Correlation between hepatitis B genotypes, 1896 precore mutation, virus loads and liver dysfunction in an Indian population

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Background/objectives: Hepatitis B virus (HBV) genotypes may differ in pathogenicity. However, the interplay between different virus characteristics such as genotypes, mutants and virus loads has not been well studied. We investigated the association between HBV genotype, presence of 1896 precore mutation and HBV viral loads in patients with HBV-related liver disease.

Methods: One hundred and sixteen HBV DNA-seropositive patients attending a gastroenterology outpatient clinic and 107 HBV DNA-seropositive blood donors were recruited. The subjects were stratified as those with normal (Group I, n=164) and elevated (Group II, n=59) ALT levels. The HBV genotype and the presence of the 1896 precore mutation were determined, and plasma HBV DNA levels measured.

Results: Genotype C was more common in Group II than in Group I (10 (17%) vs. 4 (2.4%); p<0.005). There was no relationship between the 1896 precore mutation and the HBV DNA levels. Subjects with genotype C (n=14) had higher HBV DNA levels than those with genotypes A (n=33) or D (n=158).

Conclusions: The infecting genotype, but not the presence of 1896 precore mutation, correlates with HBV load. The association of genotype C with higher virus loads and with elevated ALT may point to a greater pathogenicity of this genotype.

Original Article

Several characteristics of hepatitis B virus (HBV) appear to be associated with variations in the severity of liver disease. These include precore mutation at position 1896, core promoter mutations, and HBV genotypes and virus load.

Mutations that abrogate or reduce the production of hepatitis B e antigen (HBeAg) have been reported to be associated with a lower rate of recovery from chronic HBV infection and poorer clinical outcome.1 However, in other studies, these mutations were associated with a beneficial outcome.2

HBV genotype may determine the clinical outcome. In a Chinese study, genotype C HBV was associated with more severe liver disease as compared with genotype B.3 In addition, response to treatment also differs among HBV genotypes.4 However, the frequency of the 1896 precore mutation, associated with negative HBeAg status, varies between HBV genotypes.5,6

Serum HBV DNA level is an accurate measure of the replicative activity of HBV,7 and lower virus loads are associated with mild or no liver disease.8 Studies from southeast Asia show that HBV genotype C is associated with higher virus loads as compared with genotype B.9 Further, HBV loads correlated with the severity of liver disease and histological damage in patients infected by precore mutants.10

In a study from northern India, precore mutants of HBV were associated with a more aggressive course of liver disease.11 However, in a study from western India,12 patients with this mutant of HBV had lower HBV DNA levels. There are two published reports on HBV genotypes from the Indian subcontinent. Thakur et al13 from northern India found that genotype D was associated with severe liver disease as compared with genotype A, where-
as Gandhe et al.\textsuperscript{14} in western India found no relationship between the HBV genotypes (D and A) and clinical outcome. These conflicting findings emphasize the need for a thorough investigation of the relationship between the HBV genotype, the presence of precore mutation and HBV loads, and their relevance to HBV-related liver disease. We therefore investigated the association between the viral genotype, presence of precore mutation and HBV loads among subjects with HBV infection in an Indian tertiary care hospital.

\textbf{Methods}

\textbf{Study subjects}

Consecutive HBV DNA positive patients (n=116) aged \textgeq16 years attending the outpatient positive clinic of the Department of Gastroenterology of the Christian Medical College (CMC), a tertiary care teaching hospital in Vellore, India, between September 2000 and November 2001 were studied. All study patients had been seropositive for HBsAg for at least 6 months. In addition, voluntary or replacement blood donors in the John Scudder Memorial Blood Bank of our hospital during Monday to Friday between March 2002 and June 2003 found to be HBsAg positive (n=160) were screened; those found to be HBV DNA positive (n=107) were also included in the study. Those with other concomitant causes of liver disease, prior treatment for HBV infection, or co-infection with HCV, HDV or human immunodeficiency virus were excluded. Sixty-one patients positive for HBV DNA were excluded from the study for the following reasons: Lack of evidence of chronic HBV infection b) less than 16 years of age c) co-infection with HCV or HDV or HIV d) patients currently on antiviral therapy.

The study subjects (n=223) were classified into those with normal (Group I; n=164) or elevated ALT levels (Group II; n=59). The ALT cut-off (64 U/L) was based on mean (2 SD) of values in 134 blood donors negative for evidence of hepatitis B, hepatitis C, hepatitis G and TT viruses.\textsuperscript{15}

Group I included 53 (32\%) subjects from eastern India, 2 (1.2\%) from northern India and 109 (66.5\%) from southern India, and Group II included 30 (51\%) subjects from eastern India and 29 (49\%) from southern India (p=ns).

\textbf{Laboratory techniques}

\textbf{Serological markers.} Specimens from all subjects were tested for HBsAg using Axsym HBsAg V2 (Abbott Laboratories) and Hepanostika HBsAg Uni-Form II (BioMerieux); for HBeAg using HBe 2 (Abbott Laboratories), anti-HBe using Anti-HBe 2 (Abbott Laboratories); anti-hepatitis B core (Hbc) IgM (DiaSorin, Italy), anti-HCV antibody using the UBI HCV EIA 3.0 and Axsym HCV version 3.0 (Abbott Laboratories); and anti-delta antibody using anti-delta EIA (Abbott Laboratories). ALT levels were estimated using an automated analyzer (Hitachi 912).

\textbf{HBV DNA detection.} Plasma from anticoagulated blood was stored until testing at \textminus60 °C. DNA was extracted from 200 µL of plasma using QIAamp DNA Blood Mini Kit (Qiagen). Ten µL of DNA (equivalent to 10 µL of plasma) was used for nested PCR, using primers targeting the HBV core gene.\textsuperscript{16}

Detection of 1896 precore mutation. This was done using a PCR–restriction fragment length polymorphism (RFLP) assay, which we have previously standardized\textsuperscript{17} based on the technique originally described by Niitsuma et al.\textsuperscript{18}

\textbf{HBV genotyping.} HBV genotypes were determined using a PCR-RFLP assay previously standardized in our laboratory\textsuperscript{19} based on the method described by Lindh et al.\textsuperscript{5} For specimens that could not be typed using RFLP, the PCR products were subjected to nucleotide sequencing and genotype was assigned based on the nucleotide sequence.

\textbf{HBV DNA quantitation.} This was done by real-time PCR, using RealArt\textsuperscript{TM} HBV TM Reagents (Artus), the ABI PRISM 7000 sequence detection system (Applied Biosystems) and Rotor-Gene 3000 (Corbett Research), and primers and probe specific for HBV. Forty-five cycles of amplification were performed as per the manufacturers’ instructions. Serial dilutions of the World Health Organization (WHO) international standard for HBV DNA nucleic acid amplification technology assays (containing 1 x 10\textsuperscript{6} international units (IU)/mL) and plasma containing 1 x 10\textsuperscript{8} IU/mL of HBV DNA (courtesy: Health Protection Agency, London, UK) were used as standards, and underwent DNA extraction along with the test samples.

\textbf{Statistical Analysis}

Data were analyzed using Fisher exact test, Chi-squared test with Yates’ correction, Student \textit{t}-test, Kruskall–Wallis test and the Mann–Whitney \textit{U}-test, as appropriate. The statistical packages NCSS/PASS 2000 (Dawson edition), Epi Info (version 6.04b), Microsoft Excel (Microsoft Office XP) and STATA (version 8.0) were used. Univariate and multivariate analysis were done with HBV DNA levels and elevated ALT levels (>64 U/L) as outcome variables. A \textit{p} value <0.05 was considered as significant.

\textbf{Results}

 HBV genotypes could be assigned in 207 specimens using either RFLP (n=195) or nucleotide sequencing (n=12); the remaining 16 (7.2\%) specimens did not show any ampli-
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in the PCR used for genotyping. The distribution of genotypes is shown in Table 1. Among subjects with genotype A, 18 (55%) were from eastern India, 14 (42%) were from southern India and 1 (3%) was from northern India. Thirteen (93%) of subjects with genotype C were from eastern India and 1 (7%) was from southern India. Among those with genotype D, 44 (28%) were from eastern India, 113 (71%) were from southern India and 1 was from northern India.

Table 1 shows a comparison of host and viral characteristics between the two study groups. Subjects with elevated ALT (Group II) were older (p<0.001), and HBV DNA loads were higher in them (p<0.005).

Table 2 shows the relationship of mutation at nucleotide position 1896 with the infecting genotypes. This mutation was seen more frequently in genotype D HBV than in genotype A HBV (p<0.005), and was not seen in genotype C HBV. Infection with the 1896 wild type was seen significantly more often in subjects with genotype A HBV than those with genotype C or genotype D virus (p<0.005 and <0.001, respectively).

HBV DNA levels in relation to 1896 mutation and the infecting genotype are shown in Figure 1 respectively. The 1896 mutation and the infecting genotype in relation to ALT levels as the outcome variable are shown in Figure 2.

HBV DNA levels did not correlate with the occurrence of the 1896 mutation (Figure 1a). Subjects with genotype C HBV infection had significantly higher median HBV DNA loads (2.2 x 10^6 IU/mL) than those with genotype A (2.1 x 10^7 IU/mL) or genotype D (2.0 x 10^4 IU/mL) (p<0.001 each) HBV (Figure 1b). Mixed infection (1896 wild type and 1896 mutant) was associated with higher ALT levels as compared with infection with the 1896 wild type (p<0.01) (Figure 2a). Genotype C (median ALT = 96 U/L) was associated with higher ALT levels than genotypes A (median ALT = 35 U/L) and D (median ALT = 51 U/L) (Figure 2b). A higher proportion of subjects with genotype C infection (93%) had HBV loads >1430 IU/mL (10^4 copies/mL) than those with genotype D (55%;

**Table 1: Host–virus characteristics in the study population**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group I (ALT ≤ ULN)</th>
<th>Group II (ALT &gt; ULN)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>164</td>
<td>59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age in years (mean [SD])</td>
<td>34 (10)</td>
<td>41 (11)</td>
<td></td>
</tr>
<tr>
<td>Male: Female</td>
<td>141: 23</td>
<td>53: 6</td>
<td></td>
</tr>
<tr>
<td>HBeAg Positive</td>
<td>24 (15%)</td>
<td>14 (24%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>140 (76%)</td>
<td>45 (85%)</td>
<td></td>
</tr>
<tr>
<td>1896 nucleotide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>35</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>65</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Mixture</td>
<td>50</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>HBV genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>23</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>10</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>D</td>
<td>121</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Mixed (A+D)</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HBV DNA load (IU/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>2.1 x 10^7 (8.29 x 10^7)</td>
<td>4.9 x 10^7 (1.3 x 10^8)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Median</td>
<td>1.5 x 10^3 (1.2 x 10^3)</td>
<td>1.2 x 10^3 (7.7 x 10^3)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Range</td>
<td>0.7 x 10^7 – 5.7 x 10^8</td>
<td>1.8 x 10^2 – 7.7 x 10^6</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

* ULN upper limit of normal (64 U/L)

b Seventeen samples were not amplifiable by PCR used for detection of 1896 mutation

c Sixteen samples were not amplifiable by PCR used for HBV genotyping

**Table 2: Relationship of HBV genotype with nucleotide at position 1896**

<table>
<thead>
<tr>
<th>Nucleotide position 1896</th>
<th>Genotype</th>
<th>Genotype</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>33</td>
<td>14</td>
<td>158</td>
</tr>
<tr>
<td>Precore mutant a</td>
<td>0 (0)</td>
<td>1 (7.1)</td>
<td>39 (24.7)</td>
</tr>
<tr>
<td>Wild type b</td>
<td>29 (87.9)</td>
<td>6 (42.9)</td>
<td>50 (31.6)</td>
</tr>
<tr>
<td>Mixed infection c</td>
<td>2 (6.1)</td>
<td>6 (42.9)</td>
<td>60 (38.0)</td>
</tr>
<tr>
<td>Not typable</td>
<td>2 (6.0)</td>
<td>1 (7.1)</td>
<td>9 (5.7)</td>
</tr>
</tbody>
</table>

Values are as number (%)

a 1896 precore mutant: Genotype A vs. D (p<0.005)
b 1896 wild type: Genotype A vs. C (Yates’ corrected, p<0.005)
c 1896 wild type: Genotype A vs. D (p<0.001)
d 1896 mixed infection: Genotype A vs. C (Yates’ corrected, p<0.05)
e 1896 mixed infection: Genotype A vs. D (p<0.001)
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HBV DNA levels were associated with gender (p<0.05), ALT levels (p<0.01), HBeAg status (p<0.001), and the infecting HBV genotype (p<0.05) by univariate analysis. On multivariate regression analysis, HBeAg-positive status, and infection with genotype C were associated with high HBV DNA levels (p<0.005; p<0.005).

Univariate logistic regression analysis showed that age, geographical origin, HBV DNA levels and the infecting HBV genotype were significantly associated with elevated ALT levels (>64 U/L). Age, HBV DNA levels and infection with genotype C were associated with elevated ALT levels (>64 U/L) in a multivariate logistic regression analysis on all variables which were significant in univariate regression.

Figure 1: Relationship of HBV DNA levels with (a) mutation at nucleotide position 1896 among HBeAg-negative subjects, and (b) the HBV genotype. The boxes represent interquartile ranges (IQR; 25th to 75th percentile). The horizontal lines within the boxes represent the medians. The T-shaped lines that extend upwards from each box represent the highest value that is less than 1.5 times the 75th percentile plus 1.5 times IQR. The T-shaped lines that extend downwards from each box represent the lowest value that is greater than 1.5 times the 25th percentile minus 1.5 times IQR. Outliers are shown as black circles.

Figure 2: Relationship of ALT levels with (a) mutation at nucleotide position 1896 among HBeAg-negative subjects, and (b) the HBV genotype. The boxes represent interquartile ranges (IQR; 25th to 75th percentile). The horizontal lines within boxes represent the medians. The T-shaped lines that extend upwards from each box represent the highest value that is less than 1.5 times the 75th percentile plus 1.5 times IQR. The T-shaped lines that extend downwards from each box represent the lowest value that is greater than 1.5 times the 25th percentile minus 1.5 times IQR. Outliers are shown as black circles.
Discussion

Geographical restriction of HBV genotypes has led to most studies comparing either genotype B with genotype C or genotype A with genotype D. A few studies have compared all known HBV genotypes; however, these studies included a considerable proportion of immigrants with various ethnicities. In this Indian, non-immigrant population, we found that genotype C HBV was more often associated with elevated ALT; no such difference was observed between genotype A and genotype D. Banerjee et al detected T1762A1764 double mutation in subjects from eastern India with genotype C and elevated ALT levels.

Considering all the study subjects, median HBV DNA levels in subjects with genotype C was higher than those with genotype A or genotype D. In contrast, the HBV DNA level in subjects with genotype A and genotype D HBV were similar. Furthermore, subjects with genotype C infection more often had HBV loads >10^4 copies/mL, or >1430 IU/mL than those with genotype D or genotype A (p<0.001; p<0.001, respectively); this cut-off was based on the recommendation by Keefe et al, for identification of patients with HBeAg-negative chronic hepatitis B as also for determining the need for therapy.

By univariate analysis, gender (p<0.05), ALT levels (p<0.01), HBeAg status (p<0.001) and the infecting HBV genotype (p<0.05) were significantly associated with HBV DNA levels. Age, presence of the 1896 mutation and the geographical origin (ethnic grouping) were not associated with HBV DNA loads. A multivariate regression analysis revealed that HBeAg-positive status, and infection with genotype C were associated with high HBV DNA levels (p<0.005; p<0.005).

A multivariate logistic regression analysis showed that age, HBV DNA levels and infection with genotype C were associated with elevated ALT levels (>64 U/L).

During the course of HBV infection, immune-mediated hepatocellular damage is reflected by elevated ALT levels. Although HBV per se is not cytopathogenic, the levels of replicating virus (in the liver), as indicated by serum/plasma HBV DNA levels, may relate to the degree of virus-induced immune-mediated liver damage. Lower HBV DNA levels have been observed in individuals with normal ALT levels and minimal histological damage (‘inactive’ carriers) as compared with those with chronic hepatitis, suggesting that virus load correlates with disease progression. The high HBV loads detected in genotype C may be associated with more pronounced liver damage in subjects infected by this genotype.

The 1896 precore mutant was detected more often in subjects with genotype D HBV infection than in those with genotype A HBV (p<0.005) (Table 2). Mixed infection (with both 1896 mutant and wild type virus) was detected more often in subjects with genotype C or genotype D, than in those with genotype A (p<0.05; p<0.001) (Table 2). The 1896 precore mutant is known to be infrequent in genotype A HBV. The genotype A HBV has a cytosine at nucleotide position 1858, which is located opposite the nucleotide 1896 in the stem loop structure of pregenomic RNA. A 1896 precore mutation (guanine to adenine) in genotype A subjects thus results in an unstable C-A base pairing, with consequent disruption of the stem loop structure. The low frequency of 1896 mutant in genotype A HBV in our study is in keeping with the previous reports.

The major limitation of our study is the use of cross-sectional sampling. However, the recruitment of a blood donor cohort for longitudinal studies is extremely challenging. Another weakness of the study is the presence of genotype C in only 14 subjects. Studying of larger numbers of subjects with genotype C infection and correlation to liver biopsy results would help draw firmer conclusions. In conclusion, our results indicate that the HBV genotype, but not the presence of 1896 precore mutation, correlates with HBV DNA levels. Significant differences exist among HBV genotypes in the selection of precore mutations. The association of genotype C with higher HBV DNA levels and elevated ALT levels suggests a greater immune-mediated disease causing potential of this genotype. However, in the absence of liver histology no firm conclusions can be drawn. Long-term follow-up studies, investigating host factors such as the age at infection, duration of infection, and the role of other accompanying mutations would help in a better understanding of the pathogenesis and outcome of HBV-related liver disease in Indian subjects.

References


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