       | 1.43 ± 0.97  | NS  |
| HBV DNA (log copies/mL) | 6.21 ± 1.7 | 5.2 ± 1.57 | NS  |

### Table 3  The rate and percentage of BCP/C region mutations in HBV isolated among different clinical groups (n %)

| Mutation          | Inactive HBsAg carriers (n = 12) | Chronic hepatitis (n = 30) | Liver cirrhosis (n = 13) | HCC (n = 7) | Total rate (%) |
|-------------------|----------------------------------|----------------------------|--------------------------|-------------|----------------|
| A1757             | 12 (100)                         | 25 (83.3)                  | 11 (84.6)                | 5 (71.4)    | 85.4           |
| C1753             | 1 (8.3)                          | 5 (16.6)                   | 5 (38.46)                | 4 (57.14)   | 24.2           |
| T1762/A1764       | 5 (41.6)                         | 11 (36.6)                  | 4 (30.76)                | 3 (42.85)   | 37.1           |
| A1899             | 3 (25)                           | 9 (30)                     | 8 (61.5)                 | 3 (42.85)   | 37.1           |
| A1896             | 5 (41.6)                         | 19 (63.3)                  | 6 (46.15)                | 6 (85.71)   | 58.1           |
| T1766/A1768       | 1 (8.3)                          | 4 (13.3)                   | 2 (15.38)                | 1 (14.28)   | 12.9           |
| T1764/G1766       | 1 (8.3)                          | 5 (16.6)                   | 1 (7.69)                 | 2 (28.5)    | 14.5           |
BCP double mutation (T1762/A1764) was observed in 37.1% isolates ($n = 23$); whereas, T1762 and A1764 were also found alone. There was no significant difference in the rate of BCP double mutation between HBeAg-positive and HBeAg-negative patients. The occurrence of precore mutant plus BCP double mutation was detected in 28.2% of HBeAg-negative patients ($n = 13$); and in 12.5% ($n = 2$) HBeAg-positive individuals. The G1862T mutation was observed in 4.8% of chronic hepatitis patients ($n = 3$). This mutation, in which valine (Val) was replaced by phenylalanine (Phe) at position 17 in the PC region, was observed only in HBeAg-negative patients.

**DISCUSSION**

In the present study, we examined the clinical, serologic, virologic and phylogenetic features in patients with different clinical stages of HBV-related liver disease (inactive HBsAg carriers, chronic hepatitis B, chronic hepatitis B with cirrhosis and HCC). We believe this is the first such study from Iran. Our previous studies on the molecular analysis of HBV revealed the presence of genotype D in Iran\[^{14,16}\]. As expected, the present study also showed that genotype D with ayw2 subtype was present in all 62 patients studied. Genotype D has been reported globally\[^{21}\]; but has a high prevalence in the Mediterranean area and in the Middle East\[^{22}\].

The relationship between HBV genotype(s), and the outcome of liver disease, and the response to treatment has been well documented, and has an important impact on public health\[^{16,23}\]. For example, several studies have shown differences in disease progression between genotype B and C in Asian patients. HBV genotype C is associated with more severe cirrhosis and HCC, and poorer response to interferon therapy\[^{24,25}\].

Moreover, it has been observed that genotypes can influence HBV replication. For example, HBeAg-negative patients harbouring genotype B had lower viral replication efficiency\[^{25}\]. Since HBV genotype D was the only predominant genotype in the present study, a comparison between different genotypes was not possible. Non D HBV genotypes are not seen in Iran; whereas, different subtypes of genotype D have been reported\[^{26}\].

In the present study, amino acid mapping of the $\delta$ gene showed a high rate of homology between the sequences. It has been shown that amino acid substitutions within the “a determinant” domain in the HBV $\delta$ region may lead to conformational changes in the S protein. Some of these changes may create important medical and public health problems including vaccine escape, failure of hepatitis B immune globulin (HBIG) to protect liver transplant patients and babies born to HBV carrier mothers, and failure to detect HBV carriers with certain diagnostic tests\[^{27,28}\]. In this study, the P120T/S was the most important substitution. This substitution was located at the outside of the “a determinant” immunologic domain. The P120T/S was detected in six isolates. As previously reported, the P120T/S substitution may cause problems with diagnostic assays, and may also cause vaccine escape and poor response to HBIG therapy\[^{27,28}\].

The G1896A PC mutation truncates the HBeAg protein product by creating a stop codon at position 28 within the precore mRNA. Therefore, patients with HBV variants carrying the A1896 mutant in the genome are usually HBeAg-negative. The G1896A PC mutant may be detected in 20%-95% of HBeAg-negative patients worldwide\[^{13}\], and is highly predominant in the Mediterranean area where HBV genotype D has a high rate of infection\[^{26}\]. In a previous study, the rate of HBV precore mutant variants in Iran was reported to be 54%-16; in the present study, the rate was 58.1%. We observed that patients with a precore mutant variant were older and had a higher rate of AST and ALT elevation (but not statistically significant) compared to patients without this variant, suggesting that this variant occurs in patients with a longer history of HBV infection and worse liver disease. In this study, 85.7%
of patients with HCC (n = 6) carried A1896 variants; whereas, this rate was less in the other study groups. These results are in accordance with previous reports\(^\text{[6,30]}\). The precore variant mutants have also been reported in HBeAg-positive patients, ranging from 0%-80%\(^\text{[6,31]}\). In our study, two patients with cirrhosis were infected with the HBV A1896 variants despite HBeAg positivity.

The BCP T1762/A1764 double mutations located at the HBV X gene diminishes HBeAg production, and is associated with more active liver disease\(^\text{[2,32]}\). In the present study, BCP double mutation T1762/A1764 was detected in 37.1% of patients; but there was no association between these mutations and the status of liver disease \((P = 0.7)\). Moreover, no significant difference \((P = 0.9)\) was observed between the frequency of BCP double mutation in patients with HBeAg-positive and HBeAg-negative phenotypes. By contrast, other studies have shown a relationship between BCP double mutations and the clinical manifestations of HBV infection\(^\text{[6,30]}\). Moreover, the T1764/G1766 double mutation in the BCP region was detected in 14.5% of isolates \((n = 9)\) (Table 2). However, this study utilized a relatively small study population, and the results suggest that mutations in the PC region were related to more severe liver disease. More studies in larger populations are required to better understand these associations.

In conclusion, the present study has shown that genotype D (predominantly subtype ayw2) is the only genotype in Iranian patients. Moreover, a high rate of the precore mutation and BCP double mutation was detected in our study.

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**COMMENTS**

**Background**

Heterogeneity of hepatitis B virus (HBV) genome and its mutations may influence the outcome of liver disease as well as the response to antiviral treatment. Considering this fact, we studied the clinical, virologic and phylogenetic features of HBV infection in four groups of patients: inactive carriers, chronic hepatitis, cirrhosis and hepatocellular carcinoma.

**Research frontiers**

The present study revealed an association between certain HBV mutations and the outcome of liver disease, and the response to treatment.

**Innovations and breakthroughs**

This is the first report on the association between different clinical presentations of HBV infection and mutations in three regions of the HBV genome: basal core promoter (BCP)/Precore, P and S.

**Peer review**

The present study is relatively small, but it provides useful information on HBV characteristics in Iranian patients with chronic HBV infection.

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