Two Subtypes of Genotype B (Ba and Bj) of Hepatitis B Virus in Japan

Fuminaka Sugauchi,1,2 Hiromitsu Kumada,3 Hiroshi Sakugawa,4 Masafumi Komatsu,5 Hirofumi Niitsuma,7 Hisayoshi Watanabe,6 Yoshihiro Akahane,9 Hajime Tokita,10 Takanobu Kato,9 Hisayoshi Watanabe,6 Yoshihiro Akahane,9 Hajime Tokita,10 Takanobu Kato,9 Hisayoshi Watanabe,6 Yoshihiro Akahane,9 Hajime Tokita,10 Takanobu Kato,9

We have previously reported 2 subtypes of hepatitis B virus (HBV) genotype B, one of which has the recombination with genotype C over the precore region plus core gene (Ba) and the other of which does not (Bj). A restriction fragment–length polymorphism method with 2 endonucleases was newly developed for distinguishing between subtypes Ba and Bj and was applied to 313 carriers of HBV genotype B in Japan. Subtype Ba was detected in 38 (12%) and subtype Bj in 275 (88%) of the carriers of HBV genotype B. Hepatitis B e antigen in serum was found more frequently in patients with chronic infection with subtype Ba than in those with chronic infection with subtype Bj (8 [32%] of 25 vs. 25 [9%] of 273; \( P<.01 \)). The new method for distinguishing between Ba and Bj by restriction fragment–length polymorphism would be useful in examining the distribution of these 2 subtypes in situations in which HBV genotype B is prevalent.

Hepatitis B virus (HBV) has been classified into 7 genotypes, designated A to G, by a divergence of >8% in the entire genomic sequence, and these 7 genotypes have characteristic geographic distributions [1–3]. Very recently, an eighth genotype with a provisional designation of ‘H’ was proposed [4], but its classification as a new genotype or as a subtype of genotype F needs further phylogenetic analyses. There have been increasing lines of evidence for the influence of HBV genotypes in the manifestation of clinical liver diseases in hosts [5–11]. It has been reported that HBV genotype B, compared with genotype C, is associated with earlier seroconversion from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe) and with lower histological activity scores, and that genotype B is less prevalent than genotype C among patients with cirrhosis. These data indicate that HBV genotype B induces less-active and less-advanced liver disease than does genotype C, although recent reports [12, 13] indicate that there is no difference in long-term outcome between patients infected with genotype B and those infected with genotype C. In Taiwan, however, HBV genotype B is reported to enhance the development of hepatocellular carcinoma (HCC) in individuals younger than 50 years of age [7]; this is not the case in patients of the same age in Japan, however [8]. There is a possibility that such remarkable clinical differences among carriers of HBV genotype B are attributable to virological differences in the HBV strains that are infecting the hosts (including differences even in strains of the same genotype). In addition, host differences and the presence or absence of cofactors may make a difference.

The recombination between HBV genomes of distinct genotypes has been reported [14–17]. It is not known,
however, how they influence the outcome of HBV infections in hosts. Recently, we reported 2 subtypes of genotype B, one of which possesses the recombination with genotype C over the precore region plus core gene (Ba) and the other of which does not (Bj) [18]. By means of PCR-based restriction fragment–length polymorphism (RFLP) in the precore region, HBV of subtype Bj (HBV/Bj) was found to be endemic in Japan, and HBV of subtype Ba (HBV/Ba) is ubiquitous in the other countries in Asia [19]. Furthermore, in a case control study, HBeAg and core promoter mutation (T1762/A1764) are found to be more frequent in the carriers of HBV/Ba than in carriers of HBV/Bj [19].

Recently, 2 HBV/Bj isolates that produced discordant test results with the previously reported PCR-RFLP [19] were recovered from Japanese HBV carriers and sequenced (GenBank accession nos. AB106884 and AB106885). They possessed adenine as nucleotide (nt) 1838 or an insertion of adenine between nt 1837 and nt 1838 in the MseI restriction site used in the PCR-RFLP [19], which may give a false result for subtype Ba in a few HBV isolates of subtype Bj. To distinguish between HBV/Ba and HBV/Bj precisely, therefore, a novel PCR-RFLP method was developed involving 2 single nucleotide polymorphisms (SNPs) in the core region. The method was applied to isolates from 313 Japanese carriers of HBV genotype B to examine geographical and clinical differences between HBV/Ba and HBV/Bj infections in Japan.

**MATERIALS AND METHODS**

 Patients. A total 313 serum samples containing HBV genotype B were obtained from chronic carriers of HBV who visited 7 hospitals that were scattered from the north of the mainland of Japan to its southern islands, where HBV genotype B is prevalent [8]. The hospitals included: Yamagata University Hospital, Yamagata; Tohoku University Hospital, Sendai; Akita City Hospital, Akita; Toranomon Hospital, Tokyo; Tokyo National Hospital, Tokyo; Yamanashi Medical University Hospital, Yamanashi; and Ryukyu University Hospital, Okinawa. Serum samples from each of the hospitals were tested to determine alanine aminotransferase (ALT) and asparate aminotransferase (AST) levels and to determine the presence of HBeAg and antihBe using commercial kits (EIA, Dinabot; Tokyo, Japan). The presence of HBV genotype B in the serum samples was determined by ELISA on preS2-region products [20, 21], and the results were confirmed by PCR-RFLP of the S gene [22].

Chronic carriers were classified into 3 groups after they had been followed up for ≥12 months, as follows: (1) the asymptomatic carrier group, defined as noncirrhotic carriers who had no subjective symptoms and who maintained normal serum ALT levels throughout the follow-up period; (2) the chronic hepatitis group, defined as noncirrhotic carriers with ALT levels exceeding the upper limit of normal (defined as 35 U/L); and (3) the liver cirrhosis group, defined as patients with clinical evidence of cirrhosis revealed by ultrasonography (e.g., coarse liver architecture, nodular liver surface, and blunted liver edge) and evidence for hypersplenism (e.g., splenomegaly revealed by ultrasound and a platelet count of <100,000 platelets/mm³). The diagnosis of acute hepatitis was established by the loss of hepatitis B surface antigen from serum within 6 months after beginning the follow-up period. Patients who were coinfected with hepatitis C virus were excluded, and none had received antiviral treatments during the follow-up period. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the ethics committees of the institutions, and informed consent was obtained from each HBV carrier.

**PCR-RFLP for distinguishing between HBV/Ba and HBV/Bj.** Nucleic acids were extracted from 100 µL of serum that had been stored at −40°C using a DNA extractor kit (Genome Science Laboratory). HBV/Ba and HBV/Bj were determined using 2 kinds of PCR-RFLP. They were essentially the same method, but they used different enzymes. One of the methods was described previously and has a target in the precore region [18]. The other method was newly developed and targeted at the core region. In the new method, the first-round PCR was carried out with sense primer (PC1-HBV: 5′-CAT GCA ACT TTT TCA CCT CTG CCT-3′ [nt 1813–1836]) and antisense primer (COR-HBV: 5′-GAG TGC GAA TCC ACA CTC CA-3′ [nt 2285–2266]). The second-round PCR was performed with another sense primer (PC2-HBV: 5′-TGT TCA AGC CTC CAA GCT GTG-3′ [nt 1861–1881]) and COR-HBV. A portion (5 µg) of the amplification product of 425 base pairs (bp) in size was digested with 5 U of HpaI and Stul at 37°C for 3 h. Digests with HpaI and Stul were run on electrophoresis in 3.0% (weight/volume) agarose gel, stained with ethidium bromide, and examined for their sizes under the ultraviolet light.

For serum samples producing discrepant results by 2 different PCR-RFLP methods, the precore region plus core gene in the HBV DNA obtained from the samples was sequenced with primers reported previously for confirmation of HBV/Ba or HBV/Bj [23]. The standard precautions for avoiding contamination during PCR were exercised carefully, and a negative control serum sample was included in each run of tests to ensure the specificity.

**Statistical analyses.** Statistical differences were evaluated using the Mann-Whitney nonparametric test, Fisher’s exact probability test, and Student’s t test, when appropriate. Differences were considered significant for P values of <.05.

**RESULTS**

**PCR-RFLP for distinguishing between HBV/Ba and HBV/Bj.** When the 70 HBV genotype B isolates retrieved from the DDBJ/
Figure 1. Nucleotide sequences of a part of the core region in 40 hepatitis B virus (HBV) isolates of genotype B. Sequences of 20 HBV isolates of subtype Bj (HBV/Bj) and 20 HBV isolates of subtype Ba (HBV/Ba) are shown, which are representative of 29 HBV/Bj isolates and 41 HBV/Ba isolates reported previously [18]. All of the HBV/Bj isolates possessed nt 2020 of A and nt 2167 of T, which made a part of an Hpa I site (GTTAAC); in remarkable contrast, all HBV/Ba isolates possessed nt 2020 of G, which made a part of a Stu I site (AGGCCT), and nt 2167 of C.

EMBL/GenBank database were compared over the entire genome, it was found that all of the 29 HBV/Bj isolates possessed nt 2020 of A (A2020) and nt 2167 of T (T2167), creating an Hpa I site (GTTAAC [nt 2165–2170]). This was in remarkable contrast to all of the remaining 41 HBV/Ba isolates, which possessed G2020, giving rise to an Stu site (AGGCCT [nt 2019–2024]) in combination with C2167. Sequences of 20 HBV/Bj and 20 HBV/Ba isolates are shown in figure 1. Taking advantage of these 2 SNPs of A or G at nt 2020 and T or C at nt 2167, an RFLP method with 2 endonucleases was developed for distinguishing between subtypes Bj and Ba. PCR products of 425 bp (nt 1861–2285), amplified on HBV/Bj isolates, were split by HpaI digestion into 2 fragments of 306 bp and 119 bp, respectively, and those on HBV/Ba isolates were not. Conversely, the PCR products of 425 bp, amplified on HBV/Ba isolates, were broken down by StuI digestion into 2 fragments of 265 bp and 160 bp, respectively, and those on HBV/Bj isolates were not.

When the PCR-RFLP method was applied to 313 serum samples obtained from Japanese carriers of HBV genotype B, HBV/Ba was found in 35 (11%) of the samples and HBV/Bj was found in 253 (81%) of the samples; subtypes were indistinguishable in the remaining 25 samples (8%). To confirm the reliability of this PCR-EFLP method, the precore region plus core gene was sequenced directly on all 35 of the 35 HBV/Ba isolates, 44 of the 253 HBV/Bj isolates, and 25 of the 25 isolates of indistinguishable subtypes. All of the 44 HBV/Bj isolates determined by PCR-RFLP were confirmed for the Bj subtype by sequencing. One of the 35 (3%) isolates of HBV/Ba determined by PCR-RFLP, however, turned out to be of subtype Bj by sequencing. Of the 25 isolates for which subtypes were undistinguishable by PCR-RFLP, 4 (16%) of the isolates were classified into HBV/Ba and 21 (84%) of the isolates were classified into HBV/Bj by sequencing.

Figure 2. Geographic distribution of 298 patients who had chronic infection with hepatitis B virus (HBV) genotype B subtypes Ba and Bj and who visited 7 different hospitals throughout Japan. Subtypes of HBV genotype B were determined by the PCR restriction fragment–length polymorphism method involving 2 single nucleotide polymorphisms with restriction enzymes StuI and HpaI (see Materials and Methods).
### Table 1. Demographic, clinical, and virological characteristics of patients in Japan who were persistently infected with hepatitis B virus (HBV) genotype B, subtype Ba or Bj, by subtype.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HBV genotype B subtype</th>
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<tbody>
<tr>
<td></td>
<td>Ba (n = 25)</td>
</tr>
<tr>
<td>Age, mean years ± SD</td>
<td>42.0 ± 15.0</td>
</tr>
<tr>
<td>No. of male subjects/no. of female subjects</td>
<td>15/10</td>
</tr>
<tr>
<td>Liver disease</td>
<td></td>
</tr>
<tr>
<td>Asymptomatic carrier state</td>
<td>40</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>52</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>8</td>
</tr>
<tr>
<td>ALT level, mean U/L ± SD</td>
<td>75.8 ± 112.0</td>
</tr>
<tr>
<td>AST level, mean U/L ± SD</td>
<td>54.9 ± 63.9</td>
</tr>
<tr>
<td>Positive for HBeAg</td>
<td></td>
</tr>
<tr>
<td>All, no. positive/no. of patients (%)</td>
<td>8/25 (32)</td>
</tr>
<tr>
<td>Age &gt;30 years, no. positive/no. of patients (%)</td>
<td>6/19 (32)</td>
</tr>
<tr>
<td>Positive for anti-HBe</td>
<td></td>
</tr>
<tr>
<td>All, no. positive/no. of patients (%)</td>
<td>17/25 (68)</td>
</tr>
<tr>
<td>Age &gt;30 years, no. positive/no. of patients (%)</td>
<td>14/19 (74)</td>
</tr>
</tbody>
</table>

**NOTE.** ALT, alanine aminotransferase; anti-HBe, antibody to hepatitis B e antigen; AST, asparate aminotransferase; HBeAg, hepatitis B e antigen; NS, not significant.

### Table 2. Classification of 313 hepatitis B virus (HBV) genotype B isolates as subtype Ba or subtype Bj, as determined by PCR-RFLP methods involving either 1 single nucleotide polymorphism (previous method) or 2 single nucleotide polymorphisms (new method).

<table>
<thead>
<tr>
<th>Subtype classification by new method</th>
<th>Ba (n = 38)</th>
<th>Bj (n = 275)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba</td>
<td>34 (89)</td>
<td>10 (4)</td>
<td>44</td>
</tr>
<tr>
<td>Bj</td>
<td>0 (0)</td>
<td>238 (86)</td>
<td>238</td>
</tr>
<tr>
<td>Unclassified</td>
<td>4 (11)</td>
<td>27 (10)</td>
<td>31</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of isolates.

**chronic hepatitis and by geographic region.** There were 15 patients with acute hepatitis B and 298 patients with chronic hepatitis B who were infected with HBV genotype B and for whom subtypes Ba or Bj were determined by PCR-RFLP. Subtype Ba was detected in 13 (87%) of the 15 patients with acute hepatitis, which is significantly more frequently than it was detected among patients with chronic hepatitis (25 [8%] of 298 patients) (P < .01).

Figure 2 illustrates the geographic distribution of subtypes Ba and Bj on the basis of data reported from 7 Japanese hospitals for 298 patients with chronic hepatitis. There were regional differences in the distribution of Ba and Bj subtypes (P < .01). Subtype Ba was detected in 12 (21%) of the 58 patients in the Tokyo metropolitan area (combining data on patients from 2 hospitals), and it was detected less often in the other districts (in a total of 13 [5%] of 240 patients, with rates of detection in individual hospitals ranging from 0% to 9%).

**Comparison of HBV/Ba carriers with HBV/Bj carriers.** Table 1 compares the demographic, virological, and clinical characteristics of 25 carriers of HBV/Ba with those of 273 carriers of HBV/Bj. The mean age ± SD was significantly higher in carriers of HBV/Bj than in carriers of HBV/Ba (48.7 ± 14.4 years vs. 42.0 ± 15.0 years; P < .01). No differences were observed in clinical manifestations (in terms of transaminase levels and the distribution of chronic liver diseases) between carriers of subtype Ba and carriers of subtype Bj.

There were marked differences in HBeAg/anti-HBe status between the patients infected with the Ba subtype and those infected with the Bj subtype. The prevalence of HBeAg was significantly higher in patients infected with HBV/Ba than in those infected with HBV/Bj (32% vs. 9%; P < .01), and the difference was even more prominent among patients >30 years old (32% vs. 4%; P < .01). Conversely, anti-HBe was significantly less frequent in patients infected with HBV/Ba than in patients infected with HBV/Bj, both overall (68% vs. 88%; P < .01) and in patients >30 years old (74% vs. 94%; P < .01).

**Comparison of 2 PCR-RFLP methods for distinguishing HBV/Ba from HBV/Bj.** The previous PCR-RFLP method with restriction endonucleases SpeI and MseI [18] involved only 1 SNP of G or A at nt 1838. It was applied to the 313 isolates of HBV genotype B for which subtypes had been determined.
in this study (table 2). Of 38 HBV/Ba isolates, 34 (89%) were classified as HBV/Ba and 4 (11%) were unclassifiable using the previous PCR-RFLP method. Of the 275 HBV/Bj isolates, 238 (86%) were classified as HBV/Bj and 27 (10%) were unclassifiable using the previous PCR-RFLP method.

There were, however, 10 HBV/Bj isolates that were classified as HBV/Ba using the previous PCR-RFLP method. Sequences of a part of the precore region in the 10 HBV/Bj isolates classified as HBV/Ba by the previous PCR-RFLP are shown in figure 3. A point mutation from G to A at nt 1838 was detected in 3 of the isolates. An insertion of A between nt 1837 and 1838 in the remaining 7 isolates induced a frame-shift in the product of precore region and resulted in an HBeAg-negative phenotype. Because these mutations created a restriction site for MseI enzyme (TTAA), they gave a false result for HBV/Bj in HBV/Ba isolates by the previous PCR-RFLP method, which involved the MseI restriction site [19]. None of the 7 carriers of HBV/Ba in whom HBV DNA sequences with an insertion of A were detected had serum samples that tested positive for HBeAg; a serum sample obtained from the remaining carrier was not available for testing. Thus, the role of this single nucleotide insertion in inducing an HBeAg-negative phenotype was confirmed.

**DISCUSSION**

In this study, a novel PCR-RFLP method involving 2 SNPs, A or G at nt 2020 and T or C at nt 2167 in the core gene, was developed for distinguishing between HBV/Bj and HBV/Ba isolates. With use of this method, 288 (92%) of the 313 HBV isolates of genotype B were classified as subtype Bj or subtype Ba. The validity of this new PCR-RFLP method was confirmed by sequencing 104 HBV DNA samples, including those from all 35 HBV/Ba isolates and those from 44 of the 253 HBV/Bj isolates. Only a single HBV/Bj isolate possessed G2020 and C2167 and produced a false result for HBV/Ba by the new PCR-RFLP method. In comparison with the previous PCR-RFLP method, which involved 1 SNP [19] and which, on rare occasions, misidentified HBV/Bj isolates as HBV/Ba, the new PCR-RFLP was more specific in distinguishing between HBV/Ba and HBV/Bj isolates (table 2).

Precore sequences of 7 HBV/Bj isolates that had discordant results when analyzed using previous and new PCR-RFLP methods revealed a unique frame-shift insertion between nt 1837 and nt 1838 for aborting the expression of HBeAg (figure 3). Of the 7 individuals from whom these HBV/Bj isolates were recovered, 2 were asymptomatic carriers, and 5 were found to have chronic hepatitis; all 7 had negative results when tested for HBeAg. A similar insertion at this position in the precore region has been described in patients with HBeAg-negative HCC or chronic hepatitis [24, 25].

In the present study, HBV/Bj was detected in 275 (88%) of the 313 serum samples in Japan that contained HBV genotype B; these included 273 (92%) of the 298 serum samples obtained from individuals who were persistently infected with HBV. Patients with HBV/Ba infection were found to have clinical outcomes that were distinctly different from those of patients with HBV/Bj infection. HBV/Ba was detected significantly more frequently in the patients with acute, resolving (rather than persistent) HBV infection (13 [87%] of 15 vs. 25 [8%] of 298; P < .01). The association between recombinant genotypes of HBV and clinical manifestations has thus far not been looked into, although several recent studies indicate that HBV genotypes have clinical consequences [5–11]. The carriers of HBV genotype C have more cases of advanced liver disease than do carriers of HBV genotype B [7, 9]. Of possible relevance to this difference, the presence of the double mutation in the core promoter (T1762/A1764) is more frequent and the point mutation in the precore region (A1896) is less frequent in patients infected with HBV genotype C than in those infected with HBV genotype B [9, 10]; these mutations decrease and abolish, respectively, the expression of HBeAg. There are differences even among infections with HBV genotype B; such differences are associated with different subtypes. Thus, patients infected with HBV/Bj have a significantly lower prevalence of HBeAg and a higher prevalence of anti-HBe than do those patients with HBV/Ba infection, indicating that seroconversion takes place earlier in patients with HBV/Bj infection [19]. These results were corroborated by the findings of the present study (table 1).

The prevalence of HBeAg in carriers of HBV genotype B varies widely and depends on the geographical region from which it is reported. In Hong Kong, where HBV/Ba infection seems to account for almost all HBV genotype B infections
in patients with HCC aged >30 years old. By contrast, in Japan, where HBV/Bj infection accounts for most HBV genotype B infections, HBeAg was detected in only 10% of patients infected with HBV genotype B who were >30 years old [8]. These differences in the prevalence of HBeAg may be explained by different distributions of HBV/Ba and HBV/Bj subtypes between the 2 countries.

No significant differences were observed in the severity of liver disease between patients with HBV/Ba infection and those with HBV/Bj infection (table 1). Only a limited number of patients with HBV/Ba infection were investigated, however, none of whom had HCC. Evidence has been accumulating that indicates the influence of HBV genotypes on the development of severe chronic liver disease, including HCC [6–11], although there are some arguments against this [12, 13]. In Taiwan, HBV genotype B infection may be responsible for the development of HCC in carriers of HBV genotype B aged <50 years [7]. By outstanding contrast, in Japan, HBV genotype B is not found in patients with HCC aged <60 years [8]. Because HBV genotype B isolates from Taiwan are HBV/Ba [19], there is a possibility that the recombination between genotypes B and C in Taiwanese HBV/Ba isolates might have enhanced an early development of HCC there. It does need to be pointed out, however, that the development of HCC is probably multifactorial, given the possible presence of chronic inflammatory changes in the liver that would enhance hepatocarcinogenesis. Furthermore, specific environmental hepatotoxins, such as aflatoxin [26] and alcohol [27], probably contribute to high incidence rates of HCC in some areas of the world. HBV genotypes influence the response to lamivudine in the treatment of patients with chronic hepatitis B [28, 29]. The response may differ even among patients infected with HBV of the same genotype. Recently, Akuta et al. [30] reported that the response to lamivudine was less frequent in patients infected with HBV/Ba than in those infected with HBV/Bj. Taken altogether, a large-scale study, in collaboration with many countries in which HBV/Ba is prevalent, is required to evaluate any clinical differences between HBV/Ba and HBV/Bj infections.

In conclusion, a new PCR-RFLP method involving 2 SNPs was developed specifically for distinguishing between HBV/Ba and HBV/Bj isolates. Of these 2 subtypes of HBV genotype B, HBV/Bj was the predominant subtype throughout Japan and was associated with the development of acute liver disease less frequently than was HBV/Ba. The recombination with HBV genotype C in HBV/Ba would contribute to a delayed seroconversion of HBeAg in individuals who are infected with it. The new PCR-RFLP would be useful in evaluating clinical, epidemiological, and virological differences between HBV/Ba and HBV/Bj infections in countries in which HBV genotype B is prevalent.

References

10. Chu CJ, Hussain M, Lok AS. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. Gastroenterology 2002; 122:1756–62.