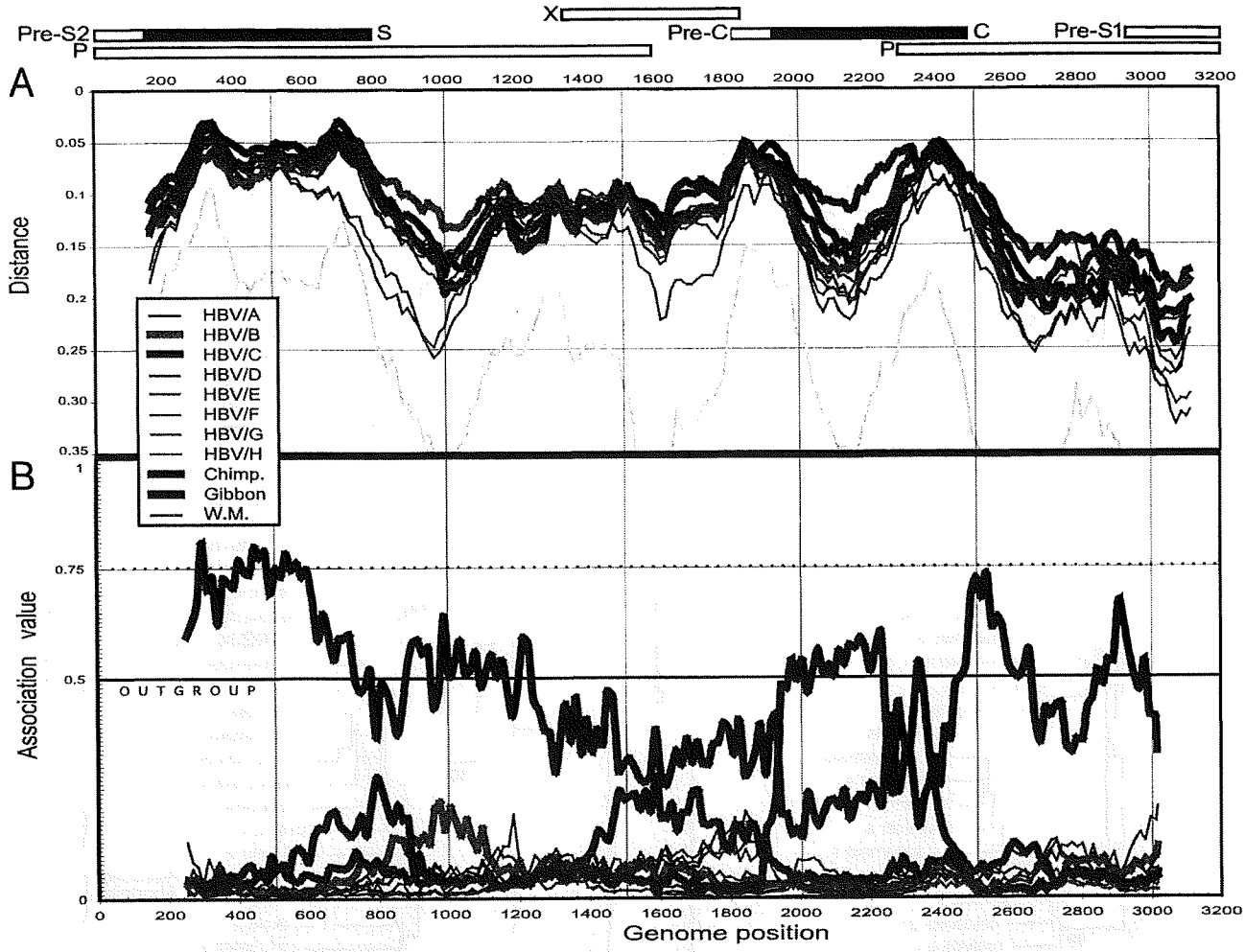

FIG. 2. Complete genome scanning carried by PHYLIP, the phylogeny inference package implemented in the Simmonic software, for the JRB34 strain versus 228 selected nonrecombinant HBV genotypes (HBV/Ba and HBV/I not included) reference strains grouped by genotype. Kimura two-parameter distance model (A) and grouping scan (B) were determined with a 300-nt size window sliding by an increment of 15 nucleotides. The *x* axis indicates the genome position (corresponding to the midpoint of the scanning fragment), and the *y* axis indicates the mean distances between JRB34 and reference groups (A). Phylogenetic association (*y* axis) was evaluated throughout entire HBV genome (*x* axis) with the same window and step size parameters (B). The association value below 0.5 was considered to represent an outgroup. The open reading frame map is shown schematically at the top of the figure.

of the Hepatitis Virus Database (39). All 1,440 complete genomes available in the DDBJ/GenBank served as references for the initial alignment in the present study. Divergence in the nucleotide sequence between a strain of provisional genotype J and previously reported strains was estimated by using MEGALIGN v.6.00 (Lazergene package; DNASTAR, Inc., Madison, WI).

Examination of recombination evidence. Evidence of possible recombination was investigated by using the software packages Simmonic 2005 v1.6 and SimPlot v3.5.1, both implementing PHYLIP (Phylogeny Inference Package v3.68; J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle [distributed by the authors]) (19, 40).

Inoculation of chimeric mice with the liver repopulated for human hepatocytes. Severe combined immunodeficiency mice transgenic for the urokinase-type plasminogen activator gene ($uPA^{+/+}/SCID^{+/+}$ mice) with the liver repopulated with human hepatocytes (chimeric mice) were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). Human serum albumin was measured by ELISA with commercial assay kits (Eiken Chemical Co., Ltd., Tokyo, Japan) for estimating the extent of repopulation. The research complied with all relevant federal guidelines and institutional policies.

Immunofluorescence. Freshly prepared liver tissues were snap-frozen in isopentane precooled in liquid nitrogen. Frozen specimens were cut at 5 to 6 μm by cryostat, mounted on glass slides, air dried, and fixed in 100% acetone at room temperature for 10 min. Sections were blocked with antibody diluent (Dako, Tokyo, Japan) and stained for hepatitis B core antigen (HBcAg). They were incubated with rabbit anti-HBc (Dako) at room temperature for 1 h, washed in phosphate-buffered saline, and then incubated with goat anti-rabbit immunoglobulin G conjugated with Cy3 (Chemicon International, Inc., Temecula, CA) or goat anti-human albumin antibody labeled with fluorescein isothiocyanate (Bethyl Laboratories, Inc., Montgomery, TX). Sections were washed with phosphate-buffered saline and observed in a fluorescence microscope (Eclipse E800M; Nikon, Tokyo, Japan).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in the present study will appear in the DDBJ/EMBL/GenBank databases under accession no. AB486012.

RESULTS

Composition of the HBV genome of genotype J. HBV DNA was extracted from serum of a patient with HCC. It was named JRB34 ("J" for Japanese; "R" after the southernmost island [Ryukyu] where the patient has spent most of his life now

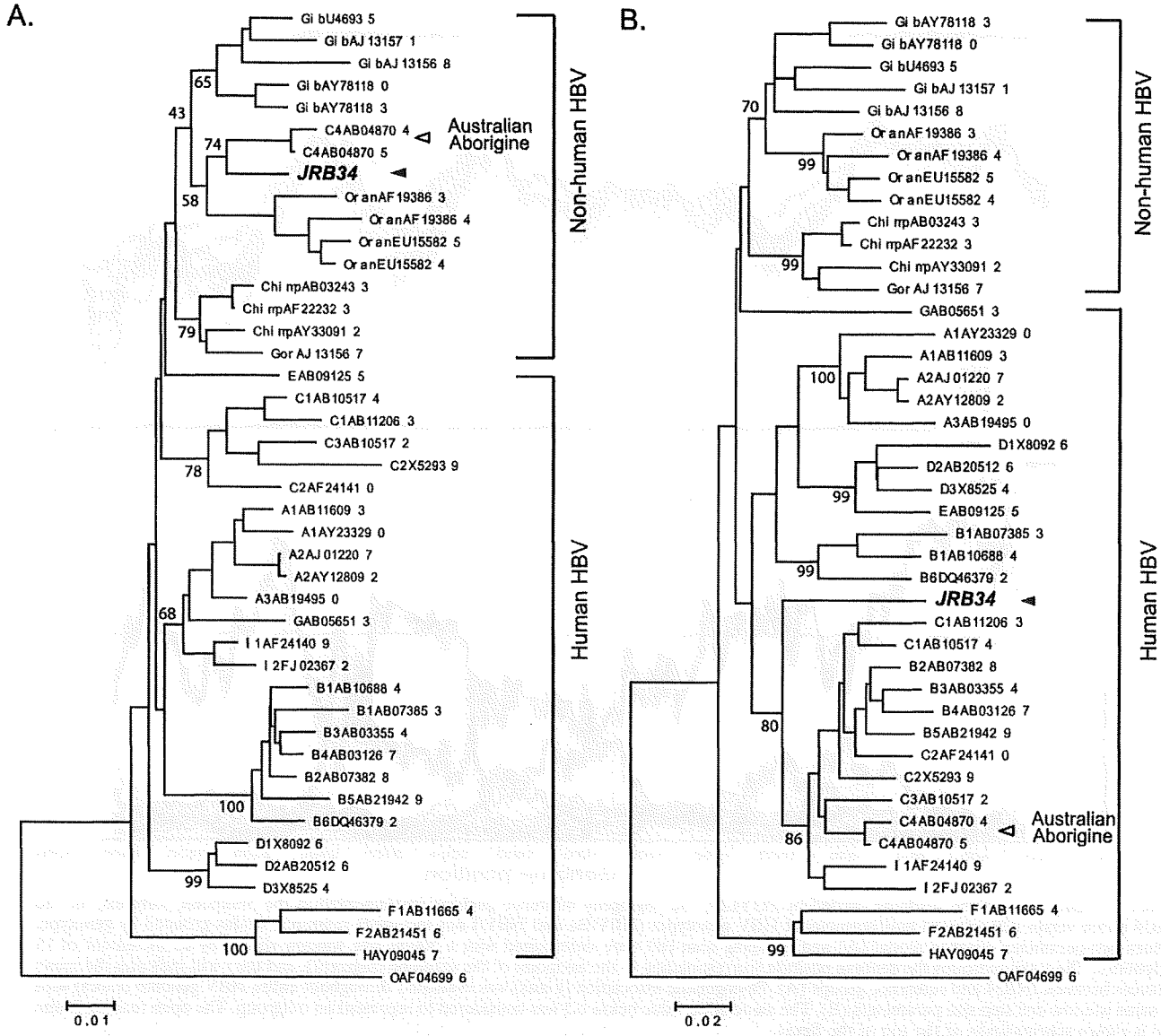

FIG. 3. Phylogenetic tree constructed on the preS/S gene (A) and C gene (B) sequences of 44 HBV isolates representing four ape and eight human genotypes. A woolly monkey HBV isolate serves as an outgroup. The HBV/J isolate (JRB34) is indicated by an arrowhead, and an HBVC4 isolate from Australian aborigine is indicated by an open triangle. The genetic distance is indicated by a bar below.

exceeding 90 years; and "B" for Borneo where he is suspected to have contracted the HBV infection). The entire nucleotide sequence was determined for the JRB34 isolate of genotype J (HBV/J). It had a genomic length of 3,182 nt, which consisted of envelope gene containing preS1 region (nt 2848 to 3171, coding for 108 amino acids [aa]), preS2 region (nt 3172 to 154 [55 aa]), and the small S gene (nt 155 to 835 [226 aa]), X gene (nt 1374 to 1838 [154 aa]), preC region (nt 1814 to 1897 [27 aa]), C gene (nt 1901 to 2452 [183 aa]), and P gene (nt 2307 to 1623 [832 aa]).

Sequence divergence of the JRB34 strain from other genotypes. The complete genome sequence of the JRB34 strain obtained in the present study was compared against those of 1,440 HBV genomes registered in the Viral Hepatitis Database (39). Estimated nucleotide sequence divergence of the JRB34 strain from four ape and nine human genotypes is summarized in the Table 1. The mean divergence by genotypes ranged from 10.7 and 10.9% (from orangutan and gibbon, respectively) to 15.6 and 15.7% (from genotypes F and H, respectively). Surprisingly, the minimum divergence of 9.9% was observed in comparison with a nonhuman HBV isolate from *Hilobates agilis* gibbon confiscated in Taiwan in 1993 (AY330917) (41). Since the sequence divergence from any documented genotypes, including recently proposed genotype I, exceeded 8%, the JRB34 strain was tentatively classified into a novel genotype J of HBV.

Phylogenetic analysis of the entire genomic sequence. In the phylogenetic tree constructed on 1,440 complete genome

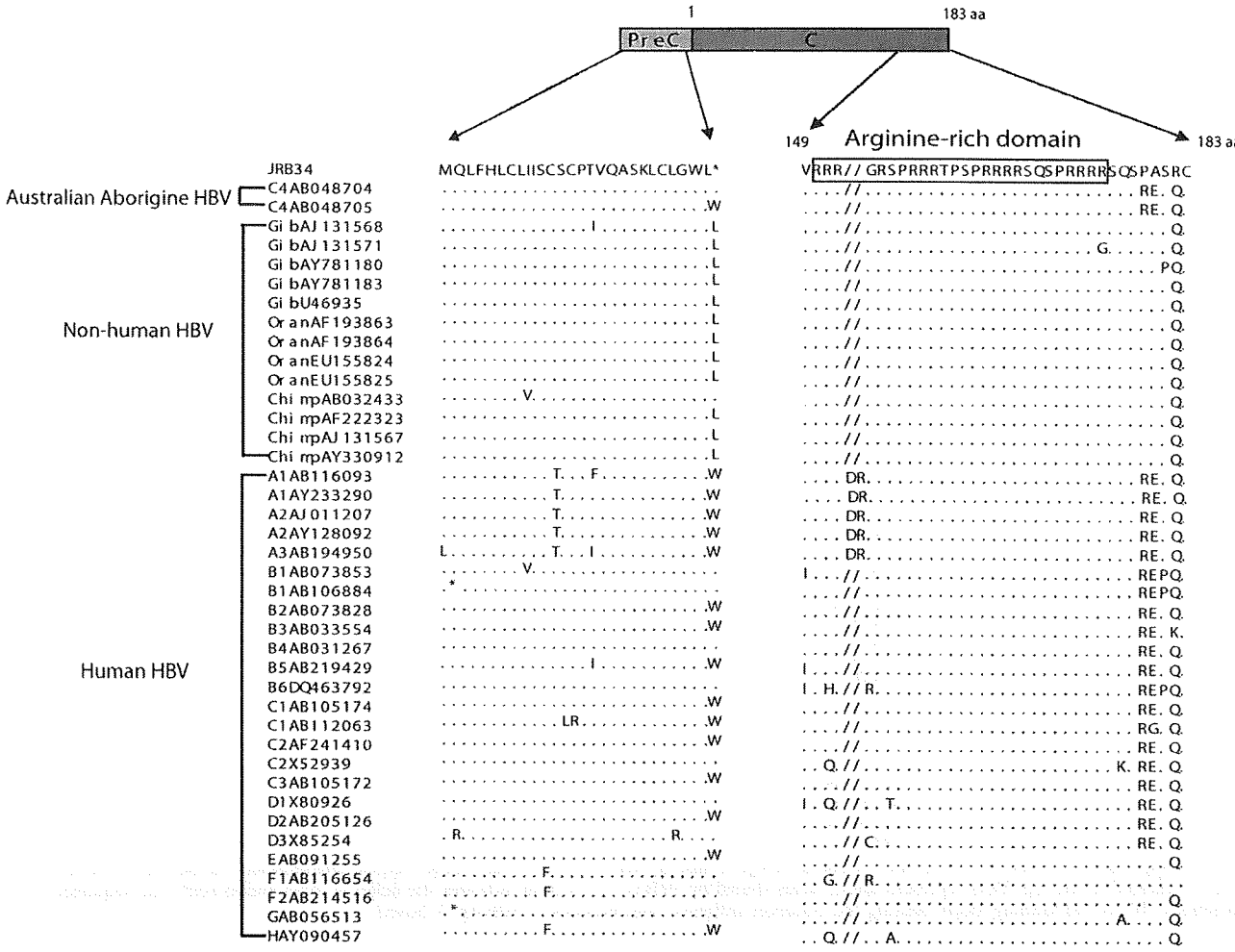

FIG. 4. Comparison of the amino acid sequence in the preC gene and carboxy-terminal amino acid sequences in the C gene of HBV isolates of various genotypes. The sequence of the HBV/J isolate (JRB34) is indicated at the top. Dots represent amino acids shared by JRB34, and a dash indicates the deletion of an amino acid. The sequence of the arginine-rich domain bearing the binding site with HBV DNA is boxed.

EMBL/DDBJ/GenBank database entries, the HBV/J strain was positioned distinctively from all known human genotypes (data not shown). It was closest to the cluster formed by gibbon- and orangutan-derived strains. However, including recombinant strains in such analyses may significantly affect the overall phylogenetic topology. This possibility was ruled out by reconstruction of the phylogeny using nonrecombinant HBV strains that further confirmed the phylogenetic peculiarity of the studied JRB34 strain (see Fig. S1 in the supplemental material). A total of 44 representative reference strains were further selected for establishing the consistency. Thus, phylogenetic topology indicating genotype-specific clustering is shown in the Fig. 1. Hence, using various sets of references, we confirmed that genotype J undoubtedly differed phylogenetically from all other known genotypes.

Lack of significant evidence of recombination with other human or ape genotypes in genotype J. To investigate possible recombination in the JRB34 genome, a window scanning analysis of aligned HBV genomes was performed by means of Simplot and Simmonics software packages. Both Bootscanning by SimPlot and GroupScanning by Simmonics showed similar output results. However, the methodological approach is different between these two software packages; GroupScanning provides more robust analysis of the phylogenetic relation between the examined strain and clusters of reference strains, whereas SimPlot does this comparison between the examined strain and parametrically generated consensus of the reference strains. The results obtained by SimPlot therefore can be significantly affected by selected parameters for the generation of consensus. This is especially undesirable when a new genotype strain (for which no references are available among known genotypes) is being analyzed (40). Figure 2 shows genome-wide distance scanning and GroupScanning plots for the JRB34 strain in comparison with a reference set consisting of 228 nonrecombinant HBV isolates retrieved from the public database (the phylogenetic tree is shown in Fig. S1 in the supplemental material). It is evident that the JRB34 strain was divergent from all known genotypes, and the closest genetic neighbors were estimated by distance and phylogenetic association scanning were the gibbon genotype (in preS, S, and P

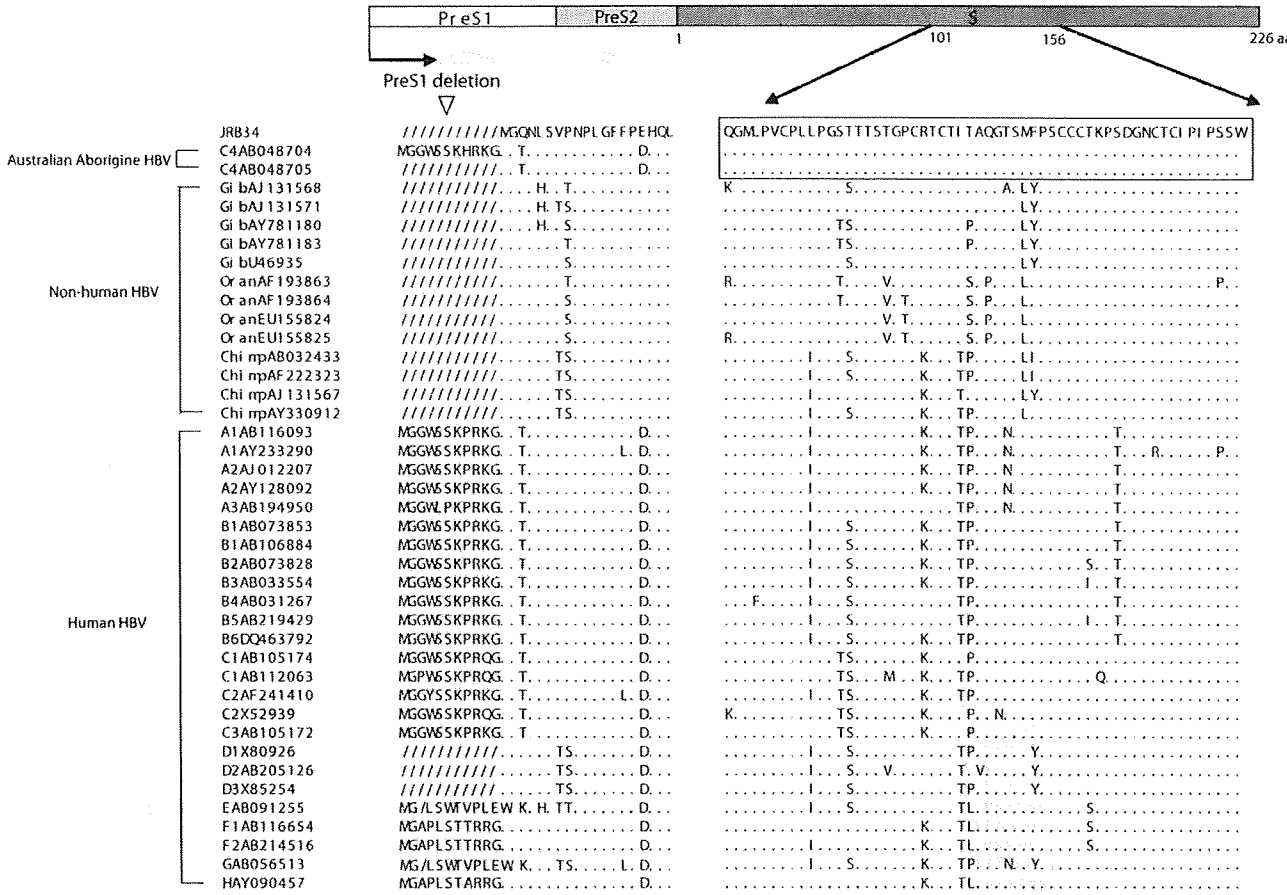

FIG. 5. Comparison of amino acid sequences of the preS/S gene among HBV isolates of various genotypes. The sequence of the HBV/J isolate (JRB34) is indicated at the top. Dots represent amino acids shared by JRB34, and a dash indicates the deletion of an amino acid. The sequence from positions 101 to 156 forming loops, bearing the common antigenic determinants of HBsAg, is boxed.

genes) and genotype C (in the core gene). However, no significant evidence of recombination between these two ape and human genotypes was revealed by the used methods. Homology scan carried out by SimPlot using the same set of reference sequences gave concordant results.

Phylogenetic analyses of the four open reading frames. Phylogenetic relationship between the JRB34 strain and other genotypes was further analyzed in four open reading frames. In the small S gene, subgenotype C4 recovered from Australian aborigines (43) changed its phylogenetic topology from the branch of human genotypes to a branch intermediate between orangutan and gibbon strains (Fig. 3A). Remarkably, genotype J and C4 strains joined together to create a clade between orangutan and gibbon strains. In contrast, genotype J clustered with human genotypes in the phylogenetic analysis of the C gene and was closely related to genotype C; it took a position outside genotype I strains, however (Fig. 3B). Genotype J was closer to gibbon and orangutan genotypes in the phylogenetic trees constructed on P and large S genes (data not shown), demonstrating its topology similar to that in the analysis of the entire genome (Fig. 1).

Amino acid sequence of the HBV/J isolate. The amino acid sequence of HBV/J was compared against those of other genotypes over three different areas of the genome. The amino acid sequence in the preC gene and arginine-rich domain in the carboxy-terminal sequence in the C gene were well conserved by genotype J (Fig. 4). In the preS1 region, genotype J had a deletion of 11 aa as gibbon and chimpanzee genotypes (Fig. 5). This deletion was shared by one of the two HBV/C4 isolates from Australian aborigines, as well as all HBV/D isolates. Amino acid sequence in the S gene of genotype J was the same as those of aborigine isolates of subgenotype C4; they would share antigenic epitopes of HBsAg. Amino acids at codons 122 and 160 were arginine (with G as nt 365) and lysine (with G as nt 479), respectively, which was consistent with subtype *ayw* of HBsAg from this patient (27).

Five domains (A to E) of DNA polymerase/reverse transcriptase in the P gene were preserved well in HBV/J, and it did not have mutations in the Tyr-Met-Asp-Asp motif in the domain C that determines the sensitivity to lamivudine (data not shown). HBV/J possessed A1762T/G1764A double mutations in the core promoter and G1896A stop codon mutation in the preC region, which was compatible with an HBeAg-minus phenotype of HBV recovered from the patient positive for anti-HBe.

Infection with HBV/J in chimeric mice with the liver repopulated for human hepatocytes. Two chimeric mice that had been transplanted with human hepatocytes were inoculated with 10^4 HBV DNA copies of genotype J. In both mice, HBV

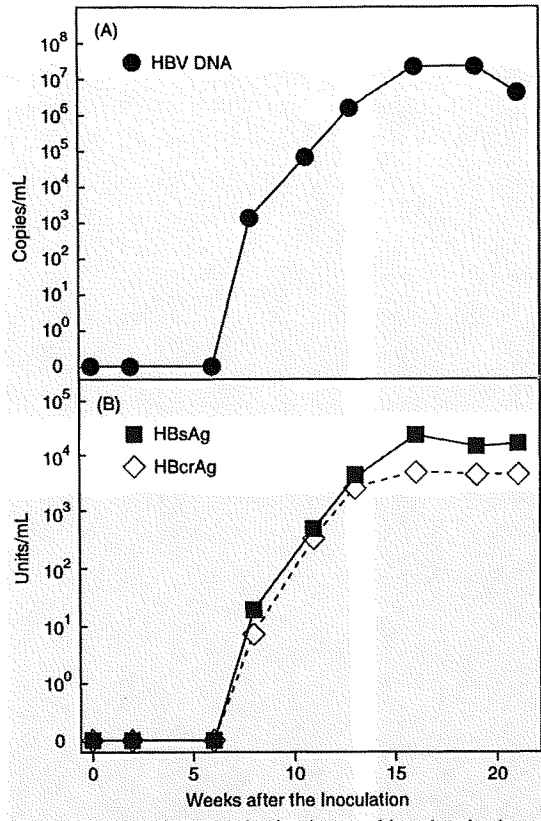

FIG. 6. Markers of HBV infection in two chimeric mice inoculated with the HBV/J isolate (JRB34). The levels of HBV DNA are illustrated in panel A, and those of HBsAg and HBcrAg are illustrated in panel B. Values represent the means for two mice.

DNA in a high titer (10^5 copies/ml) appeared in the circulation at week 7, plateaued at high levels (10^6 to 10^8 copies/ml), and stayed detectable until 22 weeks of observation after the inoculation (Fig. 6A). HBsAg and HBcrAg became detectable at week 7 and kept increasing in concentrations until week 15 when they reached a plateau at high levels (Fig. 6B). HBV strains recovered from mice at the last day of follow-up were identical in the complete genome sequence to the JRB34 strain used for inoculation.

The liver from chimeric mice infected with HBV/J was stained for HBcAg by immunofluorescence (Fig. 7A). The staining for HBcAg was confined to areas where mouse liver had been replaced for human hepatocytes, and the same areas were stained for human albumin (Fig. 7B). Colocalization of HBcAg and human hepatocytes was demonstrated by double staining for HBcAg and human albumin (Fig. 7C). Finally, expression and replication of the JRB34 strain were confirmed by successful detection of cccDNA and HBV RNA in the liver tissue from both sacrificed mice (see Fig. S2A and B in the supplemental material).

DISCUSSION

An HBV isolate (JRB34) was recovered from a male, 88-year-old Japanese patient with HCC and sequenced over the entire genome. In the full-genome sequence, the JRB34 strain had 10.9 to 15.7% divergence from 1,440 HBV strains retrieved from the DDBJ/EMBL/GenBank. The divergence exceeds 8% that has been defined originally for distinguishing between four genotypes (A to D) (29) and later for an additional four genotypes (E to H) (3, 26, 42). Phylogenetically, the sequence of JRB34 was closer to ape than human HBV genotypes. No significant evidence of recombination with eight known human and four ape genotypes was revealed by the GroupScanning analysis (40) and phylogenetic analyses. These lines of evidence have qualified the JRB34 strain to represent a possible new HBV genotype. To further confirm the epidemiological significance of this strain, capable of establishing new infections, two chimeric mice were each inoculated with 10^4 copies of JRB34 HBV DNA. They both were successfully infected with sharp increases in HBV DNA and HBsAg in serum several weeks after the inoculation. Replication in the chimeric mice was also confirmed by detection of cccDNA and HBV RNA in their liver tissues.

Recently, an HBV isolate from Vietnam (VH24 [accession no. AB231908]) was reported as a ninth human genotype (I) (12). However, VH24 differed by only 7.0% ± 0.4% from HBV isolates of genotype C and possessed complex recombination with genotypes A and G in three genomic areas. A number of sporadic HBV isolates have been reported to date that contain recombination between human genotypes (4, 24, 40), as well as between human and ape genotypes (21). Only a few recombinant variants, however, became widely spread in human populations, developing their own specific distributions and epidemiologies. This is particularly demonstrated for the B/C recombinant designated as a distinct subgenotype; Ba/B2-5 now accounts for the majority of genotype B strains in mainland Asia (44). Likewise, the C/D recombinant prevails in Tibet and northern China (50). To avoid assigning a new genotype for every newly discovered sporadic recombinant HBV variant, evidence of intergenotypic recombination should be carefully eliminated (14). However, in some cases, designation of a new genotype is proposed by a potential epidemiological significance of a novel genetic variant. Recently, a study carried out in Laos described a number of strains closely related phylogenetically with the Vietnamese genotype I strains, thereby suggesting their epidemiological significance (31). The JRB34 strain documented in the present study was genetically and phylogenetically distinct from any previously published strains, including those of genotype I from Vietnam and Laos. To avoid possible misconceptions in the future, the strain is provisionally designated genotype J.

HBV of distinct genotypes can infect great apes in the wild, including chimpanzee, gorilla, orangutan and gibbons (9, 20, 37, 51). HBV genotypes of chimpanzee and gorilla, as well as those of orangutan and gibbon, cocluster in agreement with their geographical distribution in Africa and Southeast Asia, respectively (41). Genotype J represented by the JRB34 strain clustered with gibbon/orangutan genotypes. In a phylogenetic analysis of the S region/gene sequence, JRB34 belonged to a nonhuman HBV group but was closely related to an HBV isolate of subgenotype C4 (AB048704) recovered from an Australian aborigine; C4 is most divergent from other subgenotypes of genotype C (43). In the phylogenetic analysis of the C gene, however, JRB34 clustered with human genotypes and closely related to genotype C, including C4, and was positioned

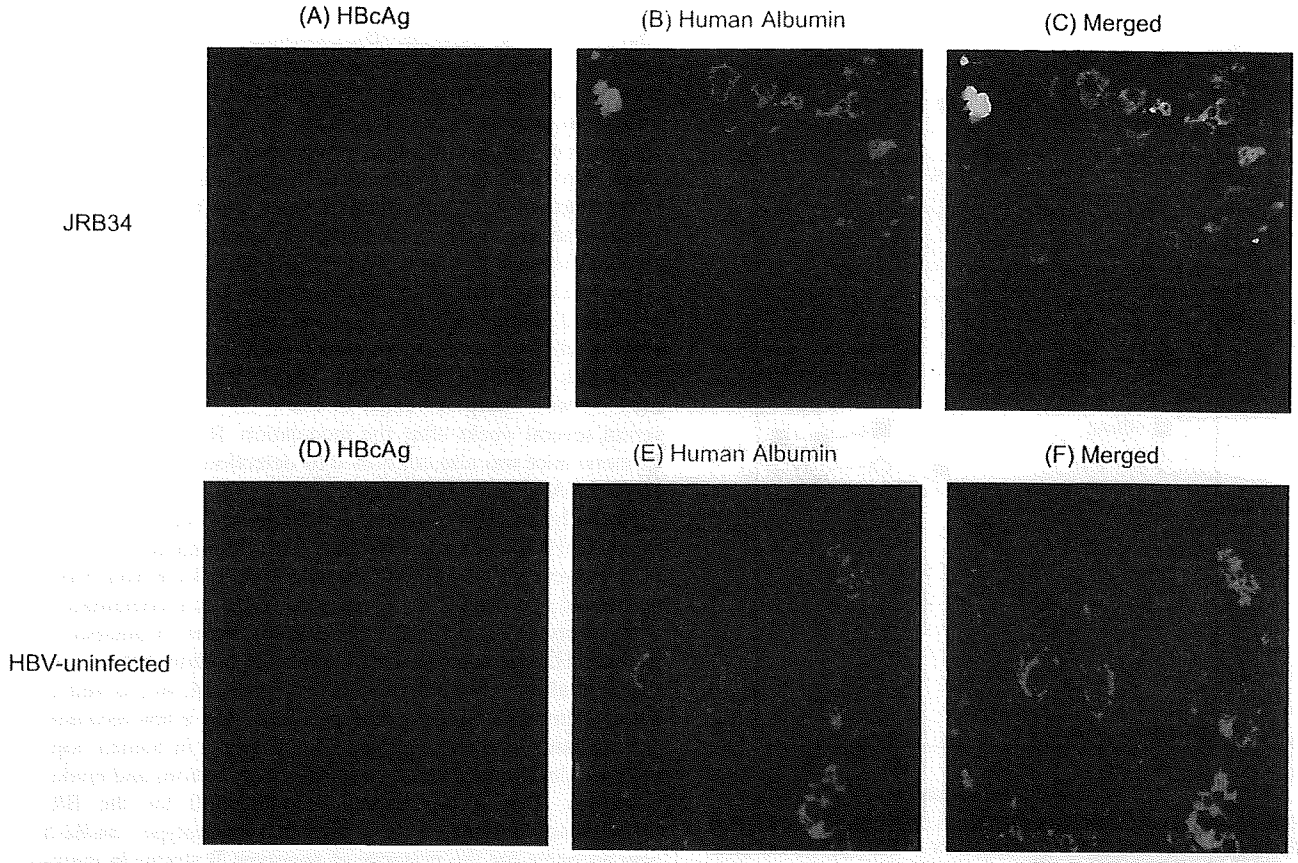

FIG. 7. (A and B) Immunofluorescent staining of a frozen liver section of a chimera mouse inoculated with the HBV/J isolate (JRB34). HBcAg is stained in panel A, and human albumin is stained in panel B. (C) Colocalization of HBcAg and human albumin is revealed by double staining. (D to F) HBV-uninfected mouse liver shows that only human albumin is stained.

outside genotype I strains (Fig. 4). Taken together, genotype J is phylogenetically close to gibbon/orangutan genotypes in the entire genome and to genotype C (C4 in particular) in the S and C genes. However, despite observed interchangeable relatedness with gibbon and genotype C/I strains, no strong evidence of recombination was confirmed in the JRB34.

In the sequence of C gene, carboxyl-terminal arginine-rich region, required for binding with HBV DNA, was preserved in JRB34. It had the G1896A stop codon in the precore region that aborts the translation of HBeAg (5, 30) and A1762T/G1764A double mutations in the core promoter that interfere with the transcription of HBeAg by downregulating preC mRNA (28, 45); they are compatible with the $HBeAg^-$ anti-HBe^+ phenotype of the patient from whom JRB34 was isolated. Since the double mutations are detected frequently in HBV DNA sequences from patients with HCC (17, 33), it could be implicated in hepatocarcinogenesis of the patient from whom JRB34 was isolated. It is not certain, however, if precore and core-promoter mutations had existed in HBV transmitted to the patient who is presumed to have been infected 60 years ago. Since amino acid sequences constituting antigenic loops of HBsAg (6) were the same as those of Australian aborigine isolates of C4, they would share antigenic epitopes of HBsAg. The amino acids at codons 122 and 160 were arginine (with G at nt 365) and lysine (with G at nt 479), respectively (27), in agreement with subtype *ayw* of HBsAg from this patient. Five domains (A to E) of DNA polymerase/reverse transcriptase in the P gene were preserved well in HBV/J, and it did not have mutations in the Tyr-Met-Asp-Asp motif in the domain C that determines the sensitivity to lamivudine (2).

How and when the patient contracted infection with HBV/J is not certain. It is very unlikely, however, that he acquired infection in Japan via perinatal or horizontal transmission. There are no wild primates in Okinawa, where the patient was originally from, and the prevalent human HBV genotypes are limited to B (60%), C (39%), and sporadic cases of A (1%) (32). Furthermore, HBV/J was not found among patient's family members who are currently alive (data not shown). The phylogenetic position within open reading frames of JRB34 in between gibbon/orangutan genotypes and human genotype C gives a clue where and when the patient had contracted HBV infection. He was drafted to Borneo during World War II (1939 to 1945); the island in the Southeast Asia is inhabited by gibbons and orangutans and has a local population mainly infected with genotypes B or C. Zoonotic infection of HBV has been previously reported (11, 46), and HBV of genotype E was recovered from a chimpanzee captured in West Africa where this genotype is common. There is a possibility that JRB34 of

genotype J had been transmitted to the study patient in Borneo during the war (38).

The origin of genotype J in gibbon/orangutan or human inhabitants in Borneo is not certain but very likely. HBV DNA and/or HBsAg was detected in 26% (55/213) and 20% (58/297) of gibbons and orangutans, respectively, captured in Southeast Asia (38). HBV is also endemic in people living there, with a prevalence of HBsAg at 2 to 8%. There would be high chances for cross-species transmission of HBV where it prevails both in human beings and nonhuman primates. Phylogenetic analysis for close relationship between human and nonhuman HBV genotypes has indicated geographical influence rather than association with particular species (41).

It is remains to be determined whether genotype J and ape-derived strains originate from species-specific convergent evolution of distant strains or whether they have diverged from a single common ancestor sometime in the past and evolved independently thereafter. The validity of cross-species infection or species-specific evolution for genotype J would be verified by sequence analysis of HBV DNA from gibbons and humans living in Borneo. If they turn out to be the same, cross-species infection will be justified. Should genotype J be restricted to human beings, in converse, species-specific infection will be confirmed.

In conclusion, a novel HBV genotype was identified in the Ryukyu isolate and provisionally named genotype J. Phylogenetic analyses over the full-length sequence and open reading frames indicate a close relationship of genotype J with gibbon/orangutan genotypes and human genotype C. The index patient would have been infected with HBV/J while he resided in Borneo inhabited by gibbons and orangutans. Although only one HBV isolate of genotype J (JRB34) has been identified, this may be only the tip of an iceberg. It would be worthwhile to examine the genotype of HBV infecting people and gibbons, as well as orangutans, living in Borneo and neighboring countries for mapping the epidemiology of genotype J and finding any clinical relevance.

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Genome-wide association of *IL28B* with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C

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The recommended treatment for patients with chronic hepatitis C, pegylated interferon-α (PEG-IFN-α) plus ribavirin (RBV), does not provide sustained virologic response (SVR) in all patients. We report a genome-wide association study (GWAS) to null virological response (NVR) in the treatment of patients with hepatitis C virus (HCV) genotype 1 within a Japanese population. We found two SNPs near the gene *IL28B* on chromosome 19 to be strongly associated with NVR (rs12980275, $P = 1.93 \times 10^{-13}$, and rs8099917, 3.11×10^{-15}). We replicated these associations in an independent cohort (combined *P* values, 2.84×10^{-27} (OR = 17.7; 95% CI = 10.0–31.3) and 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3), respectively). Compared to NVR, these SNPs were also associated with SVR (rs12980275, $P = 3.99 \times 10^{-24}$, and rs8099917, $P = 1.11 \times 10^{-27}$). In further fine mapping of the region, seven SNPs (rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917 and rs7248668) located in the *IL28B* region showed the most significant associations ($P = 5.52 \times 10^{-28}$–2.68×10^{-32}; OR = 22.3–27.1). Real-time quantitative PCR assays in peripheral blood mononuclear cells showed lower *IL28B* expression levels in individuals carrying the minor alleles ($P = 0.015$).

Hepatitis C is a global health problem that affects a significant proportion of the world's population. The World Health Organization estimated that in 1999, there were 170 million HCV carriers worldwide, with 3–4 million new cases appearing each year. HCV infection affects more than 4 million people in the United States, where it represents the leading cause of cirrhosis and hepatocellular carcinoma as well as the leading cause of liver transplantation[1]. The American Gastroenterological Association estimated that drugs are the largest direct costs of hepatitis C[1].

The most effective current standard of care in patients with chronic hepatitis C, a combination of PEG-IFN-α with ribavirin, does not produce SVR in all patients treated. Large-scale studies on 48-week-long PEG-IFN-α/RBV treatment in the United States and Europe showed that 42–52% of patients with HCV genotype 1 achieved SVR[2–4], and similar results were found in Japan. However, older patients (greater than 50 years of age) had a significantly lower rate of SVR due to poor adherence resulting from adverse events and laboratory-detectable abnormalities such as neutropenia and thrombocytopenia[5,6]. Specifically, various well-described side effects (such as a flu-like syndrome, hematologic abnormalities and adverse neuropsychiatric events) often necessitate dose reduction, and 10–14% of patients require premature withdrawal from interferon-based therapy[7]. To avoid these side effects in patients who will not be helped by the treatment, as well as to reduce the substantial cost of PEG-IFN-α/RBV treatment, it would be useful to be able to predict an individual's response before or early in treatment. Several viral factors, such as genotype 1, high baseline viral load, viral

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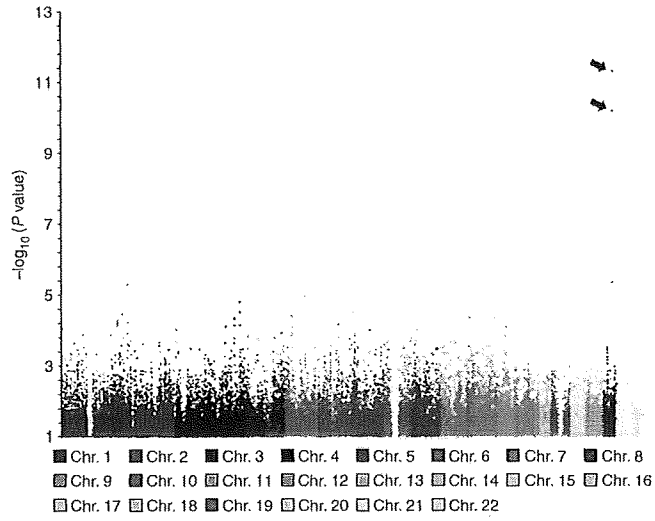

Figure 1 Genome-wide association results with PEG-IFN-α/RBV treatment in 142 Japanese patients with HCV (78 NVR and 64 VR samples). *P* values were calculated by using a χ^2 test for allele frequencies. The dots with arrows for chromosome 19 denote SNPs that showed significant genome-wide associations ($P < 8.05 \times 10^{-8}$) with response to PEG-IFN-α/RBV treatment.

kinetics during treatment, and amino acid pattern in the interferon sensitivity–determining region, have been reported to be significantly associated with the treatment outcome in a number of independent studies[8–10]. Studies have also provided strong evidence that ~20% of patients with HCV genotype 1 and 5% of patients with genotype 2 or 3 have a null response to PEG-IFN-α/RBV. No definite predictor of this resistance is currently available that make it possible to bypass the initial 12–24 weeks' treatment before deciding whether treatment should be continued. If a reliable predictor of non-response were identified for use in patients before treatment initiation, then an estimated 20%, including those who have little or no chance to achieve SVR, could be spared the side effects and cost of treatment.

Host factors, including age, sex, race, liver fibrosis and obesity, have also been reported to be associated with PEG-IFN-α/RBV therapy outcome[11,12]. However, little is known about the host genetic factors that might be associated with the response to therapy: thus far only a few candidate genes, including those encoding type I interferon receptor-1 (*IFNAR1*) and mitogen-activated protein kinase–activated protein kinase 3 (*MAPKAPK3*), have been reported to be associated with treatment response[13,14]. We describe here a GWAS for response to PEG-IFN-α/RBV treatment.

We conducted this GWAS to identify host genes associated with response to PEG-IFN-α/RBV treatment in 154 Japanese patients with HCV genotype 1 (82 with NVR and 72 with virologic response (VR), based on the selection criteria as described in Online Methods). We used the Affymetrix SNP 6.0 genome-wide SNP typing array for 900,000 SNPs. A total of 621,220 SNPs met the following criteria: (i) SNP call rate ≥95%, (ii) minor allele frequency (MAF) ≥1% and (iii) deviation from Hardy-Weinberg equilibrium (HWE) $P \geq 0.001$ in VR samples. After excluding 4 NVR and 8 VR samples that showed quality control (QC) call rates of <95%, 78 NVR and 64 VR samples were included in the association analysis. **Figure 1** shows a genome-wide view of the single-point association data based on allele frequencies. Two SNPs located close to *IL28B* on chromosome 19 showed strong associations, with a minor allele dominant model (rs12980275, $P = 1.93 \times 10^{-13}$, and rs8099917, $P = 3.11 \times 10^{-15}$, respectively), with NVR to PEG-IFN-α/RBV treatment (**Table 1**). The rs8099917 lies between *IL28B* and *IL28A*, ~8 kb downstream from *IL28B* and ~16 kb upstream from *IL28A*. These associations reached genome-wide levels of significance for both SNPs in this initial GWAS cohort (Bonferroni criterion $P < 8.05 \times 10^{-8}$ (0.05/621,220)). The frequencies of minor allele–positive patients were much higher in the NVR group than in the VR group for both SNPs (74.3% in NVR, 12.5% in VR for rs12980275; 75.6% in NVR, 9.4% in VR for rs8099917). Notably, individuals homozygous for the minor allele were observed only in the NVR group. The VR group, as compared to the NVR group, showed genotype frequencies closer to those in the healthy Japanese population[15], yet the minor allele frequencies were slightly higher in the transient virologic response (TVR) group (23.1%, 15.4%) than in the SVR group (9.8%, 7.8%) (**Table 1**). We applied the Cochrane-Armitage test on all the SNPs and found a genetic inflation factor, λ, of 1.029 for the GWAS stage (**Supplementary Fig. 1**). We also carried out principal component analysis in 142 samples for the GWAS stage together with the HapMap samples (CEU, YRI, CHB and JPT) (**Supplementary Fig. 2**); this suggested that the effect of population stratification was negligible.

Table 1 Significant association of two SNPs (rs12980275 and rs8099917) with response to PEG-IFN-α/RBV treatment

					Null responder (NVR[a], *n* = 128)			Responder (VR[a], *n* = 186)			Responder (SVR[a], *n* = 140)			NVR vs. VR		NVR vs. SVR	
dbSNP rsID	Nearest gene	MAF[b] (allele)	Allele (1/2)	Stage	11	12	22	11	12	22	11	12	22	OR (95% CI)[c]	*P* value[d]	OR (95% CI)[c]	*P* value[d]
rs12980275	*IL28B*	0.15 (G)	A/G	GWAS	20 (25.6)	54 (69.2)	4 (5.1)	56 (87.5)	8 (12.5)	0 (0.0)	46 (90.2)	5 (9.8)	0 (0.0)	20.3 (8.3–49.9)	1.93×10^{-13}	26.7 (9.3–76.5)	7.41×10^{-13}
				Replication	10 (20.0)	37 (74.0)	3 (6.0)	101 (82.8)	21 (17.2)	0 (0.0)	73 (82.0)	16 (18.0)	0 (0.0)	19.2 (8.3–44.4)	5.46×10^{-15}	18.3 (7.6–44.0)	8.37×10^{-13}
				Combined	30 (23.4)	91 (71.1)	7 (5.5)	157 (84.4)	29 (15.6)	0 (0.0)	119 (85.0)	21 (15.0)	0 (0.0)	17.7 (10.0–31.3)	2.84×10^{-27}	18.5 (10.0–34.4)	3.99×10^{-24}
rs8099917	*IL28B*	0.12 (G)	T/G	GWAS	19 (24.4)	56 (71.8)	3 (3.8)	58 (90.6)	6 (9.4)	0 (0.0)	47 (92.2)	4 (7.8)	0 (0.0)	30.0 (11.2–80.5)	3.11×10^{-15}	36.5 (11.6–114.6)	5.00×10^{-14}
				Replication	11 (22.0)	37 (74.0)	2 (4.0)	108 (88.5)	14 (11.5)	0 (0.0)	78 (87.6)	11 (12.4)	0 (0.0)	27.4 (11.5–65.3)	9.47×10^{-18}	25.1 (10.0–63.1)	1.00×10^{-14}
				Combined	30 (23.4)	93 (72.7)	5 (3.9)	166 (89.2)	20 (10.8)	0 (0.0)	125 (89.3)	15 (10.7)	0 (0.0)	27.1 (14.6–50.3)	2.68×10^{-32}	27.2 (13.9–53.4)	1.11×10^{-27}

[a]NVR, null virologic response; VR, virologic response; SVR, sustained virologic response. The 186 VRs consisted of 46 transient virologic response (TVRs) and 140 SVRs. [b]Minor allele frequency and minor allele in 184 healthy Japanese individuals[15]. The MAF of the SNPs in SVR is similar to that of TVR group, whereas that of NVR is much higher (76.6%). [c]Odds ratio for the minor allele in a dominant model. [d]*P* value by χ^2 test for the minor allele dominant model.

Table 3 Factors associated with NVR by logistic regression model

Factors	Odds ratio	95% CI	*P* value
rs8099917 (G allele)	37.68	16.71–83.85	<0.0001
Age	1.02	0.98–1.07	0.292
Gender (Female)	3.32	1.49–7.39	0.003
Re-treatment[a]	1.12	0.55–2.33	0.750
Platelet count	0.93	0.87–1.01	0.080
Aminotransferase level	1.00	0.99–1.00	0.735
Fibrosis stage[20]	1.10	0.73–1.66	0.658
HCV-RNA level	1.01	0.99–1.02	0.139

[a]Re-treatment, non-response to previous treatment with interferon-α (plus RBV).

To examine the relative contribution of factors associated with NVR, we used a logistic regression model. One tagging SNP located within *IL28B* (minor allele of rs8099917) was the most significant factor for predicting NVR, followed by gender (**Table 3**). Clinically, viral factors such as HCV genotype and HCV RNA level are important for the outcome of PEG-IFN-α/RBV therapy. Indeed, mean HCV-RNA level was significantly lower in SVR (SVR versus TVR, $P = 0.002$; SVR versus NVR, $P = 0.016$; **Supplementary Table 4**). Mean platelet count and the proportion of mild fibrosis (F1–F2) were significantly higher in SVR than in NVR.

Real-time quantitative PCR assays in peripheral blood mononuclear cells revealed a significantly lower level of *IL28* mRNA expression in individuals with the minor alleles (**Fig. 3**), suggesting that variant(s) regulating *IL28* expression is associated with a response to PEG-IFN-α/RBV treatment. *IL28B* encodes a cytokine distantly related to type I (α and β) interferons and the interleukin (IL)-10 family. This gene and *IL28A* and *IL29* (encoding IL-28A and IL-29, respectively) are three closely related cytokine genes that encode proteins known as type III IFNs (IFN-λs) and that form a cytokine gene cluster at chromosomal region 19q13 (ref. 16). The three cytokines are induced by viral infection and have antiviral activity[16,17]. All three interact with a heterodimeric class II cytokine receptor that consists of IL-10 receptor beta (IL10Rβ) and IL-28 receptor alpha (IL28Rα, encoded by *IL28RA*)[16,17], and they may serve as an alternative to type I IFNs in providing immunity to viral infection.

Notably, a recent report showed that the strong antiviral activity evoked by treating mice with TLR3 or TLR9 agonists was significantly reduced in both $IL28RA^{-/-}$ and $IFNAR^{-/-}$ mice, indicating that IFN-λ is important in mediating antiviral protection by ligands for TLR3 and TRL9 (ref. 18). IFN-λ induced a steady increase in the expression of a subset of IFN-stimulated genes, whereas IFN-α induced the same genes with more rapid and transient kinetics[19]. Therefore, it is possible that IFN-λ induces a slower but more sustained response that is important for TLR-mediated antiviral protection. This might be one of the ways that a genetic variant regulating *IL28* expression influences the response to PEG-IFN-α/RBV treatment. Further research will be required to fully understand the specific mechanism by which a genotype might affect the response to treatment.

In conclusion, the strongest associations with NVR were observed for seven SNPs, rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917 and rs7248668, that are located in the downstream flanking region, the third intron, the third exon, the first intron and the upstream flanking region of *IL28B*. Further studies following our report of this robust genetic association to NVR may make it possible to develop a pre-treatment predictor of which individuals are likely to respond to PEG-IFN-α/RBV treatment. This would remove the need for the initial 12–24 weeks of treatment that is currently used as a basis for a clinical decision about whether treatment should be continued. That would allow better targeting of PEG-IFN-α/RBV treatment, avoiding the unpleasant side effects that commonly accompany the treatment where it is unlikely to be beneficial, and reduce overall treatment costs. Because of the small number of samples in this study, we plan to conduct a further prospective multicenter study to establish these SNPs as a clinically useful marker.

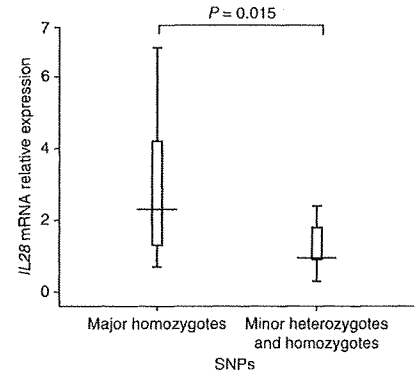

Figure 3 Quantification of *IL28* mRNA expression. The expression level of *IL28* genes was determined by real-time quantitative RT-PCR using RNA purified from peripheral blood mononuclear cells. Distribution of relative gene expression levels was compared between the individuals homozygous for major alleles ($n = 10$) and the heterozygous or homozygous individuals carrying minor alleles ($n = 10$) of rs8099917 by using the Mann-Whitney *U*-test. The bars indicate the median. All samples were obtained from HCV-infected patients before PEG-IFN-α/RBV therapy.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Study design and discussion: Y.T., N.N., N.M., K.T., M.M.; sample collection: Y.T., M.K., K.M., N.S., M.N., M.K., K.H., S.H., Y.I., E.M., E.T., S.M., Y.M., M.H., A.S., Y.H., S.N., I.S., M.I., K.I., K.Y., F.S., N.I.; genotyping: N.N.; statistical analysis: N.N., A.K., K.I.; quantitative RT-PCR: M.S.; manuscript writing: Y.T., N.N., K.T., M.M.

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ONLINE METHODS

Genotyping methods. *Illumina genotyping.* 1,968 Icelandic case and 35,382 Icelandic control samples were successfully assayed with the Infinium HumanHap300 SNP chip (Illumina) containing 317,503 haplotype tagging SNPs derived from phase I of the International HapMap project. Of the SNPs assayed on the chip, 2,906 SNPs had a yield <95%, and 271 SNPs had a minor allele frequency (in the combined set of cases and controls) <0.01 or were monomorphic. An additional 4,632 SNPs showed a significant distortion from Hardy-Weinberg equilibrium in the controls ($P < 1.0 \times 10^{-3}$). In total, 6,983 unique SNPs were removed from the study. Thus, the analysis reported in the main text uses 310,520 SNPs. Any samples with a call rate below 98% were excluded from the analysis.

Replication genotyping. Single SNP genotyping of the SNPs reported in the main text for the case-control groups from Iceland, The Netherlands, Spain and Chicago was carried out by deCODE Genetics in Reykjavik, Iceland, applying the Centaurus[20] platform. The quality of each Centaurus SNP assay was evaluated by genotyping each assay in the CEU and/or YRI HapMap samples and comparing the results with the HapMap publicly released data. Assays with >1.5% mismatch rate were not used, and an LD test was used for markers known to be in LD. We regenotyped >10% of the samples and observed a mismatch rate <0.5%. Genotyping of samples from Finland and from Nashville, Tennessee, USA, was done using the same Centaurus assays as was used in Iceland at the University of Tampere and Vanderbilt University, respectively, using standard protocols.

For each of the SNPs discussed in the main text, the yield was >95% for those samples for which genotyping was attempted in all study groups.

The SNPs rs16902094 on 8q24 and rs11228565 on 11q13 are not present on the HumanHap300 chip. Therefore, using a single SNP assay for genotyping, an attempt was made to genotype 6,900 and 800 individuals, respectively, of the 35,382 Icelandic controls as well as 1,860 Icelandic cases and all available individuals from the replication study groups.

Discovery of a previously unidentified SNP on 8q24 by Solexa resequencing. In order to search for new SNPs on 8q24, a 527-kb region (128,113,108–128,640,337 bp, Build 36) was sequenced using the Solexa resequencing platform (Illumina Inc.). From our set of ~2,000 cases, 800 were selected randomly and split into two DNA pools, each with 400 samples. Similarly, 800 control individuals not known to have prostate cancer were selected randomly and split into two DNA pools with 400 samples each. Dilutions were prepared in duplicates for the four pools and used for long-range PCR reactions (each amplimer consisted of ~10 kb). PCR fragments were run on 0.8% agarose gels, the DNA was visualized with BlueView (Sigma Inc.) under normal light and the sizes of the PCR fragments were estimated with HindIII-digested lambda size marker (Fermentas Inc). Bands of correct sizes were excised out of the agarose gels and purified with Qiagen gel extraction kit (Qiagen Inc.). The PCR products were quantified by PicoGreen assay (Invitrogen Inc.) as described by the manufacturer. The preparation of the Solexa DNA libraries, the cluster generation and the DNA sequencing was done according to a published procedure[21]. The SNP analysis pipeline is composed of four components: alignment, SNP calling, filtering and association analysis. Promising SNPs were selected for further study and confirmation using Centaurus single-track SNP assays.

Statistical analysis. *Association analysis.* For SNPs that were in strong LD, whenever the genotype of one SNP was missing for an individual, the genotype of the correlated SNP was used to provide partial information through a likelihood approach as previously described[9]. This ensured that results presented in **Supplementary Table 5** were based on the same number of individuals and allowed meaningful comparisons of results for correlated SNPs. A likelihood procedure described in a previous publication[22] and implemented in the NEMO software (deCODE) was used for the association analyses.

We tested the association of an allele to prostate cancer using a standard likelihood ratio statistic that, if the subjects were unrelated, would have asymptotically a χ^2 distribution with 1 degree of freedom under the null hypothesis. Allelic frequencies rather than carrier frequencies are presented for the markers in the main text. Allele-specific ORs and associated P values were calculated assuming a multiplicative model for the two chromosomes of each individual[23]. Results from multiple case-control groups were combined using a Mantel-Haenszel model[24] in which the groups were allowed to have different population frequencies for alleles, haplotypes and genotypes but were assumed to have common relative risks (see ref. 3 for a more detailed description of the association analysis).

The control groups from Iceland, The Netherlands, Spain and Finland included both male and female controls. No significant difference between male and female controls was detected for SNPs presented in **Tables 1–4** for each of these four groups. Controls from other study groups included only males.

In order to assess the association for the SNP rs4962416 on 10q26, which is in the CEU section of the HapMap database but is absent from the Illumina HumanHap300 chip, we used a method based on haplotypes of two markers (rs7077275 and rs893856) present on the chip. We used a method we have previously employed[25] that is an extension of the two-marker haplotype tagging method[26] and is similar in spirit to two other proposed methods[27,28]. We computed associations with a linear combination of the haplotypes chosen to act as surrogates to HapMap markers in the regions. These calculations were based on 1,724 prostate cancer cases and 35,322 controls genotyped on the HumanHap300 chip.

Multivariate analysis. In a multivariate analysis, we combined the effects of 22 variants associated with risk of prostate cancer using estimates based on data from only the Icelandic study group. A multiplicative model was assumed at each variant and between all variants. For the 21 autosomal variants, we tested for deviation from the multiplicative model when comparing it to the full model of genotypic OR. Given the number of tests performed, no significant deviation from the multiplicative model was detected (all $P > 0.0024$, corresponding to $P = 0.05$ divided by 21). For the variant located on the X chromosome (rs5945572), there exist only two male genotypes (carrier and noncarrier). Also, we tested the pairwise interactions between the 22 risk variants using logistic regressions including terms corresponding to the 231 possible pairs. Given the number of tests performed, no significant deviation from the multiplicative model was detected with a level of significance <0.00022 (corresponding to $P = 0.05$ divided by 231). Similarly, the absence of interaction between variants has previously been reported[5,16,29]. Odds ratios were calculated for all possible genotype combinations based on these 22 variants and expressed relative to the average general population risk. The combined OR estimates were then divided into OR ranges and presented along with the percentage of the population within each OR range (**Table 6**). The general population risk was determined using a frequency-weighted average risk for all possible genotypes.

The Icelandic samples were part of the initial discovery study populations for 10 of these 22 variants, and therefore the estimates for these variants may be inflated due to winner's curse (whereby marginally significant association produces inflated effect due to the fact that their only chance to be significant is to have those estimates inflated). From **Tables 1–4**, we are using the estimates for the following variants: rs10934853 on 3q21.3, rs11228565 on 11q13 and rs8102476 on 19q13.2. For the five variants (rs1447295, rs6983267, rs16901979, rs16902094 and rs445114) on 8q24.21, we are using the adjusted estimates as reported in **Supplementary Table 3b**. From **Table 5**, we use the estimates for all variants except rs1571801 on 9q33 because its effect was in the opposite direction compared to the original publication and rs10896450 on 11q13 for which data for the refinement SNP (rs11228565) in **Tables 1–4** was used. Furthermore, for the two variants on 17q12, reported in **Table 5**, we are using the estimates adjusted for each other because the two markers are located ~25 kb from each other but are not correlated ($D' = 0.03$, $r^2 = 0.0004$ according to CEU HapMap data); rs4430796 has an adjusted OR of 1.17 (95% CI: 1.08–1.26) and rs11649743 has an adjusted OR of 1.06 (95% CI: 0.99–1.14).

We applied the combined genetic relative risk to the lifetime risk by multiplying them together. Because the association with disease for these variants has not been shown to depend on age at diagnosis[3,16], we assumed that the individual estimates for each variant will have a similar effect at any given age. The lifetime risk of getting prostate cancer is estimated to be 12% on average in Iceland before the age of 75, according to Cancer Incidence, Mortality and Prevalence in the Nordic Countries (NORDCAN) (see URL section).

Analysis of the CGEMS data. For the five individual study populations from the CGEMS study[5,7] (ACS, American Cancer Society Prevention Study

II (US); ATBC, Alpha-Tocopherol, Beta-Carotene Prevention Study (Finland); FPCC, CeRePP French Prostate Case-Control Study (France); HPFS, Health Professionals Follow-up Study (US); PLCO, Prostate, Lung, Colon, Ovarian Trial (US)), when assessing the allelic effect, we used the precomputed data released in spring 2008 corresponding to 'all case versus control (dichotomous), genotype trend effect model, adjusted'. When assessing the genotypic effect at each loci for the CGEMS study we used the precomputed 'All case versus control (dichotomous), genotype-specific effect model, adjusted, ALL (ACS, HPFS, FPCC, ATBC, PLCO)'.

Correction for relatedness. Some individuals in the Icelandic case-control groups were related to each other, causing the aforementioned χ^2 test statistic to have a mean >1. We estimated the inflation factor by using a previously described procedure[30] in which we simulated genotypes through the genealogy of the 37,350 Icelanders analyzed in the present study (number of simulations = 100,000). The inflation factor was estimated to be 1.10. Results from the Icelandic samples presented in the main text are based on adjusting the χ^2 statistics by dividing each of them by 1.10.

URLs. Cancer Genetics Markers of Susceptibility (CGEMS) study, http://cgems.cancer.gov/; The Icelandic Cancer Registry, http://www.krabbameinsskra.is/indexen.jsp?icd=C61; The International HapMap Project, http://hapmap.org/index.html; a cancer stat fact sheet at NORDCAN, The Association of the Nordic Cancer Registries, http://www-dep.iarc.fr/NORDCAN/english/StatsFact.asp?cancer=241&country=352.

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Original Article

Case–control study for the identification of virological factors associated with fulminant hepatitis B

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Background: Host and viral factors can promote the development of fulminant hepatitis B (FHB), but there have been no case–control studies for figuring out virological parameters that can distinguish FHB.

Methods: In a case–control study, virological factors associated with the development of FHB were sought in 50 patients with FH developed by transient hepatitis B virus (HBV) infection (FH-T) and 50 with acute self-limited hepatitis B (AHB) who were matched for sex and age. In addition, 12 patients with FH developed by acute exacerbation (AE) of asymptomatic HBV carrier (ASC) (FH-C) were also compared with 12 patients without FH by AE of chronic hepatitis B (AE-C).

Results: Higher HBV DNA levels, subgenotype B1/B], A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent ($P < 0.05$), while hepatitis B e-antigen was less frequent in the FH-T patients than AHB. In multivariate analysis, G1896A mutation (odds ratio [OR], 13.53; 95% confidence interval [CI], 2.75–66.64), serum HBV DNA more than 5.23 log copies/mL (OR, 5.14; 95% CI, 1.10–24.15) and total bilirubin more than 10.35 mg/mL (OR, 7.81; 95% CI, 1.77–34.51) were independently associated with a fulminant outcome by transient HBV infection. On the other hand, in comparison with the patients between FH-C and AE-C groups, there was no significant difference of virological factors associated with the development of FHB.

Conclusion: A number of virological factors have been defined that may distinguish FH-T from AHB in a case–control study. The pathogenic mechanism of FHB between transient HBV infection and AE of ASC would be different.

Key words: acute exacerbation of asymptomatic hepatitis B virus carrier, fulminant hepatitis, genotypes, transient hepatitis B virus infection

INTRODUCTION

IN JAPAN, 634 patients with fulminant hepatitis (FH) were registered from 1998–2003. Of them, 41.8% were infected with hepatitis B virus (HBV) that is the most frequent cause of FH there.[1] HBV is classified into eight genotypes (A–H) based on a sequence divergence of more than 8% in the entire genome of approximately

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3200 nucleotides.[2–5] They have distinct geographical distributions and are associated with the severity of liver disease.[6,7] Furthermore, subgenotypes have been reported for HBV/A, B and C, and they are named A1/Aa (Asian/African type) and A2/Ae (European type),[8] B1/Bj (Japanese type) and B2/Ba (Asian type),[9] and C1/Cs (Southeast Asian type) and C2/Ce (East Asian type).[10,11] HBV genotypes/subgenotypes and mutations in the pre-core region and the core promoter can influence the viral replication and expression of hepatitis B e-antigen (HBeAg).[6,12]

Acute HBV infection in adulthood resolves in the most cases by far, but can induce FH or go on to become chronic in some. It has been reported that host and viral factors may influence the development of fulminant hepatitis B (FHB), but the pathogenesis of FHB remains unclear. As for virological factors associated with FHB, mutations in the core promoter (A1762T/G1764A)[13] and the pre-core region (G1896A)[14–16] have been reported in association with the development of FHB in Asia and the Middle East. Additional mutations, including T1753V, T1754V and A2339G in the core gene are implicated, also.[17,18] In regard of HBV genotypes, subgenotype B1/Bj is highly associated with the development of FHB in Japan.[15] In contrast, an association of HBV genotypes with the fulminant outcome has not been reproduced in patients from the USA and Europe.[19–22] Such a discrepancy would be attributed, at least in part, to distinct geographical distributions of HBV genotypes/subgenotypes over the world.

The original definition by Trey *et al.*[23] about fulminant hepatic failure is widely used all over the world. On the other hand, in Japan, the diagnosis of FH was contingent on a slight modification of Trey's original definition by the Inuyama Symposium (Aichi, Japan in 1981). Furthermore, the Intractable Liver Diseases Study Group of Japan modified the criteria for the etiology of FH and late-onset hepatic failure in 2002. According to the criteria of the Intractable Liver Diseases Study Group of Japan, there are two clinical entities of FHB that are induced, respectively, by transient HBV infection and acute exacerbation (AE) of an asymptomatic HBV carrier (ASC).[1]

Recently, FH developing in ASC who undergo AE is increasing in Japan.[1] In patients with hematological malignancy, in particular, rituximab and/or glucocorticoid, can reactivate HBV for the development of FHB.[24] The outcome is poor for FHB precipitating in ASC who undergo acute exacerbation,[1] but it has been difficult to identify it by clinical examinations.

As there have been no case-control studies for figuring out virological parameters that can distinguish FHB, a case-control study was conducted on the patients with FH by transient HBV infection and acute self-limited hepatitis B (AHB) in this study, for the identification of virological factors that influence a fulminant outcome. In addition, the patients with FH by AE of ASC, which is assumed as a different clinical condition from transient HBV infection, were also compared with the patients without FH by AE of chronic hepatitis B (CHB) in a case-control study.

METHODS

Patients

DURING 9 YEARS from 1998 to 2006, in twenty-six hospitals all over Japan, sera were obtained from the 50 FH patients by transient HBV infection (the FH-T group) and the 50 patients with AHB (the AHB group) who were controlled for age and sex. As the elder patients with FHB were enrolled in this study (mean age, 42.8 years), the mean age of AHB patients became relatively high (42.9 years, Table 1). Furthermore, the 12 FH patients developed by AE of ASC (the FH-C group) were also compared with the 12 patients without FH by AE of CHB who were matched by age and sex (the AE-C group).

All the serum samples tested for this study were collected at hospitalization. All 124 patients had hepatitis B surface antigen (HBsAg) in serum. Infection with hepatitis A virus and hepatitis C virus, as well as alcoholic hepatitis, were excluded in them.

The diagnosis of acute hepatitis B was based on sudden manifestation of clinical symptoms of hepatitis and detection of high-titered immunoglobulin (Ig)M anti-hepatitis B core (HBC). Patients with initial high-titered anti-HBC (>90% inhibition by a 1:200 diluted serum) were excluded. The diagnosis of FH was contingent on a slight modification by Inuyama Symposium (Aichi, Japan in 1981) of the original definition by Trey *et al.*:[23] (i) coma of grade II or higher; and (ii) a prothorombin time less than 40% developing within 8 weeks after the onset of hepatitis. To exclude AE of ASC in FH-T and AHB groups, we confirmed the negativity of HBsAg before onset of FHB or AHB and no family histories of hepatitis were found among all the patients. Furthermore, serum HBsAg in all patients with FH-T or AHB became naturally seronegative within 24 weeks. AE of ASC or CHB was defined as the elevation of alanine aminotransferase (ALT >300 IU/L) or total bilirubin (T.bil >3.0 mg/dL).[25] All 24 patients with AE of ASC or CHB could be confirmed positive for serum HBsAg before the onset of acute liver injury.

Table 1 Baseline characteristics between fulminant hepatitis B patients by transient infection (FH-T) and acute self-limited hepatitis B (AHB) patients

Features	FH-T ($n = 50$)	AHB ($n = 50$)	Differences *P*-value
Age (years)	42.8 ± 16.1	42.9 ± 14.6	Matched
Men	25 (50%)	25 (50%)	Matched
ALT (IU/L)	3788 ± 2856	2170 ± 1350	<0.001
AST (IU/L)	3131 ± 3673	1676 ± 1851	<0.05
Total bilirubin (mg/dL)	14.8 ± 8.6	9.5 ± 9.8	<0.01
Prothrombin time (%)	16.9 ± 11.2	72.8 ± 26.0	<0.001
HBeAg positive	15 (30%)	28 (56%)	<0.01
Core protein (log U/mL)	3.21 ± 1.28	3.01 ± 1.00	NS
HBcrAg (log U/mL)	5.30 ± 1.32	5.95 ± 1.13	<0.01
HBV DNA (log copies/mL)	5.97 ± 1.87	4.98 ± 1.17	<0.005
Deceased	19 (38%)	0 (0%)	<0.001

AHB, acute self-limited hepatitis B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FH-T, fulminant hepatitis B by transient HBV infection; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

Serological markers of HBV infection

Hepatitis B surface antigen, HBeAg and the corresponding antibody (anti-HBe) were determined by enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan) or chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan). Anti-HBC of IgM and IgG classes were determined by radioimmunoassay (Abbott Japan). Core protein constituting the viral nucleocapsid and HBV core-related antigen (HBcrAg), both of which correlate with HBV DNA in serum, were measured by CLEIA as described elsewhere.[26,27]

Quantification of serum HBV DNA

Hepatitis B virus DNA sequences spanning the S gene were amplified by real-time detection polymerase chain reaction (RTD-PCR) in accordance with the previously described protocol[28] with a slight modification;[6] it has a detection limit of 100 copies/mL.

Sequencing and molecular evolutionary analysis of HBV

Nucleic acids were extracted from serum samples (100 μL) using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and subjected to PCR for amplifying genomic areas bearing enhancer II/core promoter/pre-core/core regions [nt 1628–2364], as described previously.[29] The target of PCR covered several mutations which were associated with FHB. Amplicons were sequenced directly with use of the ABI Prism Big Dye ver. 3.0 kit in the AMI 3100 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were analyzed in both forward and backward directions.

Hepatitis B virus genotypes were determined by molecular evolutionary analysis. Reference HBV sequences were retrieved from the DDBJ/EMBL/GenBank database and aligned by CLUSTAL X, then genetic distances were estimated with the 6-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/).[30] Based on obtained distances, phylogenetic trees were constructed by the neighbor-joining (NJ) method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times.

Statistical analysis

Statistical differences were evaluated by the Mann–Whitney *U*-test, Fisher's exact probability test and χ^2-test, where appropriate. Differences were considered to be statistically significant at $P < 0.05$. Multivariate analyses with logistic regression were utilized to sort out independent risk factors for FHB. STATA Software ver. 8.0 was employed for all analyses.

RESULTS

Baseline characteristics of the patients with FHB by transient HBV infection and AHB

TABLE 1 COMPARES baseline clinical characteristics of the 50 FH-T patients and the 50 AHB who

were matched for age and sex. The peak ALT, AST and T.bil levels were significantly higher (3788 ± 2856 vs 2170 ± 1350 IU/L, $P < 0.001$; 3131 ± 3673 vs 1676 ± 1851 IU/L, $P < 0.05$; and 14.8 ± 8.6 vs 9.5 ± 9.8 mg/dL, $P < 0.01$, respectively), while HBeAg was less frequent (30% vs 56%, $P < 0.01$) in the FH-T patients than AHB. The level of HBcrAg was significantly lower (5.30 ± 1.32 vs 5.95 ± 1.13 log U/mL, $P < 0.01$), while HBV DNA loads were higher (5.97 ± 1.87 vs 4.98 ± 1.17 log copies/mL, $P < 0.005$), in the FH-T patients than AHB. The level of core protein in sera tended to be higher in the FH-T patients than AHB (3.21 ± 1.28 vs 3.01 ± 1.00 log U/mL). Death occurred more often in the FH-T patients than AHB (38% vs 0%, $P < 0.001$).

HBV Genotypes and enhancer II/core promoter/pre-core/core Mutations in Patients with FHB by transient HBV infection and AHB

Figure 1(a) compares the distribution of HBV genotypes/subgenotypes between the FH-T and the AHB patients. The subgenotype C2/Ce was most prevalent in both patients with FH-T and AHB (66% and 62%, respectively), whereas B1/Bj was more frequent in the FH-T patients than AHB (22% vs 6%, $P < 0.05$). Likewise, mutations in enhancer II/core promoter/pre-core/core regions are compared between the FH-T and AHB patients in Figure 1(b). A1762T/G1764A, G1896A, G1899A and A2339G mutation were more frequent in the FH-T patients than AHB (48% vs 16%, $P < 0.001$; 62% vs 6%, $P < 0.001$; 24% vs 4%, $P < 0.001$; and 8% vs 0%, $P < 0.05$, respectively).

Figure 2(a) compares various mutations between the 11 FH-T patients and the three AHB patients who were infected with B1/Bj. Only G1896A was significantly more frequent (73% vs 0%, $P < 0.05$), while the lack of any mutations was less common (0% vs 33%, $P < 0.05$) in the FH-T patients than AHB. In comparison with the 33 FH-T patients and the 31 AHB patients who were infected with C2/Ce (Fig. 2b), A1762T/G1764A (70% vs 19%, $P < 0.001$), G1896A (61% vs 6%, $P < 0.001$) and the combination of all three mutations (A1762T/G1764A and G1896A) (45% vs 6%, $P < 0.001$) were significantly more frequent, while the lack of any mutations was less common (9% vs 70%, $P < 0.001$) in the FH-T patients than AHB. Interestingly, all the AHB patients with both G1896A and A1762T/G1764A mutations suffered acute severe hepatitis B that was defined by prothrombin time less than 40% but without coma of grade II or higher.

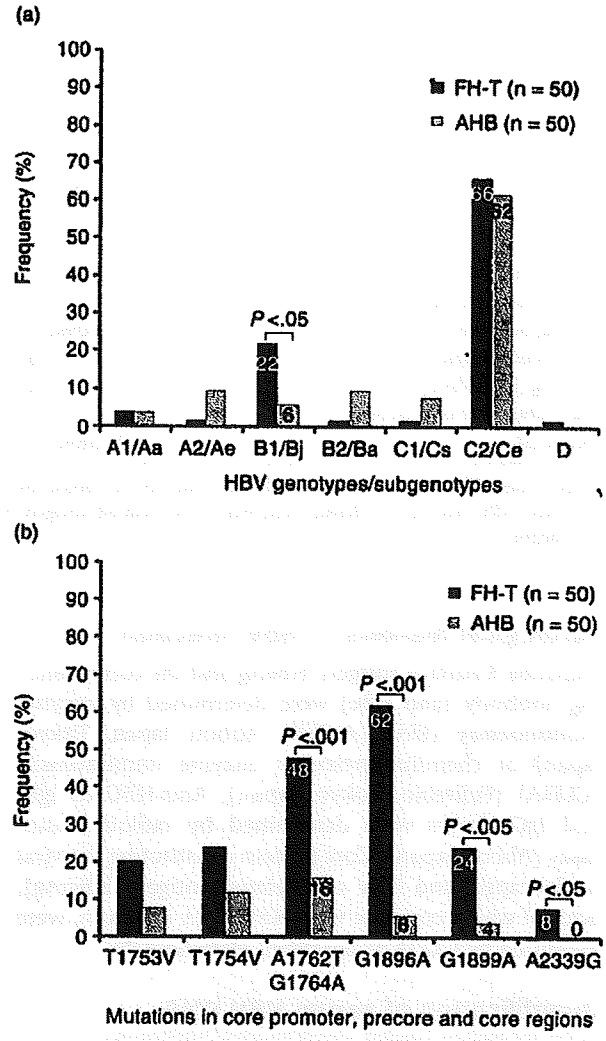

Figure 1 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 50 transient hepatitis B virus infection (FH-T) and the 50 acute self-limited hepatitis B (AHB) patients.

Factors independently associated with the development of FHB by transient HBV infection

The following independent factors, promoting the development of FHB, were evaluated by multivariate analysis: ALT, AST, T.bil, HBeAg, HBV DNA, core protein, HBcrAg, genotypes/subgenotypes (B1/Bj or not) and mutations (T1753V, T1754V, A1762T/G1764A, G1896A, G1899A and A2339G). T.bil more than 10.35 mg/dL (OR, 7.81 [95% CI, 1.77–34.51], $P = 0.0067$), G1896A mutation (OR, 13.53 [95% CI,

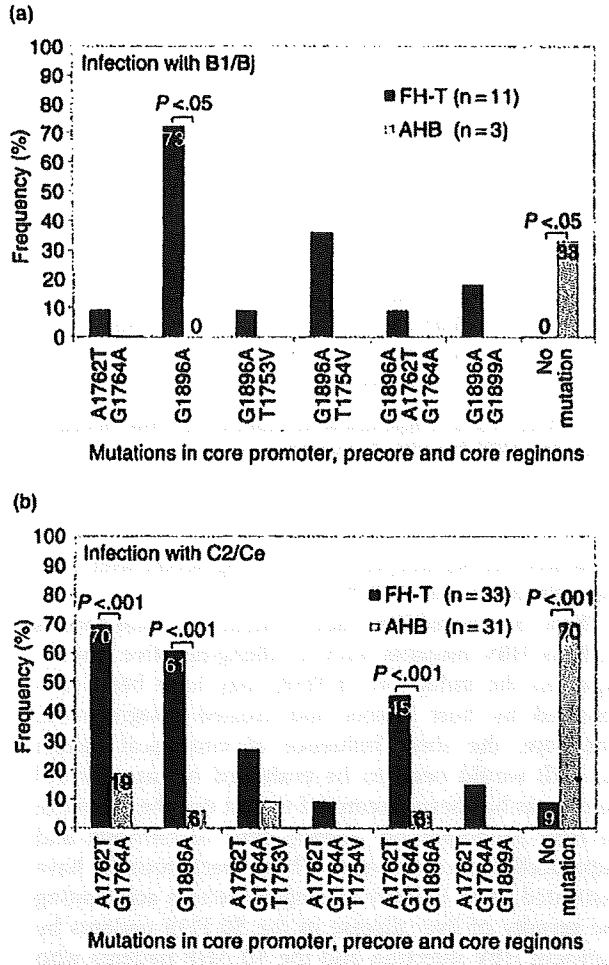

Figure 2 Frequencies of core promoter, pre-core and core mutations compared between the transient hepatitis B virus infection (FH-T) and the acute self-limited hepatitis B (AHB) patients who were infected with HBV of subgenotype B1/Bj (a) or C2/Ce (b).

2.75–66.64], $P = 0.0014$) and serum HBV DNA more than 5.23 log copies/mL (OR, 5.14 [95% CI, 1.10–24.15], $P = 0.0379$) were independent risk factors for the development of FHB by transient HBV infection (Table 2). Other mutations (T1753V, T1754V, A1762T/G1764A, G1899A and A2339G) were not significantly associated with the development of FHB by transient HBV infection, however.

Baseline clinical characteristics for distinguishing between the patients with FHB by AE of ASC (FH-C) and those without FHB by AE of CHB (AE-C)

Table 3 compares baseline clinical characteristics between the 12 FH-C patients and the 12 AE-C patients who were matched for age and sex. The levels of T.bil were significantly higher in the FH-C patients (15.0 ± 7.3 vs 7.3 ± 8.8 mg/dL, $P < 0.05$), but the peak ALT and AST levels tended to be slightly higher in the FH-C patients than AE-C (887 ± 681 vs. 641 ± 620 IU/L and 701 ± 451 vs 601 ± 753 IU/L, respectively). There were also no significant differences in levels of sera HBV DNA, core protein and HBcrAg between these two groups (7.44 ± 1.51 vs 6.60 ± 1.10 log copies/mL, 5.04 ± 1.45 vs 5.07 ± 1.07 log U/mL, and 6.35 ± 1.70 vs 6.29 ± 1.95 log U/mL, respectively).

HBV genotypes and enhancer II/core promoter/pre-core/core mutations between the patients with FH-C and those with AE-C

There were no significant differences in the frequencies of any HBV genotypes between the 12 FH-C patients and the 12 AE-C patients (Fig. 3a). In addition, there were also no significant differences in the frequencies

Table 2 Multivariate analysis for factors independently associated with fulminant hepatitis by transient HBV infection

Factors	Odds ratio	95% confidence interval	*P*-value
Total bilirubin (mg/dL)†			
<10.35	1		
≥10.35	7.81	1.77–34.51	0.0067
G1896A mutation			
Absent	1		
Present	13.53	2.75–66.64	0.0014
HBV DNA (log copies/mL)†			
<5.23	1		
≥5.23	5.14	1.10–24.15	0.0379

†Median values. HBV, hepatitis B virus.

Table 3 Baseline characteristics between patients with FH by AE of ASC (FH-C) and those without FH by AE of CHB (AE-C)

Features	FH-C ($n = 12$)	AE-C ($n = 12$)	Differences *P*-value
Age (years)	51.7 ± 14.7	49.9 ± 5.6	Matched
Male	10 (83%)	9 (75%)	Matched
ALT (IU/L)	887 ± 681	641 ± 620	NS
AST (IU/L)	701 ± 451	601 ± 753	NS
Total bilirubin (mg/dL)	15.0 ± 7.3	7.3 ± 8.8	<0.05
Prothrombin time (%)	25.8 ± 6.6	48.4 ± 21.5	<0.005
HBeAg positive	4 (33%)	3 (25%)	NS
Core protein (log U/mL)	5.04 ± 1.45	5.07 ± 1.07	NS
HBcrAg (log U/mL)	6.35 ± 1.70	6.29 ± 1.95	NS
HBV DNA (log copies/mL)	7.44 ± 1.51	6.60 ± 1.10	NS

AE, acute exacerbation; ALT, alanine aminotransferase; ASC, asymptomatic HBV carrier; AST, aspartate aminotransferase; CHB, chronic hepatitis B; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

of any specific mutations between these two groups (Fig. 3b).

DISCUSSION

THE MAGNITUDE OF liver injuries depends on the replication level of HBV and cytotoxic immune responses of the host raised against viral epitopes in general.[31] Various viral factors have been proposed that promote the development of FHB, represented by pre-core (G1896A) and core promoter (A1762T/G1764A) mutations.[13–16] Impact of virological factors on the development of FHB has remained controversial, however, especially because these mutations are rarely detected in the patients from the USA and France.[19–21] It has been argued that the development of FHB is not promoted by these mutations and is dependent on host factors including the human leukocyte antigen (HLA) environment.[22]

The expression of HBeAg is terminated by G1896A mutation in the pre-core region at the translation level,[32] and downregulated by the A1762T/G1764A double mutation at the transcription level.[33,34] Lamberts *et al.* are the first to implicate a negative influence of HBeAg on the replication of HBV.[35] Should HBeAg suppress the replication of HBV, presumably by inhibiting the encapsidation of pre-genome,[35] the lack or decrease of HBeAg would enhance the reproduction of HBV. Furthermore, HBeAg acts as a tollerogen to T cells recognizing epitopes on core protein, thereby, obviating immune injury of hepatocytes.[36,37] In the absence or decrease of HBeAg, therefore, hosts would mount vigor cytotoxic T-cell responses to core epitopes excessively presented on hepatocytes, and develop severe liver injuries culminating in FHB.[38]

There is a possibility that influence of viral factors such as HBV mutants with a HBeAg-negative phenotype, on the induction of FHB, may have been confounded by host factors and created disagreement. Therefore, the sheer influence of virological factors on FHB would need to be evaluated in case-control studies, as has been attempted to sort out the influence of HBV genotypes on development of cirrhosis and hepatocellular carcinoma.[8] These backgrounds have instigated us to identify virological factors accelerating the severity of liver disease in the 50 FHB patients by transient HBV infection and the 50 AHB patients who were of the same ethnicity and matched for age as well as sex.

In this case controlled study, A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent in the patients with FH-T than AHB, providing further corroboration of previous studies;[13–16] these mutations could enhance viral replication. Interestingly, our recent study using an *in vitro* replication model, showed that A2339G mutation in the core region enhanced viral replication and the effect of A2339G mutation may be associated with inhibition of the cleavage of the core protein by a furin-like protease, resulting in the high expression of the complete core protein.[18] Such enhanced HBV would induce significant immune response, resulting in development of FHB.

In multivariate analysis, higher levels of serum HBV DNA and G1896A mutation were independent virological risk factors for the development of FHB by transient