Molecular epidemiology and genetic characterization of hepatitis B virus in the Indian subcontinent

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**S U M M A R Y**

Background: Hepatitis B virus (HBV) is a gradually evolving virus. The aim of this study was to characterize the distribution pattern of HBV genotypes and subgenotypes and HBsAg subtypes in chronic hepatitis B subjects from the Indian subcontinent. We also sought to investigate the genetic diversity of HBV genotypes and its influence on the therapeutic response.

Methods: A total of 295 chronic hepatitis B subjects were studied. HBV genotypes and subgenotypes were determined using the generated HBV reverse transcriptase (rt) sequences. HBsAg subtypes were predicted using a newly developed automated program in Microsoft Visual Basic (VB6). Genetic diversity was characterized by calculating the mean genetic distance (d), the number of synonymous substitutions per synonymous site (dS), and the number of non-synonymous substitutions per non-synonymous site (dN). The virological response was measured by HBV DNA levels.

Results: In southern India, the predominant HBV genotype/subtype was D2/ayw3 (79.1%). In eastern India, C1/adr (28.2%) was found to be the predominant genotype/subtype, followed by A1/adw2 (25.4%). In the north-eastern region, C2/adr, D2/ayw3, and D5/ayw2 were predominant and were each identified in 20.8% of subjects. In treatment-naïve subjects, the d, dS, and dN of genotype D sequences were higher compared to genotypes C and A. Additionally, the d, dS, and dN of HBV rt sequence were higher in subjects who subsequently showed a virological response to nucleos(t)ide analogues as compared to non-responders, irrespective of the genotypes tested (p = 0.014 to p < 0.0001).

Conclusions: We have described the distribution of HBV genotypes and subgenotypes and HBsAg subtypes in three major regions of the Indian subcontinent. HBV genetic diversity may play a pivotal role in the clinical outcome of chronic hepatitis B.

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1. Introduction

Hepatitis B virus (HBV) is a circular, partially double-stranded DNA virus of the family Hepadnaviridae. HBV replicates through a reverse-transcribed RNA intermediate, and the low fidelity rates of the reverse transcriptase enzyme lead to highly error-prone nucleotide synthesis. Hepadnaviruses are known to have originated more than 19 million years ago, and the evolutionary rate of HBV is estimated to be 7.72 × 10⁻⁴ substitutions/site/year. Together with the higher evolutionary rate, selection pressure imprints a high viral genetic diversity.

HBV is classified into genotypes and subgenotypes with an intergenotypic diversity of 8% and intragenotypic diversity of 4%, respectively. Accordingly, eight major HBV genotypes (A–H), two novel genotypes (I and J), and several subgenotypes are well described. HBV strains are also categorized into nine major hepatitis B surface antigen (HBsAg) subtypes based on their antigenic determinants in the major hydrophilic region. Further extensive investigations may lead to the recognition of yet unidentified HBV variants. HBV genotypes are known to show a geographical pattern in their distribution and have been used to trace the migration of populations from geographically distant regions. Moreover, HBV genotypes and HBsAg subtypes have been reported to influence disease progression and treatment response. Therefore, the molecular characterization of HBV strains is important for disease monitoring and clinical outcomes.

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Given the importance of HBV classification, we investigated the distribution pattern of HBV genotypes and subgenotypes and HBsAg subtypes in chronic hepatitis B subjects from the Indian subcontinent. We also characterized the genetic diversity between HBV genotypes and its influence on the subsequent therapeutic response.

2. Materials and methods

2.1. Study subjects

A total of 295 subjects with chronic hepatitis B were enrolled in this study. The study subjects comprised individuals attending the liver clinic of a tertiary care hospital in South India. The subjects were recruited between January 2007 and November 2011 and were part of our HBV antiviral resistance mutations study.14,15

2.2. HBV polymerase/reverse transcriptase gene amplification and sequencing

The HBV polymerase gene covering the entire reverse transcriptase (rt) region was amplified using Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA). PCR and the bi-directional sequencing reaction were performed as described previously.14

The nucleotide sequences generated in this study have been deposited in the GenBank database under accession numbers GU798963 to GU799059 and JQ514280 to JQ514554.

2.3. Determination of HBV genotypes

HBV genotypes were determined using the HBVseq program in the Stanford database.

2.4. Determination of HBV subgenotypes

The study sequences were aligned with published sequences representing all known HBV subgenotypes.3,16–19 Multiple sequence alignment was performed using the built-in ClustalW integrated in MEGA4.20 HBV subgenotypes were determined by phylogenetic analysis in MEGA4 using the neighbour-joining method with a bootstrap test of 1000 replicates and maximum composite likelihood algorithm.

2.5. Determination of HBsAg subtypes

An automated computer programme for HBsAg subtype determination was developed in Microsoft Visual Basic (VB6). The overlapping surface gene sequence of HBV rt (155–835 nucleotides) was translated to the corresponding surface gene amino acids using the BioEdit tool. The HBsAg subtypes were then determined by the subtype program that examines every combination of amino acids at positions 122, 160, 127, 159, and 140 (in that order), as deduced by Purdy et al.8

2.6. HBV genetic diversity

An analysis of the number of base substitutions per site between sequences (genetic distance (d)) was conducted using the maximum composite likelihood method in MEGA4. The number of synonymous substitutions per synonymous site (dS) and the number of non-synonymous substitutions per non-synonymous site (dN) were calculated using the Nei–Gojobori model with Jukes–Cantor correction in MEGA4. The baseline analysis was performed for a total 197 treatment-naive chronic hepatitis B subjects.

2.7. Statistical analysis

Categorical variables were compared using the Chi-square test. The d, dS, and dN were compared using the Kruskal–Wallis test. A p-value of <0.05 was considered statistically significant. All analyses were done using Stata 12 (StataCorp, College Station, TX, USA).

3. Results

The study subjects came from four regions of the Indian subcontinent: southern, western, eastern, and north-eastern. Subjects from the states of Tamil Nadu, Kerala, Karnataka, and Andhra Pradesh and one subject from the Maldives in the south-west of India represented the southern Indian subcontinent population (n = 92). Two subjects were from the western Indian states of Maharashtra and Rajasthan. The subjects of eastern India came from West Bengal, Bihar, Jharkhand, and Oriissa states and Bangladesh (n = 177). Subjects from the states of Assam, Manipur, Meghalaya, Tripura, and Arunachal Pradesh and the adjacent country of Bhutan represented the north-eastern region (n = 24).

3.1. HBV genotypes

Three major HBV genotypes – A, C, and D – were found in this population. HBV genotype D (n = 175; 59.3%) was found to be the predominant circulating genotype, followed by genotypes C (n = 67; 22.7%) and A (n = 53; 18.0%).

3.1.1. Regional distribution of HBV genotypes

There existed distinct patterns of HBV genotype circulation in the study populations of the three main regions. Among the 92 subjects from the southern region, HBV genotype D was the predominant genotype, circulating in 82 (89.1%) subjects. Genotypes A and C were less prevalent and were identified in six (6.5%) and four (4.4%) subjects, respectively (p < 0.0001). Among the 177 subjects from the eastern region, HBV genotype D was predominant, identified in 79 (44.6%) subjects. The other common genotypes C and A were identified in 52 (29.4%) and 46 (26%) subjects, respectively (p = 0.0004). Among the 24 subjects from the north-eastern region, genotype D was identified in 12 (50%) subjects, genotype C in 11 (45.8%) subjects, and genotype A in one (4.2%) subject (p = 0.001).

Genotype D was identified in the two subjects from the western region.

3.2. HBV subgenotypes

All HBV genotype A sequences were identified as subgenotype A1 (Figure 1). Among the 67 genotype C sequences, 58 (86.6%) clustered to the C1 subgenotype and six (8.9%) to C2 (Figure 2). The subgenotypes could not be determined in three (4.5%) genotype C subjects.

HBV genotype D study sequences clustered into subgenotypes D1 (n = 20, 11.4%), D2 (n = 112, 64%), D3 (n = 15, 8.6%), and D5 (n = 27, 15.4%) (Figures 3 and 4). The subgenotype could not be determined for one (0.6%) genotype D sequence.

3.2.1. Regional distribution of HBV subgenotypes

The frequency and distribution of HBV subgenotypes are shown in Table 1. There existed a significant difference between subgenotypes and study population regions (p < 0.001). To some extent, the subgenotypes of genotypes C and D differed in their regional distribution. The frequency of subgenotype C1 was higher in the eastern region (86.2%). Among six subgenotype C2, five
(83.3%) were found to be circulating in Arunachal Pradesh state of the north-eastern region. Subgenotypes D1, D3, and D5 were identified in 80%, 93.3%, and 74.1% of subjects from the eastern region, respectively. HBV subgroup D2 was found to be predominant in the southern region (68.7%).

3.3. HBsAg subtypes

A schematic representation of the newly developed automated program for HBsAg subtype determination is illustrated in Figure 5.

In the subjects studied, six HBsAg subtypes – adw, adw2, adw3, ayw1, ayw2, and ayw3 – were found to be circulating in the Indian subcontinent. HBsAg subtypes ayw3, adw, adw2, and ayw2 were the most common subtypes and were identified in 134 (45.4%), 66 (22.4%), 52 (17.6%), and 36 (12.2%) of the study subjects, respectively. HBsAg subtypes ayw1 and adw1 were identified in one (0.3%) subject each. HBsAg subtypes could not be determined for five (1.7%) treatment-experienced subjects, as they presented with unusual amino acid substitutions at surface gene positions that are crucial for subtype determination.

3.3.1. HBsAg subtypes and genotype/subgenotype association

There existed a significant association between HBV genotypes and subtypes (p < 0.0001). In the analysis performed, HBsAg subtypes adw2 and adw were always found to co-exist with genotypes A and C, respectively. Likewise, HBsAg subtypes ayw2 and ayw3 always presented in genotype D subjects. HBsAg subtype adw3 was identified in one subject carrying subgenotype D2. HBsAg subtype ayw1 was detected in one subject with genotype A.

3.4. Regional distribution of HBV genotypes and subgenotypes and HBsAg subtypes

3.4.1. HBV genotype A and HBsAg subtypes

In 53 subjects identified with HBV genotype A, 52 (98.1%) specifically carried subtype adw2 and the remaining one (1.1%) subject presented with an unusual ayw1 subtype that is more common in South African countries. Among these subjects, 46 (86.8%) were from the eastern region, six (11.3%) from the southern region, and one (1.9%) from the north-eastern region of the Indian subcontinent.

3.4.2. HBV genotype C and HBsAg subtypes

HBV genotype C was always associated with subtype adr and was identified in 52 (77.6%) subjects from the eastern region, 10 (14.9%) subjects from the north-eastern region, and four (6%) subjects from the southern region. The subtype of one (1.5%) genotype C subject could not be assigned.

3.4.3. HBV genotype D and HBsAg subtypes

One subject from the southern region was identified with subtype adw3. Most of the subtype ayw2 associated with genotype
D was identified in subjects from the eastern region (n = 83.3%), followed by three (8.3%) from the southern region, two (5.6%) from the north-eastern region, and one (2.8%) from the western region. Subtype ayw3 was spread throughout the study population and was identified in 75 (55.9%) subjects in the south, 48 (35.8%) in the east, 10 (7.5%) in the north-east, and one (0.7%) in western India. The HBsAg subtype could not be determined for four genotype D subjects.

The overall distribution pattern of HBV subgenotypes and HBsAg subtypes identified in this study is shown in Figure 6. In southern India, the predominant HBV subgenotype/subtype was D2/ayw3 (79.1%). In eastern India, C1/adr (28.2%) was the predominant subgenotype/subtype, followed by A1/adw2 (25.4%), D2/ayw3 (15.3%), D5/ayw3 (11.3%), D1/ayw2 (8.5%), and D3/ayw2 (7.9%). In the north-eastern region, C2/adr, D2/ayw3, and D5/ayw3 were predominant, each identified in 20.8% of subjects. Two subjects from western India had subgenotype/subtype D1/ayw2 and D2/ayw3, and one subject from the Maldives of south-west India was identified with D2/ayw3.

### 3.5. HBV genetic diversity

In order to identify the genetic diversity of HBV genotypes, d, ds, and dN were studied in 197 treatment-naive subjects. There existed a significant difference between the three major HBV genotypes studied (p < 0.0001). The d, ds, and dN of genotype D were higher as compared to genotypes C and A (Table 2).

Further, to determine whether there is a significant association between the baseline sequence and treatment response, baseline samples of subjects who subsequently showed a response (n = 79) and those who did not respond (n = 44) to nucleos(t)ide analogues (lamivudine or adefovir or entecavir) were analysed. The d, ds, and dN were higher in responders as compared to non-responders, irrespective of the genotypes tested (p = 0.014 to p = 0.0001; Table 3). Responders were subjects who subsequently showed a ≥1 log10 IU/ml of HBV DNA reduction with a median treatment duration of 6 months, or undetectable HBV DNA (<82 IU/ml) after a median treatment duration of 12 months. Non-responders were subjects who subsequently showed a < 1 log10 IU/ml reduction in HBV DNA after a median treatment duration of 6 months, or continued to be positive for HBV DNA after a median treatment duration of 12 months.

### 4. Discussion

Our study enrolled a total of 295 subjects with chronic hepatitis B infection from 15 states within India and three adjacent countries, i.e., Bangladesh, Bhutan, and the Maldives. Most of
the subjects represented three major regions: southern India, eastern India, and the north-eastern region. Additionally, a few subjects were from south-west India and western India.

4.1. HBV genotypes and subgenotypes and HBsAg subtypes

The most common genotypes identified in India are A, C, and D. HBV genotypes A and D were shown to be predominant in northern and southern India, and genotype C in eastern India.21-25 Previously, our laboratory has reported the prevailing circulation of HBV genotype D, followed by genotypes A and C in chronic hepatitis B subjects.23,24 The present study also showed a preponderance of genotype D (59.1%), but the frequency of genotype C (22.6%) exceeded that of genotype A (17.9%). This may be due to the inclusion of more subjects from eastern India. Thus we found distinct patterns of HBV genotype circulation in the study populations of the three main regions. Our data also showed increasing evidence of genotype C circulation in southern India, suggesting transmission routes from other regions. The progression of chronic infection is unfavourable in HBV genotype A and D infections.26-28 Advanced stages of liver disease, including hepatocellular carcinoma, are more often seen in chronic infection with genotypes C and D.29-31 Given the clinical importance of genotypes A, C, and D and their predominant circulation in the Indian subcontinent, the utmost standard of care is warranted.

In our study, all HBV genotype A samples were identified as subgenotype A1. Most of the genotype C was identified as subgenotype C1 (86.6%), with a few subgenotype C2 (8.9%). Among the six subgenotype C2, five (83.3%) were found to be circulating in the Arunachal Pradesh state of north-east India, which is bordered by China to the north, where subgenotype C2 is more prevalent. This clearly suggests the transmission route of subgenotype C2 to India. The subgenotypes of three (4.5%) genotype C sequences and one (0.6%) genotype D sequence could
not be assigned with the surface gene sequences, and as suggested in the recent guidelines, further analysis is required to confirm the newer subgenotype circulation in the Indian subcontinent.7,19

We have developed a new program to determine HBsAg subtypes. The program uses the algorithm determined by Purdy et al.8 to identify the nine major HBsAg subtypes currently known. This tool enables the determination of HBsAg subtypes automatically and reduces the time and error rates of manual procedures. Hence it should be a useful tool, especially in the clinical setting and for epidemiological studies. The program was validated by

Table 1
Regional distribution pattern of HBV subgenotypes

<table>
<thead>
<tr>
<th>HBV subgenotype</th>
<th>Total</th>
<th>Southern (n = 92), n (%)</th>
<th>Western (n = 2), n (%)</th>
<th>Eastern (n = 115), n (%)</th>
<th>North-eastern (n = 87), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>53</td>
<td>6 (11.3)</td>
<td>0</td>
<td>46 (86.8)</td>
<td>1 (1.9)</td>
</tr>
<tr>
<td>C1</td>
<td>58</td>
<td>4 (6.9)</td>
<td>0</td>
<td>50 (86.2)</td>
<td>4 (6.9)</td>
</tr>
<tr>
<td>C2</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1 (16.7)</td>
<td>6 (83.3)</td>
</tr>
<tr>
<td>C (unassigned)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1 (33.3)</td>
<td>2 (66.7)</td>
</tr>
<tr>
<td>D1</td>
<td>20</td>
<td>2 (10)</td>
<td>1 (5)</td>
<td>16 (80)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>D2</td>
<td>112</td>
<td>77 (68.7)</td>
<td>1 (0.9)</td>
<td>29 (25.9)</td>
<td>5 (4.5)</td>
</tr>
<tr>
<td>D3</td>
<td>15</td>
<td>1 (6.7)</td>
<td>0</td>
<td>14 (93.3)</td>
<td>0</td>
</tr>
<tr>
<td>D5</td>
<td>27</td>
<td>2 (7.4)</td>
<td>0</td>
<td>20 (74.1)</td>
<td>5 (18.5)</td>
</tr>
<tr>
<td>D (unassigned)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (100)</td>
</tr>
</tbody>
</table>

HBV, hepatitis B virus.

* Three HBV genotype C sequences and one genotype D sequence could not be assigned to any known subgenotypes using the surface gene sequences.
comparing the results generated by this tool with our earlier published HBsAg subtypes. In the previous study, the subtypes of 97 subjects were determined manually by positioning the HBV surface gene amino acids using BioEdit. In the present study, the deduced subtypes were re-analysed using the new subtyping program, and all 97 subtypes showed concordance to those of the previously determined HBsAg subtypes.

In our study, we identified six HBsAg subtypes to be circulating in the Indian subcontinent. The presence of the majority of HBsAg subtypes in this population indicates a diverse virus population. To our knowledge this is the first report to show the prevalence of circulating HBsAg subtypes across three regions of the Indian subcontinent. The subtypes could not be determined in five (1.7%) treatment-experienced subjects, as they presented with unusual amino acid substitutions at surface gene positions that are crucial for subtype determination. One reason could be the selection pressure of antiviral drugs, which would have enabled these variants to occur. As showed by Purdy et al.8 we also noticed a significant association between genotypes and HBsAg subtypes (p < 0.0001). Therefore, distribution patterns of HBsAg subtypes were mostly similar to those of the closely related genotypes.

4.2. HBV genetic diversity

HBV genotypes have been shown to be associated with disease progression and the therapeutic response to immunomodulatory drugs, indicating that the genetic heterogeneity of viral genotypes may play a role in the virus–host relationship. The d, ds, and dN were higher in genotype D sequences when compared to genotypes A and C. This finding has also been evidenced in two other studies; De Maddalena et al.12 showed high genetic heterogeneity in genotype D as measured by higher dN values.
Figure 6. Regional distribution pattern of HBV subgenotypes and HBsAg subtypes in the Indian subcontinent subjects with chronic HBV. The subgenotype/subtype in two subjects from western India (D1/ayw2 and D2/ayw3) and one subject from the Maldives, south-west India (D2/ayw3) are not shown (untyp = untypeable).

Table 2
Genetic diversity of HBV genotypes in treatment-naive subjects. The table shows the mean genetic distance (d), the number of synonymous substitutions per synonymous site (dS), and the number of non-synonymous substitutions per non-synonymous site (dN) of HBV reverse transcriptase sequences in treatment-naive subjects (N=197)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>d (10⁻² substitutions/site)</th>
<th>dS (10⁻² substitutions/site)</th>
<th>dN (10⁻² substitutions/site)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n=32)</td>
<td>1.262 (0.019)</td>
<td>2.988 (0.055)</td>
<td>0.719 (0.129)</td>
</tr>
<tr>
<td>C (n=40)</td>
<td>1.640 (0.038)</td>
<td>4.460 (0.119)</td>
<td>0.641 (0.016)</td>
</tr>
<tr>
<td>D (n=125)</td>
<td>2.213 (0.0156)</td>
<td>6.049 (0.046)</td>
<td>0.814 (0.006)</td>
</tr>
</tbody>
</table>

HBV, hepatitis B virus.

Table 3
Genetic diversity of HBV genotypes in treatment-naive subjects and subsequent response to nucleos(t)ide analogues. The table shows the mean genetic distance (d), the number of synonymous substitutions per synonymous site (dS), and the number of non-synonymous substitutions per non-synonymous site (dN) of baseline HBV reverse transcriptase sequences in responders and non-responders to nucleos(t)ide analogues.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Responders</th>
<th>Non-responders</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n=15)</td>
<td>2.24 (0.223)</td>
<td>0.943 (0.494)</td>
<td>0.0001</td>
</tr>
<tr>
<td>dS (10⁻² substitution/site)</td>
<td>5.406 (0.565)</td>
<td>2.389 (0.174)</td>
<td>0.0001</td>
</tr>
<tr>
<td>dN (10⁻² substitution/site)</td>
<td>1.277 (0.131)</td>
<td>0 (0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>C (n=14)</td>
<td>1.277 (0.131)</td>
<td>0 (0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>d (10⁻² substitution/site)</td>
<td>3.273 (0.252)</td>
<td>1.728 (0.188)</td>
<td>0.0001</td>
</tr>
<tr>
<td>dS (10⁻² substitution/site)</td>
<td>8.884 (0.632)</td>
<td>4.453 (0.575)</td>
<td>0.0001</td>
</tr>
<tr>
<td>dN (10⁻² substitution/site)</td>
<td>1.455 (0.144)</td>
<td>0.819 (0.086)</td>
<td>0.014</td>
</tr>
<tr>
<td>D (n=50)</td>
<td>1.480 (0.225)</td>
<td>0.978 (0.350)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

HBV, hepatitis B virus.

a Values are the mean with the standard error in parenthesis.
b Nucleos(t)ide analogues: lamivudine, adefovir, or entecavir.
compared to other genotypes, and Solmone et al. showed higher numbers of amino acid substitutions in genotype D than genotype A. In our analysis, the d and dS were higher in genotype C than genotype A. However, the dN was higher in genotype A than genotype C.

Intriguingly, on analysis of the baseline sequence and subsequent response to nucleos(t)ide analogues, the d, dS, and dN were higher in responders as compared to non-responders, irrespective of the genotypes tested. These results contradict the findings of Chen et al. In their analysis, the d, dS, and dN were significantly higher in non-responders after 4 weeks of lamivudine treatment. They suggested that the antiviral pressure could have led to the selection of mutants, and hence the viral genetic diversity in non-responders was higher than in lamivudine responders. Another study comparing the evolution of viral quasi-species between hepatitis B e antigen (HBeAg) seroconversion and non-steroconversion showed higher viral genetic diversity among responders than non-responders. Fukai et al. showed a higher number of HBV rt substitutions in the lamivudine responders than in non-responders. These earlier published findings corroborate the findings of the present study.

The concept of intermediate antiviral pressure and selection of mutants can be extended in terms of host immune pressure and adaptive mutations to explain this finding. At low immune pressure, the virus replication is active and under selection only a few adaptive mutations. When immune pressure is high, there is a complete suppression or very low levels of viral replication and therefore no chance of mutant selection. However, at intermediate levels of immune response, the virus evolves strategies to counteract the selection pressure and therefore higher numbers of mutations are observed. This hypothesis has also been proposed by Lim et al. and others. We speculate that the selection pressure of the host immune response could have led to high viral genetic diversity in responders, which is supported by our earlier study in which responders showed a better anti-HBe response and elevated serum aminotransferases at baseline.

In summary, we have described the distribution of HBV genotypes and subgenotypes and HBSAg subtypes in three major regions of the Indian subcontinent. The newly developed automated program for HBSAg subtype determination would be a useful tool for epidemiological and clinical studies. We hypothesize that high viral genetic diversity, elevated baseline serum aminotransferases, and spontaneous anti-HBe seroconversion, suggesting a high immune response, combined with the action of nucleos(t)ide analogues will yield a better virological response.

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Ethical approval: The study was approved by the Institutional Review Board (EC Min. No. IRB (EC)-10-16-01-2008) and informed written consent was obtained from all of the subjects.

Conflict of interest: The authors declare no conflicts of interest.

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