

Figure 6. ERK5 is phosphorylated during the G2/M phases of the cell cycle. (A) Immunoblot analysis to detect protein levels of total ERK5 and β -actin, an internal control, in SNU449 cells that were synchronized at the G1/S, early S, or M phases using a double-thymidine, aphidicolin, or nocodazole block, respectively, or were untreated and used as an asynchronous (ASY) population. (B) Levels of phosphorylated ERK5 (p-ERK5). ERK5 was immunoprecipitated (IP) from lysates of SNU449 cells that were synchronized at the M phase (M) or from asynchronous cells (ASY). The samples were split and analyzed by immunoblotting (IB) for p-ERK5 and total ERK5. Normal rabbit immunoglobulin (normal IgG) was used as a negative control for immunoprecipitation. (C) Flow cytometric analysis. SNU449 cells were synchronized to the G1/S boundary using a double-thymidine block. Synchronized cells were released from the block and harvested at the indicated time points. The X-axis indicates DNA content and the Y-axis indicates the number of cells. (D) Time course of changes in the level of total ERK5 after release from the double-thymidine block. The level of β -actin was used as an internal control. (E) Time course of changes in the level of p-ERK5 after release from the double-thymidine block. ERK5 was immunoprecipitated from

lysates of SNU449 cells harvested at the indicated times after release from the double-thymidine block. The samples were split and analyzed by immunoblotting for p-ERK5 and total ERK5. SNU449 cells, synchronized at the M phase with nocodazole, were also examined as described in (A) and (B). Normal rabbit IgG was used as a negative control for immunoprecipitation. (F) Representative images of mitotic cells in an SNU449 cell population that was transfected with MAPK7- or control-siRNA. SNU449 cells were treated with siRNA targeting MAPK7, negative control siRNA, or the transfection agent alone (Lipofectamine). Untreated cell were maintained under identical conditions. These cells were synchronized at the G1/S boundary using a double-thymidine block. The synchronized cells were released from the block and stained with anti-phospho-histone H3 9 hr after release, a time corresponding to the G2/M phase as shown in (C). Mitotic cells were identified by positive staining for phospho-histone H3 (green). Nuclear DNA was stained with propidium iodide (red). (G) The mitotic index was scored as described in Materials and Methods section. Data are presented as means \pm SD (ANOVA; * $P < 0.05$).

expression using RNAi and assessed its effect on mitosis. SNU449 cells were transfected with siRNA targeting *MAPK7* and synchronized at the G1/S-phase boundary by a double-thymidine block. The synchronized cells were released from the block and harvested 9 hr after release, a time which corresponds to the G2/M phase (Fig. 6C). Finally, harvested cells were stained with anti-phospho-histone H3 antibody, which specifically detects mitotic cells (Fig. 6F). Compared with a control siRNA or transfection agent alone, transfection of *MAPK7* siRNA significantly reduced the mitotic index (Fig. 6G). These findings suggest that ERK5 regulates mitotic entry in the HCC cells.

DISCUSSION

High-density SNP arrays are powerful tools for high-resolution analysis of DNA copy number aberrations in cancers. In the present study, using the Affymetrix GeneChip 100K and 250K SNP arrays we detected a novel amplification in HCC cells at 17p11. We were able to narrow the amplification to a 750-kb region. Notably, the amplification might have been missed using conventional analyses such as CGH. Amplification at 17p11.2-p12 has been detected in high-grade osteosarcoma using CGH (Forus et al., 1995; Tarkkanen et al., 1995). The group of van Dartel et al., (2002) established 17p11.2-p12 amplification profiles by semi-quantitative PCR using 15 microsatellite markers and seven candidate genes to assay amplification in this tumor type. They found that most of the tumors had complex amplification profiles, suggesting that multiple amplification targets, including *MAPK7*, might be present in region 17p11.2-p12. In contrast, we were able to define a smaller common region of amplification at 17p11 in two HCC cells and to determine the expression status of all genes in the amplicon. Three of the seven genes in the amplicon; *EPN2*, *EPPB9*, and *MAPK7*, were always overexpressed in cells that showed amplification in the 17p11 region. Thus, we considered these three genes as candidate targets for amplification. The function of *EPPB9* (B9 protein) is not known, and the protein encoded by *EPN2* (epsin 2) is similar to epsin 1, which plays a putative role in clathrin-mediated endocytosis (Rosenthal et al., 1999). Therefore, we focused on *MAPK7* as a target for the amplification.

Several lines of evidence implicate ERK5, which is encoded by *MAPK7*, in tumorigenesis

(Wang and Tournier, 2006): (a) the ERK5 pathway is activated by Ras (English et al., 1999), ErbB (Esparis-Ogando et al., 2002; Yuste et al., 2005), Src (Sun et al., 2003), Cot (Chiariello et al., 2000), Bcr-Abl (Buschbeck et al., 2005), insulin-like growth factor-II (Linnerth et al., 2005), and interleukin-6 (Carvajal-Vergara et al., 2005); (b) ERK5 is involved in the control of breast cancer cell proliferation (Esparis-Ogando et al., 2002); (c) ERK5 mediates a survival signal that confers chemoresistance to breast cancer (Weldon et al., 2002); (d) insulin-like growth factor-II promotes cell survival via the ERK5 pathway in lung cancer cells (Linnerth et al., 2005); (e) the level of ERK5 contributes to the survival of Bcr/Abl-positive leukemic cells (Buschbeck et al., 2005); (f) ERK5 regulates cell proliferation and antiapoptotic responses in multiple myeloma (Carvajal-Vergara et al., 2005); and (g) an elevated level of MEK5, a specific activator of ERK5, is associated with metastasis and a poor prognosis in prostate cancer (Mehta et al., 2003).

The present study is the first to show the status of amplification and expression of *MAPK7* and its functional role in HCC. We found that *MAPK7* is amplified in 35 of 66 HCC tumors (53%). However, we could not determine the copy number of *MAPK7* in the nontumorous counterparts of the samples assayed because these samples were not available. Therefore, we cannot exclude the possibility that copy number polymorphism might influence the results of copy number analysis. We studied the expression of ERK5 using immunohistochemical analysis in primary HCCs and their surrounding nontumorous liver tissues. In nontumorous liver tissues, ERK5 was weakly expressed in the cytoplasm of non-neoplastic hepatocytes. Intriguingly, it was more strongly expressed in bile ducts, bile ductules, and a few small hepatocytes. In HCC tumor tissues, ERK5 was expressed in the cytoplasm of tumor cells. The level of ERK5 was elevated in 11 of 43 HCC tumors compared with their nontumorous counterparts. However, we did not observe a significant link between the level of ERK5 and any clinicopathological parameters. A recent report showed that, in prostate cancer, an increase in ERK5 cytoplasmic signals correlates with advanced disease and that strong nuclear ERK5 localization correlates with poor survival (McCracken et al., 2008).

We examined the functional roles of ERK5 in HCC cells using RNAi. Downregulation of *MAPK7* by siRNA suppressed the growth of

SNU449 cells, which had the greatest amplification and overexpression of *MAPK7* of all of the cell lines tested. These findings suggest that increased levels of ERK5 enhance the growth of HCC cells. Moreover, our results indicate that ERK5 is phosphorylated during the G2/M phases of the cell cycle and that it regulates entry into mitosis, which may explain how it promotes the growth of HCC cells.

Conflicting results have been reported by different investigators regarding the role of ERK5 in cell cycle progression. Some investigators have reported that ERK5 regulates the G1/S transition: expression of a dominant-negative form of ERK5 prevents cells from entering the S-phase of the cell cycle (Kato et al., 1998), and ERK5 can drive cyclin D1 expression (Mulloy et al., 2003). In contrast, Cude et al., (2007) and Gírio et al., (2007) recently reported that ERK5 is activated at the G2/M phases and is required for mitotic entry, findings that agree with our results.

Few molecules have been identified as direct downstream targets of ERK5. The transcriptional factors of the monocyte enhancer factor 2 family are among the best characterized substrates of ERK5. Phosphorylation of monocyte enhancer factor 2C by ERK5 enhances its transcriptional activity and subsequently leads to an increase in c-Jun gene expression (Kato et al., 1997; Wang and Tournier, 2006). A more complete identification of components downstream of ERK5 will be necessary to fully understand the role of ERK5 in carcinogenesis.

In summary, using high-density SNP arrays, we identified *MAPK7* as a probable target for the amplification events at 17p11 in HCCs. Our results suggest that the ERK5 protein product of the *MAPK7* gene plays a role in proliferation of HCC cells by regulating mitotic entry and may therefore be an optimal target for the development of novel therapies for this widespread type of cancer.

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Two cases of development of entecavir resistance during entecavir treatment for nucleoside-naïve chronic hepatitis B

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Abstract

Background Entecavir (ETV) is a potent nucleoside analogue against hepatitis B virus (HBV), and emergence of drug resistance is rare in nucleoside-naïve patients

because development of ETV resistance (ETVr) requires at least three amino acid substitutions in HBV reverse transcriptase. We observed two cases of genotypic ETVr with viral rebound and biochemical breakthrough during

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ETV treatment of nucleoside-naïve patients with chronic hepatitis B (CHB).

Results Case 1: A 44-year-old HBeAg-positive man received ETV 0.1 mg/day for 52 weeks and 0.5 mg/day for 96 weeks consecutively. HBV DNA was 10.0 log₁₀ copies/ml at baseline, declined to a nadir of 3.1 at week 100, and rebounded to 4.5 at week 124 and 6.7 at week 148. Alanine aminotransferase (ALT) level increased to 112 IU/l at week 148. Switching to a lamivudine (LVD)/adefovir-dipivoxil combination was effective in decreasing HBV DNA. **Case 2:** A 47-year-old HBeAg-positive man received ETV 0.5 mg/day for 188 weeks. HBV DNA was 8.2 log₁₀ copies/ml at baseline, declined to a nadir of 2.9 at week 124, and then rebounded to 4.7 at week 148 and 6.4 at week 160. ALT level increased to 72 IU/l at week 172. The ETVr-related substitution (S202G), along with LVD-resistance-related substitutions (L180M and M204V), was detected by sequence analysis at week 124 in both case 1 and case 2.

Conclusions ETVr emerged in two Japanese nucleoside-naïve CHB patients after prolonged therapy and incomplete suppression and in one patient after <0.5 mg of dosing. ETV patients with detectable HBV DNA or breakthrough after extended therapy should be evaluated for compliance to therapy and potential emergence of resistance.

Keywords Entecavir · HBV · Chronic hepatitis B · Drug resistance · Nucleoside-naïve

Introduction

Hepatitis B virus (HBV) infection is a serious health problem because of its high prevalence, estimated to be infecting more than 350 million people worldwide, and its potential for inducing chronic hepatitis, cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC) [1, 2]. It has been demonstrated that the most potent risk factor for development of cirrhosis or HCC is serum HBV DNA level [3, 4], and it seems that suppressing serum HBV load is essential for improving the prognosis of HBV carriers. Treatment of chronic hepatitis B (CHB) has evolved markedly with the introduction of nucleoside-analogue antivirals, that is, lamivudine (LVD), adefovir-dipivoxil (ADV), entecavir (ETV), and telbivudine, to clinical practice. LVD, the first approved nucleoside analogue against HBV, was shown to be effective in suppressing HBV DNA replication, improving transaminase levels, improving liver histology, inducing hepatitis B e antigen (HBeAg) seroconversion, and suppressing hepatic insufficiency and hepatocarcinogenesis in CHB and compensated cirrhosis [5, 6]. However, the effectiveness of LVD is limited because of frequent development of drug resistance

followed by a hepatitis flare and, occasionally, hepatic failure [7, 8].

ETV, a novel anti-HBV nucleoside analogue, has more than 1,500 times greater potency than LVD in vitro [9]. In clinical trials, ETV administration demonstrated potent anti-HBV activity with a marked decline in serum HBV DNA level and a significant improvement in liver histology than LVD in nucleoside-naïve HBeAg-positive and -negative patients [10, 11]. In addition, emergence of ETV resistance (ETVr) or viral rebound was shown in these studies to be rare. From these results, recent treatment guidelines have recommended ETV as the first-line nucleoside analogue for nucleoside-naïve CHB patients, including those with cirrhosis [12, 13].

It has been reported that the development of ETVr in nucleoside-naïve patients is very rare, even after 4 years of therapy. Recently, however, rare cases of ETVr, which developed in nucleoside-naïve patients in clinical studies, have been reported [14–16]. We also observed two patients who developed ETVr-associated HBV reverse transcriptase (RT) substitutions, followed by *virologic rebound*, defined as an elevation in serum HBV DNA of more than 1 log₁₀ copy/ml from nadir, and biochemical breakthrough in long-term ETV treatment of nucleoside-naïve CHB patients. In this article, we report these two cases in detail.

Case report

Case 1

A 44-year-old Japanese male CHB patient was positive for hepatitis B surface antigen (HBsAg), HBeAg, serum HBV DNA, and had HBV genotype C, had elevated alanine aminotransferase (ALT) levels, and had no history of nucleoside analogue treatment. The patient had a history of acute appendicitis at age 30, ureteral stone at age 35, and hyperlipidemia at age 43. He had a habit of drinking alcohol (700 ml) daily but did not smoke. At age 27, he was diagnosed for the first time by health screening as an asymptomatic HBV carrier in the immune-tolerant phase, defined by HBsAg positivity and normal liver enzymes, and he was followed up regularly elsewhere with blood tests for liver enzymes. He was found to have ALT elevation. He was referred to our hospital at age 44 and was diagnosed with CHB. Serum HBV DNA level determined by Roche Amplicor™ Monitor PCR assay (lower limit of detection is 2.6 log₁₀ copies/ml = 400 copies/ml; Roche Diagnostics K.K., Tokyo, Japan) [17] was 10.0 log₁₀ copies/ml and serum ALT level was 199 IU/l. Histologic diagnosis by percutaneous liver biopsy at baseline revealed chronic hepatitis with mild fibrosis and mild activity (CH F1/A1, according to the New Inuyama Classification) [18].

Table 1 Baseline characteristics

	Normal range	Unit	Case 1	Case 2
Age	–	–	44 years	47 years
Gender	–	–	Male	Male
T. Bil	0.2–1.0	mg/dl	0.8	0.5
AST	10–40	IU/l	113	48
ALT	5–40	IU/l	199	74
ALP	115–359	IU/l	268	216
BUN	6–20	mg/dl	9.5	15.9
CREA	0.61–1.04	mg/dl	0.95	0.83
ALB	4.0–5.0	g/dl	4.2	4.3
WBC	3500–8500	/ μ l	6,800	5,650
Hb	13.5–17.0	g/dl	15.7	14.8
PLT	13.1–36.2	10^4 / μ l	18.9	14.5
Prothrombin time	10–13	second	10.8	11.2
INR	–	–	1.0	0.9
HBsAg (CLIA)	0–0.05	IU/ml	>100 (positive)	>100 (positive)
anti-HBs (CLIA)	0–10	IU/ml	0 (negative)	0 (negative)
HBeAg (CLIA)	0–1		120 (positive)	190 (positive)
anti-HBe (CLIA)	0–50	%	<35 (negative)	0 (negative)
HBV DNA (PCR)	<2.6	\log_{10} copies/ml	10.0	8.2
HBV genotype			Genotype C	Genotype C
YMDD (sequencing)			YMDD+	YMDD+
			YVDD–	YVDD–
			YIDD–	YIDD–
Liver histology ^a			CH F1/A1	CH F2/A2

^a Diagnosed according to New Inuyama classification. T. Bil: total bilirubin, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkalinephosphatase, BUN: blood urea nitrogen, CREA: serum creatinine, ALB: serum albumin, WBC: white blood cell count, Hb: hemoglobin, PLT: platelet count, INR: international normalized ratio, HBsAg: hepatitis B surface antigen, CLIA: chemiluminescent immunoassay, anti-HBs: antibody to hepatitis B surface antigen, HBeAg: hepatitis B e antigen, anti-HBe: antibody to hepatitis B e antigen, HBV: hepatitis B virus, PCR: polymerase chain reaction, YMDD: tyrosine-methionine-aspartate-aspartate motif, YVDD: tyrosine-valine-aspartate-aspartate motif, YIDD: tyrosine-isoleucine-aspartate-aspartate motif, CH F1/A1: chronic hepatitis with mild fibrosis and mild activity, CH F2/A2: chronic hepatitis with moderate fibrosis and moderate activity

Other baseline characteristics are shown in Table 1. He was enrolled in a phase II clinical trial of ETV and was randomized into 0.1- and 0.5-mg dosage groups. The trial was conducted in Japan in compliance with the ethical principles of the Declaration of Helsinki, Good Clinical Practice guidelines, and Articles/Notifications of the Ministry of Health, Labor and Welfare (H. Kobashi et al., *J Gastroenterol Hepatol*, in press). He was assigned into the 0.1-mg dosage group and administered ETV at daily dose of 0.1 mg for an initial 52 weeks. Subsequently, he was administered ETV continuously at a daily dose of 0.5 mg for the following 96 weeks. The serum HBV DNA level, which was 10.0 \log_{10} copies/ml at baseline, declined to a nadir of 3.1 \log_{10} copies/ml at week 88 of ETV treatment. Thereafter, HBV DNA level increased from 4.5 \log_{10} copies/ml at week 124 to 6.3 \log_{10} copies/ml at week 140 and 6.7 \log_{10} copies/ml at week 148. ALT levels increased

from 28 IU/l at week 144 to 112 IU/l at week 148. The patient discontinued ETV therapy at week 148, and then received a combination therapy of 100 mg of LVD and 10 mg of ADV per day. Afterwards, HBV DNA level dropped to below 2.6 \log_{10} copies/ml and ALT level was normalized after 28 weeks of LVD/ADV dosing (Fig. 1).

HBV DNA sequence analysis was performed using PCR-amplified HBV DNA from preserved serum samples at baseline and at every 24 weeks via HBV DNA polymerase sequence assay (developed at SRL, Inc., Tokyo, Japan). Although sequence analysis of the baseline isolate revealed no substitution in the RT domain of the HBV DNA polymerase gene, analysis of the isolates collected over time revealed the M204I substitution at week 100 and the L180M, S202G, and M204V substitutions at weeks 124 and 144, respectively (Table 2). In addition, a polymorphic residue N238 was found as mixed N238 N/H at week 100 and thereafter. The

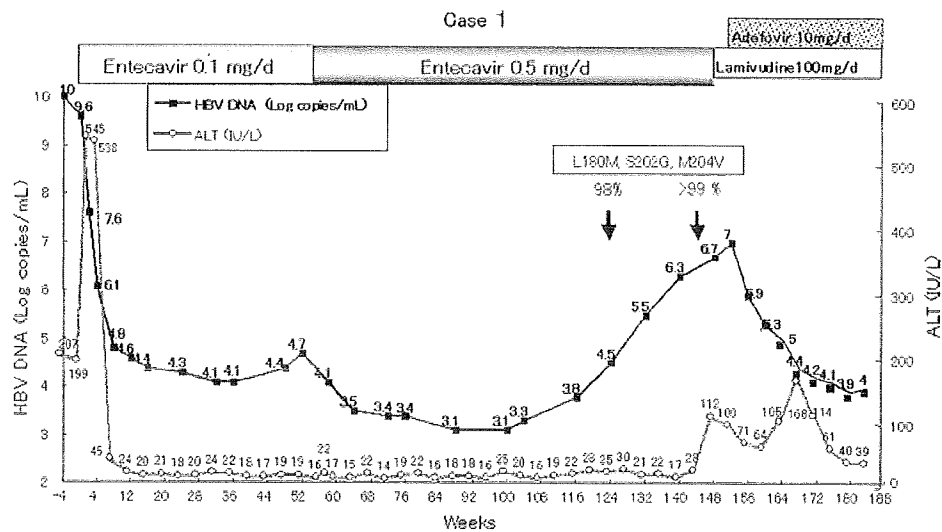


Fig. 1 Clinical course of case 1, a 44-year-old man with nucleoside-naïve CHB. ETV treatment reduced ALT levels to below the upper normal limit at week 12 and reduced HBV DNA load to a nadir of 3.1 log₁₀ copies/ml at week 88. However, HBV DNA re-elevated to 4.5 log₁₀ copies/ml at week 124 (virologic breakthrough) and 6.3 log₁₀ copies/ml at week 140, as well as ALT level re-elevated at week 148 (biochemical breakthrough). Sequence analysis of the

HBV DNA polymerase gene using serum sample obtained at weeks 124 and 144 revealed the emergence of L180M, M204V (related to LVD resistance), and S202G (related to ETVr) substitutions. SNP-PCR assay revealed that LVDr M204V and ETVr S202G substitutions were detected first at week 124 (98%) and increased at week 148 (>99%). Switching from ETV to LVD/ADV combination treatment at week 148 was successful in reducing HBV DNA load and ALT again

Table 2 Population sequence analysis of isolates from case 1 on ETV therapy

Week	Reverse transcriptase position				
	180	202	204	223	238
0	L	S	M	S/A	N
24	L	S	M	S/A	N
100	L	S	M/I	S/A	N/H
124	M	G	V	S	N/H
144	M	G	V	S	N/H

polymorphic residue S223, which was mixed as S/A at baseline, was found to be only S at weeks 124 and 144.

In addition, preserved serum samples from this patient at baseline and at every 24 weeks were analyzed by an ultrasensitive, single-nucleotide-polymorphism (SNP)-PCR assay, using a method similar to Punia et al. [19] for identification of resistance substitutions, as well as analyzing the sequence of individual clones to determine the genetic linkage of substitutions. SNP-PCR analysis was performed for the two LVD-resistance (LVDr) substitutions, M204V (codon GTG) and M204I (codons ATA and ATT), and the ETVr substitution S202G. Both wild-type and positive control plasmids containing the correct sequence were used at various concentrations to establish the background level as well as the level of detection for each substitution. For clonal analysis, the amplified RT

gene from the patient's HBV was cloned into plasmids, as well as 22 to 24 individual clones were selected and sequenced, to determine the genetic linkage of the different substitutions observed.

SNP-PCR analysis for ultrasensitive detection of the resistance substitutions revealed that the LVDr M204V(GTG) and ETVr S202G(GGT) substitutions were not detected (<0.1%) at baseline, week 24, or week 100. The M204I substitution (codon ATA) was detected at low levels at week 24 (0.4%), increased levels at week 100 (6.6%), and was present but at reduced levels at weeks 124 and 148 (0.4% at both time points). The LVDr M204V and ETVr S202G substitutions were detected first at week 124 (98%) and increased levels at week 148 (>99%). The levels of M204I(ATA) were lower at weeks 124 and 144, likely as a result of the dominant M204V/S202G virus (Table 3). Samples at weeks 48 and 76 could not be analyzed conclusively because of low yields of HBV DNA from serum samples.

Clonal analysis revealed that position 223 was a mixture of S and A residues at baseline, the LVDr substitutions L180M and M204V, as well as the ETVr substitution S202G, all emerged simultaneously and were linked in the same virus isolate clones at week 124, isolates that also contained S at position 223. These substitutions did not appear to arise from the LVDr isolates with M204I because the M204I substitution emerged in an isolate with substitution S223A.

Table 3 SNP-PCR analysis of case 1 isolates

Week	M204V		S202G		M204I (ATA)		M204I (ATT)	
	Mut/WT	Ave (%)	Mut/WT	Ave (%)	Mut/WT	Ave (%)	Mut/WT	Ave (%)
0	1/5,424	0.018	1/15,453	0.0065	1/4,199	0.024	1/37,940	0.0026
24	1/5,655	0.018	1/19,000	0.0052	<u>1/243</u>	<u>0.410</u>	1/46,518	0.0021
100	1/3,846	0.026	1/16,038	0.0062	<u>1/14</u>	<u>6.569</u>	1/50,456	0.0020
124	<u>48/1</u>	<u>97.973</u>	<u>59/1</u>	<u>98.327</u>	<u>1/265</u>	<u>0.377</u>	1/12,879	0.0078
144	<u>706/1</u>	<u>99.859</u>	<u>1,250/1</u>	<u>99.920</u>	<u>1/237</u>	<u>0.421</u>	1/10,573	0.0095

Cells with bold and underlined font are considered positive (>1/1000 or >0.1% mutant/wild-type)

Mut/WT, mutant/wild type, mean ($N = 3$)

Ave %, average % in total HBV DNA

Case 2

A 47-year-old Japanese male CHB patient was positive for HBsAg, HBeAg, serum HBV DNA, and had HBV genotype C, had elevated ALT levels, and had no history of nucleoside analogue treatment. At age 33, he was diagnosed for the first time as an asymptomatic HBV carrier in the immune-tolerant phase because of positive HBsAg and normal liver enzymes. At age 44, he was found to have ALT elevation, referred to our hospital, and diagnosed with CHB. Histologic diagnosis by percutaneous liver biopsy revealed chronic hepatitis with moderate fibrosis and moderate activity (CH F2/A2 according to the New Inuyama Classification). He was treated with ursodeoxycholic acid at a daily dose of 600 mg orally and glycyrrhizin preparation (stronger Neo-Minophagen CTM) 40 ml i.v. thrice per week for 3 months. However, liver enzymes did not normalize. Interferon- α 2b administration, three mega units i.m. thrice per week, was started at age 45 and continued for 24 weeks. Although HBV DNA level was reduced transiently to below 3.7 log₁₀ copies/ml at the end of therapy, it rose 9 months after cessation of interferon therapy to 8.2 log₁₀ copies/ml and ALT level increased to 483 IU/l. At age 47, the patient was started on ETV treatment as the subject enrolled in the ETV clinical trial (ETV-053) in Japan at a daily oral dose of 0.5 mg and continued for 188 weeks. A liver biopsy performed 1 month before starting the ETV treatment showed chronic hepatitis with moderate fibrosis and moderate activity (CH F2/A2, according to the New Inuyama Classification). The baseline serum HBV DNA level was 8.2 log₁₀ copies/ml, ALT level was 74 IU/l, and other baseline characteristics were as shown in Table 1. The serum HBV DNA level declined to 3.2 log₁₀ copies/ml and ALT level decreased to below the upper limit of normal at week 32. Liver histology improved to mild-to-moderate fibrosis and mild activity (CH F1-2/A1) at week 48 and chronic hepatitis with mild-to-moderate fibrosis and mild activity (CH F1/

A1) at week 148. HBV DNA level was suppressed to a nadir of 2.9 log₁₀ (794) copies/ml at week 124 and rose again to 4.7 log₁₀ copies/ml at week 148, 5.4 log₁₀ copies/ml at week 152, and 6.4 log₁₀ copies/ml at week 160 and 7.0 log₁₀ copies/ml at week 164. ALT level rose to 79 IU/l at week 172 and remained between 40 and 50 IU/l thereafter. ETV at 0.5 mg/day was continued until this time (Fig. 2).

HBV DNA sequence analysis revealed no resistance substitutions in the patient's baseline virus. However, the LVDr-related substitutions L180M and M204V, as well as ETVr-related substitution S202G, were detected at week 124, as a mixed population with wild type, and at week 148, as a pure population (Table 4). In addition, the patient displayed evidence of several polymorphic substitutions at baseline, indicating a mixed quasi-species, which became enriched for those with the resistant virus over time.

SNP-PCR analysis was used to determine the first appearance of the resistance substitutions, using the same method as for case 1. There was no antiviral resistance detected at baseline (<0.1%). The M204V (0.65%) and S202G substitutions were detected first at week 24 but not again until week 124. At weeks 124 and 148, the resistant isolate had become enriched to 43% (M204V) and 98% (M204V), respectively (Table 5).

Clonal analysis was performed to determine the genetic linkage of the various substitutions observed, using the same method as for case 1. The amplified RT gene from the patient's virus was cloned into plasmids, and 24 to 27 individual clones were selected and sequenced. From the clonal analysis, it can be seen that there are three positions that contain mixtures at baseline; position 55 is a mixture of H and R residues, position 221 is a mixture of Y and F residues, and position 269 is a mixture of I and L residues. The substitutions L180M and M204V, as well as the ETVr-related substitution S202G, all emerge simultaneously and in an isolate with H at position 55, Y at position 221, and I at position 269.

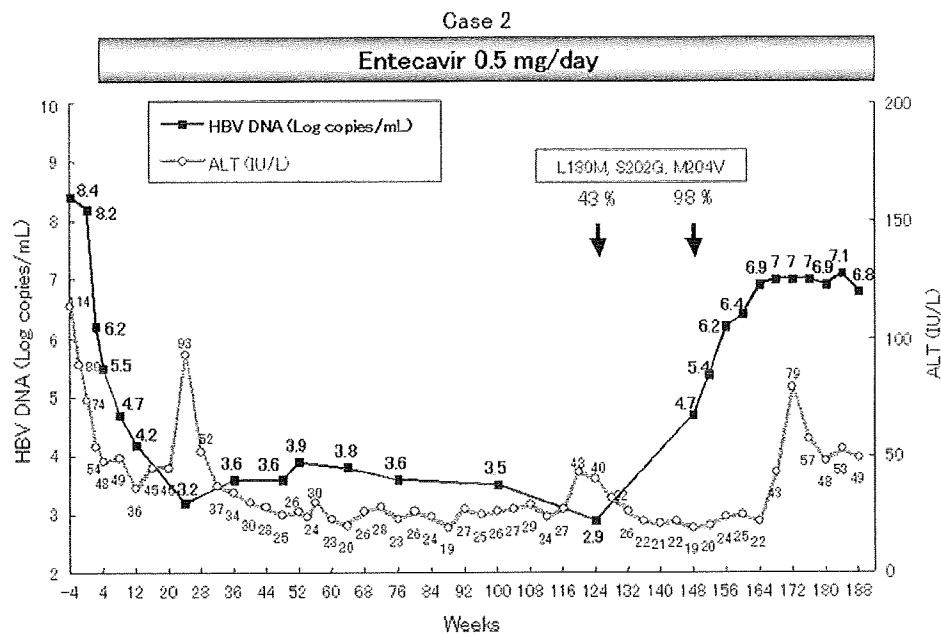


Fig. 2 Clinical course of case 2, a 47-year-old man with nucleoside-naïve CHB. ETV treatment reduced ALT level to below the upper normal limit at week 30 and reduced serum HBV DNA level to a nadir of 2.9 log₁₀ copies/ml at week 124. However, HBV DNA level re-elevated to 4.7 log₁₀ copies/ml (virologic breakthrough) at week 148 and 7.0 log₁₀ copies/ml at week 168, as well as ALT level re-

elevated to 79 IU/l at week 172. Sequence analysis of the HBV DNA polymerase gene using serum sample obtained at weeks 124 and 148 revealed the emergence of L180M, M204V (related to LVD resistance), and S202G (related to ETVr) substitutions. SNP-PCR assay revealed that the resistant isolate was enriched to 43 (M204V) and 98% (M204V), respectively

Table 4 Population sequence analysis of isolates from case 2 on ETV therapy

Week	RT position									
	55	76	180	191	195	202	204	221	269	
0	H/R	S	L	V	F	S	M	Y/F	I/L	
24	H/R	S	L	V	F	S	M	Y/F	I/L	
52	H/R	S	L	V	F	S	M	Y/F	I/L	
100	H	S	L	V	F	S	M	Y/F	I/L	
124	H	S/T	L/M	V/I	F/S	S/G	M/V	Y	I/L	
148	H	S	M	V	F	G	V	Y	I	

Table 5 SNP-PCR analysis of case 2 isolates

Week	S202G ^a	M204V (GTG, %)	M204I (ATA, %)	M204I (ATT, %)
0	Negative	0.016	0.020	0.0065
24	Positive	0.65	0.029	0.018
52	Negative	0.021	0.020	0.018
100	Negative	0.020	0.021	0.010
124	Positive	43	0.33	0.010
148	Positive	98	2.9	0.016

^a S202G PCR was non-quantitative. A positive indicates 4-fold, 5085-fold, and 10475-fold the wild-type background for weeks 24, 124, and 148, respectively. The baseline isolate gave 1.1-fold the wild-type background

Discussion

The most important limitation of long-term nucleoside analogue treatment for CHB is the emergence of drug-resistant mutant HBV followed by viral breakthrough and hepatitis flare [12]. The most common mutation associated with LVD_r involves substitution of methionine in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV DNA polymerase gene RT domain with valine or isoleucine (M204V/I), with or without a leucine-to-methionine substitution in an upstream region (rtL180M) [20]. It was reported that LVD_r was detected at a rate of 14 to 32% after 1 year and 60 to 70% after 5 years of LVD treatment [12]. The substitutions conferring resistance to ADV are asparagine to threonine (N236T) and alanine to valine or threonine (A181V/T) [21], and the cumulative probability of ADV resistance with elevation of HBV DNA level has been reported to be 20% at 5 years in HBeAg-negative patients [22] and as high as 42% in HBeAg-positive patients [23].

In the case of ETV, it has been reported that resistance to the drug requires at least one of three substitutions in HBV RT, that is, rtT184, rtS202, and rtM250, as well as LVD_r-related substitutions rtL180M and M204V [24]. Phenotypic analyses of samples associated with virologic breakthrough confirmed that ETV susceptibility correlates

with the spectrum of these additional substitutions conferring genotypic resistance and the increased level of circulating HBV DNA [25].

There is a high genetic barrier to resistance to ETV in nucleoside-naïve patients and <1% experience virologic breakthrough with ETVr through 4 years of therapy [15]. However, in LVD-refractory patients, the barrier to resistance is lower because the suppression of HBV replication is not as great and these patients mostly harbor virus with two of the three substitutions required for high-level ETVr [26]. This results in virologic breakthrough with ETVr in LVD-refractory patients at 1% in the first year but increasing to 39.5% after 4 years of therapy [15].

In this article, we report two cases with confirmed genotypic resistance to ETV, virologic rebound, and biochemical breakthrough during long-term ETV treatment for nucleoside-naïve CHB patients. In the first case, the patient received a lower dose of ETV (0.1 mg daily for 52 weeks) than is currently recommended in product labeling. It was shown that LVD-ADV combination therapy was apparently effective for the ETV-resistant strain, presumably because there is no cross-resistance between ETV and ADV [26, 27].

SNP-PCR analysis for resistance substitutions revealed that the LVDr M204V(GTG) and the ETVr S202G(GGT) substitutions were negative at baseline and emerged simultaneously at week 124 in both patients. The three resistance substitutions L180M, M204V, and S202G appeared to be genetically linked and did not arise in a stepwise manner in nucleoside-naïve patients, as has been described previously.

ETV displays several properties for consideration as the first-line nucleoside analogue because of its potent antiviral activity and a lower frequency of drug resistance than LVD, ADV, or telbivudine [13]. Although ETV is effective in LVD-refractory patients, the potency is reduced somewhat and the barrier to resistance is diminished by the presence of rtM204I/V and rtL180M substitutions. The fact that ETVr may develop in nucleoside-naïve patients, even if the chance is small, is noteworthy. In case 1, the patient received a lower dose of ETV (0.1 mg daily), which may be a possible contributing factor to resistance. The common features of our two cases were: HBeAg-positivity, male, high viral load, slow decrease of HBV DNA, and persistently detectable HBV DNA by PCR ($>2.6 \log_{10}$ copies/ml) during the treatment course; however, these characteristics were also present in some other patients who did not develop ETVr. Patient compliance with prescribed therapy also should be assessed in such situations. It is believed that some subpopulations of HBV that proliferate very actively and are not completely suppressed by ETV may have a chance of being selected for the resistance substitutions required for ETV virologic failure. Accordingly, such cases

with persistent HBV DNA after extended ETV treatment should be evaluated for emergence of drug-resistance substitutions with close monitoring of HBV DNA level, even in nucleoside-naïve patients.

The rate at which resistant mutants are selected is related to pretreatment serum HBV DNA level, rapidity of viral suppression, duration of treatment, and prior exposure to nucleoside analogue therapies [12]. For the management of the emergence of drug resistance in nucleoside analogue treatment of CHB and cirrhosis, prediction and early detection of drug-resistant HBV by close monitoring of serum viral load and genotypic resistance are necessary. Keeffe et al. [28] reported the “road-map concept,” that is, on-treatment monitoring strategy, for selection of nucleoside analogues by early prediction of efficacy and resistance using assessment of viral responses at weeks 12 and 24. Although this “concept” seems imperfect because the probability of emergence of resistance to particular nucleoside analogue is not taken into account, a similar strategy for management of drug resistance by close monitoring of viral load and confirming genotypic resistance with consideration of the property of each nucleoside analogue should be established for antiviral treatment of CHB using nucleoside analogues.

Conclusions

We reported two cases of emergence of genotypic resistance to ETV accompanied by virologic breakthrough in nucleoside-naïve CHB patients. One patient was treated with a lower than recommended dose of ETV. Although development of ETVr-related gene mutations is rare in nucleoside-naïve patients, the patients with a slow decline of HBV DNA or persistent HBV DNA ($>2.6 \log_{10}$ copies/ml) after ETV administration should be evaluated carefully for the potential emergence of ETVr.

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Distribution of Hepatitis B Virus Genotypes among Patients with Chronic Infection in Japan Shifting toward an Increase of Genotype A[†]

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Acute hepatitis B virus (HBV) infection has been increasing through promiscuous sexual contacts, and HBV genotype A (HBV/A) is frequent in patients with acute hepatitis B (AHB) in Japan. To compare the geographic distribution of HBV genotypes in patients with chronic hepatitis B (CHB) in Japan between 2005 and 2006 and between 2000 and 2001, with special attention to changes in the proportion of HBV/A, a cohort study was performed to survey changes in genotypes of CHB patients at 16 hospitals throughout Japan. Furthermore, we investigated the clinical characteristics of each genotype and examined the genomic characteristics of HBV/A isolates by molecular evolutionary analyses. Of the 1,271 patients, 3.5%, 14.1%, and 82.3% were infected with HBV/A, -B, and -C, respectively. In comparison with our previous survey during 2000 and 2001, HBV/A was twice as frequent (3.5% versus 1.7%; $P = 0.02$). The mean age was lower in the patients with HBV/A than in those with HBV/B or -C. Based on phylogenetic analyses of 11 full-length genomes and 29 pre-S2/S region sequences from patients, HBV/A isolates were imported from Europe and the United States, as well as the Philippines and India. They clustered with HBV/A from AHB patients and have spread throughout Japan. HBV/A has been increasing in CHB patients in Japan as a consequence of AHB spreading in the younger generation through promiscuous sexual contacts, aided by a tendency of HBV/A to induce chronic hepatitis. The spread of HBV/A infection in Japan should be prevented by universal vaccination programs.

Hepatitis B virus (HBV), a member of the *Hepadnaviridae*, is a circular, partially double-stranded DNA virus and is one of the major causes of chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC).

The HBV genome is composed of approximately 3,200 nucleotides. HBV is classified into eight genotypes, designated A to H, based on an intergroup divergence of 8% or more in the complete nucleotide sequence (3, 23, 26, 37). They have dis-

tinct geographical distributions and are associated with differences in clinical and virological characteristics, such as severity of liver disease and response to antiviral therapies (7, 8, 12, 13, 22, 28). Furthermore, subgenotypes have been reported for HBV/A, -B, and -C and named A1 to -3 (17, 38), B1 to -6 (31, 32, 40), and C1 to -6 (20, 31, 45). Equally, other genotypes are classified into subgenotypes. There have been increasing lines of evidence to indicate influences of HBV subgenotypes on the outcome of liver disease and the response to antiviral therapies (1, 39, 44).

In 2001, we reported the geographic distribution of HBV genotypes in Japan (27). Of the 720 Japanese patients with chronic HBV infection (CHB), 12 (1.7%) harbored HBV/A, 88 (12.2%) HBV/B, 610 (84.7%) HBV/C, 3 (0.4%) HBV/D, and 7 (1.0%) mixed genotypes. HBV/C was detected in over 94%

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of patients on the Japanese mainland, while HBV/B was found in 64% of those in Okinawa, the southernmost islands, and 44% of those in the Tohoku area in the northern part of the mainland.

Recently, acute HBV infection (AHB) has been increasing in Japan, predominantly through promiscuous sexual contacts. In addition, it was reported that HBV/A was more frequent in patients with acute hepatitis than in those with chronic hepatitis (29, 41, 49). Recent studies suggest that the chances for progression to chronic disease may differ among patients acutely infected with HBV of distinct genotypes (21, 25); patients infected with HBV/A run an increased risk of becoming HBV carriers. Hence, it is of utmost concern whether chronic HBV/A infection is increasing in Japan.

In the present study, we compared the geographic distribution of HBV genotypes in Japan during 2005 and 2006 with 2000 and 2001, with special attention to changes in the proportion of HBV/A. Furthermore, we investigated the clinical characteristics of each genotype and examined the genomic characteristics of HBV/A isolates by molecular evolutionary analyses.

MATERIALS AND METHODS

Patients. From September 2005 to October 2006, sera were collected from 1,370 consecutive patients with CHB at 16 representative hospitals that were liver centers in their respective regions throughout Japan for the purpose of investigating the geographic distribution of HBV genotypes in Japan. All of the patients were diagnosed after they had been followed for at least 12 months. Patients diagnosed with AHB were excluded from the study; they had a sudden onset of clinical symptoms of hepatitis, along with high-titer antibody to HBV core antigen of the immunoglobulin M class in serum. Their sera were tested for alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (γ -GTP), and hepatitis B e antigen (HBeAg), as well as antibody to HBeAg (anti-HBe) (Dinabot, Tokyo, Japan). Four clinical diagnoses were established for them. The inactive carrier state was defined by the presence of HBV surface antigen (HBsAg) with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension. Chronic hepatitis was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/liter]) persisting over 6 months (with at least three bimonthly tests). Cirrhosis was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges, and hypersplenism), platelet counts of $<100,000/\text{cm}^3$, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy, or a combination thereof.

The study protocol conformed to the 1975 declaration of Helsinki and was approved by the ethics committees of the respective institutions. Every patient or his/her next of kin gave informed consent to the purpose of the study.

Genotypes and subgenotypes of HBV. The six HBV genotypes (A to F) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV Genotype EIA; Institutes of Immunology Co., Ltd., Tokyo, Japan). The method depends on the combination of epitopes on pre-S2 region products detected by monoclonal antibodies that were specific for each of them (46, 47). Subgenotypes of HBV/A, designated A1 and A2, were determined by direct sequencing of the pre-S2/S gene, followed by a phylogenetic analysis.

Quantification of HBV DNA and sequencing. HBV DNA levels in sera were quantitated with a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Basel, Switzerland) with a detection range from 2.6 to 7.6 log copies/ml. Nucleic acids were extracted from 100 μl of serum using the Qiaamp DNA Blood Minikit (Qiagen GmbH, Hilden, Germany). Eleven complete HBV/A genomes and 29 pre-S2/S region sequences were amplified by PCR with appropriate primer sets, as described previously (40). The amplified HBV DNA fragments were directly sequenced using the ABI Prism Big Dye kit version 3.0 (Applied Biosystems, Foster City, CA) in an ABI 3100 automated DNA sequencer (Applied Biosystems). All sequences were analyzed in both forward and reverse directions. Complete and partial HBV genome sequences were aligned using GENETYX version 11.0 (Software Development Co., Ltd., Tokyo, Japan).

TABLE 1. Characteristics of 1,271 CHB patients

Parameter	Value
Characteristic	
Male gender [no. (%)]	766 (60.3)
Age (yr; mean \pm SD)	51.4 \pm 14.0
Diagnosis	
Inactive carrier state [no. (%)]	206 (16.2)
Chronic hepatitis [no. (%)]	786 (61.8)
Cirrhosis [no. (%)]	175 (13.8)
HCC [no. (%)]	104 (8.2)
Antiviral treatment [no. (%)]	577 (45.4)
Blood tests	
Platelets ($10^4/\text{mm}^3$)	21.4 \pm 30.2
ALT (IU/liter)	59.8 \pm 103.0
ALP (IU/liter)	270.4 \pm 136.0
γ -GTP (IU/liter)	47.4 \pm 66.1
HBV markers	
HBeAg [no. (%)]	399 (31.4)
HBV DNA (median [range] [log copies/ml])	4.2 (<2.6 to >7.6)

Molecular evolutionary analysis of HBV. Reference sequences were retrieved from the DDBJ/EMBL/GenBank databases with their accession numbers for identification. To investigate the relationship between HBV isolates from patients with chronic and acute hepatitis B in Japan, HBV/A isolates (AH1 to -10) were randomly retrieved from them and sequenced in our previous study (29). Nucleotide sequences of HBV DNA were aligned by the program CLUSTAL X, and genetic distance was estimated by the six-parameter method (10) in the Hepatitis Virus Database (36). Based on these values, phylogenetic trees were constructed by the neighbor-joining method (30) with the midpoint rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1,000 times.

Statistical analysis. Categorical variables were compared between groups by the χ^2 test or Fisher's exact test and noncategorical variables by the Mann-Whitney U test. A *P* value of less than 0.05 was considered significant.

Nucleotide sequence accession numbers. The DDBJ/EMBL/GenBank accession numbers of the complete genome sequences of HBV isolates JPN_CH1 to -11 are AB453979 to AB453989.

RESULTS

Distribution of HBV genotypes among patients with CHB.

Of the 1,370 serum samples, the genotype could not be determined for 99 (7.2%) by EIA due to low HBsAg levels, leaving 1,271 for analysis in this study (Table 1). Of these, 206 (16.2%) were inactive carriers, 786 (61.8%) had chronic hepatitis, 175 (13.8%) cirrhosis, and 104 (8.2%) HCC. They had a mean age of 51.4 \pm 14.0 years and included 766 (60.3%) men. They had a median HBV DNA level of 4.2 log copies/ml, and 399 (31.4%) of them were positive for HBeAg. Antiviral treatment had been given to 577 (45.4%) of them with interferon, lamivudine, adefovir piroxil, or entecavir.

The genotypes were HBV/A in 44 (3.5%), HBV/B in 179 (14.1%), HBV/C in 1,046 (82.2%), and HBV/D in 2 (0.2%) (Table 2). In comparison with our previous report on the distribution of genotypes in Japan in 2001 (27), HBV/A was more frequent in this study (3.5% versus 1.7%; *P* = 0.02). Of the 16 hospitals in this study, 10 overlapped with those in our previous report from 2001. In these 10 hospitals, HBV/A was more frequent in the present than in the previous survey (3.6% versus 1.7%; *P* = 0.04).

The distribution of HBV genotypes in Japan differed by

TABLE 2. Distribution of HBV Genotypes

Genotype	No. (%)	
	2005–2006 (<i>n</i> = 1,271)	2000–2001 ^a (<i>n</i> = 720)
A	44 (3.5 ^b)	12 (1.7)
B	179 (14.1)	88 (12.2)
C	1,046 (82.3)	610 (84.7)
D	2 (0.2)	3 (0.4)
Mixed	0 (0.0)	7 (1.0)

^a From Orito et al. (27).^b *P* = 0.02.

geographic location (Fig. 1). HBV/C was the most prevalent in the majority of areas. In the Tohoku area, the northern part of the Japanese mainland (Honshu), HBV/B was more prevalent than in the other areas of the Japanese mainland. In Okinawa, the southernmost islands of Japan, HBV/B was predominant. Of note, HBV/A was more frequent in the Kanto area (9.5%), the metropolitan area, and Okinawa (9.1%) than in the other areas.

Clinical differences among HBV/A, -B, and -C. Clinical backgrounds were compared among the patients infected with HBV/A, -B, and -C (Table 3). HBeAg was significantly less prevalent in the patients infected with HBV/B than in those infected with HBV/A or -C (*P* < 0.01 for each). When the positivity of HBeAg was stratified by age, HBeAg was markedly less common in patients infected with HBV/B than in those infected with HBV/A or -C who were older than 40 years of age (7/157 [4.5%] versus 4/19 [21.1%] [*P* < 0.05] or 215/755 [28.5%] [*P* < 0.01]) (Fig. 2). There were no significant differences in HBV DNA levels among patients infected with the three genotypes. As antiviral treatments might have influenced the severity of liver disease, clinical states were compared among patients infected with HBV/A, -B, and -C who did and

did not receive it; antiviral treatments did not affect the above-mentioned trends represented in Table 3 in age, diagnosis, and HBeAg, as well as ALT and HBV DNA levels (data not shown).

Additionally, we compared the distributions of age and liver diseases in patients infected with HBV/A, -B, and -C. In patients infected with HBV/C, the prevalence of cirrhosis and HCC increased in those older than 50 years of age compared to younger patients (Fig. 3), whereas in the patients infected with HBV/B, cirrhosis and HCC were rare in elderly patients. The proportion of patients younger than 40 years of age was higher in those infected with HBV/A than in those infected with HBV/B or -C (25/44 [56.8%] versus 22/179 [12.3%] or 288/1,046 [27.5%]; *P* < 0.01 for each), while cirrhosis and HCC were also found in those older than 50 years of age infected with HBV/A.

Coinfection with human immunodeficiency virus type 1 (HIV-1) was found in 6 of the 44 (13.6%) patients infected with HBV/A compared to only 3 of the 1,046 (0.3%) patients infected with HBV/C (*P* < 0.0001); it occurred in none of the 179 patients infected with HBV/B.

Phylogenetic analyses. Among the 44 HBV/A isolates, the complete genome was sequenced successfully in 11 (JPN_CH1 to -11). Seven of them were classified as HBV/A2 and four as HBV/A1. A phylogenetic tree was constructed based on the complete genome sequences of these 11 isolates, along with those from two patients with AHB and those from 40 HBV/A isolates retrieved from the database (Fig. 4). Of the seven HBV/A2 isolates, the four from patients with CHB in this study formed a cluster with the Japanese isolates retrieved from the database and two from patients with AHB. Of the other three isolates, JPN_CH5 clustered with French and U.S. isolates, JPN_CH6 with German isolates, and JPN_CH7 with

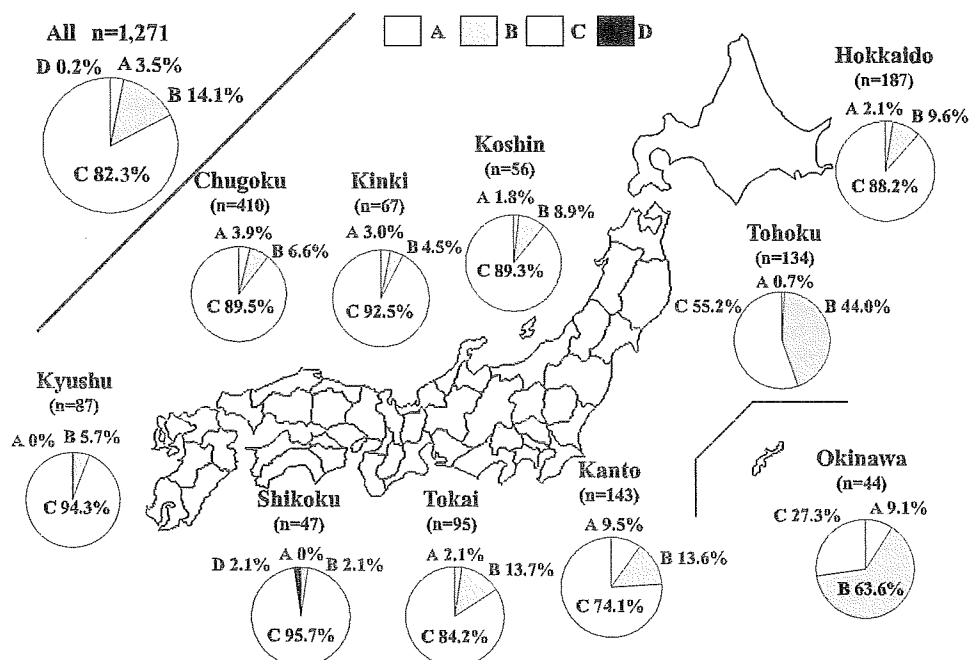


FIG. 1. Geographic distribution of HBV genotypes in patients with chronic HBV infection in Japan during 2005 and 2006.

TABLE 3. Clinical characteristics of individuals chronically infected with HBV of different genotypes

Parameter	Value for genotype:		
	A (n = 44)	B (n = 179)	C (n = 1,046)
Male gender [no. (%)]	32 (72.7)	112 (62.6)	621 (59.4)
Age (yr [mean \pm SD])	41.3 \pm 14.9 ^a	55.8 \pm 13.7 ^b	48.8 \pm 13.3
Diagnosis			
Inactive carrier state [no. (%)]	13 (29.5) ^c	63 (35.2) ^b	129 (12.3)
Chronic hepatitis [no. (%)]	26 (59)	103 (57.5)	656 (62.7)
Cirrhosis [no. (%)]	3 (6.8)	10 (5.6) ^b	162 (15.5)
HCC [no. (%)]	2 (4.5)	3 (1.7) ^b	99 (9.5)
Anti viral treatment [no. (%)]	13 (29.5) ^d	48 (26.8) ^b	516 (49.3)
Blood tests			
Platelet (10 ⁴ /mm ³)	23.3 \pm 21.9	25.9 \pm 35.9 ^e	20.6 \pm 29.5
ALT (IU/liter)	56.2 \pm 83.8	42.2 \pm 104.2 ^c	63.0 \pm 103.3
ALP (U/liter)	247.1 \pm 123.0	255.5 \pm 97.9	273.9 \pm 141.9
γ -GTP (U/liter)	39.6 \pm 34.6	49.3 \pm 63.4	47.5 \pm 67.6
HBV markers			
HBeAg [positive rate(%)]	15 (34.0) ^f	17 (9.5) ^b	367 (35.1)
HBV DNA (median [range]) (log copies/ml)	4.2 (<2.6- >7.6)	4.1 (<2.6- >7.6)	4.2 (<2.6- >7.6)

^a $P < 0.01$, A versus B or C.

^b $P < 0.01$, B versus C.

^c $P < 0.01$, A versus C.

^d $P < 0.05$, A versus C.

^e $P < 0.05$, B versus C.

^f $P < 0.01$, A versus B.

Spanish and Italian isolates. All four HBV/A1 isolates in this study formed a cluster with Philippine and Indian isolates.

In addition, the pre-S2/S region sequences of a total of 29 isolates were determined, including the 11 isolates whose complete genomes were sequenced. Of these, 21 (72%) were classified as HBV/A2 and the remaining 8 as HBV/A1. A phylogenetic tree was constructed based on the pre-S2/S region sequences from the 29 isolates, along with those from 10 patients with AHB infected with HBV/A and 47 HBV/A isolates retrieved from the database (Fig. 5). The 21 HBV/A2 isolates in the present study formed a cluster with Japanese, American, and European isolates retrieved from the database and those from patients with acute hepatitis. In addition, some of them were highly homologous with each other. Likewise, HBV/A1 isolates from eight patients with chronic hepatitis in this study

were highly homologous with those from two patients with acute hepatitis and isolates from the Philippines and India. Based on the phylogenetic analyses, HBV/A isolates were imported from Europe and the United States, as well as the Philippines and India, and had infiltrated throughout Japan.

DISCUSSION

Perinatal transmission from carrier mothers to their babies has been the principal route for establishing persistent HBV infection in Asian countries (19). In Japan, passive and active immunoprophylaxis with HBV immune globulin and vaccine has been mandated for babies born to HBeAg-positive carrier mothers since 1986; this was extended to HBeAg-negative carrier mothers in 1995. As a result, HBsAg has become rare in Japanese born after 1986; it was detected in only 0.2% of first-time blood donors younger than 19 years of age in 2000 (24). However, AHB has been increasing in Japan, predominantly through promiscuous sexual contacts.

In Japan, HBV/A is detected rarely among patients with CHB but is frequent in those with acute hepatitis (14, 25, 29, 41, 43). Yotsuyanagi et al. reported the distribution of genotypes in 145 Japanese patients with AHB and found HBV/A in 27 (19%), HBV/B in 8 (5%), and HBV/C in 109 (75%) (49). HBV/A is more frequent in metropolitan areas than other areas. The majority of patients with HBV/A infection in metropolitan areas have had extramarital sexual contacts with multiple irregular partners, through which they could have contracted infection. In support of this view, among men who have sex with men (MSM) who are coinfecting with HBV and HIV-1 in Tokyo, most were infected with HBV/A (15, 35).

In Japan, AHB in adulthood becomes chronic in only ~1%

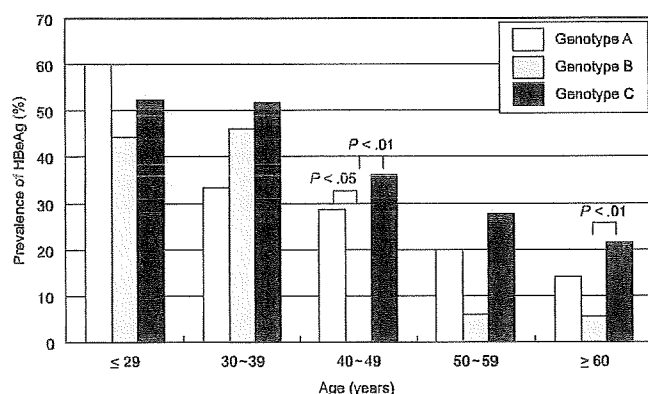


FIG. 2. Prevalence of HBeAg among patients infected with HBV of different genotypes stratified by the age.

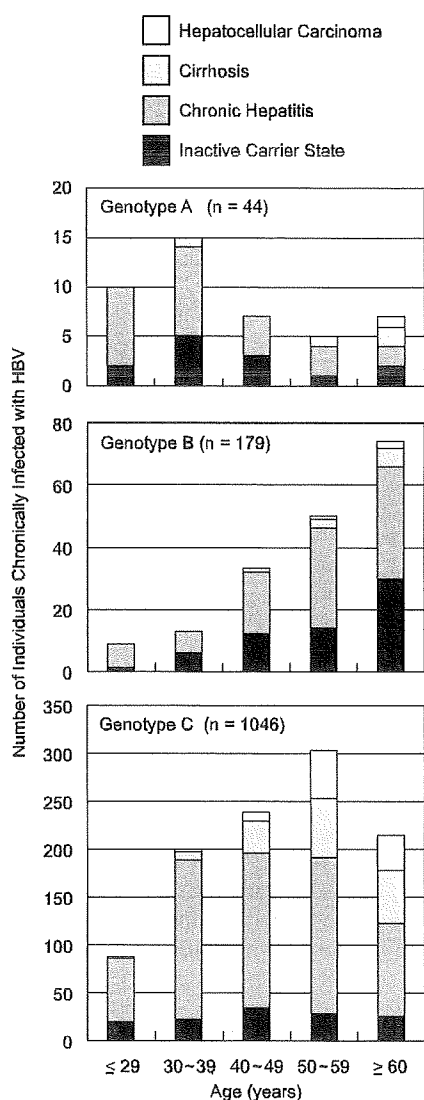


FIG. 3. Distribution of HCC, cirrhosis, chronic hepatitis, and inactive carrier state among the 1,271 patients infected with HBV of different genotypes stratified by the age.

of cases. This is much less than the progression to chronic disease (close to 10%) in Europe and the United States, where HBV/A prevails (34). Recent studies have suggested that the chances for persistence may differ among patients acutely infected with HBV of distinct genotypes (21, 25). In particular, acute infection with HBV/A may bring about an increased risk of progression to chronic disease. Therefore, an increase of acute infection with HBV/A would result in a surge of HBV/A among patients with CHB in Japan. In actuality, in comparison with our previous results during 2000 and 2001 (27), HBV/A was twice as frequent in this study (3.5% versus 1.7%; $P = 0.02$). HBV/A has been increasing in patients with CHB in the Kanto area, where HBV/A in patients with acute hepatitis is more frequent than in the other areas. In the islands of Okinawa, also, HBV/A was found to be prevalent in this study. Of the four patients infected with HBV/A there, two were coinfecting with HIV-1. They were both MSM, and they were sus-

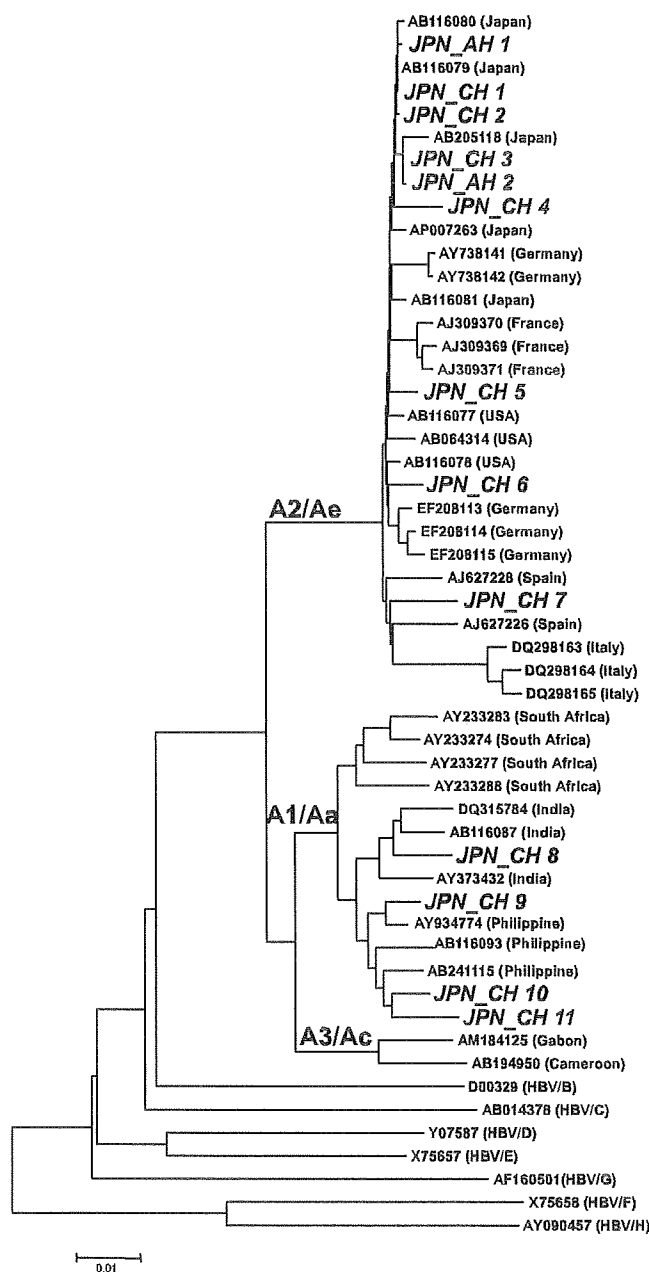


FIG. 4. Phylogenetic tree constructed based on the complete genome sequences of HBV/A isolates. Those from 11 patients with chronic infection in this study are shown in boldface italic (JPN_CH1 to -11), along with two isolates (JPN_AH1 and -2) from patients with acute hepatitis in Japan reported in our previous study (17). Representative isolates were retrieved from the DDBJ/EMBL/GenBank databases, including 21 HBV/Ae, 10 HBV/Aa, and 2 HBV/Ac isolates, along with 7 HBV isolates representative of the other seven genotypes. Isolates from the databases are identified by accession numbers, followed by the country of origin. The bar at the bottom spans 0.01 nucleotide substitutions per site.

pected to have been infected with HIV through sexual contacts on the Japanese mainland. It has been reported that HIV infection increases the probability that AHBs will become chronic (2, 11, 33, 48). Because they share routes of transmission and the risk for HIV-1 and HBV infections, approximately

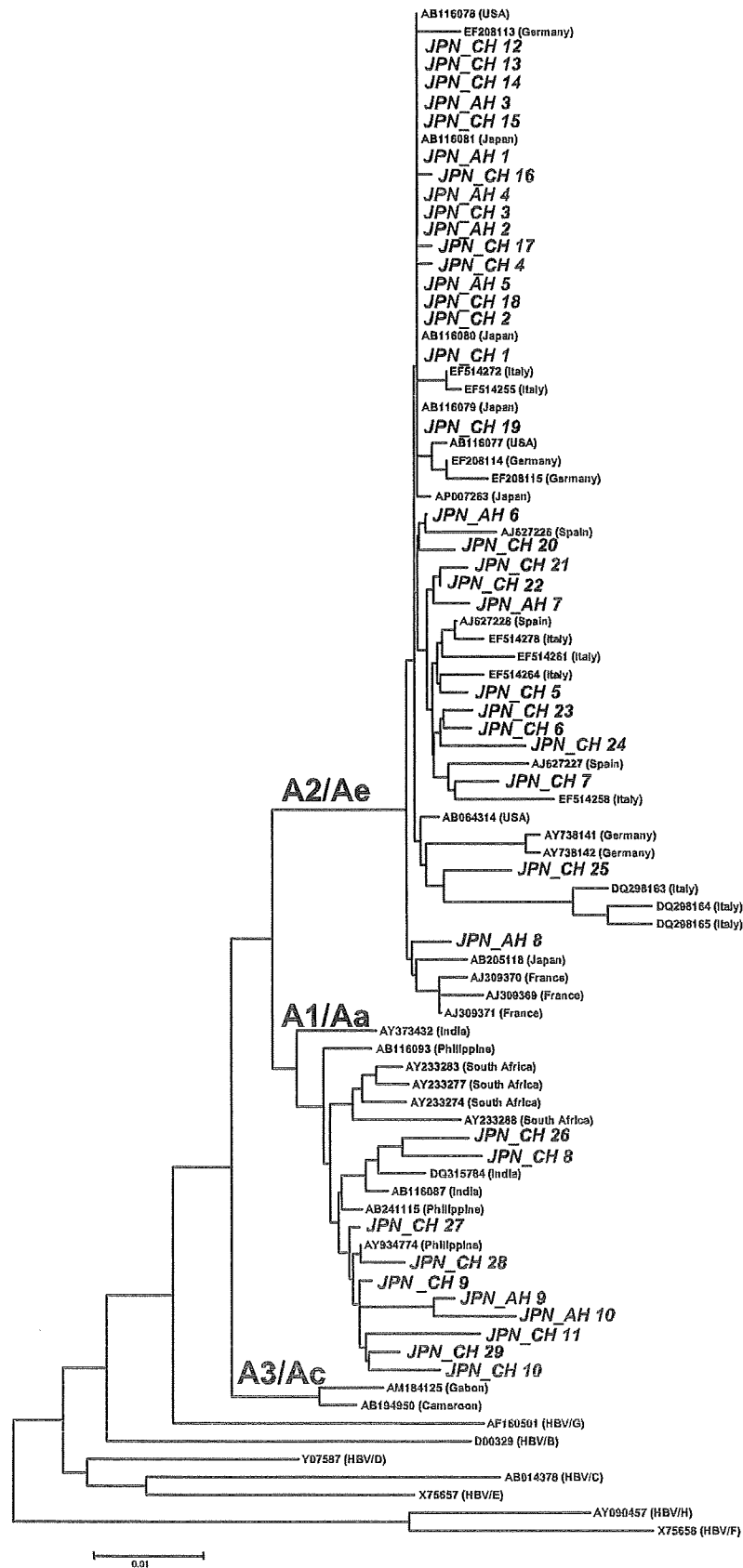


FIG. 5. Phylogenetic tree constructed based on pre-S2/S region sequences of HBV/A isolates. Those from 29 patients with chronic infection in this study are shown in boldface italic (*JPN_CH 1 to -29*), along with 10 isolates (*JPN_AH 1 to -10*) from patients with acute hepatitis in Japan reported in our previous study (17). Representative isolates were retrieved from the DDBJ/EMBL/GenBank databases, including 28 HBV/Ae, 10 HBV/Aa, and 2 HBV/Ac isolates and 7 HBV isolates representative of the other seven genotypes. Isolates from the databases are identified by accession numbers, followed by the country of origin. The bar at the bottom spans 0.01 nucleotide substitutions per site.

90% of patients with AIDS have markers of past or ongoing HBV infection (18). Thus, HBV carriers are more frequent in the HIV-1-positive than in the HIV-1-negative population (4, 9). Among patients with HIV infection in Japan, 6.3% are HBsAg positive, in particular, 8.3% of HIV-infected MSM (16). In this study, coinfection with HIV was found in 6 of the 44 (13.6%) patients infected with HBV/A. All of them were men. Their median age was 27.7 ± 4.1 years, and five patients were positive for HBeAg. Thus, there is a possibility that HIV-1 and HBV/A coinfections are increasing among young people in Japan, and the high rate of HBeAg positivity may be influenced by immune suppression due to HIV infection.

In the phylogenetic analysis, the HBV/A2 isolates recovered in this study were homologous to those from Europe and the United States, and some of them clustered with the Japanese isolates. On the other hand, there were HBV/A1 isolates that formed a cluster with those from the Philippines and India. Furthermore, some isolates from patients with acute hepatitis who were infected with HBV/A in Japan were highly homologous to HBV/A isolates from patients with chronic hepatitis. This invites speculation that some HBV/A isolates were introduced into Japan from foreign countries, while others have already settled down there and spread from patients with chronic infection to their contacts. HBV/A would have been infiltrating throughout Japan by these two different routes.

Clinical differences among patients infected with HBV/A, -B, and -C were observed. The mean age was lower in the patients infected with HBV/A than in those infected with HBV/B or -C. As mentioned above, AHB patients infected with HBV/A have been increasing in the younger generation in Japan, and around 10% of them would have progressed to chronic infection. This is one of the reasons why the patients infected with HBV/A are younger than those infected with HBV/B or -C. Most patients infected with HBV/B were negative for HBeAg, while a high proportion of the patients infected with HBV/A and -C had it. In particular, this difference was remarkable in the patients who were older than 40 years of age. Thus, the seroconversion rate for the loss of HBeAg among younger people may be higher in infection with HBV/B than in that with HBV/A or -C. Inactive carriers were commoner in HBV/A than in HBV/C infection, as well.

These lines of evidence indicate that the activity of hepatitis is lower in HBV/B than HBV/C infection, and patients with HBV/B seroconvert from HBeAg to anti-HBe at young ages. In addition, cirrhosis and HCC were less frequent in the patients infected with HBV/B than in those infected with HBV/C. Therefore, the prognosis would be better in the patients infected with HBV/B than in those infected with HBV/C. These results are in accord with previous reports (5, 13, 28, 42). There have been few reports on the clinical features of patients with chronic hepatitis infected with HBV/A in Japan. Chu et al. have reported the distribution of HBV genotypes with reference to clinical characteristics in the United States (6). They have shown that HBV/A and HBV/C infections are accompanied by a higher frequency of HBeAg than HBV/B infection, while HBV/B is associated with a lower rate of hepatic decompensation than HBV/A and -C. In our study, inactive carriers were commoner, while cirrhosis and HCC were found less often in HBV/A than in HBV/C infection. HBeAg was more prevalent in the patients infected with HBV/A than in those

infected with HBV/B who were older than 40 years of age. Therefore, it can be said that the prognosis is better for patients infected with HBV/A than for those infected with HBV/C; it may be poorer than for those infected with HBV/B.

In conclusion, HBV/A has been increasing among CHB patients in Japan. On the basis of phylogenetic analyses, some HBV/A isolates appear to have been imported from foreign countries. They clustered with HBV/A from AHB patients and have infiltrated throughout Japan. It is very likely that acute and chronic infections with HBV/A have been increasing in Japan. Obviously, immunoprophylaxis of perinatal HBV infection, implemented since 1986 on a national basis, has been insufficient to prevent horizontal HBV/A infection diffusing among high-risk groups by transmission routes shared by HIV infection. The foreseeable spread of HBV/A infection in Japan should be prevented by universal vaccination programs extended to high-risk groups or the general population.

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