Comparison of genetic and derived amino acid changes in full-length hepatitis B virus genome between the patients with self-limited acute hepatitis B and those with acute exacerbation (AE) in chronic hepatitis B (CHB) during HBeAg seroconversion

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Abstract

The aim of study was to compare the genetic differences in full-length HBV DNA between the patients with acute hepatitis B (AHB) and those with acute exacerbation (AE) in chronic hepatitis B (CHB) during HBeAg seroconversion. The sequence was determined from serial sera of AHB (n=3) and CHB (n=3) at presentation and after HBeAg seroconversion. All patients have been infected by subgenotype C2 of HBV. There was no marked genetic diversity in full-length genome of HBV between AHB and CHB during AE (CHB vs. AHB (%): 1.33 ± 0.49 vs. 1.35 ± 0.35). However, in C and S gene, the genetic diversity was higher in patients with CHB than in those with AHB at AE (CHB vs. AHB (%): C gene, 1.23 ± 0.23 vs. 0.40 ± 0.17; S gene, 1.03 ± 0.55 vs. 0.57 ± 0.38). Interestingly, the substitutions in these genes were likely to occur on putative HLA class I/II restricted epitopes, and the changes of gene in core and surface epitope-related codon were higher in CHB than AHB. In AHB, genetic change of C gene was higher after HBeAg seroconversion than in acute status (HBeAg (-) vs. acute status (%): C gene, 1.20 ± 0.71 vs. 0.40 ± 0.17). In contrast, there were no remarkable differences in CHB before or after HBeAg seroconversion. The different pattern of genetic variation that related to immune escape between AHB and CHB might be associated with the different clinical consequences after HBeAg seroconversion. (J Med Life Sci 2009;6:342-350)

Key Words : Hepatitis B virus, Acute hepatitis B, Chronic hepatitis B, Acute exacerbation, HBeAg seroconversion

INTRODUCTION

Hepatitis B virus (HBV) is one of the major causes of various liver disease including acute hepatitis B (AHB), chronic hepatitis B (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC) in endemic areas. In adults, over 90% of AHB resolves with rare reactivation. In contrast, during the chronic HBV infection, acute exacerbation (AE) of CHB, defined as defined as elevation of serum ALT levels to more than 10 times upper limit of normal and more than twice the baseline value, and following spontaneous HBeAg seroconversion, defined as HBeAg negative, anti-HBe positive, and normal ALT, frequently occurred and accompanied with increasing genetic mutations. In addition, reactivation of hepatitis frequently observed in patients with CHB.

The mechanism of AE during HBeAg seroconversion was not clear yet and has been suggested to be associated with the imbalance between host factor (immune system) and viral factor (HBV genotype and genetic variants in HBV DNA).

Because HBV polymerase lacks proofreading function, mutations in HBV genome naturally occurred during viral replication throughout the whole HBV genome.

As a non-cytopathic pathogen, the clearance of HBV was mostly dependent on virus-specific T-cell response. Recently, different frequency of virus-specific CD4' helper- and CD8' cytotoxic/T lymphocyte (CTL) had been found in AHB and CHB and assumed to be associated with various clinical courses of HBV infection. Moreover, residue substitution in the epitopes recognized by virus-specific CTL, mutations in HBV genes, such as precore/basal core promoter (PC/BCP) relating to viral...
replication or in genes encoding the protein (core and surface protein) that mainly affected by the immune system had also been reported to relate to the persistence of chronic HBV infection\textsuperscript{26-30}.

The purpose of present study was to compare the genetic and derived amino acid changes in full-length genome of HBV before and after HBeAg seroconversion from the patients with AHB or CHB.

**PATIENTS AND METHODS**

**Patients**

Consecutive six patients who were diagnosed as self-limited acute hepatitis B (AHB n=3) and chronic hepatitis B (CHB n=3) with AE followed by HBeAg seroconversion were selected. Their serial serum samples were collected at clinical presentation of hepatitis and after spontaneous HBeAg seroconversion. The clinical data of these 6 patients are presented in Table 1.

To evaluate the genetic diversity, the individual HBV DNA sequence was compared with the consensus HBV DNA sequence (subgenotype C2) deduced from eight of HBV DNA registered in GenBank using Molecular Evolutionary Genetics Analysis (MEGA4) software (http://www.megasoftware.net) (GenBank No.: AY641558: AY641560: AY64156l: AF286594: DQ683578: X01587: AY123041: D000630).

Patients were excluded if they had any of the following: history of receiving anti-virus therapy or immunosuppressive therapy before collecting serum sample, concomitant hepatitis C or D virus infection, and history of heavy alcohol drinking. Ethics committee approved this study and patients gave written informed consent.

**Serologic testing**

Hepatitis B surface antigen (HBsAg), anti-HBs, HBeAg.

Table 1. The basic clinical characteristics of the study patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex/Age</th>
<th>Diagnosis</th>
<th>ALT (IU/L)</th>
<th>HBV DNA (pg/ml)</th>
<th>HBeAg/anti-HBe</th>
<th>ALT (IU/L)</th>
<th>HBV DNA (pg/ml)</th>
<th>HBeAg/anti-HBe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F/43</td>
<td>AHB</td>
<td>646</td>
<td>&lt;0.5</td>
<td>/-/-</td>
<td>55</td>
<td>ND</td>
<td>/-/-</td>
</tr>
<tr>
<td>2</td>
<td>F/26</td>
<td>AHB</td>
<td>1680</td>
<td>0.9</td>
<td>+/-/-</td>
<td>76</td>
<td>ND</td>
<td>+/-/-</td>
</tr>
<tr>
<td>3</td>
<td>F/41</td>
<td>AHB</td>
<td>5185</td>
<td>NA</td>
<td>+/-/-</td>
<td>15</td>
<td>ND</td>
<td>+/-/-</td>
</tr>
<tr>
<td>4</td>
<td>F/26</td>
<td>CHB</td>
<td>159 (534)</td>
<td>0.9</td>
<td>+/-/-</td>
<td>27</td>
<td>ND</td>
<td>+/-/-</td>
</tr>
<tr>
<td>5</td>
<td>M/37</td>
<td>CHB</td>
<td>430</td>
<td>59</td>
<td>+/-/-</td>
<td>20 (88)</td>
<td>ND (147)</td>
<td>+/-/-</td>
</tr>
<tr>
<td>6</td>
<td>F/61</td>
<td>CHB</td>
<td>126 (472)</td>
<td>0.8</td>
<td>+/-/-</td>
<td>24</td>
<td>ND</td>
<td>+/-/-</td>
</tr>
</tbody>
</table>

Abbreviation: AHB, Acute Hepatitis B; CHB, Chronic Hepatitis B; ALT, Alanine aminotransferase; ND, not detected.

The purpose of present study was to compare the genetic and derived amino acid changes in full-length genome of HBV before and after HBeAg seroconversion from the patients with AHB or CHB.

**Amplification and sequencing analysis of the full-length of HBV DNA**

HBV nucleic acids were extracted from 200 μl of serum that had been stored at -80 °C using a High Pure Viral Nucleic Acid Kit (Roche, Penzberg, Germany). The full-length of the HBV genome was amplified with two overlapping fragments by nested polymerase chain reaction (PCR). The primers introduced by Gunther et al., Takahashi et al. and Sugauchi et al. were modified and used for amplification of the full-length of HBV DNA and sequencing PCR (Table 2). In brief, the long fragment about 3.2kb long was amplified with the primer pair, P1/P2, and the short fragment referred to the nick site including P1 and P2 primer regions was amplified with primer pair S1/S2. The second PCR were carried out with four primer pairs that overlapped and covered the whole length of the long fragment and short fragment. Briefly, the first round PCR was carried out in a tube containing 50 μl, which was composed of the following components: 0.2 μM concentration of each of the external primer, 0.2mM concentration of each of the four dNTP, 25 μl of 2×PCR buffer (Takara LA taq with GC buffer, Japan) and 10 μl of solution extracted from serum. The first round PCR was programmed to the first incubation of the samples at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, at 60°C for 1 min and then at 72°C for 1 min, with a 10 minutes extension step at 72°C. The
second PCR was programmed same to the first PCR except annealing temperature (52°C or 60°C) and the amounts of templates (5 μl of the first PCR products). Five micro liters of the second round PCR products were analyzed by electrophoresis in a 1% Agarose gel stained with ethidium bromide and visualized with an ultraviolet transilluminator. The size of PCR products was estimated according to the migration pattern of a 1 Kb DNA ladder (Promega Co. USA). Then, the second PCR products were purified from 1% agarose gel using QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany).

The purified PCR product was used as template for

Table 2. Primer pairs for the full-length of HBV DNA amplification

<table>
<thead>
<tr>
<th></th>
<th>First PCR (Full-length genome)</th>
<th>Second PCR</th>
<th>Sequencing PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long fragment</td>
<td>P1 (forward: nt 1821-1841)</td>
<td>T713 (forward: nt 1421-1440)</td>
<td>F1 (nt 1321-1841)</td>
</tr>
<tr>
<td></td>
<td>P2 (reverse: nt 1823-1806)</td>
<td>T717 (reverse: nt 1872-1892)</td>
<td>HCl1 (nt 2191-2210)</td>
</tr>
<tr>
<td>Short fragment</td>
<td>T511 (forward: nt 1255-1274)</td>
<td>T712 (forward: nt 1421-1440)</td>
<td>T53 (nt 2816-2835)</td>
</tr>
<tr>
<td></td>
<td>HIC24 (reverse: nt 2048-2072)</td>
<td>T711 (forward: nt 1421-1440)</td>
<td>#52-2 (reverse: nt 668-687)</td>
</tr>
</tbody>
</table>

Table 3. Comparison of genetic diversity of HBV DNA between AHB and CHB

<table>
<thead>
<tr>
<th></th>
<th>Full Genome</th>
<th>Core gene</th>
<th>Surface gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AHB</td>
<td>CHB</td>
<td>AHB</td>
</tr>
<tr>
<td>At clinical presentation</td>
<td>1.0</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.03±0.25</td>
<td>1.33±0.49</td>
<td>0.40±0.17</td>
</tr>
<tr>
<td>After HBeAg seroconversion</td>
<td>1.1</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.35±0.35</td>
<td>1.40±0.61</td>
<td>1.20±0.71</td>
</tr>
</tbody>
</table>

Abbreviation: AHB, Acute Hepatitis B; CHB, Chronic Hepatitis B; ALT, Alanine aminotransferase; NA, not available
sequencing PCR, which was carried out with the 11 primers using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, USA) by an automatic sequencing machine—ABI PRISM 3100 Genetic Analyzer. (HITACHI, Tokyo, Japan) All necessary precautions to prevent cross-contamination were performed, and negative controls were included in each assay.

**Phylogenetic analysis**

The genotype of the HBV was determined by phylogenetic analysis comparing with 34 reference strains from GenBank. The alignment of the full-length sequencing was performed by cluster method and the phylogenetic tree was constructed by neighbor joining method.

**RESULTS**

HBV DNA from all the six patients was extracted at clinical presentation and after HBeAg seroconversion except patient 3. In patient 3, its nucleotide sequence was determined only in clinical presentation because failure of HBV DNA amplification by PCR at the time of HBeAg seroconversion. All patients have been infected with genotype C2 and subtype adr of HBV.

To evaluate the genetic diversity between AHB and CHB,

**Figure 1.** Determinant of HBV genotype by phylogenetic analysis

![Phylogenetic tree](image)

HBV genotype was determined by phylogenetic analysis comparing with 34 reference strains from GenBank. The study subjects were labeled with.

we compared the full-length genome, core (C) gene and surface (S) gene of HBV according to clinical status. Because of lacking the serum sample before the onset of acute manifestation in AHB and CHB, we compared the genes with consensus HBV DNA sequences, which were deduced from eight HBV DNAs (Genotype C2) registered in GenBank. As results, the average genetic diversity of full-length HBV DNA was slightly higher in CHB than that in AHB at acute status (CHB vs. AHB (%): 1.35 ± 0.49 vs. 1.03 ± 0.25), and there were no remarkable differences after HBeAg seroconversion (CHB vs. AHB (%): 1.40 ± 0.61 vs. 1.35 ± 0.35) (Table 3 and Fig. 2). In contrast, in C and S gene, the average genetic diversity was markedly higher in patients with CHB than in those with AHB at presentation (CHB vs. AHB (%): C gene, 1.23 ± 0.23 vs. 0.40 ± 0.17 S gene, 1.03 ± 0.55 vs. 0.57 ± 0.38). But it didn’t show marked genetic distances after HBeAg seroconversion (CHB vs. AHB (%): core gene, 1.23 ± 0.31 vs. 1.20 ± 0.71 surface gene, 0.97 ± 0.61 vs. 0.75 ± 0.35) (Fig. 1). In AHB, average genetic diversity of full-length HBV DNA was slightly higher in sera after HBeAg seroconversion than in acute status (HBeAg (−) vs. acute status (%): 1.35 ± 0.35 vs. 1.03 ± 0.25). Especially, in C gene, the genetic diversity after HBeAg seroconversion was about 3 times higher than that in acute status (HBeAg (−) vs. acute status (%): C gene, 1.20 ± 0.71 vs. 0.40 ± 0.17). In S gene, it was also obtained similar result (HBeAg (−) vs. acute status (%): S

**Figure 2.** Genetic diversity between AHB and CHB during HBeAg seroconversion

![Genetic diversity graph](image)

The individual HBV DNA sequence was compared with the consensus HBV DNA sequence (genotype C2) deduced from eight of HBV DNA registered in GenBank using Molecular Evolutionary Genetics Analysis (MEGA4) software (http://www.megasoftware.net) (GenBank No.; AY641558: AY641560: AY641561: AF286594: DQ638978: X01587: AY123041: D000630). Abbreviation: AHB, Acute Hepatitis B; CHB, Chronic Hepatitis B
gene, 0.75 ± 0.35 vs. 0.57 ± 0.38), but not much like that in C gene (Fig. 2). In contrast, in CHB, there were no remarkable genetic differences in full-length HBV DNA, core and S gene between acute status and after HBeAg seroconversion (Fig. 2).

A total of 20 and 39 of nucleotide changes in core gene as well as 47 and 96 of nucleotide changes in surface gene were detected in AHB and CHB, respectively. Among them, 50% (10/20), 56.4% (22/39), 42.5% (20/47) and 45.8% (44/96) were non-synonymous mutations in these regions, respectively. Interestingly, the non-synonymous mutations detected in CHB were seen more frequent on putative human leukocyte antigen (HLA) class I/II restricted epitope-related codons than that in AHB (CHB vs. AHB: C gene, 68.2% (15/22) vs. 30% (3/10); S gene, 34% (15/44) vs. 14% (3/20) (Fig. 3).

In the study, we also analyzed the genetic diversity in a reactivated patient (Patient 5). In full-length HBV DNA and surface gene, the genetic diversity was not much different during HBeAg seroconversion and reactivation. In C gene, the genetic diversity was slightly higher after HBeAg seroconversion (1.1%) and lower after reactivation (1.3%) than in acute status (0.9%) (Fig. 4).

The basal core promoter/precore (BCP/PC) mutations were broadly detected in study patients and didn't show marked distinction in AHB and CHB (Fig. 5). The A1762T/G1764A double mutations in BCP region, which produce K130M and R131I amino acid substitutions in HBx protein, were found in the Patient 2 and 5. The G1764A single mutation was detected in two AHB patients (Patient 2 and 3) and in one CHB (Patient 5). However, alternatively C1766T/T1768A mutation was determined in these three patients. The PC mutation (G1896A), which makes a stop codon at codon 28 to eliminate the HBe protein production, was observed in the Patient 6.

**DISCUSSION**

In the study, the genetic diversity of full-length HBV DNA was not much different between CHB and AHB regardless of clinical status while comparing with consensus HBV DNA. However, there had existed less genetic variants in AHB compared with CHB in core and surface gene. In core gene, the genetic variants were more frequent in acute status of CHB than AHB, but not much different after HBeAg seroconversion. Especially, these variants tended to center on the genes coding core- or surface-epitope and were more frequent in CHB than that of AHB regardless of clinical status. This finding suggested that genetic mutations on these epitope-related codons might be mostly selected, not randomly, during the viral clearance under the immunopressure. Because the changes of amino acid on epitope decreased the binding capacity with HLA complex or recognition by T cell receptor. HBV with this kind of...
changes, in viral aspect, might escape from the immune system to survive. Interestingly, different pattern of genetic changes existed between AHB and CHB during HBeAg seroconversion. In AHB, the frequency of genetic or amino acid substitution was higher in sera after HBeAg seroconversion than in acute status. Particularly, this difference was remarkable in core gene. On the contrary, in CHB, there were no remarkable differences in full-length HBV DNA, core and surface gene before or after HBeAg seroconversion and still maintained the high frequency of genetic variants. Core protein was known as the main target of immune system and the mutations in core epitope-related codon were frequently detected during immune tolerance phase around the time of HBeAg clearance. Previously, Whalley SA et al., reported that the most variants in AHB appeared transiently and were rapidly replaced by wild-type sequences in hepatitis B resolvers that achieved clearance of serum HbsAg. This phenomenon might be related to the vigorous, efficient and short period of immune response, compared with CHB, or virus in AHB. Therefore, this different immune response might result in different pattern of genetic changes and it might be one of the mechanisms of the frequent reactivation after spontaneous HBeAg seroconversion in CHB.

Figure 5. Residue substitutions in core and surface protein.

Residue substitutions were compared with the consensus amino acid sequence (genotype C2) deduced from eight of HBVs registered in GenBank (GenBank No.: AY641558; AY641560; AY641561; AP286594; DQ683578; X01587; AY123041; D000630). Abbreviation: AHB, Acute Hepatitis B:

<table>
<thead>
<tr>
<th>Core protein</th>
<th>Core 1-20, 11-17, 18-27</th>
<th>Core 50-69</th>
<th>Core 111-125, 115-124, 117-125, 123-139, 136-140, 140-151</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
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<tr>
<td>Patient 2</td>
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<td></td>
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<tr>
<td>Patient 3</td>
<td></td>
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<td>Patient 4</td>
<td></td>
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<td></td>
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<td>Patient 5</td>
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<td>Patient 6</td>
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</table>

<table>
<thead>
<tr>
<th>Surface protein</th>
<th>HBs 1-20, 22-29</th>
<th>HBs 41-49</th>
<th>HBs 68-96, 95-104</th>
<th>HBs 172-180</th>
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<td>Patient 2</td>
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<td>Patient 3</td>
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<td>Patient 4</td>
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<td>Patient 5</td>
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<td>Patient 6</td>
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</table>

Figure 6. Nucleotide mutations in basal core promoter and precore region

Nucleotide sequences in basal core promoter were compared with the consensus HBV DNA sequence (genotype C2) deduced from eight of HBV DNA registered in GenBank (GenBank No.: AY641558; AY641560; AY641561; AP286594; DQ683578; X01587; AY123041; D000630). Abbreviation: AHB, Acute Hepatitis B; CHB, Chronic Hepatitis B.
that already possessed high frequency of genetic mutations before AE, although AE during CHB had similar clinical course to AHB that could lead to spontaneous HBeAg seroconversion. This speculation also might be explained by the result from a CHB patient (Patient 5), who reactivated with similar genetic diversity to the other two clinical statuses (Figure 5). Although, the genetic changes slightly decreased after reactivation, it still had higher genetic diversity than that in acute status of AHB.

Out of the mutations in core and surface gene, the BCP (A1762T/G1764A) mutation had been supposed to associate with severity and progression of liver disease and was detected in up to 90% of CHB infected with genotype C of HBV[27, 34]. In In vitro study, this double mutation increased viral replication and reduced HBeAg production[26-37]. In the present study, BCP mutation was broadly detected throughout the patients and seemed not to be related to different outcome between AHB and CHB.

In the present study, there were some limitations. First, lack of the HLA genotyping, we could not exactly explain the detected mutations in epitope-related codon, most of which were HLA-A2 restricted epitope, were result of the immune selection. However, considering the HLA-A2 haplotypic allele is one of the major HLA allele in Korea, it could be indirectly explained that such mutations in epitope might be due to the immune selection[38, 39]. Second, owing to the small size of samples and PCR based direct sequencing method, the statistics analysis of genetic diversity and the proportion of mutations between wild type and mutant type had not been available in this study.

Overall, the different consequences in AHB and CHB during HBeAg seroconversion might be associated with different pattern of genetic variations in HBV DNA that related to immune escape or viral replication.

Nucleotide changes were located within core or surface gene, in which resides several important immunogenic epitopes. Only a few HLA class-I restricted T-cell epitopes for HBV have been identified until now, and most are HLA-A2 restricted[40].

However, such phenomenon may be a result that coordinated by multiple mutations in epitope region to escape from immune system for survive or in some specific region that influencing the viral transcription and replication.

References

1) Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention


