Genotype and phylogenetic characterization of hepatitis B virus among multi-ethnic cohort in Hawaii

Mayumi Sakurai, Fuminaka Sugauchi, Naoky Tsai, Seiji Suzuki, Izumi Hasegawa, Kei Fujiwara, Etsuro Orito, Ryuzo Ueda, Masashi Mizokami

AIM: Hepatitis B virus (HBV) genotypes in carriers from Hawaii have not been evaluated previously. The aim of the present study was to evaluate the distribution of HBV genotypes and their clinical relevance in Hawaii.

METHODS: Genotyping of HBV among 61 multi-ethnic carriers in Hawaii was performed by genetic methods. Three complete genomes and 61 core promoter/precore regions of HBV were sequenced directly.

RESULTS: HBV genotype distribution among the 61 carriers was 23.0% for genotype A, 14.7% for genotype B and 62.3% for genotype C. Genotypes A, B and C were obtained from the carriers whose ethnicities were Filipino and Caucasian, Southeast Asian, and various Asian and Micronesian, respectively. All cases of genotype B were composed of recombinant strains with genotype C in the precore plus core region named genotype Ba. HBeAg was detected more frequently in genotype C than in genotype B (68.4% vs 33.3%, P<0.05) and basal core promoter (BCP) mutation (T1762/ A1764) was more frequently found in genotype C than in genotype B. Twelve of the 38 genotype C strains possessed C at nucleotide (nt) position 1858 (C-1858). However, there was no significant difference in clinical characteristics between C-1858 and T-1858 variants. Based on complete genome sequences, phylogenetic analysis revealed one patient of Micronesian ethnicity as having C-1858 clustered with two isolates from Polynesia with T-1858. In addition, two strains from Asian ethnicities were clustered with known isolates in carriers from Southeast Asia.

CONCLUSION: Genotypes A, B and C are predominant types among multi-ethnic HBV carriers in Hawaii, and distribution of HBV genotypes is dependent on the ethnic background of the carriers in Hawaii.
Genotyping of HBV
The serum samples were stored at -20 °C until assay was performed. Serum DNA was extracted from 100 µL of serum using a DNA extractor kit (Genome Science Laboratory, Fukushima, Japan). The genotypes of HBV were determined by enzyme-linked immunosorbent assay (ELISA) (HBV GENOTYPE EIA, Institute of Immunology Co., Ltd., Tokyo, Japan) with monoclonal antibodies that are type-specific to epitope in the preS2-region product[15]. If the result of ELISA was indeterminate, the genotypes were detected by restriction fragment length polymorphism (RFLP), as previously described[16]. Genotype B was classified into 2 subgroups, “Ba” which has a recombinant sequence of genotype C in the precore/core region, or “Bj” which does not have it, by the method reported previously[12]. Genotype G of HBV was detected by PCR with hemi-nested primers deduced from the fragment length polymorphism (RFLP), as previously was indeterminate, the genotypes were detected by restriction method reported previously[12]. Genotype G of HBV was detected by PCR with hemi-nested primers deduced from the unique insertion of 36 nucleotides (nt) in the core gene that is specific to this genotype[15].

Sequencing of HBV genome
Three complete genomes and 61 core promoter/precore regions of HBV were amplified by polymerase chain reaction with several primer sets, as previously described[18]. Amplified HBV DNA fragments were sequenced directly by dideoxy sequencing using a Taq Dye Deoxy Terminator cycle sequencing kit with an automated fluorescent 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). These nucleotide sequences were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers AB105172- AB105174.

Molecular evolutionary analysis of HBV
The pairwise nucleotide sequences were aligned using the CLUSTAL W program[19]. The genetic distances were calculated with the 6-parameter method, and the phylogenetic tree was constructed by the neighbor-joining method[20] using the ODEN program (version 1.1.1)[21]. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1 000 times. Reference sequences of HBV, shown as accession numbers, were obtained from the DDBJ/EMBL/ GenBank database.

Statistical analyses
Statistical differences were evaluated using the Mann-Whitney nonparametric test, the Fisher’s exact probability test and the Student’s t-test where appropriate. Differences were considered significant for a P-value less than 0.05.

RESULTS
Genotypes of HBV
The distribution of HBV genotypes among 61 multi-ethnic carriers in Hawaii was 14(23.0%) for genotype A, 9(14.7%) for genotype B and 38(62.3%) for genotype C. All the 9 genotype B strains were found to be Ba that has a recombinant sequence of genotype C in precore/core region. Genotypes D, E, F and G were not found in this study.

Comparison with clinical characteristics among HBV genotypes
Clinical and serological characteristics were compared among the patients infected with genotypes A, B and C (Table 1). Patients with HBV genotype A were of Filipino (n=10), Caucasian (n=3), and Hawaiian/Chinese (n=1) ethnicities. Genotype B was found in patients of Chinese (n=4), Taiwanese (n=2), Vietnamese (n=2), and Hawaiian/Chinese (n=1) ethnicities. Genotype C was found in the patients whose ethnic backgrounds were various Asian (n=33), Micronesian (n=3), and Caucasian (n=2). There were no significant differences in terms of the age, gender, serum AST, ALT, ALP, γ-GTP and the clinical stages of liver disease among them. The proportion of HBeAg positive-phenotype in patients with genotypes A, B

Table 1 Comparison of clinical backgrounds in carriers with HBV genotypes A, B and C

| Features             | Genotype n(%) |
|----------------------|---------------|
|                      | A (n=14)      | B (n=9)      | C (n=38)     |
| Age (mean±SD, yr)   | 49.1±14.5     | 46.9±13.0    | 43.0±11.9    |
| Gender (male:female)| 10:4          | 4:5          | 21:17        |
| Race                 |               |              |              |
| Asian                |               |              |              |
| Mainland Chinese     | 0(0)          | 5(33)        | 10(67)       |
| Philippine           | 10(83)        | 0(0)         | 2(40)        |
| Taiwanese            | 0(0)          | 2(50)        | 2(50)        |
| Hong Kong            | 0(0)          | 0(0)         | 8(100)       |
| Vietnamese           | 0(0)          | 2(40)        | 3(60)        |
| Korean               | 0(0)          | 0(0)         | 7(100)       |
| Japanese             | 0(0)          | 0(0)         | 1(100)       |
| Micronesian           | 0(0)          | 0(0)         | 3(100)       |
| Polynesian            | 1(100)        | 0(0)         | 0(0)         |
| Caucasian            | 3(60)         | 0(0)         | 2(40)        |
| Liver disease        |               |              |              |
| ASC                  | 2(14)         | 3(33)        | 9(24)        |
| CH                   | 10(71)        | 5(56)        | 24(63)       |
| LC                   | 2(14)         | 1(11)        | 1(3)         |
| HCC                  | 0(0)          | 0(0)         | 4(11)        |
| HBeAg (+)            | 8(57)         | 3(33)*       | 26(68)*      |
| Laboratory finding   |               |              |              |
| AST (U/ L)           | 63.3±42.2     | 56.6±58.7    | 54.7±81.9    |
| ALT (U/ L)           | 41.4±31.6     | 34.8±27.0    | 37.3±59.7    |
| ALP (U/ L)           | 359.9±139.4   | 287.3±140.2  | 328.0±166.2  |
| γ-GTP (U/ L)         | 121.0±121.3   | 55.9±37.2    | 66.8±103.0   |

* P<0.05 between genotypes B and C. ASC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; γ-GTP: γ-glutamyl transpeptidase.
and C was 57.1%, 33.3% and 68.4% respectively, with a significant difference observed between genotypes B and C (P<0.05). HCC was found in 10.5% of patients with genotype C.

**Comparison with core promoter/precore sequences**
The frequency of mutation in core promoter (nt 1762/1764) and precore (nt 1858 and nt 1896) region was compared among genotypes A, B and C (Table 2). No significant differences in the frequency of the core promoter mutants were found among genotypes A, B and C (21.4%, 44.4%, and 57.9% respectively). Precore stop mutation (A-1896) was detected in genotypes B and C (33.3% and 21.1%) but not detected in genotype A. Sequence analysis of the mutation at nt 1858 in the precore region showed that all genotype B strains and 68% of genotype C strains possessed a T nucleotide (T-1858). In contrast, all genotype A strains had a C nucleotide in this region (C-1858).

**HBV genotype C with C-1858 or T-1858**
In order to clarify the significance of nucleotide variety (C or T) at nt 1858 in clinical and virological characteristics, 12 genotype C strains with C-1858 were compared to that of 26 strains of genotype C with T-1858 (Table 3). All strains of both groups were obtained only from carriers whose ethnic background is Asian. HBeAg positive-phenotype was more frequent in genotype C patients with C-1858 than in those with T-1858 (83.3% vs 61.5%). The precore stop mutation (A-1896) was found in 30.8%(8/26) of those with genotype C with nucleotide T-1858, but not in those subjects with the C-1858 nucleotide. There were no significant differences in frequencies in terms of the age, gender, serum AST, ALT, ALP, γ-GTP and the clinical stages of liver disease and BCP mutation between them.

**Phylogenetic analysis**
To clarify the phylogenetic characterization of genotype C with C-1858, the complete genome of three HBV strains in carriers was sequenced. These subjects were from Micronesia (HI-1), Hong Kong (HI-2) and Vietnam (HI-3). Molecular evolutionary analysis was also conducted (Figure 1). Of them, one strain (HI-1) was clustered into a subgroup of genotype C with T-1858 from Polynesian with significant bootstrap values. The HI-2 and HI-3 strains were clustered into a subgroup of genotype C from Thailand and Vietnam, and separated from

### Table 2
Comparison of core promoter/precore sequences between genotypes A, B and C

| Mutation | Genotype n (%) |
|----------|----------------|
|          | A (n=14) | B (n=9) | C (n=38) |
| CP mutation | Double mutation | 3(21) | 4(44) | 22(58%) |
| PC mutation nt 1858 | Cytosine (C) | 14(100) | 0(0) | 12(32) |
|            | Thymine (T) | 0(0) | 9(100) | 26(68) |
| nt 1896 | Guanine (G) | 14(100) | 6(67) | 30(79) |
|            | Adenine (A) | 0(0) | 3(33) | 8(21) |

CP: Core promoter; PC: Precore; nt: Nucleotide.

### Table 3
Clinical and virological characteristics in carriers of genotype C with C-1858 or T-1858

| Mutation n(%) |
|--------------|
| C-1858 (n=12) | T-1858 (n=26) |
| Age (mean±SD, yr) | 41.7±13.0 | 43.5±11.6 |
| Gender (male:female) | 9:3 | 12:14 |
| Race |Asian | 3(30) | 7(70) |
|      |Mainland Chinese | 0(0) | 2(100) |
|      |Filipino | 0(0) | 2(100) |
|      | Taiwanese | 0(0) | 2(100) |
|      | Hong Kong | 3(38) | 5(63) |
|      | Vietnamese | 2(67) | 1(33) |
|      | Korean | 1(14) | 5(66) |
|      | Japanese | 0(0) | 1(100) |
|      | Micronesian | 2(100) | 0(0) |
|      | Polynesian | 0(0) | 0(0) |
|      | Caucasian | 1(50) | 1(50) |
| Liver disease |ASC | 2(17) | 10(29) |
|                |CH | 8(67) | 21(60) |
|                |LC | 1(8) | 1(3) |
|                |HCC | 1(8) | 3(9) |
| Laboratory finding |HBeAg (+) | 10(83) | 16(62) |
|                  |AST (U/L) | 34.9±17.0 | 63.8±97.6 |
|                  |ALT (U/L) | 23.2±11.2 | 43.8±71.3 |
|                  |ALP (U/L) | 334.6±186.3 | 324.9±157.0 |
|                  |γ-GTP (U/L) | 87.8±167.3 | 57.2±48.4 |
| CP mutation | Double mutation | 6(50) | 16(62) |
| PC mutation |A-1896 | 0(0) | 8(31) |

ASC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; γ-GTP: γ-glutamyl transpeptidase; CP: Core promoter; PC: Precore.
those strains of China and Japan. Moreover, the strains from Thailand and Vietnam had separate branches, and Hawaiian strains were clustered into the branch with Vietnam strains. Interestingly, all strains from Vietnam had C-1858, and those from Thailand had T-1858.

### DISCUSSION

The findings of the present study indicate that HBV genotypes A, B and C are prevalent in Hawaii, and genotype C is the major genotype. Most cases of genotype A were found in immigrants from the Philippines and countries known to be prevalent regions for genotype A. Genotype B was found only in immigrants from Asian regions where genotype B was endemic. In addition, genotype C was obtained from immigrant who came from various Asian countries, where genotype C was prevalent. These results indicate that the distribution of HBV genotypes in Hawaii is associated with their respective ethnic background.

Recently, it was reported that genotype B could be classified into the Bj (j standing for Japan) and Ba (a standing for Asia) subgroups. Ba shared a genomic sequence with genotype C in the precore/core region, which was prevalent in Asian countries. Bj was restricted to Japan, and did not have this recombination. It was shown that Ba induces more severe liver disease than Bj due to delayed seroconversion of HBeAg. In this study, we found that genotype B, prevalent in Hawaii, was classified as Ba because they were all obtained from carriers with Asian ethnicity (excluding Japanese). In addition, the rate of positive HBeAg (33.3%) and basal core promoter (BCP) mutation (44.4%) in patients of Hawaii infected with genotype B were higher than those in Japanese patients with genotype B. This result is also consistent with a previous report.

The double mutation in the core promoter, A-to-T mutation at nt 1762 and G-to-A mutation at nt 1764, was associated with reduced synthesis of precore mRNA. In addition, it has been reported that the BCP mutation was associated with the progression of liver disease. In this study, although it was not significant, BCP mutation was detected more frequently in genotype C than in genotype B. This result supports our previous observation that the BCP mutation was significantly more frequent in genotype C patients than in genotype B patients. In addition, the present study demonstrated that the proportion of HBeAg positivity in genotype C was significantly higher than that in genotype B (68.4% vs 33.3%). However, our study could not show the clinical difference between genotypes B and C most likely due to the small number of patients studied. In the future, a case-controlled study in multi-ethnic carriers with larger samples is required to confirm

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**Figure 1** A phylogenetic tree based on the complete genome sequences of hepatitis B virus with 27 reference strains. Isolate names of HI-1, HI-2 and HI-3 were sequenced in this study. The length of the horizontal bar indicates the number of nucleotide substitutions per site. Numbers beside the main roots are the results of bootstrap analysis.
if genotype C could induce more severe liver disease than genotype B\textsuperscript{9,10}.

Interestingly, we detected 12 strains of genotype C possessing C-1858 in Hawaii. HBV strain with C-1858 could prevent the A-1896 precore mutation from shutting off the synthesis of \textit{HBcAg}\textsuperscript{26}. This C-1858 variant was frequently found in genotypes A and F\textsuperscript{26}. In genotype C, the C-1858 variant was observed in Southeast Asian patients, and the phylogenetic origin of genotype C with C-1858 variant has been reported from Vietnam recently. In this study, the complete genomes of 3 genotype C strains with C-1858 were sequenced. One strain obtained from a Micronesian patient with C-1858 was clustered with previously reported Polynesian strains with T-1858. This indicates that both the C-1858 and T-1858 strains of genotype C are endemic to South Pacific countries. Two other strains obtained from patients with Hong Kong and Vietnamese ethnicities were clustered with the strains of genotype C from Southeast Asian countries. This result is consistent with geographic distribution of HBV genotype\textsuperscript{18}. Interestingly, in this subgroup, there were 2 variants of strains, one had C-1858, prevalent in Vietnam, and the other had T-1858, prevalent in Thailand.

The clinical significance of C-1858 or T-1858 among genotype C is not well known. In this study, we compared the clinical and laboratory characteristics between C-1858 and T-1858 variant, but there were no significant differences between them. The number of patients was not enough to clarify the importance of this variation, and its significance for clinical characteristics remains unknown. Further studies would be required using larger numbers of samples.

In conclusion, genotypes A, B and C are the predominant types among multi-ethnic HBV carriers in Hawaii, and the distribution of these genotypes is dependent on the ethnic origin of the carriers in Hawaii. The influence of these genotypes on the clinical manifestations of these HBV carriers in Hawaii is not well defined due to the current small sample size. Case-controlled study with larger cohorts from our unique community is needed.

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