

平成18年度 研究成果

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IV. 研究成果の刊行物・別刷

Short
CommunicationMolecular tracing of Japan-indigenous hepatitis E
viruses

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The ancestor(s) of apparently Japan-indigenous strains of *Hepatitis E virus* (HEV) was probably of foreign origin, but it remains unclear when and from where it made inroads. In this study, 24 genotype 3 and 24 genotype 4 HEV strains recovered in Japan each showed a significant cluster, clearly distinct from those of foreign strains, in the phylogenetic tree constructed from an 821 nt RNA polymerase gene fragment. The evolutionary rate, approximately 0.8×10^{-3} nucleotide substitutions per site per year, enabled tracing of the demographic history of HEV and suggested that the ancestors of Japan-indigenous HEV had made inroads around 1900, when several kinds of Yorkshire pig were imported from the UK to Japan. Interestingly, the evolutionary growth of genotype 3 in Japan has been slow since the 1920s, whereas genotype 4 has spread rapidly since the 1980s. In conclusion, these data suggest that the indigenization and spread of HEV in Japan were associated with the popularization of eating pork.

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Transmission of *Hepatitis E virus* (HEV) occurs primarily by the faecal–oral route through contaminated water supplies in developing countries (Purcell & Emerson, 2001). Additionally, increasing evidence has indicated that hepatitis E is a zoonosis (Harrison, 1999; Kabrane-Lazizi *et al.*, 1999; Meng *et al.*, 1997, 1998, 2002; Nishizawa *et al.*, 2003;

Okamoto *et al.*, 2001; Tei *et al.*, 2003; Yazaki *et al.*, 2003). It has recently been suggested that zoonotic, food-borne transmission of HEV from domestic pigs, wild boars or wild deer to humans plays an important role in the occurrence of domestic infections of hepatitis E in Japan, where people have unique habits of ingesting raw fish (sushi or sashimi) and uncooked or undercooked meat (also organ meats, such as raw liver) (Matsuda *et al.*, 2003; Tamada *et al.*, 2004). Thus, it seems that HEV infection is now autochthonous in Japan. It remains unclear, however, when and from where the ancestral HEV strains made inroads and have spread in

The GenBank/EMBL/DDBJ accession numbers for the HEV nucleotide sequences reported in this paper are shown in Fig. 1.

Supplementary tables are available in JGV Online.

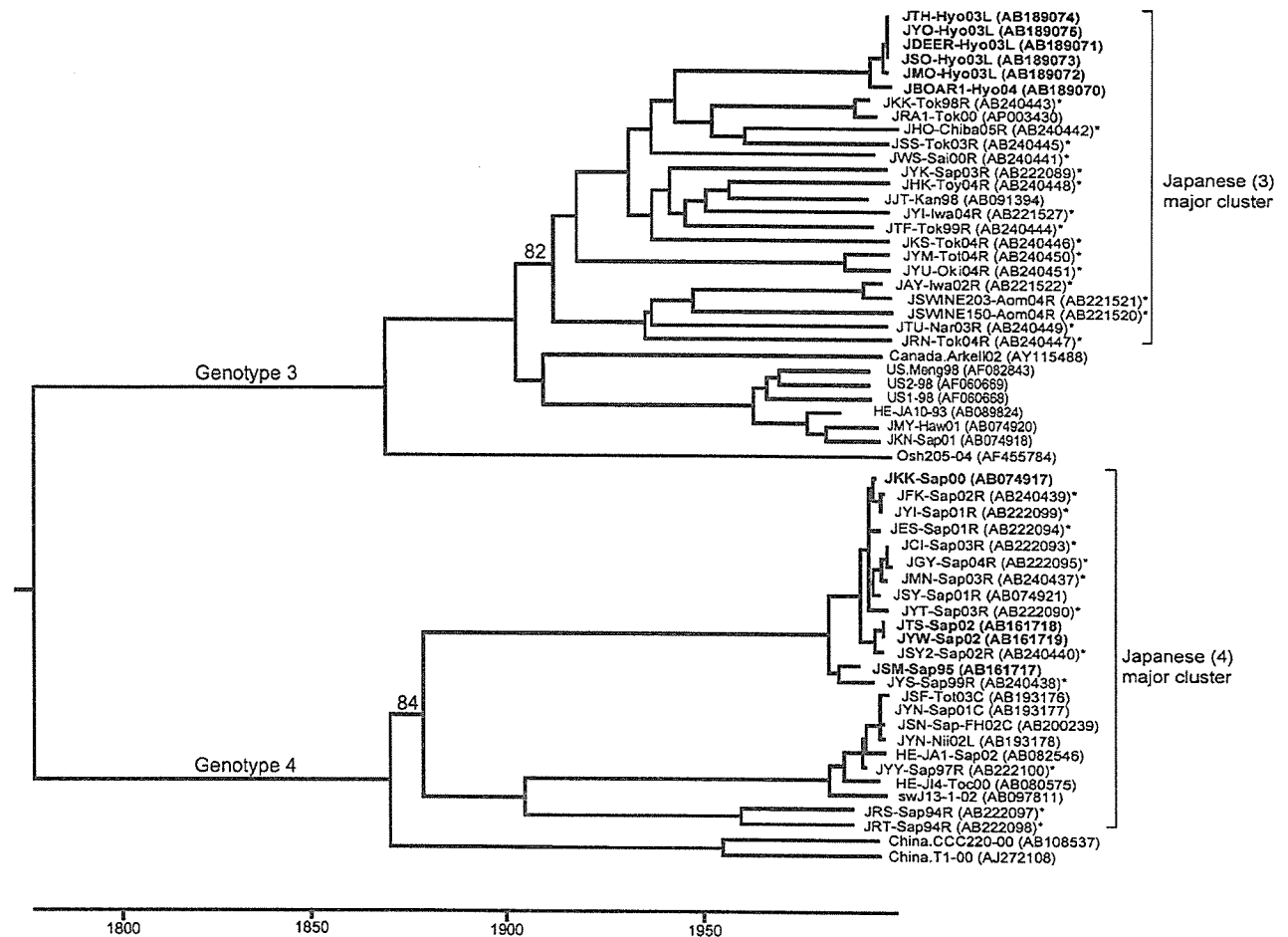


Fig. 1. Phylogenetic tree of the partial RNA polymerase region of the HEV genome. Twenty-four genotype 3 and 24 genotype 4 strains in Japan showed each significant cluster to have a high bootstrap value and to be distinct from other reference sequences (USA, Canada and Japanese minor strains in genotype 3; Chinese strains in genotype 4). Genetic distances have been transformed into a time scale of years by using estimates of the molecular clock (0.84×10^{-3} nucleotide substitutions per site per year). Ten strains in bold are used for linear regression in Fig. 2. Strain names are followed by prefecture or city names in Japan: Hyo, Hyogo; Tok, Tokyo; Sai, Saitama; Sap, Sapporo; Iwa, Iwate; Kan, Kanagawa; Oki, Okinawa; Aom, Aomori; Nar, Nara; Tot, Tottori; Nii, Niigata; Toc, Tochigi; Toy, Toyama. Asterisks indicate strains that were newly sequenced in this study.

Japan. In this study, we first estimated the evolutionary rate of HEV by using Japan-indigenous genotype 3 and genotype 4 strains, which were phylogenetically distinct from the other strains in foreign countries. Then, based on this evolutionary rate, we traced the demographic history of HEV in Japan.

For linear-regression analyses within significant clusters, two independent datasets were applied: one was a Hyogo cluster (genotype 3) with JMO-Hyo03L, JTH-Hyo03L, JSO-Hyo03L, JYO-Hyo03L, JDEER-Hyo03L (these five isolates were obtained in April 2003) and JBOAR1-Hyo04 (April 2004) (Takahashi *et al.*, 2004a), and another was a Sapporo cluster (genotype 4) with JSM-Sap95 (March 1995), JKK-Sap00 (November 2000), JYWSap02 (August 2002) and

JTS-Sap02 (September 2002) (Takahashi *et al.*, 2004b). GenBank accession numbers for these strains are given in Fig. 1. To elucidate the epidemiological history of the HEV population in Japan, 48 known and newly sequenced HEV strains ($n=24$ for each of genotype 3 and 4) were used for molecular-evolutionary analyses. The nucleotide sequences of 28 strains for the molecular-clock analyses were determined in this study (the other 20 sequences dealt with in this paper were available from GenBank).

Nucleic acids were extracted from serum samples (50 μ l) by using a commercial Smitest EX-R & D kit (Genome Science) and precipitated in a 2 ml tube. The pellet was air-dried for 15 min and then suspended in 10 μ l autoclaved distilled water containing 10 U RNase inhibitor ml^{-1} (TaKaRa

Shuzo). A sequence spanning 821 nt in the RNA-dependent RNA polymerase region (corresponding to nt 3961–4781 of the prototype Burmese HEV strain; GenBank accession no. M73218), including the GDD motif, was amplified by PCR in three overlapping regions with 20-mer primers deduced from known HEV sequences. Reverse transcription was performed at 50 °C for 60 min with the Thermo-Script RT system (Invitrogen), and the first- and second-round PCRs were carried out in the presence of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). The final products were sequenced in an ABI 377 DNA sequencer (PE Biosystems) with an ABI Prism BigDye kit (Applied Biosystems). The sequences determined were utilized to confirm HEV genotypes and to construct phylogenetic trees. The reliability of the phylogenetic tree was assessed by bootstrap-resampling tests.

A reconstructed tree was built on the RNA polymerase region by using a heuristic maximum-likelihood (ML) topology search with stepwise addition and nearest neighbour-interchange algorithms. Tree likelihood scores were calculated by using the HKY85 model (Hasegawa *et al.*, 1985) with the molecular clock enforced, using PAUP version 4.0b8. Using the estimated topology, all possible root positions were evaluated under a single-rate dated-tips (SRDT) model with the computer software TipDate v1.2 and the root that yielded the highest likelihood was adopted (Rambaut, 2000). The program provided an ML estimate of the rate and also the associated date of the most recent common ancestor of the sequences, using a model that assumed a constant rate of nucleotide substitution. The molecular clock was tested by a likelihood-ratio test between the SRDT model and a general unconstrained branch-length model [different-rate (DR) model].

For estimates of demographic history, a non-parametric function $N(t)$, also known as a skyline plot, was obtained by transforming the coalescent intervals of an observed genealogy into a piecewise plot that represented an effective population size through time (Pybus *et al.*, 2001; Pybus & Rambaut, 2002). A parametric ML was estimated by several models with the computer software GENIE v3.5 to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed from sampled DNA sequences (Pybus & Rambaut, 2002). This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model fitting was evaluated by likelihood-ratio tests of the parametric ML estimates (Lemey *et al.*, 2003; Pybus *et al.*, 2003; Tanaka *et al.*, 2005). Approximate 95 % confidence intervals for the parameters were estimated by using the likelihood-ratio test statistics.

A phylogenetic tree in the partial RNA polymerase region of the HEV genome is represented in Fig. 1. A functional gene, such as the RNA polymerase gene, is suitable for molecular-evolutionary analyses based on the neutral theory, because the substitution of functional genes is based on the neutral theory. The 24 genotype 3 and 24 genotype 4 strains in Japan

showed a significant cluster with a high bootstrap value, which was the major Japanese cluster distinct from other strains found in foreign countries by molecular-evolutionary analyses. Such a significant cluster is suitable for the following coalescent analysis. Additionally, the tree topology based on the RNA polymerase region, including functional genes, was quite similar to that based on complete genomes (data not shown).

To determine the evolutionary rate of HEV, the 48 Japanese HEV strains (Fig. 1) were subjected to further molecular-evolutionary analyses. The molecular-evolutionary rate was estimated by two independent methods. In brief, linear-regression analyses using highly similar strains, i.e. six genotype 3 strains in Hyogo and four genotype 4 strains in Sapporo, indicated that a molecular-evolutionary rate was $(0.81-0.88) \times 10^{-3}$ nucleotide substitutions per site per year (Fig. 2). Second, TipDate (v1.2) was used to compare the DR model with the single-rate (SR) and SRDT models. The SRDT model provided an adequate fit to the data ($P > 0.05$; see Supplementary Table S1, available in JGV Online). Based on the SRDT model, the mean rate of nucleotide substitutions was estimated to be $(0.81-0.94) \times 10^{-3}$ nucleotide

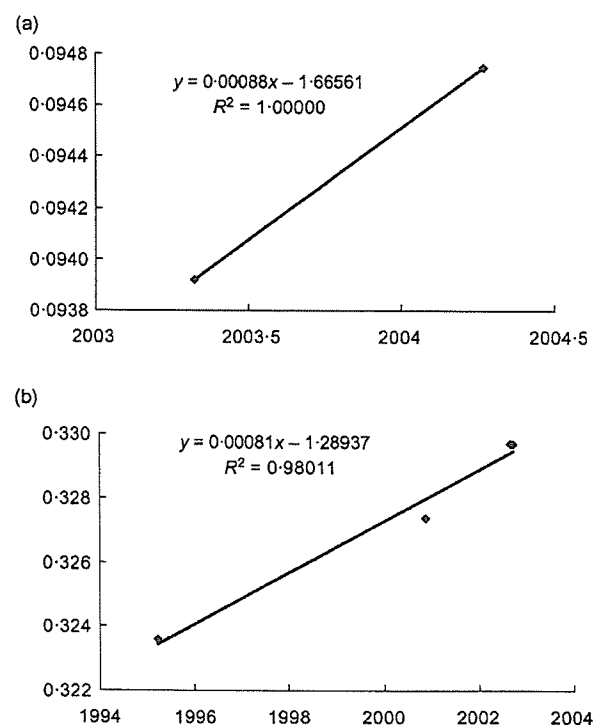


Fig. 2. Linear-regression analyses within the partial RNA polymerase region for evolutionary rate of HEV. (a) The evolutionary rate of genotype 3 in the Hyogo cluster is estimated to be 0.88×10^{-3} nucleotide substitutions per site per year; (b) the evolutionary rate of genotype 4 in the Sapporo cluster is estimated to be 0.81×10^{-3} nucleotide substitutions per site per year.

substitutions per site per year, which was similar to the rate for *Hepatitis C virus* (Ina *et al.*, 1994; Tanaka *et al.*, 2002). When we used 0.84×10^{-3} nucleotide substitutions per site per year, which was based on all 48 sequences (24 genotype 3 and 24 genotype 4), the time of the most recent common ancestor of Japan-indigenous genotype 3 was estimated to be in the 1900s (95% confidence interval, 1902–1917) and that of genotype 4 was approximately in the 1880s (1881–1898) (Fig. 1).

Based on the phylogenetic tree, the effective number of HEV infections through time, $N(t)$, was analysed by using a skyline plot for the Japan-indigenous HEV strains. The parameters for several models in GENIE v3.5 were examined (see Supplementary Table S2, available in JGV Online). Time t was then transformed to year by using the constant rate (0.84×10^{-3} nucleotide substitutions per site per year), assuming the collecting time to be the present. Fig. 3 shows the skyline plots and population growth for the HEV strains, according to a specific demographic model in GENIE v3.5 with three parameters and a piecewise-expansion growth model, which was evaluated by likelihood-ratio testing (Ina *et al.*, 1994; Lemey *et al.*, 2003; Pybus *et al.*, 2003; Tanaka *et al.*, 2005). Our estimates of the effective numbers of HEV infections showed a transition from constant size to exponential growth in the 1920s (95% confidence interval, 1916–1930) among the genotype 3 population (Fig. 3a), whereas the rapid exponential growth among the genotype 4 population was dated in the 1980s (1978–1990) (Fig. 3b).

Because the natural course of HEV infection in human beings and animals is usually transient, not persistent as in the cases of hepatitis B and C viruses, it is almost impossible to estimate the molecular-evolutionary rate of HEV by using serial samples from an individual host. However, even though HEV does not persist in individual hosts, it could persist in the community by hopping from host to host successively. The first study attempting to estimate the number of synonymous mutations per synonymous site (k_s) of *Hepatitis A virus* (HAV) was reported by Sánchez *et al.* (2003). The estimated k_s values from HAV strains isolated from a clam-associated outbreak varied from 0.038 for VP0 to 0.29 for VP1. Similarly, we estimated the evolutionary rate of HEV by using Japan-indigenous genotype 3 and genotype 4 strains isolated over time. The rate was estimated to be approximately 0.8×10^{-3} nucleotide substitutions per site per year by two independent methods, which was around half of our previously estimated rate (Takahashi *et al.*, 2004b). One of the reasons is that the molecular-evolutionary rate would depend on estimated genes; the previous report (Takahashi *et al.*, 2004b) used complete sequences, whereas this study used only RNA polymerase sequences. Another reason is that the previous extrapolation of substitution rate on pairwise (direct) comparisons can give overestimates of the molecular clock and hence divergent times of HEV species, as reported previously (Ina *et al.*, 1994). Based on the molecular clock, we traced the demographic history of HEV in Japan and the indigenization time

was suggested to be similar (approx. 1900), but the spread time was quite different, between HEV genotypes 3 and 4 (1920s versus 1980s). Interestingly, in addition, the evolutionary growth of genotype 3 has been quite slow since the 1920s, whereas genotype 4 strains have spread rapidly in Sapporo since the 1980s.

Zoonosis has been implicated in HEV transmission. The first animal strain of HEV to be isolated and characterized was a swine HEV from a pig in the USA in 1997 (Meng *et al.*, 1997). Since then, many swine HEV strains, which exhibit extensive genetic heterogeneity, have been identified worldwide and shown to be genetically related closely to strains of human HEV (Chandler *et al.*, 1999; Hsieh *et al.*, 1999; Huang *et al.*, 2002; Okamoto *et al.*, 2001; Wang *et al.*, 2002). Recent findings suggested an interspecies HEV transmission between boar and deer in their wild life (Takahashi *et al.*, 2004a) and that both animals might serve as an infection source for human beings. More recently, wild mongoose was newly added to the list of HEV-reservoir animals in Japan

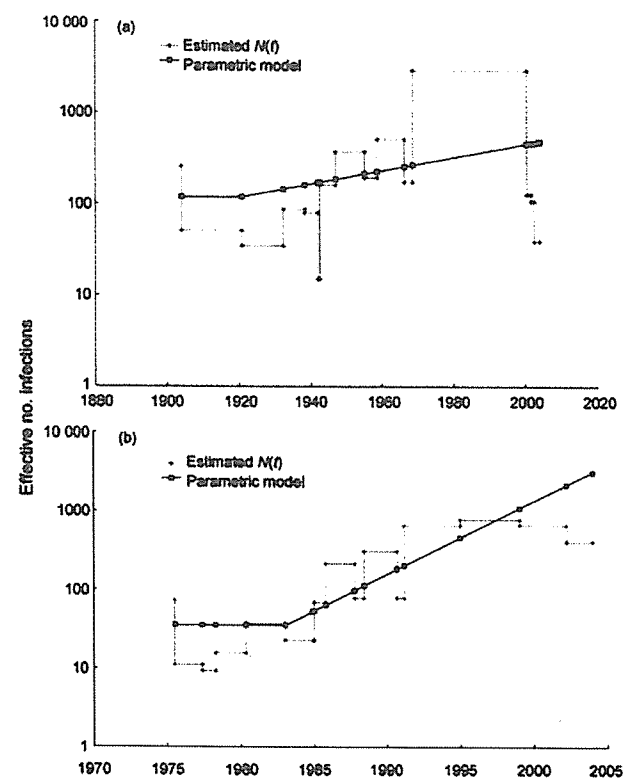


Fig. 3. ML estimates of $N(t)$ on the effective number of (a) HEV genotype 3 and (b) HEV genotype 4 infections in Japan. The parametric model is indicated by the black line and stepwise plots by the grey line, which represents corresponding non-parametric estimates of $N(t)$ (number as a function of time). Genetic distances have been transformed into a time scale of years by using estimates of the molecular clock in the partial RNA polymerase region of HEV.

(Nakamura *et al.*, 2006). Notwithstanding the importance of these wild animals, pigs for food must be the major reservoirs of HEV: a recent Japanese study indicated that anti-HEV antibodies were detected in 1448 (58%) of 2500 pigs from 2 to 6 months of age at 25 commercial swine farms in Japan (Takahashi *et al.*, 2003). The importance of transmission of HEV from pigs to humans was further supported by a recent field study in Indonesia: Muslim people, for whom it is a taboo to eat or contact pigs, were significantly less frequently positive for anti-HEV than Hindu people (2.0 vs 20%) (Surya *et al.*, 2005).

Our molecular-evolutionary analyses suggested that HEV entered Japan around 1900. If we have traced the origin of Japan-indigenous HEV correctly back to about 100 years ago, what happened at that time in relevance to HEV's indigenization? Several kinds of Yorkshire pig were imported for the first time in the history of Japan from the UK in 1900, by the Japanese government's policy to introduce excellent domestic animals for food in Western countries to Japan, as a measure to nutritionally strengthen the people (especially soldiers) of this formerly vegetarian country. Since then, the Yorkshire pigs have been propagated in Japan and, in the 1930s, thousands of pigs were reported all over Japan (<http://okayama.lin.go.jp/history/2-3-1-2.htm>), suggesting that the domestic spread of HEV might have been associated with the popularization of pigs for food in Japan. Indeed, a previous phylogenetic analysis of a 304 bp nucleotide sequence (ORF2) obtained from the two UK swine strains showed a close relationship with Japanese swine strains in genotype 3 (Banks *et al.*, 2004), indicating that Japanese genotype 3 may have been imported from the UK. On the other hand, Japanese genotype 4 strains were related phylogenetically to Asian strains in Taiwan and China. As the HEV found in wild boars living in the Iriomote Island, near Taiwan, was of genotype 4 (unpublished results), the source of Japanese genotype 4 might be from Taiwan or the mainland of China. Note that a phylogenetic analysis showed that the Japanese swine and human HEV strains segregated into four clusters [three genotype 3 clusters (one major Japanese and two minor clusters) and one genotype 4 cluster], with the highest nucleotide identity being 94.4–100% between swine and human strains in each cluster (Takahashi *et al.*, 2003), suggesting that swine have served as one of the most important reservoirs for HEV to be transmitted to humans. The possible risk factor for transmission of HEV was to have eaten uncooked or undercooked pig liver and/or intestine 1–2 months before the onset of hepatitis E in Hokkaido, Japan (Mizuo *et al.*, 2005). Such eating habits, which are particularly unique to those living in Hokkaido (Sapporo is one of the big cities there) in recent decades, might be one of the reasons that HEV has been widespread in this area since 1990, as supported by our molecular-evolutionary analyses in this study.

In conclusion, based on our present data, the indigenization and domestic spread of HEV in Japan are proposed to have been associated with the importation and popularization of

pigs for food in Japan. However, there still remains a possibility of different scenarios. Another animal(s) might have carried the virus to Japan: for example, mongoose was imported from India to Japan in 1910 (Nakamura *et al.*, 2006).

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Influence of Genotypes and Precore Mutations on Fulminant or Chronic Outcome of Acute Hepatitis B Virus Infection

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The outcome of acute hepatitis B virus (HBV) infection is variable, influenced by host and viral factors. From 1982 through 2004, 301 patients with acute HBV infection entered a multi-center cross-sectional study in Japan. Patients with fulminant hepatitis (n = 40) were older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P < .0017$), less predominantly male (43% vs. 71%, $P = .0005$), less positive for hepatitis B e antigen (HBeAg) (23% vs. 60%, $P < .0001$), less infected with subgenotype Ae (0% vs. 13%, $P < .05$), and more frequently with Bj (30% vs. 4%, $P < .0001$) than those with acute self-limited hepatitis (n = 261). Precore (G1896A) and core-promoter (A1762T/G1764A) mutations were more frequent in patients with fulminant than acute self-limited hepatitis (53% vs. 9% and 50% vs. 17%, $P < .0001$ for both). HBV infection persisted in only three (1%) patients, and they represented 2 of the 23 infected with Ae and 1 of the 187 with the other subgenotypes (9% vs. 0.5%, $P = .032$); none of them received antiviral therapy. In multivariate analysis, age 34 years or older, Bj, HBeAg-negative, total bilirubin 10.0 mg/dL or greater, and G1896A mutation were independently associated with the fulminant outcome. In *in vitro* transfection experiments, the replication of Bj clone was markedly enhanced by introducing either G1896A or A1762T/G1764A mutation. **In conclusion**, persistence of HBV was rare (1%) and associated with Ae, whereas fulminant hepatitis was frequent (13%) and associated with Bj and lack of HBeAg as well as high replication due to precore mutation in patients with acute HBV infection. *Supplementary material for this article can be found on the HEPATOLOGY website (<http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html>). (HEPATOLOGY 2006; 44:326-334.)*

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBc, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; EIA, enzyme immunoassay; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ALT, alanine aminotransferase.

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Approximately 3 billion people, one half of the world population, have been exposed to hepatitis B virus (HBV), of whom approximately 350 million are persistently infected with it.¹ Acute infection with HBV resolves in the great majority but can induce fulminant hepatitis or go on to become chronic. Host and viral factors may influence fulminant or chronic outcome of acute HBV infection, but they are not fully defined.

Eight genotypes have been detected by a sequence divergence greater than 8% in the entire HBV genome of approximately 3,200 nucleotides (nt), and designated by capital alphabet letters from A (HBV/A) to H in the order of documentation.²⁻⁵ They have distinct geographical distributions associated with severity of liver disease as well as response to antiviral therapies.⁶⁻⁸ Furthermore, subgenotypes have been reported for HBV/A, B, and C and named Aa/A1 (Asian/African type) and Ae/A2 (European type),⁹ Bj/B1 (Japanese type) and Ba/B2 (Asian type),¹⁰ as well as Cs/C1 (Southeast Asian type) and Ce/C2 (East Asian type).¹¹⁻¹³ Increasing lines of evidence indicate that subgenotypes of HBV/A and B influence the replication of HBV and bear clinical relevance.¹⁴⁻¹⁶ Furthermore, genotypes affect mutations in precore region and core promoter, thereby influencing the expression of hepatitis B e antigen (HBeAg).^{8,17}

During the 23 years from 1982 to 2004, a multi-center cross-sectional study was conducted throughout Japan on 301 patients with acute hepatitis B. We examined the influence of genotypes/subgenotypes on their fulminant or chronic outcome. Furthermore, the influence of G1896A or A1762T/G1764A on replication of HBV was evaluated in an *in vitro* replication model.

Patients and Methods

Patients With Acute Hepatitis B. During 1982 through 2004, 336 consecutive cases of acute hepatitis B were registered in 16 hospitals throughout Japan. These hospitals were from the following eight areas: Hokkaido (represented by J.-H. K. and S.H.), Tohoku (T.K. and K.S.), Kanto (H.T., Y.A. and K.I.), Koshin (E.T. and S.O), Tokai (A.O., Y.T., E.O., M.S., R.U., M.M., and S.K.), Kinki (T.O.), Honshu/Shikoku (Y.M., K.H., and M.O.), and Kyushu (H.Y. and H.S.). The diagnosis of acute hepatitis B was contingent on a sudden onset of clinical symptoms of hepatitis and detection of high-titered antibody to hepatitis B core antigen (anti-HBc) of IgM class in serum. Patients with initial high-titered anti-HBc ($\geq 90\%$ inhibition by a 1:200 diluted serum) were excluded; they were diagnosed as exacerbation of chronic hepatitis B. Patients with acute hepatitis A, hepatitis C, or human immunodeficiency virus co-infection, and drug-

or alcohol-induced acute hepatitis also were excluded; hepatitis D virus infection was not examined because of its extreme rarity in Japan.¹⁸ Most of them were followed for clinical outcomes until the disappearance of hepatitis B surface antigen (HBsAg) during 24 weeks or longer after the presentation. The criteria of fulminant hepatitis are based on the report by Trey et al.,¹⁹ with a slight modification in 1981 (Inuyama symposium, Aichi, Japan): coma of grade II or higher and prothrombin time less than 40% developing within 8 weeks after the onset. Serum samples were collected at the presentation and had been stored at -80°C . HBV genotypes, HBV DNA, and HBeAg were determined, and clinical outcomes of acute hepatitis were analyzed. The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the Ethics Committees of the institutions. Every patient gave an informed consent for this study.

Serological Markers of HBV Infection. HBsAg was determined by hemagglutination (MyCell; Institute of Immunology Co., Ltd., Tokyo, Japan) or enzyme immunoassay (EIA) (Axsym; Abbott Japan, Tokyo, Japan), and HBeAg by enzyme-linked immunosorbent assay (F-HBe; Kokusai Diagnostic, Kobe, Japan) or chemiluminescent EIA (Fujirebio Inc., Tokyo, Japan). Anti-HBc of IgM and IgG classes were determined by radioimmunoassay (Abbott Japan).

Genotypes and Subgenotypes of HBV. The six major HBV genotypes (A-F) were determined serologically by EIA using commercial kits (HBV GENOTYPE EIA; Institute of Immunology). The method depends on the combination of epitopes on preS2-region products detected by monoclonal antibodies, which is specific for each of them.²⁰ HBV/G was determined by a slight modification of the polymerase chain reaction (PCR) with specific primers.²¹

Subgenotypes of HBV/A designated Ae prevalent in Europe and Aa frequent in Africa as well as Asia,⁹ which corresponds to subgroup A' originally reported by Bowyer et al.,²² were determined by PCR restriction fragment length polymorphism (RFLP) involving nucleotide conversions in an immediate upstream of the precore region that are specific for each of them.^{16,23} HBV/Bj (Japanese type) lacking the recombination with C over the precore region and the core gene and Ba (Asian type) with the recombination were determined by its absence or presence on HBV DNA sequences, as well as RFLP based on specific nucleotide substitutions, after the methods described previously.^{15,24}

Subgenotypes of HBV/C, Cs (Southeast Asian type) found only in Southeast Asia, including Vietnam, Myanmar, Thailand, Laos, Bangladesh, Hong Kong, and Southern China, and Ce (East Asian type), found in Far

East Asia, including Japan, Korea, and Northern China, were determined by the PCR-RFLP method described previously.¹²

Quantification of HBV DNA and Sequencing. HBV DNA sequences spanning the S gene were determined by real-time detection PCR according to the method of Abe et al.,²⁵ with the detection limit of 100 copies/mL. HBV DNA sequences bearing core promoter, precore region, and the core gene were amplified by PCR with hemi-nested primers by the method described previously.¹⁰ Negative samples were tested by another more sensitive second-round PCR with HB7F and HBV1917R (5'-CTC CAC AGT AGC TCC AAA TTC TTT A-3'). Thereafter, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer.

Construction of Plasmid and Site-Directed Mutagenesis of HBV DNA. Serum samples were obtained from two patients infected with HBV/Bj and a patient with Ce. HBV DNA was extracted from 100 μ L serum using QIAamp DNA blood kit (QIAGEN, GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments covering the entire HBV genome. Amplified fragments were inserted into pGEM-T Easy Vector (Promega, Madison, WI) and cloned in DH5a competent cells (TOYOBO, Osaka, Japan). At least five clones of each fragment were sequenced and the consensus sequence determined. Among them, those containing the consensus sequence were identified and adopted as templates for further construction. Finally, 1.24-fold the HBV genome (nt 1413-3215/1-2185), just enough to transcribe oversized pregenome and precore mRNA, was constructed into pUC19 vector (Invitrogen Corp., Carlsbad, CA). For site-directed mutagenesis, the wild-type HBV was digested by *HindIII* and *EcoO65I* and ligated with the fragment carrying T1762/A1764 to produce 1.24-fold the genome carrying the core-promoter double mutation. Similarly, 1.24-fold the HBV genome with the precore stop-codon mutation (1896A) was generated. Further details are available online at: <http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html>.

Cell Culture and DNA Transfection. For the standard replication assay, 10-cm-diameter dishes were seeded with 1×10^6 Huh7 cells each. After 16 hours of culture, cells were transfected with 5 μ g DNA construct using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and harvested 3 days later. Transfection efficiency was measured by cotransfection with 1 μ g reporter plasmid expressing secreted alkaline phosphatase and estimating its enzymatic activity in the culture supernatant.

Southern Blot Hybridization. HBV DNA samples

from cells at day 3 in culture were separated on 1.2% (wt/vol) agarose gel, transferred to a positive-charged nylon membrane (Roche Diagnostics), and hybridized with full-length HBV DNA labeled with alkaline phosphatase. Detection was performed with CDP-star (Amersham Biosciences, Piscataway, NJ), and signals were analyzed in the LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Statistical Analysis. Categorical variables were compared between groups by the chi-squared test and non-categorical variables by the Mann-Whitney *U*-test. A *P* value less than .05 was considered significant. Multivariate analyses with logistic regression were used to determine independent factors for fulminant hepatitis. STATA Software (StataCorp LP, College Station, TX) version 8.0 was employed for analyses.

Results

Demographic and Clinical Differences in Patients Infected With Various HBV Genotypes/Subgenotypes. Genotypes of HBV were not classifiable in 28 (8%), and sufficient clinical data were not available in 7 (2%) of the 336 patients with acute hepatitis B. Exclusive of these 35 patients, 301 (90%) were left for evaluation of HBV genotypes in reference to clinical outcome.

HBV genotypes/subgenotypes were Aa in 10 (3%), Ae in 33 (11%), Ba in 22 (7%), Bj in 22 (7%), Cs in 11 (4%), Ce in 192 (64%), D in 5 (2%), and G in 6 (2%); none of them were infected with F or H (Table 1). All six patients with HBV/G were co-infected with another genotype; Ae in two, Ba in two, and Ce in the remaining two. The mean age was lower in the patients with HBV/Ae than Ba ($P = .0001$), Aa ($P < .01$), Bj or Cs ($P < .05$ for each) and Ce than Ba ($P < .05$). Men predominated in HBV infections with foreign (Ae and Ba) compared with domestic genotypes (Bj and Ce) ($P < .05$).

HBeAg was detected in 79% of patients with HBV/Ae at a frequency much higher than that with Bj ($P < .005$), Ce ($P < .001$) or Ba ($P < .05$). HBeAg in four of the six (67%) patients with HBV/G was coded for by HBV of the other genotypes co-infecting them, because it has two stop codons and an insertion in the core gene that prohibit encoding HBeAg.²¹ HBV DNA levels as well as HBeAg-positive rates at the presentation were higher in HBV/Ae than Ce ($P < .005$) or Bj ($P < .05$) infection.

The peak alanine aminotransferase (ALT) level was higher in HBV/Bj than Ae infection ($P < .05$). Fulminant hepatitis was significantly more frequent in patients infected with HBV/Bj (55%) than the other genotypes ($P < .05$); it occurred in two of the five (40%) patients with HBV/D, also. In reflection of severe clinical course,

Table 1. Clinical Characteristics of Patients Acutely Infected With HBV of Distinct Genotypes/Subgenotypes

Features	Genotypes/Subgenotypes							
	Aa (n = 10)	Ae (n = 33)	Ba (n = 22)	Bj (n = 22)	Cs (n = 11)	Ce (n = 192)	D ^a (n = 5)	G ^{a,b} (n = 6)
Age (years)	42.2 ± 13.1	31.2 ± 10.3 ^d	41.5 ± 10.7 ^e	43.5 ± 19.1	38.5 ± 11.1	36.3 ± 15.0	38.6 ± 20.8	42.7 ± 17.5
Men	8 (80%)	30 (91%) ^f	19 (86%) ^g	9 (41%)	7 (64%)	122 (64%)	2 (40%)	6 (100%)
HBeAg positive	7 (70%)	26 (79%) ^h	11 (50%)	8 (36%)	8 (73%)	101 (53%)	1 (20%)	4 (67%)
ALT (IU/L)	1875 ± 759	2070 ± 1113 ⁱ	2523 ± 1185	3472 ± 2720	2269 ± 995	2610 ± 1719	2559 ± 1672	2142 ± 722
Duration of elevated ALT (weeks) ^c	7.9 ± 5.8	9.5 ± 6.2	8.8 ± 3.7 ^j	6.0 ± 2.5	10.1 ± 7.5	7.7 ± 5.1	5.7 ± 2.1	9.8 ± 1.5
Total bilirubin (mg/dL)	14.1 ± 10.3	9.0 ± 7.2	9.3 ± 5.9	10.9 ± 9.0	11.0 ± 13.8	9.8 ± 10.7	8.2 ± 2.2	13.0 ± 7.8
HBV DNA (log copies/mL)								
Median	4.76	6.08 ^k	5.15	4.93	5.61	4.94	5.91	5.97
(range)	(2.90-8.08)	(2.00-8.46)	(2.00-8.19)	(2.00-8.44)	(2.00-8.50)	(2.00-9.06)	(2.00-8.37)	(3.35-7.11)
<2.00 (undetectable)	0 (0%)	1 (3%)	2 (9%)	3 (14%)	2 (18%)	28 (15%)	1 (20%)	0 (0%)
Medication with								
Lamivudine	1 (10%)	9 (27%)	2 (9%)	5 (23%)	2 (18%)	28 (15%)	4 (80%)	2 (33%)
Steroid	0	3 (9%)	0	5 (23%)	1 (9%)	16 (8%)	0	0

^aPatients with HBV genotype D or G were not included in the analysis.

^bAll patients with HBV genotype G were co-infected with HBV of another genotype; Ae in two, Ba in two, and Ce in two.

^cExclusive of the 16 patients who died of fulminant hepatitis, 3 receiving liver transplantation and 10 without clinical data available.

^d $P = .0001$, Ae vs. Ba. $P < .01$, Ae vs. Aa. $P < .05$, Ae vs. Bj or Cs.

^e $P < .05$, Ba vs. Ce.

^f $P = .0001$, Ae vs. Bj. $P < .005$, Ae vs. Ce.

^g $P < .005$, Ba vs. Bj. $P < .05$, Ba vs. Ce.

^h $P < .005$, Ae vs. Bj. $P < .01$, Ae vs. Ce. $P < .05$, Ae vs. Ba.

ⁱ $P < .05$, Ae vs. Bj.

^j $P < .01$, Ba vs. Bj. $P < .05$, Ba vs. Ce.

^k $P < .005$, Ae vs. Ce. $P < .05$, Ae vs. Bj.

the peak ALT level tended to be high in patients with HBV/Bj.

Presumed infection routes of 301 patients were sexual transmission in 172 (57%), blood transfusion in 4 (1%), medical accidents in 17 (6%), and unknown in the remaining 108 (36%).

Clinical Outcome of Patients With Acute Hepatitis B. Fulminant hepatitis developed in 40 (13%) patients. To cope with severe acute liver disease, lamivudine and steroid were administered to 53 (18%) and 25 (8%) patients, respectively. Fulminant hepatitis led to death in 16 (5%) patients, and three (1%) received liver transplantation. Exclusive of the 40 patients with fulminant hepatitis who received various treatments and five without clinical data, 256 (85%) were followed for the chronic outcome (Fig. 1). Serum ALT levels stayed elevated for longer than 24 weeks for the diagnosis of chronic hepatitis in eight (3%) of them. Among them, five had cleared HBsAg from serum until then, and therefore, their liver function abnormality was not attributed to persistent HBV infection. Table 2 summarizes persistence of HBV infection in the 256 patients with acute hepatitis; 253 (99%) lost serum HBsAg by 6 months. Hence, HBV infection evolved into chronicity in only 3 of the 256 (1%) patients, representing 2 of the 32 (6%) infected with HBV/Ae and 1 of the 21 (5%) with Ba. All of the three with chronic outcome had low-titered IgG anti-HBc at the presentation, and

two of them had been negative for HBsAg before the presentation. None of them had received lamivudine or steroid treatment during their acute phase of illness. Of the patients without antiviral therapy, chronic outcome was significantly more frequent in those infected with HBV/Ae than non-Ae genotypes (9% ^{2/23} vs. 0.5% ^{1/187}, $P = .032$).

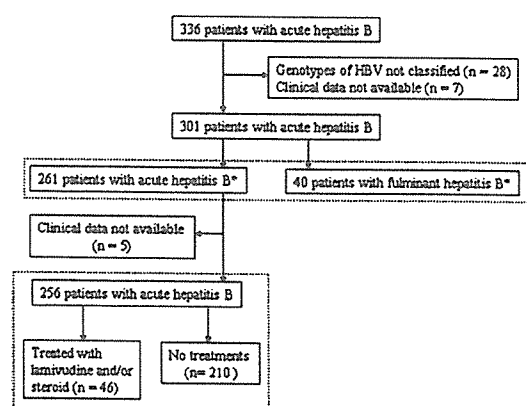


Fig. 1. A flow diagram of 336 patients studied. Comparison was made between patients with fulminant and acute self-limited hepatitis (upper dotted area), and the chronicity was compared between patients with and without treatments (lower dotted area). *Of 301 patients, 37 were negative for HBV DNA, including 27 with acute and 10 with fulminant hepatitis.

Table 2. Persistence of HBV Infection in the Patients With Acute Hepatitis Who Did or Did Not Receive Lamivudine or Steroid

Treatment	Total	Genotypes/Subgenotypes							
		Aa (n = 8) ^a	Ae (n = 32) ^a	Ba (n = 21) ^a	Bj (n = 10) ^a	Cs (n = 10) ^a	Ce (n = 167) ^a	D (n = 3) ^a	G (n = 5) ^a
Total (n = 256)	3/256 (1.2%)	0	2/32 (6%) ^c	1/21 (5%)	0	0	0	0	0
Lamivudine (n = 36) ^b	0/36 (0%)	0/1 (0%)	0/9 (0%)	0/2 (0%)	0	0/1 (0%)	0/19 (0%)	0/2 (0%)	0/2 (0%)
Steroid (n = 16) ^b	0/16 (0%)	0	0/3 (0%)	0	0	0/1 (0%)	0/12 (0%)	0	0
Neither	3/210 (1.4%)	0/7 (0%)	2/23 (9%) ^c	1/19 (5%)	0/10 (0%)	0/8 (0%)	0/139 (0%)	0/1 (0%)	0/3 (0%)

^aExclusive of 40 patients with fulminant hepatitis and 5 without clinical data available.

^bSix patients received steroid along with lamivudine.

^c $P < .05$, Ae vs. non-Ae.

Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis. Table 3 compares demographic, clinical, and virological characteristics between the 40 patients with fulminant and the 261 with acute self-limited hepatitis for whom analysis was feasible. Patients with fulminant hepatitis were significantly older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P = .0017$), less predominantly male (43% vs. 71%, $P = .0005$) and less often positive for HBeAg (23% vs. 60%, $P < .0001$) than those with acute hepatitis. Peak ALT and total bilirubin levels were higher for fulminant than acute hepatitis ($P < .0001$), reflecting severe hepatic lesions. Notably, the median HBV DNA level was lower in patients with fulminant than acute hepatitis (4.89 vs. 5.19 log copies/mL, $P = .0178$); the frequency of unde-

tectable HBV DNA at the presentation was higher in fulminant hepatitis (25% vs. 10%, $P = .0086$). Lamivudine or steroid was given significantly more often to patients with fulminant hepatitis.

There were marked differences in the distribution of genotypes between patients with fulminant and acute hepatitis. HBV/Ae was less frequent (0% vs. 13%, $P = .0121$), whereas Bj was more often (30% vs. 4%, $P < .0001$) in patients with fulminant than acute hepatitis. Although HBV/Ce tended to be less frequent in patients with fulminant than acute hepatitis (55% vs. 65%), the difference fell short of being significant.

Precore stop-codon mutation (G1896A) and core-promoter double mutation (A1762T/G1764A) were more

Table 3. Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis Who Were Infected With HBV

Features	Fulminant (n = 40)	Acute (n = 261)	P Value
Age (years)	44.7 ± 16.3	36.0 ± 14.3	.0017
Men	17 (43%)	186 (71%)	.0005
HBeAg positive	9 (23%)	157 (60%)	<.0001
ALT (IU/L)	4315 ± 2889	2284 ± 1221	<.0001
Total bilirubin (mg/dL)	20.5 ± 16.4	8.3 ± 7.3	<.0001
HBV DNA (log copies/mL)			
Median	4.89	5.19	.0178
(range)	(2.00-8.44)	(2.00-9.06)	
<2.00 (undetectable)	10 (25%)	27 (10%)	.0086
Treatment			
Lamivudine	16 (40%)	37 (14%)	.0003
Steroid	9 (23%)	16 (6%)	.0022
Genotypes/subgenotypes			
Aa	1 (2.5%)	9 (3%)	NS
Ae	0 (0%)	33 (13%)	.0121
Ba	1 (2.5%)	21 (8%)	NS
Bj	12 (30%)	10 (4%)	<.0001
Cs	1 (2.5%)	10 (4%)	NS
Ce	22 (55%)	170 (65%)	NS
D	2 (5%)	3 (1%)	NS
G	1 (2.5%)	5 (2%)	NS
Mutations ^a			
nt 1753 and/or nt1754 ^b	11/30 (37%)	28/234 (12%)	.0003
A1762T/G1764A	15/30 (50%)	39/234 (17%)	<.0001
G1896A	16/30 (53%)	21/234 (9%)	<.0001
G1899A	7/30 (23%)	8/234 (3%)	<.0001

^aExclusive of 37 patients in whom precore region and core-promoter could not be amplified by PCR.

^bT1753C/A/G and/or T1754C/A/G.

Table 4. Multivariate Analysis for Factors Independently Associated With Fulminant Hepatitis

Factors	Odds Ratio	95% Confidence Interval	P Value
Age (yr)			
<34 ^a	1		
≥34	3.472	1.094-11.023	.0347
Sex			
Male	1		
Female	2.272	0.780-6.613	.1323
HBeAg			
Positive	1		
Negative	3.344	1.065-10.506	.0387
ALT (IU/L)			
<2200 ^a	1		
≥2200	2.094	0.683-6.414	.1957
Total bilirubin (mg/dL)			
<10.0 ^a	1		
≥10.0	18.818	4.320-81.980	<.0001
HBVDNA (log copies/mL)			
<5.00 ^a	1		
≥5.00	1.042	0.367-2.961	.9383
Treatment			
Lamivudine (-)	1		
Lamivudine (+)	2.650	0.814-8.625	.1056
Steroid (-)	1		
Steroid (+)	2.515	0.668-9.472	.1728
Genotypes/Subgenotypes			
Non-Bj	1		
Bj	7.001	1.737-28.228	.0062
Mutations			
nt 1753 and/or 1754 ^b			
Absent	1		
Present	2.316	0.698-7.683	.1700
A1762T/G1764A			
Absent	1		
Present	1.013	0.295-3.478	.9841
G1896A			
Absent	1		
Present	4.157	1.265-13.657	.0189
G1899A			
Absent	1		
Present	2.525	0.534-11.949	.2427

^aMedian values.^bT1753C/A/G or T1754C/A/G.

frequent in patients with fulminant than acute hepatitis (53% vs. 9% and 50% vs. 17%, respectively, $P < .0001$ for each). Likewise, mutations in core-promoter at nt 1753 or nt 1754, and G1899A mutation were more frequent in patients with fulminant than acute hepatitis ($P = .0003$ and $P < .0001$, respectively).

Factors Independently Associated With the Development of Fulminant Hepatitis. Various factors found in association with fulminant hepatitis were evaluated for the independence in multivariate analysis (Table 4). Age 34 years or older (odds ratio 3.47 [95% confidence interval 1.09-11.02], $P = .035$), HBV/Bj (7.00 [1.74-28.23], $P = .006$), HBeAg-negative (3.34 [1.07-10.51], $P = .039$), total bilirubin ≥ 10.0 mg/dL (18.82 [4.32-81.98], $P < .0001$) and G1896A (4.16 [1.27-13.66], $P = .019$)

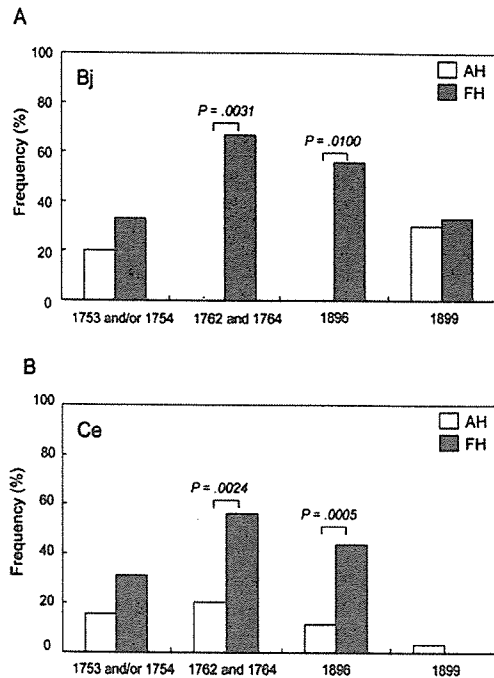


Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

were independent risk factors for the development of fulminant hepatitis.

In view of the majority of Japanese patients who were infected with Bj or Ce, mutations in the precore region and core-promoter were compared between those with fulminant and acute self-limited hepatitis for each subgenotype (Fig. 2). G1896A and A1762T/G1764A were significantly more frequent in patients with fulminant than acute hepatitis infected with either HBV/Bj or Ce (56% vs. 0% and 67% vs. 0% for Bj or 44% vs. 11% and 56% vs. 22% for Ce, respectively, $P \leq .01$ for all). For the patients infected with HBV/Bj, in particular, precore and core-promoter mutations were highly frequent in those with fulminant hepatitis (56% and 67%, respectively), whereas they occurred in none of those with acute hepatitis. G1899A was equally frequent in both patients with fulminant and acute hepatitis infected with HBV/Bj; it was rarely seen in those with Ce. Mutations involving nt 1753 or nt 1754 tended to be more frequent in patients with fulminant than acute hepatitis.

Replication of the Wild-Type HBV as Well as Precore and Core-Promoter Mutants In Vitro. Full-length HBV DNA of the wild-type HBV/Bj from a patient with chronic hepatitis B was incorporated with G1896A or A1762T/G1764A mutation *in vitro*. Another plasmid was constructed with HBV/Bj_58 carrying G1896A from a fulminant patient. Figure 3 compares

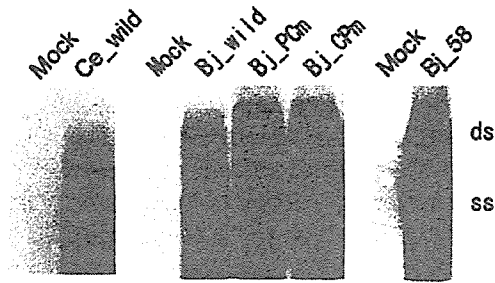


Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PcM) or core-promoter (Bj_CpM) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

densities of migration patterns of the wild-type, precore, and core-promoter mutants in Southern blotting analysis. The wild-type HBV/Bj displayed a band for single-stranded (ss) HBV DNA and an additional band for double-stranded (ds) HBV DNA. Of note, the densities of these bands were far greater for HBV/Bj mutants incorporated with precore or core-promoter mutation, as well as Bj_58 with the precore mutation, thereby indicating much enhanced replicative activity of precore or core-promoter mutant *in vitro*. Although the intracellular HBV DNA level for the wild-type HBV/Bj was comparable with that for the wild-type Ce (Fig. 3), the extracellular HBV DNA level in culture media was approximately threefold higher for Bj than Ce ($P < .01$) (Sugiyama M et al., manuscript in submission).

Discussion

A nationwide survey of genotypes/subgenotypes in patients with acute HBV infection from Japan during the past 2 decades has examined their influence on fulminant and chronic outcomes. The study was feasible in a country where mass vaccination has not been performed because of an extremely high efficacy of immunoprophylaxis on babies born to carrier mothers; it has decreased the persistent HBV carrier rate from 1.4% to 0.3%.²⁶ Acute HBV infection keeps increasing, however, predominantly through promiscuous sexual contacts in Japan.

Fulminant hepatitis developed rather frequently in 40 of the 301 (13%) patients. This is likely due to selection bias because the study included only patients who were hospitalized for acute hepatitis B. Exclusion of subclinical cases of acute HBV infection would have overestimated the incidence of fulminant hepatitis. Regardless of such a selection bias, influence of HBV genotypes/subgenotypes was evident in comparison with the 40 patients with fulminant and the 261 with acute self-limited hepatitis. Remarkably, none of the 33 patients infected with HBV/Ae

developed fulminant hepatitis. In sharp contrast, 12 of the 22 (55%) patients infected with HBV/Bj developed it. Furthermore, both precore (G1896A) and core-promoter (A1762T/G1764A) mutations were detected significantly more frequently in patients with fulminant than acute self-limited hepatitis. In infection with HBV/Bj, in particular, the frequency of core-promoter mutation was much higher in the patients with fulminant (67%) than that reported in those with chronic hepatitis (16%).²⁷ Precore and core-promoter mutations are very frequent in patients with fulminant hepatitis from Asia²⁸⁻³⁰ and the Middle East.³¹ The failure in detecting these mutations in Western countries³²⁻³⁵ could be attributed to frequent HBV/Ae and rare Bj there. In multivariate analysis, HBeAg-negative, HBV/Bj, and the precore stop-codon mutation for G1896A were independent risk factors for the development of fulminant hepatitis (Table 4). Various mutations at nt 1753 for enhanced HBV replication,³⁶ as well as those adjacent at nt 1754 prevailing in patients with fulminant hepatitis,³⁷ occurred more frequently in patients with fulminant than acute self-limited hepatitis. Host factors, such as age and total bilirubin, contributed to the development of fulminant hepatitis as well (Table 4).

In vitro replication analysis demonstrated the intracellular HBV DNA level of the wild-type HBV/Bj comparable with that of the wild-type Ce (Fig. 3). The extracellular HBV DNA level of HBV/Bj-clone, however, was much higher than those of the other genotypes, indicating its strong inclination to be secreted from cells (Sugiyama et al., manuscript in submission). Such a high concentration of HBV/Bj in the circulation of patients would rapidly and extensively promote infection of hepatocytes.

Enhanced replication capacities of precore (G1896A) and core-promoter (A1762T/G1764A) mutants for HBeAg-minus and -reduced phenotypes, respectively, were demonstrated in a replication model *in vitro* (Fig. 3). These observations were concordant with those in previous reports^{38,39}; however no data are available on the replication of HBV/Bj *in vitro*, either of the wild-type or variants with these mutations. Extremely high intracellular and extracellular expressions of viral DNA were observed for the HBV/Bj clone with precore stop-codon mutation from a patient with fulminant hepatitis. These results might implicate high replication due to mutations of precore region and core-promoter in the induction of fulminant hepatitis. In support of this view, Bocharov et al.⁴⁰ have proposed that enhanced HBV replication would efficiently stimulate immune reactions, represented by the cytotoxic T lymphocyte response, suggesting that enhanced replication by HBV/Bj or precore/