Clinical and epidemiological characteristics of HBV genotype A carrier donors in Iwate Red Cross Blood Center

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Abstract

Among 492 donors identified as hepatitis B virus (HBV) carrier during the period between June 1994 and August 2000 in Iwate Red Cross Blood Center, randomly selected 200 underwent HBV genotyping. 8 (4%) had genotype A and 76 (38%) and 80 (40%) had genotype B and C, respectively. 36 (18%) could not be assigned to any HBV genotype because of failure in a HBV-DNA amplification procedure by polymerase chain reaction. Genotype A comprised 2 males and 6 females with average age of 28.6 ± 7.5 years and no regional deviation in residential district distribution were observed. In genotype A, frequency of donors below 30 years was significantly higher than that in genotype B and C (75% vs. 34%, 53%, P < 0.05) suggesting that spread of genotype A infection in the community might occur later than that of genotype B and C. In genotype A, although frequency of anti-HBe was 78% which stands between that of genotype B and C, all donors had wild type sequence at nt1896 (G) with nt1858 (C) regardless of HBe/anti-HBe status. Additionally subtyping of genotype A revealed that all of them belonged to HBV/Ae and also had nt1809 (G) and nt1812 (C) which agreed with previously reported sequences. Further investigation is needed to elucidate clinical and epidemiological features of HBV genotype A infection.

Key words: HBV, genotype A, subtype, HBV/Ae, HBV/Ae

Introduction

So far eight hepatitis B virus (HBV) genotypes, A to H, were isolated based on a divergence in entire nucleotide sequence of HBV genome\textsuperscript{10}. Previously, we reported that in Iwate prefecture most of HBV carrier donors belonged to genotype B or C and only less than 10% of them did to genotype A which is a predominant strain in North America\textsuperscript{a}. Although, to date, it has been widely accepted that maternal transmission at birth is the main cause for HBV carrier reproduction, recently Kobayashi et al\textsuperscript{a} reported that frequency of genotype A has been increasing among patients with acute hepatitis in Tokyo metropolitan area and following observation revealed some of them led to a persistent HBV infection. These findings have brought up a current problem which should be elucidated in relation to the mechanisms of spread of HBV infection in the community and prophylactic strategy in our country. Recently Hasegawa et al\textsuperscript{a} demonstrated a novel subtyping assay method of genotype A distinguishing HBV/Aa\textsuperscript{a} for African and Asian from HBV/Ae\textsuperscript{e} for European and its usefulness in epidemiological studies. In the present study, we tried to determine a subtype of eight HBV carrier donors who were proved to be infected with genotype A at blood donation to Iwate Red Cross Blood Center and investigate clinical and epidemiological features among them.
Materials and method

Donors
Among donors identified as hepatitis B virus (HBV) carrier during the period between June 1994 and August 2000, randomly selected 200 with equal distribution in sex, age and residential district underwent HBV genotyping. 8 (4%) had genotype A and 76 (38%) and 80 (40%) had genotype B and C, respectively. 36 (18%) could not be assigned to any HBV genotype because of failure in HBV-DNA amplification by polymerase chain reaction (PCR).

Serum HBV markers
The HBsAg titer was determined by reversed passive hemagglutination and HBeAg and anti-HBe were determined by enzyme-linked immunosorbent assay.

Quantification of HBV-DNA
HBV DNA was quantified using a TMA kit (Chugai Diagnostic Science Co. Ltd., Tokyo, Japan) according to the method of Kamisango et al. Briefly, from a 300 μl serum sample, the HBV specific base sequence was amplified as RNA mainly using two enzymes, two primers and a substrate. Furthermore, RNA was quantified based on chemiluminescence induced by single-stranded DNA probes labeled with acridinium ester that were complementary to the amplified RNA. The results were expressed as log genome equivalent (LGE) per ml.

HBV genotyping by RFLP analysis
HBV genotyping were performed using restriction fragment length polymorphism (RFLP) according to Mizokami et al. In brief, from a 100 μl of serum sample, DNA was extracted using SMITEST EX-R&D (Genome Science Laboratories Co. Ltd., Fukushima Japan). 85 S gene sequences were aligned and analyzed to determine the genotype-specific conserved sequences. After identifying the restriction enzyme sites, HBV DNA extracted were amplified by nested PCR with the first-round (sense :HBMF1 and antisense:HBMR2 primers) and then the second-round (inner sense :HBMF2 and antisense:HBMR2 primers). Restriction digestions were carried out using second-round PCR product and reactions were carried out with 10 units of AlwI, HphI, NciI, NlaIV or EarI (New England BioLabs). The digested PCR products were electrophoresed on agarose gel containing ethidium bromide. The RFLP pattern was then evaluated under ultraviolet light.

HBV genotype A subtyping
DNA amplification and direct sequencing in preS2/S region (nt3205-835) were performed according to Bowyer et al., which was followed by a construction of phylogenetic tree by six-parameter and neighbor-joining methods. An alignment of preS2/S region of 42 sequences obtained from GenBank was used as references of HBV/Ae or HBV/Aa, respectively. Then, to distinguish the subtype of genotype A, the computer program Hepatitis Virus Database Server System (http://c2as02.genes.nci.nih.gov/) was used.

Nucleotide sequencing in core promoter and precore region
Core promoter and precore genes were amplified and sequenced directly according to previously reported procedures.

Statistical analysis
Statistical analysis was performed using SPSS 12.0J for Windows (SPSS Inc., IL, USA). Fischer's exact probability test, Scheffe's test and Student's t-test were used to determine significant differences, and P values of less than 5% were considered significant.

Ethical considerations
Present study was carried out in obedience to the guideline of epidemiological studies devised by Ministry of Health, Labor and Welfare to prevent leakage of personal information and were conducted to dedicate to social profit.
Result

1) Comparison of the average age and age distribution in each genotype (Table 1, 2)

Table 1. Average age in each genotype

<table>
<thead>
<tr>
<th>Geotype</th>
<th>n</th>
<th>mean ± S.D. years</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>28.6 ± 7.5</td>
</tr>
<tr>
<td>B</td>
<td>76</td>
<td>37.7 ± 14.1</td>
</tr>
<tr>
<td>C</td>
<td>80</td>
<td>30.0 ± 12.8</td>
</tr>
</tbody>
</table>

\[**P<0.01\]

\[***P<0.001\]

However, no significant difference in the HBsAg titer was observed between genotype A and C.

3) Relationship between a genotype and HBeAg/anti-HBe status (Table 4)

Table 4. Frequency of HBeAg/anti-HBe in each genotype

<table>
<thead>
<tr>
<th>genotype</th>
<th>HBeAg n (%)</th>
<th>anti-HBe n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2 (25)</td>
<td>6 (75)</td>
</tr>
<tr>
<td>B</td>
<td>13 (17)</td>
<td>63 (83)</td>
</tr>
<tr>
<td>C</td>
<td>26 (35)</td>
<td>52 (65)</td>
</tr>
</tbody>
</table>

\[*P<0.05\]

The average age in genotype A was significantly younger than in genotype B (28.6±7.5 vs. 37.7±14.1 years, \(p<0.001\)). The difference of that between genotype B and genotype C also was significant (37.7±14.1 vs. 30.0±12.8 years, \(p<0.001\)). Moreover, frequency of donors less than 30 years in genotype A was significantly higher than that in genotype B and C (75% vs. 34%, 53%, \(p<0.05\)).

2) Comparison of the HBsAg titer in each genotype (Table 3)

Table 3. Average HBsAg titer in each genotype (RPHA)

<table>
<thead>
<tr>
<th>Geotype</th>
<th>n</th>
<th>mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>13.9 ± 2.5</td>
</tr>
<tr>
<td>B</td>
<td>76</td>
<td>9.6 ± 2.5</td>
</tr>
<tr>
<td>C</td>
<td>80</td>
<td>12.4 ± 1.8</td>
</tr>
</tbody>
</table>

\[**P<0.001\]

The HBsAg titer was significantly higher in genotype A and C than in genotype B (13.9±2.5, 12.4±1.8, vs. 9.6±2.5, respectively, \(p<0.001\)).

4) Comparison of the serum ALT level in each genotype (Table 5)

Table 5. Average serum ALT level each genotype

<table>
<thead>
<tr>
<th>genotype</th>
<th>n</th>
<th>mean ± S.D. (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>23.5 ± 16.6</td>
</tr>
<tr>
<td>B</td>
<td>76</td>
<td>26.2 ± 22.1</td>
</tr>
<tr>
<td>C</td>
<td>80</td>
<td>30.0 ± 31.7</td>
</tr>
</tbody>
</table>

No significant difference was observed among three genotypes (23.5±16.6 IU/L, 26.2±22.1 IU/L, 30.3±31.7 IU/L, respectively).

5) Backgrounds of genotype A donors (Table 6)

2 were males and 6 were females. No regional deviation in residential district, defined as the north, the south, the center and the coast in Iwate prefecture, was observed. Of 8 donors, 2 had HBeAg and 6 had anti-HBe. Non of them had neither or both. All but one had HBsAg titer of 213 and only one had that of 220 with HBeAg.

Direct sequencing of core promoter genes revealed that the mutations at nt1762 /1764 (A/G to T/A) was observed in 3 of 6 and all of
Table 6. Backgrounds of genotype A donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Sex</th>
<th>Age</th>
<th>District</th>
<th>ALT(U/L)</th>
<th>HBeAg</th>
<th>anti-HBe</th>
<th>HBsAg</th>
<th>nt.1762/1764</th>
<th>1809</th>
<th>1812</th>
<th>1858</th>
<th>1896</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M.</td>
<td>28</td>
<td>North</td>
<td>25</td>
<td>(-)</td>
<td>(+)</td>
<td>2^2</td>
<td>T/A</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>2</td>
<td>M.</td>
<td>25</td>
<td>Coast</td>
<td>63</td>
<td>(+)</td>
<td>(-)</td>
<td>2^8</td>
<td>A/G</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>3</td>
<td>F.</td>
<td>24</td>
<td>Center</td>
<td>19</td>
<td>(-)</td>
<td>(+)</td>
<td>2^2</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>4</td>
<td>F.</td>
<td>26</td>
<td>South</td>
<td>14</td>
<td>(-)</td>
<td>(+)</td>
<td>2^2</td>
<td>T/A</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>5</td>
<td>F.</td>
<td>22</td>
<td>Center</td>
<td>16</td>
<td>(-)</td>
<td>(+)</td>
<td>2^2</td>
<td>T/A</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>6</td>
<td>F.</td>
<td>31</td>
<td>Center</td>
<td>24</td>
<td>(-)</td>
<td>(+)</td>
<td>2^2</td>
<td>A/</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>7</td>
<td>F.</td>
<td>27</td>
<td>South</td>
<td>15</td>
<td>(-)</td>
<td>(+)</td>
<td>2^2</td>
<td>/</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>8</td>
<td>F.</td>
<td>46</td>
<td>North</td>
<td>12</td>
<td>(+)</td>
<td>(-)</td>
<td>2^2</td>
<td>A/G</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
</tbody>
</table>

them had anti-HBe. In remaining 3, 2 had wild type sequence at nt1762/1764 (A/G) with HBeAg and one had nt1762 (A) with anti-HBe. In the other two, nt1762/1764 could not be determined and both of them had anti-HBe.

Direct sequencing of precore genes was successfully performed in 7 of 8 and revealed that they all had wild type sequence at nt1896 (G) with nt1858 (C). Furthermore they all also had nt1809 (G) and 1812 (C).

6) A phylogenetic analysis of genotype A subtype (Figure 1).

All eight donors were assigned to HBV/Ae.

Discussion

In Japan, most HBV infection are caused by genotype B or C, while genotype A accounts for a small number of HBV carriers. Moreover the common transmission responsible for HBV carrier reproduction has been thought to occur via a vertical (mother-to-child) route at birth and most of the HBV carriers spend their entire lives as asymptomatic carriers presenting continuous anti-HBe. However, some carriers, especially who did not display a seroconversion from HBeAg to anti-HBe, continue to suffer liver disease. These carriers frequently develop advanced liver disease in which genotype C was more likely to be observed.

Kikuchi et al.* first reported the adults case who converted from acute initial genotype A infection to a HBV carrier state. In Europe, most HBV infections are genotypes A and D, and the likelihood of developing chronic liver disease is significantly higher in genotype A compared with geno-type D*. Therefore, although the number of HBV genotype A carriers in Japan is low, it is necessary to elucidate whether clinical and epidemiological differences between genotype B, C and A are present, especially in clinical course following the initial exposure to HBV.

The result of our present study demonstrated that genotype A had higher HBsAg titer and was significantly more prevalent in the age below 30 years compared with genotype B and C suggesting that spread of genotype A infection in the community might occur later than that of genotype B and C. Commonly it is known that HBsAg titer usually decreases according to age, namely, in inverse proportion to a term of persistent HBV infection, while the frequency of seroconversion from HBeAg to anti-HBe generally increases according to age. The former may support that occurrence of genotype A infection is a more recent event. However, the latter is a little different between genotype B and C, that is, genotype B carriers underwent seroconversion from HBeAg to anti-HBe earlier than genotype C carriers*. In our study, although a few samples were investigated, the frequency of anti-HBe in genotype A stands between that of genotype B and C. Although from only this small cross-sectional study, we could not speculate about long-term outcome of genotype A carriers, it was noteworthy that all donors had wild type sequence at nt1896 (G) regardless of HBe/anti-HBe status as far as we could investigate.
Figure 1. A phylogenetic tree constructed using HBV preS2/S sequences (nt3205-835) with known reference isolates.
Usually HBV carriers with genotype B or C who have had spent healthy lives have continuous anti-HBe and have mutation at nt1896 (A) that is a stop codon responsible for disturbance of HBeAg production. Trepo et al noted that in genotype A carriers, wild type sequence at nt1896 (G) is dominant because nt1838 directly opposite to nt1896 in encapsidation sequences in stem loop conformation in HBV genome is usually C (cytosin) which makes strong combination between them. Ohnuma et al also reported that 9 of 10 donors who were considered to be in a window period following acute genotype A infection belonged to HBV/Ae and presented with the wild type sequence at nt 1896 (G).

Hasegawa et al reported that all of HBV/Ae had nt1809 (G) and 1812 (C), whereas a majority of HBV/Aa had nt1809 (T) and 1812 (T) which suggests that these sequences are specific for each subtype of genotype A. All of our HBV/Ae donors had nt1809 and 1812 which agreed with previously reported sequences. These differences in the nucleotide sequence between HBV/Ae and HBV-Aa might be responsible for the clinical and epidemiological characteristics in each subtype.

Kobayashi et al investigated the familial clustering of HBV in genotype A patients’ family members and suggested that also in Japan, transmission of HBV genotype A likely to occur via horizontal rather than vertical route. Further clinical and epidemiological investigations are needed to clarify this problem including to check up the cases who shifts to chronic carrier state from initial infection. Blood donors usually consist of healthy individuals who have not experienced blood transfusion, hepatic injury or surgical treatment. Therefore among genotype A donors, it is also necessary to analyze familial occurrence of HBV infection by investigating serum HBV markers of family members. When the HBV carrier is identified among them, genotyping of HBV will give an important suggestion for the manner of genotype A transmission.

Hasegawa et al described that in the United States the majority of HBV isolates from African-Americans, Caucasians, and Hispanics were HBV/Ae and those from Asians and black South Africans were mainly HBV/Aa. Whereas, all our eight donors were HBV/Ae which was similar to African-Americans, Caucasians, and Hispanics in the United states. Up to present the reason HBV/Ae prevails in Japan including Iwate Prefecture remains unknown. The discussion about HBV genotype A infection hereafter will grow heated on various studies which will bring us the latest knowledge useful for clinical and epidemiological treatment of this agent.

Acknowledgment
The authors thank Dr. Masashi Mizokami, Department of Clinical Molecular Informative Medicine, Nagoya City University, Graduate School of Medical Sciences, for his kind advice in constructing a phylogenetic tree.

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和文要旨
岩手県赤十字血液センターにおけるHBV genotype A献血者の臨床的、疫学的特徴
1994年6月から2000年8月の期間に、岩手県赤十字血液センターで献血した492例のHBVキャリアの中から、性、年齢および居住地を均等に分布させ任意に抽出した200例を対象にし、genotypeを測定した。genotype Bが76例（38%）、genotype Cが90例（40%）、genotype Aが8例（4%）、PCRで増幅されず判定不能の例が36例（18%）であった。genotype Aは男性2例、女性6例、平均年齢は28.6±7.5歳、居住地は地域差はみとめられなかったが、30歳未満例の頻度が75%でgenotype B（34%）およびgenotype C（53%）に比し有意（P <0.05）に高率で、genotypeAの感染の拡大はgenotype Bおよびgenotype Cに比し後から起こった可能性が示唆された。またHBe抗原が23%、HBe抗体が75%に認められ、それらの頻度はいずれもgenotype Bとgenotype Cの中間であった。さらにHBe抗原・抗体と無関係にpre-core領域のnt1896は測定し得た7例全例がG（guanine）の野生型で、対のnt1858は全例C（cytosine）であった。Core promoter領域のnt1762/1764は測定し得た5例において、A/G（adenine/guanine）の野生型は2例でいずれもHBe抗原陽性、T/A（thymine/adeneine）の変異型は3例でいずれもHBe抗体陽性であった。subtype分類では全例HBV/Ac（ヨーロッパ型）で、nt1809は全例G（guanine）、nt1812は全例C（cytosine）で、報告されている既知のHBV Acと同様の塩基配列を示した。今後HBV感染の家族集積性の有無や初感染例の経過を追跡することにより、genotypeAの疫学的、臨床的特徴が明らかにされるものと考えられる。