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Complete Genome Sequence and Phylogenetic Relatedness of Hepatitis B Virus Isolates in Papua, Indonesia

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Each hepatitis B virus (HBV) genotype and subgenotype is associated with a particular geographic distribution, ethnicity, and anthropological history. Our previous study showed the novel HBV subgenotypes C6 (HBV/C6) and D6 (HBV/D6), based on the S gene sequences of isolates in Papua, Indonesia. The present study investigated the complete genome sequence of 22 strains from Papua and subjected them to molecular evolutionary analysis. A phylogenetic analysis revealed that 9 out of 22 strains were classified as HBV/C6, 3 strains as HBV/D6, and 9 strains as HBV/B3. A particular strain positioned between HBV/B3 and HBV/B5 remained unclassifiable into any known subgenotypes. This strain showed high homology with HBV/C5 from the Philippines in the core region and was thought to have undergone genetic recombination with HBV/C5. Further studies are needed to determine whether this strain belongs to a new subgenotype of HBV/B. Based on the amino acid alignment, HBV/C6 has subgenotype specific variations (G18V and V47M) in the S region. HBV/C6 strains were more closely related in terms of evolutionary distance to strains from the east Asia and Pacific regions than those found in southeast Asia. HBV/D6 strains were most closely related to strains from the Western countries (HBV/D3) rather than those from Asia and Papua New Guinea. In conclusion, we have confirmed by complete sequence analysis that two novel HBV subgenotypes, HBV/C6 and HBV/D6, are prevalent in Papua, Indonesia.

Hepatitis B virus (HBV) is an etiologic agent of chronic liver disease, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, and this poses major health problems worldwide, especially in Asian Pacific countries (7, 10).

HBV strains that infect humans show genetic and antigenic heterogeneity, and eight genotypes, designated A to H, have been identified so far by molecular evolutionary analysis (19). The HBV genotypes have distinct geographical distributions, which are associated with anthropological history (4, 13, 20, 31). Furthermore, previous studies have demonstrated the presence of several subgenotypes within the widely spread genotypes. HBV genotype B (HBV/B) is classified into six subgenotypes, B1 to B6, B1 dominating in Japan, B2 in China and Vietnam, B3 in Indonesia, B4 in Vietnam, B5 in the Philippines, and B6 in the Arctic (3, 16, 23, 24, 26, 27). As for HBV/C, C1 is common in southeast Asia, C2 in east Asia, C3 in Oceania, C4 in Aborigines, and C5 in the Philippines (15, 26). HBV/D has a worldwide distribution, with its highest prevalence in the Mediterranean region, and is classified as D1 to D5 (1, 15, 17). Our previous study revealed novel subgenotypes (HBV/C6 and HBV/D6) based on the S gene sequence of HBV isolates in Papua, Indonesia, where HBV infection is endemic (9).

HBV genotyping with the S gene sequence is, in general, consistent with the genotyping of the full genomic sequence, and therefore, HBV genotypes can be assigned based upon S gene sequences (11, 16, 19). Subgenotype classification, however, may not be applicable to some HBV strains on the basis of the S region sequence alone (9, 14, 15). Accordingly, complete genome sequences are more reliable for the analysis of genotype and subgenotype classification for HBV (14). The data on the complete genome sequences of the HBV strains found in Papua are scant. The present study aimed to evaluate the HBV genotypes and subgenotypes present among the Papuan population using complete genome sequences. In addition, the phylogenetic relatedness of HBV strains isolated from Papua was assessed.

MATERIALS AND METHODS

Source of HBV DNA. A total of 45 HBsAg-positive serum samples were obtained from blood donors screened at the Red Cross Blood Center, Papua, Indonesia. Twenty-seven (2 HBV/B, 23 HBV/C, and 2 HBV/D) of them were derived from the previous study, in which 2 novel subgenotypes were identified on the basis of S gene sequences (9). To examine more isolates of HBV/B and HBV/D, 18 samples (14 HBV/B and 4 HBV/D) were analyzed for their HBV subgenotypes on the basis of their S gene sequence and enrolled in this study. Ethnically, 13 samples (5 HBV/B and 8 HBV/C) were from Papuan inhabitants, and 9 samples (5 HBV/B, 1 HBV/C, and 3 HBV/D) were from non-Papuan inhabitants (Table 1). The national census showed that about 80% of the population in this area was Papuan. Ethnicity was determined by birthplace through three generations. Informed consent for participation in this study was obtained from each individual.
Complete genome sequencing. DNA was extracted from 100 μl of serum that had been stored at −80°C using a DNA extractor kit (QIAamp DNA blood mini kit; Qiagen, Tokyo, Japan). The complete HBV genome sequences were determined by the method reported previously (28). In brief, the complete genome of HBV was first amplified as two overlapping fragments, a 3,200-bp amplicon (fragment A) and a 462-bp amplicon (fragment B) that covers the remaining HBV genetic recombination was investigated using the bootscan analysis implemented in the SimPlot software (25). The result obtained was consistent with the data from the SimPlot analysis (data not shown).

Phylogenetic analysis. Reference sequences were retrieved from DDBJ/EMBL/GenBank databases. Alignments were done using CLUSTAL X software, the phylogenetic trees were constructed by the neighbor-joining method (22), and bootstrap resampling was performed 1,000 times. These analyses were carried out using the Molecular Evolutionary Genetics Analysis (MEGA) software program (http://www.megasoftware.net). Subgenotypes were assigned as described previously (15, 18, 23, 24, 29).

Evidence for HBV genetic recombination. HBV genetic recombination was investigated using the bootscan analysis implemented in the SimPlot software program (2, 6, 8, 12, 21, 32). Four sequences were used to detect phylogenetically informative sites: the putative recombinant sequence (P18; HBV/B), two consensus sequences of the parental genotypes (D23678 for HBV/B and AB241109 for HBV/C), and a consensus sequence as an outgroup (X75658 for HBV/F) (27, 30). Informative sites were identified where two sequences shared.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank databases under accession no. AB493827 to AB493848.

RESULTS

Genotypes and subgenotypes of HBV based on the complete genome sequences and phylogenetic relatedness. Of 45 serum samples obtained from blood donors in Papua, HBV complete genome sequences were successfully determined for 22 samples (10 HBV/B, 9 HBV/C, and 3 HBV/D; 21 males and 1 female; mean age, 31.5 years; age range, 18 to 45 years). Their demographic and genetic characteristics are summarized in Table 1. Phylogenetic analyses of the complete genome sequences of the 22 strains were conducted by comparing them with the complete genome sequences of 52 HBV strains from DDBJ/EMBL/GenBank (Fig. 1A). They were classified as HBV/B (10 strains), HBV/C (9 strains), and HBV/D (3 strains).

**HBVB in Papua.** Nine of the 10 HBV/B strains were grouped into subgenotype B3, which formed a cluster including previously reported Indonesian isolates (Fig. 1A). The remaining one strain (P18) was distinctly positioned between HBV/B3 and HBV/B5 and had a high homology with HBV/C5 in the core region (Fig. 1B). SimPlot analysis was applied to determine any possible recombination and its sites between HBV/B and HBV/C5. The bootscan analysis revealed that the P18 strain had undergone a recombination event with HBV/C5 in the pre-C/C region (Fig. 2A). The recombination breakpoints were estimated at nucleotides 1873 and 2437, respectively. To further confirm the recombination event, we performed analysis using a Web-based genotyping resource (Genotyping tool, NCBI [http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi]). The result obtained was consistent with the data from the SimPlot analysis (data not shown).

A previous study from Nusa Tenggara, Indonesia, reported the presence of the novel HBV subgenotype B7 (18). Strains of this subgenotype, however, are genetically close to the HBV/B3 strains in this study. Five HBV/B3 strains in particular (25UC, 33UC, P14, P41, and P48) showed high homology with the strains described as HBV/B7 (Fig. 1A), with 1.9 to 2.9% divergence. To confirm the relationship, we also analyzed each open reading frame, core (Fig. 1B), large S (pre-S1 to S gene) (Fig. 1C), P, and X gene (data not shown). The results were mostly consistent with that obtained for the complete genome sequence analysis. No specific mutations in the small S gene, including residues 124 to 147 of HBsAg of the HBV/B strains, were found in the strains from Papua or those from Nusa Tenggara (see Fig. S1 in the supplemental material). Therefore, the HBV/B strains from Nusa Tenggara, which were previously reported to be a novel subgenotype (18), can be included in HBV/B3 along with the strains in this study.

**HBVC in Papua.** All nine HBV/C strains formed a novel cluster, separated from the other HBV/C strains (C1 to C5) based on the complete genome sequences (Fig. 1A). This result confirms our previous observation and proposal of a novel subgenotype HBV/C6 based on the S gene analysis (9). With a high divergence from other subgenotypes, specific amino acid substitutions in the small S gene, G18V and V47M, were found (see Fig. S1 in the supplemental material). These nine strains in Papua were shown to be more closely related to the HBV/C3 strains from New Caledonia and the HBV/C2 strains from east Asia than to the HBV/C1 strains from southeast Asia (Fig. 1A to C).

**HBVD in Papua.** All three HBV/D strains also formed a novel cluster, separated from the other HBV/D strains (D1 to D5) with significant bootstrap values, based on the complete genome sequences (Fig. 1A). This result again confirms our
FIG. 1. Phylogenetic trees of HBV strains isolated from 22 blood donors in Papua along with 52 reference strains. (A) Complete genome; (B) pre-C/C gene; (C) S gene. The number in the tree indicates the bootstrap reliability. The lengths of the horizontal bars indicate the number of nucleotide substitutions per site.

* HBV/C including P18 in this study cluster in the same branch as HBV/B due to the genetic recombination with HBV/B over the pre-C/C gene. Isolates from the database are indicated with accession numbers, and relevant country names are added to each HBV/B, HBV/C, and HBV/D strain. The nucleotide sequence accession numbers used as references in the phylogenetic trees are as follows: for HBV/B, EF473976, EF473977, M54923, AB033554, D00331, AB219426 through AB219429, AB241116, AB241117, DQ463791, DQ463792, DQ463787 through DQ463794, AY033073, AB073835, D00329, D23678, AF121243, and AF121251; for HBV/C, D23684, M38636, X75656, X75665, AB111946, AB112472, AB241109, AB241113, AB048704, and AB048705; for HBV/D, DQ315779, DQ315780, AB048701, AB048703, AB043359, AJ161157, AF157355, AB092690, AB078032, AB078033, AJ132335, and AY902776; for HBV/A, AB014370; for HBV/E, EX75657; for HBV/F, EX75658; for HBV/G, GA160501; and for HBV/H, HAY100457.
previous observation and proposal of the novel subgenotype HBV/D6 based on S gene analysis (9). The three strains in Papua were shown to be closely related to the HBV/D3 strains from the Western countries (Fig. 1A).

**Divergences of the entire nucleotide sequences among HBV/C6 and HBV/D6 strains.** Divergences in the entire genome sequences of the novel subgenotypes HBV/C6 and HBV/D6 were examined by comparing with the reference sequences of the other subgenotypes within a given genotype. The seven HBV/C6 strains showed 0.1 to 3.2% divergence from each other. On the other hand, they showed divergences of 6.2 to 6.7% with HBV/C5, 6.6 to 6.9% with HBV/C4, 4.4 to 4.9% with HBV/C3, 4.7 to 5.1% with HBV/C2, and 6.2 to 6.6% with HBV/C1. Similarly, the three HBV/D6 strains showed 1.4 to 2.0% divergence from each other but showed divergences of 4.7 to 6.3% with HBV/D5, 4.1 to 5.6% with HBV/D4, 3.3 to 4.7% with HBV/D3, 3.8 to 5.3% with HBV/D2, and 3.4 to 4.8% with HBV/C1.

**DISCUSSION**

In our previous report on HBV in Papua, Indonesia, we provisionally proposed novel subgenotypes HBV/C6 and HBV/D6, and other ambiguous subgenotypes of HBV/B, based on the S gene sequence variations (9). Needless to say, HBV phylogenetic analysis based on the complete genome sequences is more reliable than that of the S gene alone (14, 20). In this study, therefore, we performed complete genome sequence analysis and confirmed the presence of novel subgenotypes HBV/C6 and HBV/D6 in Papua (Fig. 1A). Subgenotypes are determined, in general, on the basis of sequence divergence from the complete genome sequence by 4% or greater (16, 19, 23). The HBV/C6 and HBV/D6 strains in this study showed ca. 4% or greater divergence from the complete genome sequence in comparison with other existing subgenotypes HBV/C1 to HBV/C5 and HBV/D1 to HBV/D5, respectively. In addition to the new subgenotypes, a unique HBV/B strain that has recombination with HBV/C5 in the pre-C/C region (P18) was found (Fig. 1 and 2). This strain was distinctly positioned between HBV/B3 and HBV/B5.

Of the 10 HBV/B isolates examined, 9 were classified as HBV/B3, together with the strains from the other place (Nusa Tenggara) in Indonesia (18). This HBV/B3 cluster can now be divided into two groups, although high homology is indicated between the two groups. We suggest that the strains from Nusa Tenggara can be considered a subgroup of HBV/B3 due to its comparatively low divergence (1.9 to 3.0%) from the other HBV/B3 strains. In general, subgenotypes are divided according to 4% or greater difference in the entire nucleotide sequences (16, 19, 23).

Recombination between HBV genotypes is a common event in countries where different genotypes are prevalent. Moreover, the recombination between HBV/B and HBV/C was found frequently in southeast Asia, and the breakpoint of recombination has been reported to be in the pre-C/C region (5, 8, 12, 25, 27, 30). Consistent with the previous reports, we found in this study the possible recombination in the pre-C/C region of the P18 strain (HBV/B) with HBV/C (Fig. 2). It is increasingly accepted that recombination between genotypes generates novel variants that contribute to
the genetic diversity of HBV. Thus, genetic recombination is a significant and relatively frequent event in the evolution of HBV (26).

HBV/D was also found in parts of Asia and in aboriginal populations in Papua New Guinea (28). The phylogenetic position of HBV/D6 (7UC, 17UC, and P7 strains) appears to be closer to HBV/D3 strains isolated from the Western countries than those from Asia and Papua New Guinea (Fig. 1). The significance of this relationship, however, remains unclear.

In conclusion, HBV genotypes B, C, and D are prevalent in Papua, Indonesia. We confirmed the presence of the novel subgenotypes HBV/C and HBV/D on the basis of the complete genome analysis. Our study will lead to interesting
findings on the genetic variety of HBV, as well as its clinical relevance in Papua, Indonesia, a multiethnic nation.

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REFERENCES