Table 4B. (B) Comparison of HBeAg-negative patients with or without virological rebound by univariate analysis.

Virological rebound	No	Yes	*P-values*
Number	56	22	
Age (years)	54 ± 11	54 ± 10	*N.S.*
Gender (male)	40	12	*N.S.*
HBV DNA (log IU/mL)	5.9 ± 1.4	5.9 ± 1.0	*N.S.*
ALT (IU/L)	163 ± 179	137 ± 163	*N.S.*
US: Cirrhosis (+)	30	11	*N.S.*
Periods to undetectable HBV DNA (months)	7.3 ± 14.8	3.1 ± 2.1	*N.S.*

Data are expressed as mean ± SD. *P*-values, *P*-values between patients with or without virological rebound groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 4C. (C) Factor associated with virological rebound among HBeAg-positive patients treated with LAM by multivariate analysis.

Factor	Category	Odds ratio	95% CI	*P-value*
Age ≤ 44.5 (years)	(+/-)	0.222	0.0547-0.9023	0.0354

DISCUSSION

To date, there is not much data regarding virological rebound after achieving HBV DNA negativity in the use of ETV or LAM. A recent report supported the merit of the change from LAM to ETV [14]. This study concluded that prior optimal viral suppression with ETV did not confer any significant advantage for patients who switched to LAM.

The present study revealed that ETV could suppress HBV replication after achieving HBV DNA negativity, although additional longer follow-up studies will be needed. On the other hand, LAM could not suppress HBV replication even after achieving HBV DNA negativity (Figure 1), although most cases with virological rebound were observed within 2 years of the start of LAM medication. We could not check the emergence of YMDD motif mutations [19] in all of the cases because the present study was performed as part of regular clinical practice. Of 2 of the HBeAg-positive patients at baseline with virological rebound, one showed YVDD motif (50%). In 4 of the HBeAg-negative patients at baseline with virological rebound, one YVDD motif (25%) and three YIDD motifs (75%) were seen. Virological rebound may not mean the emergence of NA-resistance mutations [12].

We do not know the reason why virological rebound was attained independently of age in HBeAg-positive patients treated with LAM. HBeAg to anti-HBe antibody seroconversions were found in 20 and 11 patients with and without virological rebound, that is, the HBeAg to anti-HBe antibody seroconversion rates were similar in the two groups (data not shown), although the number of study patients seemed small in the present study. Further studies might be needed. In any event, it might be important to consider the LAM-to-ETV switch in HBeAg-positive patients treated with LAM, although some of our patients in the LAM group remained HBV-negative throughout the observation period.

In the present study, 95.3% (122 of 128), 82.3% (14 of 17) and 89.2% (25 of 28) had an adherence rate >90% [16] in ETV-treated, LAM-treated with virological rebound and LAM-treated patients without virological rebound, respectively. These results supported our previous study that viral breakthrough associated with poor adherence could be a more important issue in the treatment with especially stronger NAs, such as ETV [12,16], although we cannot ensure durable HBV negativity after NAs are discontinued. We and others reported that HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection [16,20,21]. Of interest, the virological rebound with the use of LAM seemed unrelated to the HBeAg status, suggesting that it was dependent on resistant mutation.

Recently, other effective antiviral therapies such as peginterferon [22,23] and tenofovir [24,25] were reported to be useful for the control of HBV infection. These drugs might also be candidates for treating virological rebound. Fung et al. [14] reported that prior optimal viral suppression with ETV did not confer any significant advantage for patients who switched to LAM. Our results also supported the previous studies that ETV was much more efficient than LAM [26-29]. In conclusion, ETV could inhibit HBV replication if HBV DNA negativity had been achieved. In contrast, LAM could not inhibit HBV replication even if HBV negativity was achieved in the early phase. Attention should be paid to these features in clinical

practice.

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CONFLICT OF INTEREST

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ABBREVIATIONS

ALT: alanine aminotransferase; ETV: Entecavir; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LAM: lamivudine; NA: nucleos(t)ide analogue.

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Research Paper

Adherence to Medication Is a More Important Contributor to Viral Breakthrough in Chronic Hepatitis B Patients Treated with Entecavir Than in Those with Lamivudine

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Abstract

Viral breakthrough is related to poor adherence to medication in some chronic hepatitis B patients treated with nucleos(t)ide analogues (NAs). Our study aimed to examine how adherence to medication is associated with viral breakthrough in patients treated with NAs. A total of 203 patients (135 ETV and 68 LAM) were analyzed in this retrospective analysis. Physical examination, serum liver enzyme tests, and hepatitis B virus marker tests were performed at least every 3 months. We reviewed medical records and performed medical interviews regarding to patients' adherence to medication. Adherence rates <90% were defined as poor adherence in the present study. Cumulative viral breakthrough rates were lower in the ETV-treated patients than in the LAM-treated patients ($P<0.001$). Seven ETV-treated (5.1%) and 6 LAM-treated patients (8.8%) revealed poor adherence to medication ($P=0.48$). Among ETV-treated patients, 4 (3.1%) of 128 patients without poor adherence experienced viral breakthrough and 3 (42.8%) of 7 patients with poor adherence experienced viral breakthrough ($P<0.001$). Only 3 of 38 (7.8%) LAM-treated patients with viral breakthrough had poor adherence, a lower rate than the ETV-treated patients ($P=0.039$). Nucleoside analogue resistance mutations were observed in 50.0% of ETV- and 94.1% of LAM-treated patients with viral breakthrough ($P=0.047$). Viral breakthrough associated with poor adherence could be a more important issue in the treatment with especially stronger NAs, such as ETV.

Key words: Adherence, Entecavir, Lamivudine, Hepatitis B, Viral Breakthrough.

INTRODUCTION

Two billion people have been exposed to hepatitis B virus (HBV), and 350-400 million people remain chronically infected worldwide. In Japan, the prevalence of HBV carriers is estimated at ~1% of the pop-

ulation, but HBV is a major health issue because it causes acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [1, 2].

Lamivudine (LAM) is a reverse-transcriptase inhibitor of HBV DNA polymerase that possesses excellent profile of safety and tolerability and causes inhibition of viral replication. LAM was the first nucleos(t)ide analogue (NA) to be approved for antiviral treatment of hepatitis B patients [3, 4]. Entecavir (ETV), a deoxyguanosine analogue, is a potent and selective inhibitor of HBV replication. The in vitro potency of ETV is 100- to 1,000-fold greater than that of LAM, and it has a selectivity index (concentration of drug required to reduce viable cell number by 50% [CC_{50}] / concentration of drug required to reduce viral replication by 50% [EC_{50}]) of approximately 8,000 [5, 6]. LAM (until 2005) and ETV (from 2006) have been used as first-line NAs for most patients with chronic hepatitis B in Japan. Most patients with chronic hepatitis B have been undergoing treatment for longer durations, and prolonged treatment is associated with increasing rates of viral breakthrough [7]. It has been reported that not all cases are associated with resistance mutations [8, 9]. We have also reported that some cases of viral breakthrough during ETV treatment were related to poor adherence to medication [10].

Adherence rates are usually lower in patients with long-term treatment regimens, such as for hypertension, than in patients with short-term regimens, such as for gastric ulcers [11]. It has been reported that 74.8% of patients with hypertension were determined to have an adherence rate ≥80% [12], and that 55.3% of patients with chronic hepatitis B had an adherence rate >90% [8].

In the present study, we aimed to investigate whether drug adherence is related to viral breakthrough in chronic hepatitis B patients treated with LAM or ETV. We also investigated the pattern of poor adherence and suggested how adherence to medication could be improved.

MATERIALS AND METHODS

Patients

Two hundred seventy-five NA-treated naïve patients (185 ETV- and 90 LAM-treated patients), who were admitted to Chiba University Hospital between April 2000 and September 2011, were enrolled (Figure 1). Some of these patients had already been included in a previous report [10]. Between November 2011 and April 2012, doctors performed medical interviews of those patients to determine their adherence to medication. Seventy-two patients (50 ETV- and 22 LAM-treated patients) were excluded from this retrospective analysis, because their adherence to medication could not be confirmed. One hundred thirty-five patients were administered 0.5 mg of ETV daily and 68 patients were administered 100 mg of LAM daily (Table 1). In all patients, serum hepatitis B surface antigen (HBsAg) and HBV DNA were positive. All patients had negative results for hepatitis C virus or human immunodeficiency virus antibodies. Physical examinations, serum liver enzyme tests, and HBV marker tests were performed at least every 3 months. The study was carried out in accordance with the Helsinki Declaration, and was approved by the Ethics Committee of Chiba University, Graduate School of Medicine (No. 977).

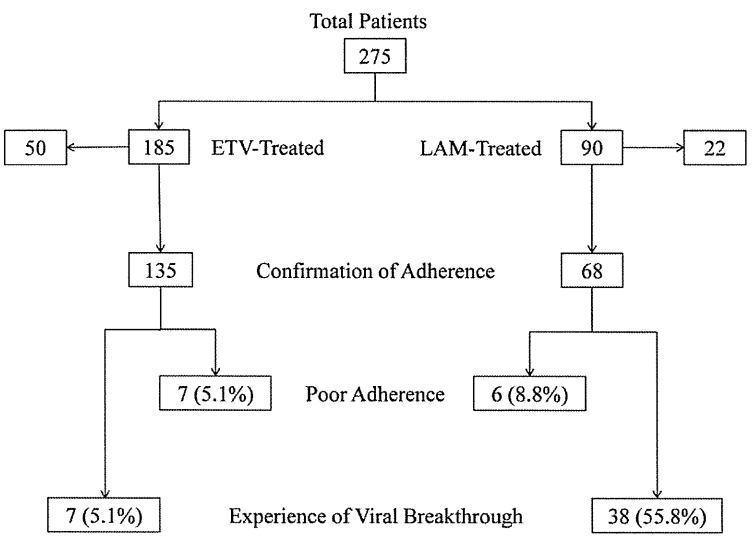


Figure 1. Patients, adherence rates, and the prevalence of viral breakthrough in this study. ETV, entecavir; LAM, lamivudine.

Table 1. Baseline characteristics of patients.

	ETV	LAM	P-values
Number of cases	135	68	
Age (years)	51.7 ± 11.7	45.5 ± 12.1	<0.001
Gender (male/female)	83/52	49/19	0.135
HBeAg (+/-)	64/71	45/23	0.011
Genotype (A/B/C/unknown)	0/11/78/46	1/6/57/4	0.427
HBV DNA (log IU/mL) (≤5.0/> 5.0/unknown)	27/108/0	3/55/10	0.009
ALT (IU/L)	161 ± 195	353 ± 394	<0.001
Platelets ($\times 10^4/mm^3$)	16.3 ± 5.9	16.9 ± 7.0	0.556
APRI	2.49 ± 4.19	6.52 ± 6.98	<0.001
Follow-up period (months)	26.9 ± 21.6	49.0 ± 39.7	<0.001

ETV, entecavir; LAM, lamivudine; HBeAg, hepatitis B e antigen; N.D., not determined; HBV DNA, hepatitis B virus deoxyribonucleic acid; ALT, alanine aminotransferase; APRI, aspartate aminotransferase platelet ratio index. Continuous variables are expressed as mean ± standard deviation.

Blood examinations

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, and platelet counts were reviewed in the present study. We also calculated the aspartate aminotransferase platelet ratio index [APRI: AST (IU/L)/ 35/platelet count ($10^3/\mu L$) x 100], which is significantly correlated with the staging of liver fibrosis, with a higher correlation coefficient than platelet count or AST level alone [13].

Detection of HBV markers

HBsAg, hepatitis B e antigen (HBeAg) and anti-HBe antibody were determined by ELISA (Abbott, Chicago, IL, USA) or CLEIA (Fujirebio, Tokyo, Japan)[14]. HBV genotype was determined by ELISA (Institute of Immunology, Tokyo, Japan) [15]. HBV DNA was measured by Roche Amplicor PCR assay (detection limits: 2.6 log IU/mL; Roche Diagnostics, Tokyo, Japan).

Follow-up period

The follow-up period ended when the NA was switched to another NA or another NA was added, or it was discontinued for various reasons.

Definition of adherence to medication

To obtain information regarding adherence to medication, we reviewed medical records. We also interviewed patients about their adherence to medication. We expressed the rate of adherence to medication as a percentage calculated by the number of days of taking a pill divided by the follow-up period (days). Adherence rates <90% were defined as poor adherence in the present study.

Definition of viral breakthrough

Viral breakthrough was defined as an increase of ≥ 1 log IU/mL in serum HBV DNA level from nadir.

Sequence analysis of HBV DNA

The YMDD motif was analyzed by PCR-ELMA in sera of patients who had experienced viral breakthrough, as reported by Kobayashi et al [16]. HBV polymerase/reverse transcriptase (RT) substitutions were also analyzed in sera of ETV-treated patients who had experienced viral breakthrough. Briefly, HBV DNA was extracted from 100 μL of sera using SepaGene (Sanko Junyaku, Tokyo, Japan). Nested PCR was performed using LA Taq polymerase (Takara Bio, Otsu, Shiga, Japan) under the following conditions: 5-min denaturation at 94°C, 35 cycles with denaturation at 94°C for 40 s, annealing at 58°C for 1 min, and extension at 68°C for 1.5 min [2]. An 862 base-pair fragment (nt 242-1103) containing the polymerase RT domain was amplified on the PCR Thermal Cycler Dice Model TP600 (Takara Bio). The primers for the first PCR were 5'-CAG AGT CTA GAC TCG TGG-3' (sense, nt 242-258) and 5'-GGC GAG AAA GTG AAAGCC-3' (antisense, nt 1103-1086). The PCR product was sequenced using the primers: 5'-TGG CTC AGT TTA CTAGTG CC -3' (nt 668-687) and 5'-GGC ACT AGT AAA CTGAGC CA-3' (nt 687-668), and these primers were also used for the second PCR. To prepare the sequence template, PCR products were treated with ExoSAP-ITR (Affymetrix, Inc., Santa Clara, CA, USA), and then sequenced using the BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan). Sequences were performed with Applied Biosystems 3730xl (Life Technologies) [17].

Statistical analysis

Statistical analyses were performed using SAS 9.3 Software (SAS Institute, Cary, NC, USA). Continuous variables were expressed as mean ± standard deviation and were compared by Student's t-test or

Welch's t-test. Categorical variables were compared by chi-square test or Fisher's exact probability test. The Kaplan-Meier method was used to calculate viral breakthrough rates. Baseline was taken as the date when the first dose of LAM or ETV was taken. Statistical significance was considered at a *P-value* < *0.05*.

RESULTS

Baseline characteristics of patients

Baseline characteristics of patients are shown in Table 1. In ETV-treated patients, the age was higher, the prevalence of HBeAg-negative patients was higher, HBV DNA was lower, ALT levels were lower, and APRI was lower (ie., liver fibrosis was milder) than in LAM-treated patients. HBV genotype C was dominant in both groups. The follow-up period in ETV-treated patients was shorter than that in LAM-treated patients, based on the fact that ETV was a newer drug and many ETV-treated patients had started treatment more recently.

Adherence to medication, and viral breakthrough between ETV- and LAM-treated patients

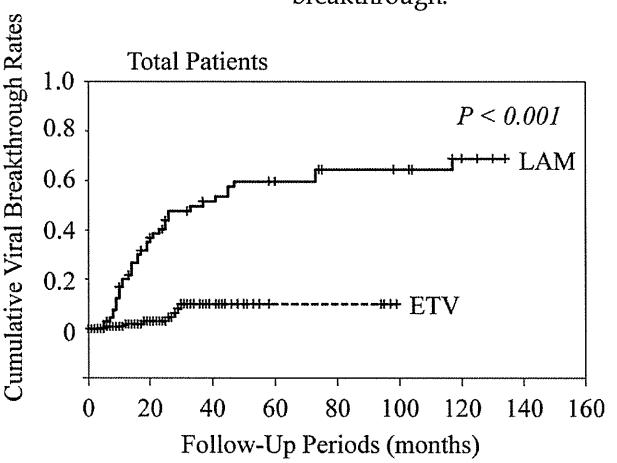
Most patients presented good adherence to medication in the present study. Seven ETV-treated (5.1%) and 6 LAM-treated patients (8.8%) had poor adherence (Figure 1). The number of patients with poor adherence was not significantly different between the ETV- and LAM-treated groups (*P=0.48*). The characteristics of the 13 patients with poor adherence are shown in Table 2. Cumulative viral breakthrough rates were lower in the ETV-treated patients than in the LAM-treated patients (*P<0.001*) (Figure 2).

Viral breakthrough in HBeAg-positive and -negative patients

Among the LAM-treated patients, cumulative viral breakthrough rates in HBeAg-positive patients at baseline (n=45; 25.0% at 1 year, 55.1% at 3 years, and 67.0% at 5 years) were similar to those in HBeAg-negative patients at baseline (n=23; 9.5% at 1 year, 38.2% at 3 years, and 44.4% at 5 years; *P=0.16*). Among the ETV-treated patients, cumulative viral breakthrough rates in HBeAg-positive patients at baseline (n=64; 2.2% at 1 year, 18.1% at 3 years, and 18.1% at 5 years) were also similar to those in HBeAg-negative patients at baseline (n=71; 1.6% at 1 year, 1.6% at 3 years, and 1.6% at 5 years; *P=0.050*).

Among the LAM-treated patients who were HBeAg-positive at baseline, cumulative viral breakthrough rates in patients who converted to HBeAg-seronegative were lower than those in patients who maintained HBeAg seropositivity (*P<0.001*) (Figure 3). All LAM-treated patients who did not become HBeAg-seronegative experienced viral breakthrough. Among the ETV-treated patients who were positive for HBeAg at baseline, conversion to HBeAg seronegativity did not affect the rate of viral breakthrough (*data not shown*).

There were no differences in HBV viral loads at study entry between HBeAg-positive patients with and without viral breakthrough. There were also no differences in HBV viral loads between HBeAg-negative patients with and without viral breakthrough.



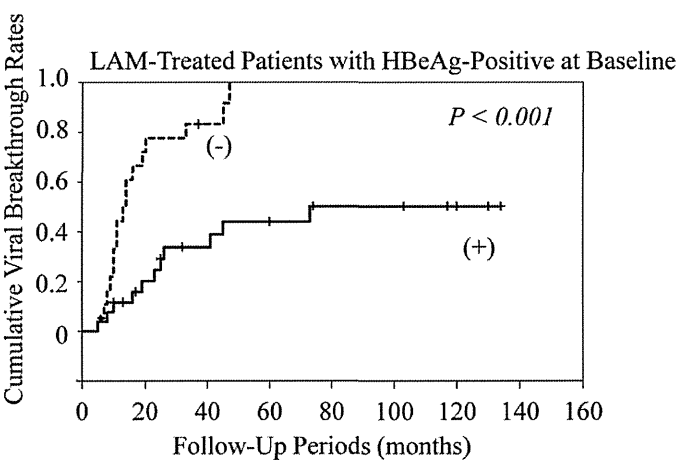
	Drug	Baseline	12	24	36	48	60
Number of Patients	LAM	68	52	34	27	20	19
	ETV	135	95	69	34	19	5

Figure 2. Cumulative viral breakthrough rates. ETV, entecavir; LAM, lamivudine.

Table 2. Patients with poor adherence to medication.

Case	Drug	Adherence rate (%)	Age (years)	Gender	Genotype	HBeAg	HBV DNA (log IU/mL)	ALT (IU/L)	APRI	HBeAg-seronegative	HBV DNA negativity	VT	Duration of treatment before VT (months)	Resistance mutations	Treatment after VT	Clinical outcome
1	ETV	50	55	F	B	-	3.8	16	0.33	N.A.	+	+	6	-	ETV	good
2	ETV	75	49	M	C	+	7.3	107	1.60	+	+	+	28	+	LAM+ADV	good
3	ETV	85	38	M	C	+	6.9	59	2.80	-	+	+	29	N.D.	ETV	good
4	ETV	80	39	M	C	+	5.8	51	0.63	+	+	-	N.A.	N.A.	ETV	good
5	ETV	85	37	F	C	+	6.9	160	2.25	+	+	-	N.A.	N.A.	ETV	good
6	ETV	85	66	M	N.D.	+	7.7	68	0.95	-	-	-	N.A.	N.A.	ETV	good
7	ETV	85	38	M	C	+	6.5	478	7.94	-	+	-	N.A.	N.A.	ETV	good
8	LAM	50	47	F	C	+	6.5	455	2.54	+	+	+	45	-	LAM	good
9	LAM	80	36	M	C	+	7.0	110	4.25	+	+	+	41	+	LAM+ADV	good
10	LAM	85	23	M	C	+	>7.6	161	3.53	-	+	+	11	-	cessation	flare
11	LAM	85	32	M	C	+	>7.6	343	1.30	+	+	-	N.A.	N.A.	LAM	good
12	LAM	85	54	F	C	-	4.1	196	2.68	N.A.	+	-	N.A.	N.A.	LAM	good
13	LAM	85	36	M	C	+	6.7	1576	15.78	+	+	-	N.A.	N.A.	LAM	good

Cases 2 and 3 had already been included in a previous report.[10] HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid, ALT, alanine aminotransferase; APRI, aspartate aminotransferase platelet ratio index; VT, viral breakthrough; ETV, entecavir; LAM, lamivudine; ADV, adefovir; F, female; M, male; N.D., not determined; N.A., not available; HBeAg-seronegative, conversion to HBeAg-seronegative after administration of a nucleoside analogue; HBV DNA negativity, achieving HBV DNA negativity after administration of a nucleoside analogue; flare, fluctuating ALT after treatment after VT.



HBeAg-Seronegative	Baseline	12	24	36	48	60
Number of Patients (-)	19	10	4	3	0	0
Number of Patients (+)	26	22	17	13	11	11

Figure 3. Cumulative viral breakthrough rates in lamivudine (LAM)-treated patients with HBe antigen (HBeAg)-positive at baseline. (-), maintaining HBeAg seropositivity; (+), conversion to HBeAg-seronegative.

Viral breakthrough in patients who achieved, and did not achieve HBV DNA negativity

Among the LAM-treated patients, cumulative viral breakthrough rates in patients who did not achieve HBV DNA negativity were higher than in those who achieved HBV DNA negativity ($P<0.001$) (Figure 4). All patients who did not achieve HBV DNA negativity experienced viral breakthrough. In

contrast, among the ETV-treated patients, cumulative viral breakthrough rates in patients who did not achieve HBV DNA negativity were similar to the rates in those who achieved HBV DNA negativity (*data not shown*).

Correlation between adherence to medication and viral breakthrough

We also compared viral breakthrough rates according to adherence to medication. Among 62 LAM-treated patients who did not have poor adherence, 35 patients (56.4%) experienced viral breakthrough (Figure 5). Among 6 LAM-treated patients with poor adherence, 3 patients (50.0%) experienced viral breakthrough. In LAM treatment, poor adherence did not contribute to viral breakthrough (*P=0.89*). However, among 128 ETV-treated patients who did not have poor adherence, 4 patients (3.1%) experienced viral breakthrough. Among 7 ETV-treated patients with poor adherence, 3 patients (42.8%) experienced viral breakthrough. In the treatment with ETV, poor adherence contributed to viral breakthrough (*P<0.001*).

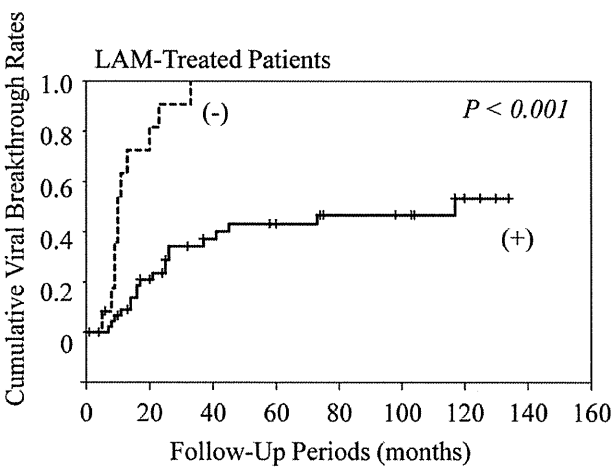
Resistance mutations

Resistance mutations were analyzed in some patients who experienced viral breakthrough. They were analyzed in 34 LAM-treated patients and 4 ETV-treated patients (Table 3). Thirty-two LAM-resistant patients had 10 YVDD, 17 YIDD, and 5 YV/IDD motifs, and 2 ETV-resistant patients had two YVDD motifs. Resistance mutations were not observed in 2 LAM-treated patients (5.8%) and 2 ETV-treated patients (50.0%) (*P=0.047*).

Table 3. Patients with viral breakthrough.

	ETV		LAM	
Adherence rate	≥90%	<90%	≥90%	<90%
Resistance mutation (+)	1	1	31	1
L180M	1	1	N.D.	N.D.
T184A	1	0	N.D.	N.D.
S202G	0	1	N.D.	N.D.
M204V	1	1	9	1
M204I	0	0	17	0
M204V/I	0	0	5	0
M250V	0	0	N.D.	N.D.
Resistance mutation (-)	1	1	0	2

ETV, entecavir; LAM, lamivudine; N.D., not determined. Numbers of amino acid positions were according to Refs. 2 and 10.



	HBV DNA Negativity	Baseline	12	24	36	48	60
Number of Patients	(-)	12	4	1	0	0	0
	(+)	47	39	30	23	19	18

Figure 4. Cumulative viral breakthrough rates in lamivudine (LAM)-treated patients who achieved HBV DNA negativity and those who did not. (-), maintaining HBV DNA positivity; (+), achieving HBV DNA negativity. HBV DNA negativity was unknown in 9 patients because of lack of data.

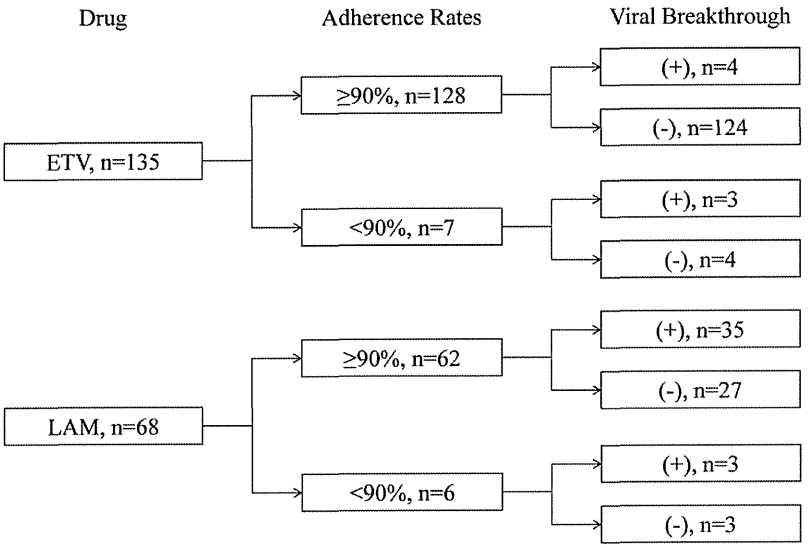


Figure 5. Association between adherence to medication and viral breakthrough.

DISCUSSION

The current study found that ETV-treated patients were not likely to acquire any resistance mutations and experience an ALT flare. Therefore, patients with poor liver residual function, such as liver cirrhosis, were likely to be administered ETV rather than LAM. Unexpectedly, HBsAg loss was observed in 3 of 28 LAM-treated patients without viral breakthrough (10.7%) and in 3 of 118 ETV-treated patients without viral breakthrough (2.5%). Long-term treatment with these drugs might result in HBsAg loss, although several reports have stated that one-year treatment with peg-interferon led to more HBsAg loss than these drugs [18-25].

In the current study, adherence to medication of most patients was excellent. The reasons for this might be as follows: (1) Our setting was a University Hospital, and this may have strengthened their will to succeeded with the treatment; (2) some patients with poor adherence might have been excluded because they did not see a doctor during the interview period; and (3) the rate of adherence to medication was based on patient self-assessment. A previous report showed that adherence might be underestimated by the Medication Event Monitoring System, a system that automatically records whenever a drug bottle is opened, and might be overestimated by pill counting and at interviews [26]. We classified the adherence rate as good at 90% or more, and as poor at less than 90%. However, we could not prove any significant influence of this classification on viral breakthrough as well as resistance mutation.

In the 13 patients with poor adherence (Table 2), we examined the reasons for their failure to take the pills. All 13 patients displayed some carelessness about taking pills. Two ETV-treated patients did not see a doctor and could not take pills continuously for a certain period of time, which particularly appeared to affect their viral breakthrough.

In LAM-treated patients, conversion of HBeAg to seronegative and achieving HBV DNA negativity was one of the important factors for successful treatment (Figures 3 & 4). In contrast, among ETV-treated patients, maintaining HBeAg seropositivity or HBV DNA positivity was not associated with viral breakthrough in the present study. Because of the stronger effect of ETV, it has been reported that long-term ETV treatment leads to a viral response in the vast majority of patients with detectable HBV DNA after 48 weeks [27]. Moreover, in the current study, poor adherence to medication was a major factor of viral breakthrough in the ETV-treated patients, but not in the LAM-treated patients. Ha et al. [9] also reported that medication non-adherence is likely to be a more important contributor to treatment failure than antiviral resistance, especially with new anti-HBV agents such as ETV and tenofovir. In LAM-treated or ETV-treated patients, viral breakthrough without resistance mutations might occur to some degree because of poor adherence to medication. In the present study, in LAM-treated patients, emergence of viral breakthrough with resistance mutations was common. Therefore, viral breakthrough due to poor adherence to LAM might not be important, compared with ETV-treated patients. However, in ETV-treated pa-

tients, viral breakthrough with resistance mutations was rare, and therefore, viral breakthrough due to poor adherence to ETV might be important.

In conclusion, viral breakthrough associated with poor adherence could be an important issue in the treatment with strong nucleoside analogues, such as ETV.

ABBREVIATIONS

ALT: alanine aminotransferase
ETV: entecavir
HBeAg: hepatitis B e antigen
HBsAg: hepatitis B surface antigen
HBV: hepatitis B virus
HCC: hepatocellular carcinoma
NA: nucleos(t)ide analogue
LAM: lamivudine

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Contributors

HK, TK, FI, and OY designed the study. HK, TK, MA, TC, HM, KF, FK, FI, FN and OY saw patients and conducted the interview. HK, TK, WS, and SN analyzed the data. HK and TK drafted the paper and all authors approved the paper.

COMPETING INTERESTS

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Original Article

Novel hepatitis B virus strain developing due to recombination between genotypes H and B strains isolated from a Japanese patient

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Aim: In Japan, genotypes B and C are the predominant genotypes isolated from patients with chronic hepatitis B, while genotype A predominates in patients with acute hepatitis B. Globalization, however, appears to have changed the distribution of the hepatitis B virus (HBV) genotypes. Thus, the viral characteristics of HBV genotypes other than genotypes A, B and C were examined.

Methods: Screening of genotypes was performed by enzyme immunoassay and/or polymerase chain reaction INVADER method in 222 patients with HBV. The full-length nucleotide sequences of unusual strains were compared to those in the database, followed by construction of a phylogenetic tree.

Results: Unusual HBV strains were isolated from two patients: a 27-year-old Japanese bisexual man with acute hepatitis B with HIV co-infection and a 52-year-old Japanese man with chronic hepatitis B. The former strain was classified as genotype H, showing an overall identity of 99.8% to the Thailand strain (EU498228), while the nucleotide sequence of the latter strain showed similarity to the genotype B strains isolated in Malaysia (JQ027316) and Indonesia (JQ429079) between DR2 and DR1 in the X region, with identities of 96.9%. However, this strain was classified as genotype H by full-length sequence analysis, and the sequence between nt2023 and nt2262 showed no similarity to that in any previously reported strains.

Conclusion: HBV strains showing recombination between genotype B and H strains were found even in chronic hepatitis patients in Japan. Globalization may yield HBV strains of possible novel genotypes containing novel nucleotide sequences in the precore/core region.

Key words: genotype, globalization, hepatitis B virus, nucleotide sequence, recombination

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a global health problem with an estimated 400 million people worldwide showing persistent infection.[1] These patients are at a serious risk of developing the complication of liver cirrhosis and hepatocellular carcinoma (HCC),[2] and approximately 1 million deaths per year are attributed to cirrhosis and HCC caused by HBV infection.[3] In Japan, more than 30 000 people die of HCC each year,[4] and in 15% of these cases, the etiology has been shown to be HBV infection.[5] On the other hand, patients with persistent HBV infection serve as a source of HBV transmission to the healthy population, resulting in the occurrence of acute liver diseases with fatal outcomes. According to a nationwide survey of fulminant hepatitis and late-onset hepatic failure in Japan, acute liver failure is caused by HBV infection, either transient infection or acute exacerbation of persistent infection, in approximately 40% of cases.[6–8]

Hepatitis B virus is a double-stranded DNA virus belonging to the *Hepadnaviridae* family; the genome is composed of approximately 3200 nucleotides organized into four open reading frames (ORF) for the P, C, S and X genes.[9] According to the results of full-length nucleotide sequence analysis of the entire genome, HBV has been classified into at least eight genotypes, A–H,

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showing nucleotide differences of more than 8% from each other.[10] The frequency of each genotype among isolates from patients with HBV infection differs depending on the geographic area of the world;[11] genotype A HBV strains prevail in Africa, Europe and India, while genotype B and C strains are frequent in Asia, and genotype E strains in sub-Saharan Africa. On the other hand, genotype D strains are distributed all over the world, and genotype F and H strains are found exclusively in Central and South America. It has been demonstrated that the clinical features of patients with HBV infection, including their responses to antiviral therapies, differ depending on the genotype of the viral strain causing the infection,[12] suggesting that identification of the HBV genotype causing the infection, in addition to determination of the serum HBV DNA levels and mutation profile of the viral genome is crucial to establish the therapeutic strategy in patients with both acute and chronic liver diseases caused by HBV.

However, it has been reported recently that globalization of the world may have altered the geographic distribution of HBV genotypes, including in Asian countries. In Japan, genotypes B1/Bj and C2 strains are the predominantly isolated strains from patients with both acute and chronic liver diseases caused by HBV infection; the distribution of the HBV genotypes has been reported to differ depending on the geographic areas even within Japan; genotype B strains are found more frequently in Okinawa islands and northeastern areas of Honshu island, while genotype C strains are more prevalent in other areas of Japan.[13] It has been suggested that such a distribution pattern may be upset in the near future, because genotype A strains have begun to be isolated more frequently from patients with acute liver diseases caused by HBV infection in Japan, especially in metropolitan cities such as Tokyo, Osaka and Nagoya,[14,15] and this genotype strain is known to produce persistent infection even in elderly patients contracting the infection.[16] Furthermore, the occurrence of recombination among different genotypes may also influence the geographic distribution patterns. HBV strains resulting from genome recombinations among genotype A, C and G strains have been found in Laos and Vietnam, and been tentatively proposed as "genotype I" strains.[17,18] Moreover, a HBV strain positioned between the human and ape genotypes on the phylogenetic tree has been isolated from a Japanese patient with HCC who had previously lived in Borneo.[19]

Thus, we screened the genotypes of the HBV strains isolated from patients with acute and chronic liver diseases caused by HBV, and the full-length nucleotide sequences of the strains other than genotype A, B and C strains found in the screening examination were analyzed and compared with those in the database. In the present paper, we report on the viral characteristics of such unusual strains detected in Japanese patients with HBV infection.

METHODS

Patients and experimental designs

THE SUBJECTS WERE 222 Japanese patients with acute or chronic hepatitis seen first between May 2011 and December 2012 at the outpatient clinic of Saitama Medical University Hospital. All the patients tested positive for serum hepatitis B surface antigen (HBsAg), and the HBV genotypes were screened by enzyme immunoassay (EIA)[20,21] or the polymerase chain reaction (PCR)-INVADER method.[22] The full-length nucleotide sequence was analyzed when genotypes other than A, B or C were identified from the patients. The screening examinations for the HBV genotypes were done under the assurance of national health insurance coverage. Written informed consent was obtained from each of the patients prior to the analysis of the full-length nucleotide sequences of the isolated HBV strains. The characteristics of the viral genotypes other than A, B or C identified through the screening examination were analyzed after obtaining the approval of the institutional review board of Saitama Medical University Hospital.

DNA extraction and direct nucleotide sequencing of the HBV strains

Nucleic acids were extracted from 200 μL of serum samples QIAamp MinElute Virus Spin Kits (Qiagen, Tokyo, Japan). The virus DNA was eluted in RNase-free water at a volume of 100 μL and maintained at −20°C until use. To obtain a full-length nucleotide sequence of HBV DNA, a long-distance nested PCR was performed to amplify two overlapping fragments according to the methods of Takahashi *et al.*[23] using oligonucleotide primers shown in Table S1.

A fragment with a length of 3040 bases (WA2) corresponding to oligonucleotides from 1908–1780 nt of a standard genotype C HBV isolate was amplified using two primer sets, external WA-L (1859–1882 nt) and WA-R (1805–1828 nt) primers and internal WA2-L (1887–1908 nt) and WA2-R (1780–1801 nt) primers, and PrimeSTAR GXL DNA Polymerase (TaKaRa, Shiga, Japan) with the primer annealing at 60°C for 35 cycles

in the first PCR and 30 cycles in the second PCR. A fragment with a length of approximately 378 bases (gN2) corresponding to the residue from 1702–2081 nt was amplified similarly using two primer sets, external gN1-L (1606–1625 nt) and gN1-FR/gN1-HR (2160–2179 nt) primers and internal gN2-L/gN2-HL (1683–1702 nt) and gN2-FR/gN2-HR (2081–2100 nt) primers, and TaKaRa Ex Taq Hot Start Version (TaKaRa) with the primer annealing at 55°C for 35 cycles in the first PCR and 30 cycles in the second PCR. PCR conditions for PrimeSTAR GXL DNA Polymerase and PrimeSTAR GXL DNA Polymerase were specified according to the protocol of the manufacturer.

Both WA2 and gN2 fragments were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the BigDye Teminator version 3.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA) using the internal primers shown in Table S1, according to the protocol of the manufacturer. The nucleotide sequences of the amplified products were directly sequenced with a 3130 Genetic Analyzer (Applied Biosystems), and the obtained data for nucleotide sequences were connected using ATGC version 7 (GENETYX, Tokyo, Japan).

Whole-genome cloning of HBV strains

To obtain a whole-genome clone of HBV strains, an additional PCR and In-Fusion reactions were performed. The WA2 and gN2 fragments were amplified using Prime STAR MAX DNA Polymerase (TaKaRa) and primer sets, WA2-Sap I-L (1943–1960 nt) and WA2-Sap I-R (1689–1708 nt) primers and gN2-Sap I-L (1704–1723 nt) and gN2-Sap I-R (1940–1957 nt) primers, respectively (Table S1), with the primer annealing at 55°C for 35 cycles. T-Vector pMD20 (TaKaRa) was amplified using a primer set, pMD20-Sap I-L (1705–1708 nt) and pMD20-Sap I-R (1704–1707 nt) primers, at conditions similar to that in amplification of both fragments. All PCR conditions were specified according to the protocol of the manufacturer. Both fragments and the vector were purified using the QIAquick PCR Purification Kit (Qiagen). WA2-Sap I fragment (100 ng), 50 ng of gN2-Sap I fragment and 100 ng of T-Vector pMD20-Sap I were mixed in a tube with In-Fusion HD Enzyme Premix (Clontech, Mountain View, CA, USA) at a total volume of 10 μL. The reaction mixture was incubated at 50°C for 15 min, and then transferred to ice. Reaction mixture (2.5 μL) was transformed into Stellar Competent Cell (Clontech) followed by mini-prepping and was subjected to nucleotide sequencing. Both conditions for In-Fusion reaction and transformation were specified according to the protocol of the manufacturer.

SimPlot analysis and construction of the phylogenetic tree

The complete full-genome sequences of the isolated HBV strains were compared with those of the 35 reference sequences retrieved from the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank database. The full-genome sequences of the following HBV strains shown in the database (represented by their accession numbers) were used in the SimPlot analysis, followed by construction of the phylogenetic tree: genotype A, *AB076678*, *AF090838* and *M57663*; genotype B, *AB010291*, *AB033554*, *AF121249*, *D00329* and *D50521*; genotype C, *AB049609*, *AB049610*, *AB112063*, *AB112066*, *AB112471* and *AB115417*; genotype D, *AB033559*, *AB126581* and *Z35716*; genotype E, *AB091255*, *AB106564* and *X75657*; genotype F, *AB166850*, *AY090459* and *X69798*; genotype G, *AB056513*, *AB064310* and *AF160501*; genotype H, *AB179747*, *AY090454*, *AY090457* and *AY090460*; genotype I, *EU833891*, *GU357844*, *JF899337* and *JF899338*; and genotype J, *AB486012*.

The nucleotide sequences were multiple-aligned using GENETYX for Windows version 11 software (GENETYX) and the genotype was specified using Kimura's two-parameter method.[24] A phylogenetic tree was constructed by the neighbor-joining method.[25] To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and resampling were carried out 1000 times. The subtypes of the strains used for the comparison were obtained from published articles.[26,27] Moreover, the recombination of the HBV genomes among strains of different genotypes was examined by the SimPlot program (available at http://sray.med.som.jhmi.edu/SCRoftware/) and boot scanning analysis.[25,28]

RESULTS

Genotypes of HBV strains obtained from patients with acute and chronic liver diseases

THE HBV STRAINS isolated from the 222 patients were classified according to the screening examinations carried out by EIA and/or the PCR-INVADER method as follows: genotype A, 21 (9.4%) strains; genotype B, 66 (29.7%) strains; and genotype C, 112 (50.5%) strains. The HBV genotype was indeterminate in 21 patients (9.4%) due to the low titers of serum HBsAg and/or HBV DNA. When the total subject population was stratified further, genotypes A, B, C and the

indeterminate genotype were found in 15 (50.0%), three (10.0%), 11 (36.7%) and zero (0%) of the 30 patients with acute liver diseases, and six (3.1%), 63 (32.8%), 101 (52.6%) and 21 (11.0%) of the 192 patients with chronic liver diseases, respectively. In contrast, one each of the patients (1.0%) with acute (case 1) and chronic (case 2) liver diseases had a HBV genotype other than A, B or C. The demographic and clinical features of the two patients were as follows.

A 27-year-old bisexual man (case 1) working in the adult entertainment industry was diagnosed as having acute hepatitis caused by HBV, and the genotype of the infecting HBV strain was identified as genotype H by the PCR-INVADER method. He received highly active antiretroviral therapy because of co-infection with HIV, and the serum HBV DNA titers decreased to less than the detectable level, with positivity for serum anti-HBs antibody developing 25 months later.

A 57-year-old man (case 2) was diagnosed as having chronic hepatitis caused by HBV, and the infecting HBV strain was classified as genotype F by the PCR-INVADER method, despite the genotype being classified as indeterminate by the EIA method. His deceased father had lived in Brazil in his youth and his elder brother had been diagnosed as being a HBV carrier at another hospital. He received oral entecavir at a daily dose of

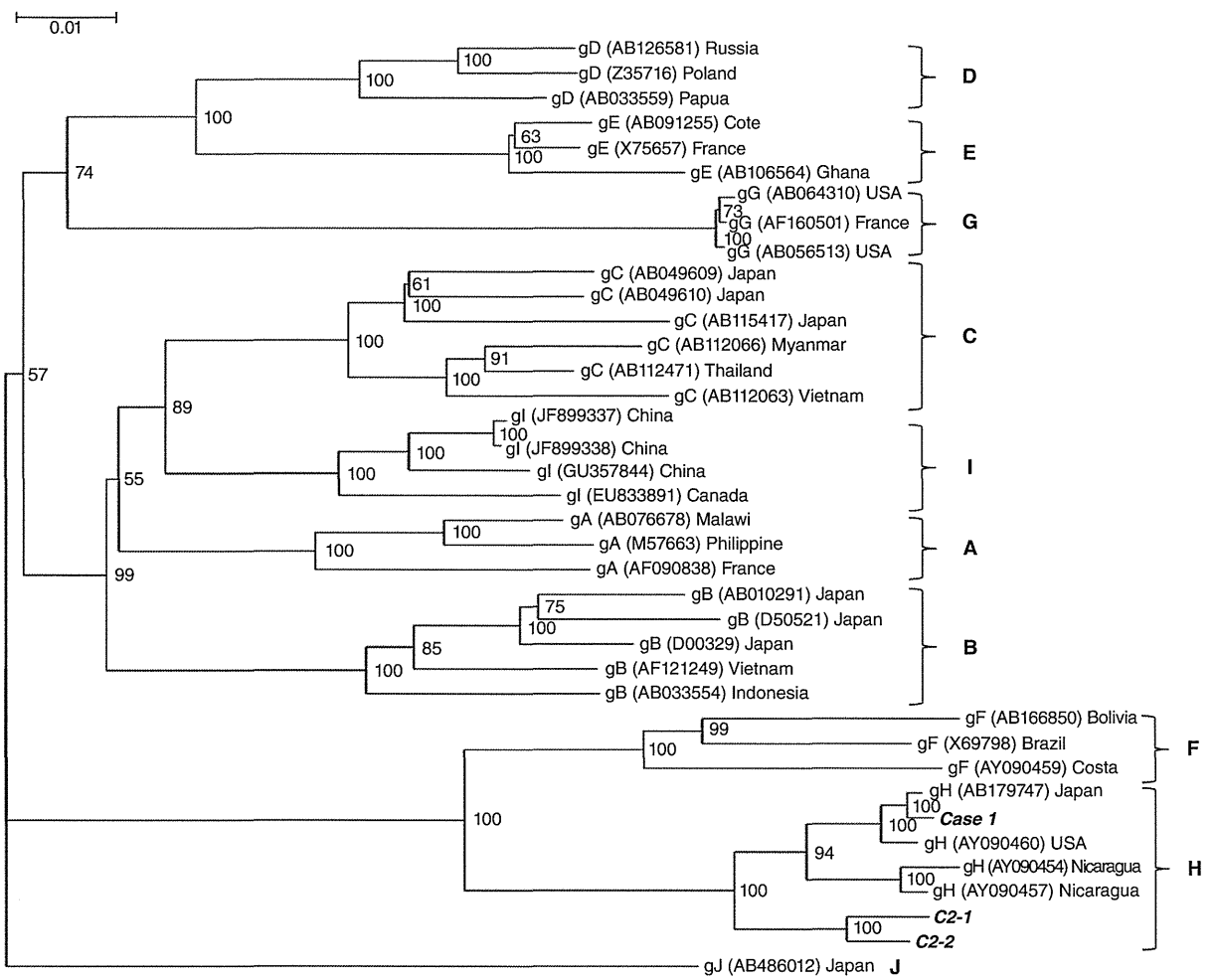

Figure 1 A phylogenetic tree constructed based on the full-length sequence of hepatitis B virus (HBV) strains isolated from case 1 and case 2 in comparison with that of 35 reference strains. The bootstrap values are indicated at each tree root and the genotypes are on the right. The horizontal bar provides a genetic distance.

0.5 mg, and the serum HBV titers decreased from 5.3 log copies/mL to a level less than 2.1 log copies/mL by 3 months of treatment.

Full-length nucleotide sequences of the isolated HBV strains that were different from genotypes A, B and C

The nucleotide sequences of the HBV strains isolated from cases 1 and 2 were analyzed. A phylogenetic tree constructed based on the full-length sequence of HBV genome led to classification of the HBV strain isolated from case 1 as genotype H, showing an overall identity of 99.8% (3210/3215 bp) to the Thailand strain of genotype H (EU498228) (Figs 1,2). A similar analysis using a phylogenetic tree led to classification of the HBV strain isolated from case 2 as genotype H (Figs 1,3) despite it being classified as indeterminate and genotype F by EIA and PCR-INVADER assay, respectively. The full-length nucleotide sequence analysis showed an overall identity of 97.1% (3125/3218 bp) to genotype H strain isolated from a patient in Mexico (AB375164).

The nucleotide sequence of the HBV strains isolated from case 2 was further analyzed depending on the ORF, because the identity of the full-length nucleotide sequences to that of previously reported strains was less in case 2 than that in case 1. Consequently, the nucleotide sequence between DR2 (1590–1600 nt) and DR1 (1824–1834 nt) in the X region showed a similarity to that of the corresponding region of a genotype B strain isolated in Malaysia (JQ027316) and Indonesia (JQ429079), with identities of 98.4% (241/245 bp) and 98.0% (240/245 bp) (Fig. 4a). Moreover, analysis of the nucleotide sequence between 2023 and 2262 nt in the precore/core regions revealed that several different clones existed as quasispecies among HBV strains isolated from case 2, and two major clones, C2-1 and C2-2, were separated following cloning and sequencing of whole-genome nucleotides. Both C2-1 and C2-2 clones

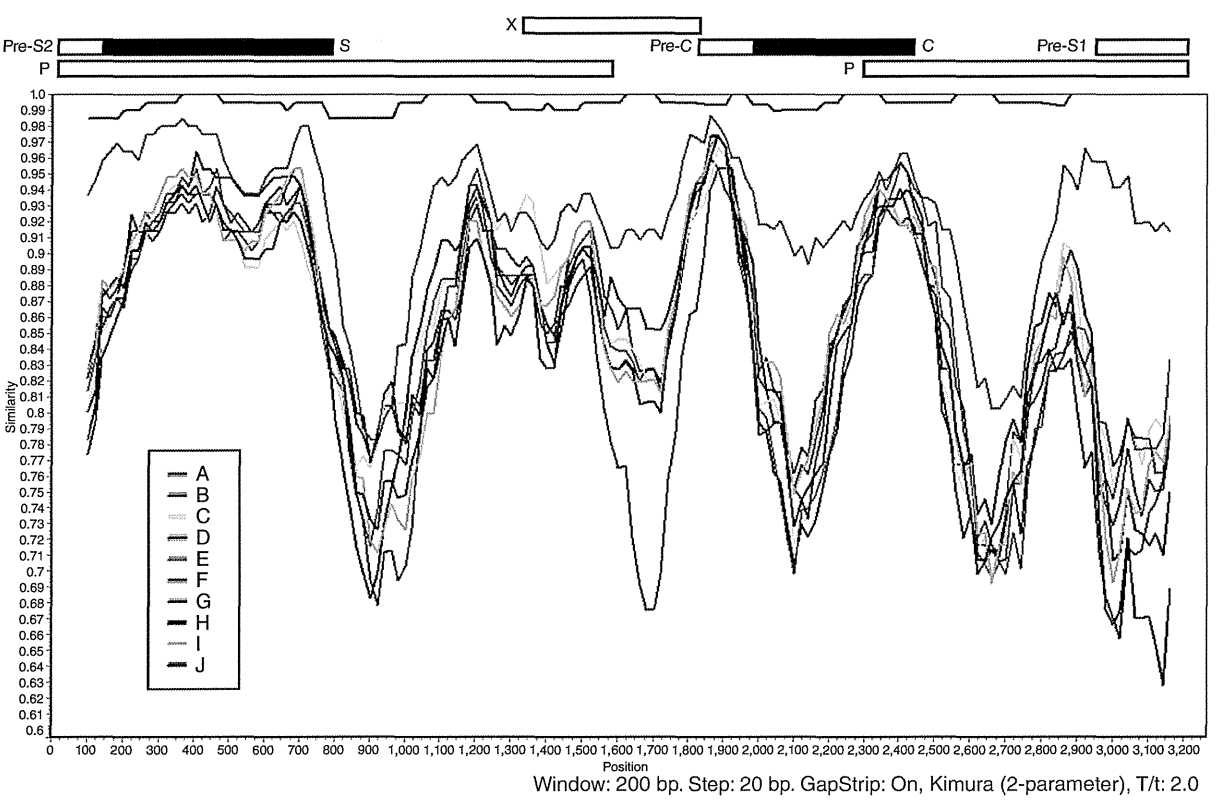

Figure 2 Nucleotide similarity comparison of a full-length sequence of hepatitis B virus (HBV) strains isolated from case 1 in reference to previously reported HBV genotypes A–J. The parameters used for the analysis are shown at the bottom of the figure (200-bp window size, 20-bp step size and gap-stripped alignments).

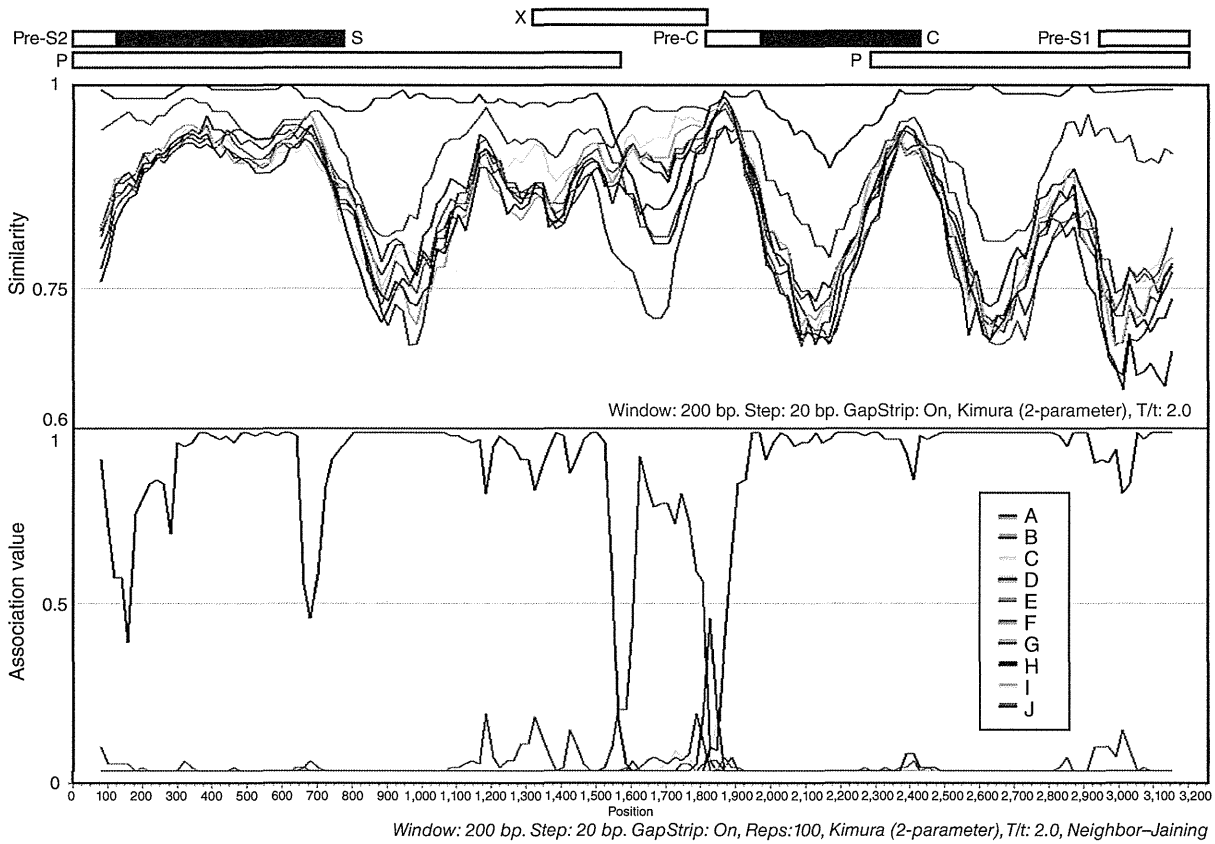

Figure 3 Nucleotide similarity comparison of the full-length sequence of the hepatitis B virus (HBV) strain isolated from case 2 in reference to previously reported HBV genotypes A–J. The parameters used for the analysis are shown at the bottom of the figure (200-bp window size, 20-bp step size, 100 bootstrap replicates, gap-stripped alignments and neighbor-joining algorithm).

were classified as genotype H according to full-length nucleotide sequence analysis, with an identity of 96.4% to 95.8% to each other, and as genotype B based on analysis of the nucleotide sequence between DR2 and DR1, with an identity of 96.9% to 95.8%, respectively. However, the nucleotide sequence between 2023 and 2262 nt in the precore/core regions showed no similarity to that of any previously reported HBV strains. In these regions, the C2-1 and C2-2 clones showed nucleotide sequences with an identity of 98.6% to each other, and the nucleotide divergences in comparison to strains of genotypes A–J ranged 9.6–30.0% in the C2-1 clone and 8.1–28.5% in the C2-2 clone (Table 1). A phylogenetic tree constructed based on these regions revealed that both strains may be classified into the novel cluster of HBV (Fig. 4b). Also, the amino acid sequence divergences from previously reported HBV strains ranged from 18.1% to 27.9% in the C2-1 clone and 17.1% to 26.9% in the C2-2 clone.

The nucleotide sequence data reported in the present study will appear in the DDBJ/EMBL/GenBank databases under accession number AB818694 for case 1, AB819065 for the C2-1 and AB819066 for the C2-2 strain.

DISCUSSION

IN THE PRESENT paper, the genotypes of the HBV strains isolated from 222 patients with acute and chronic hepatitis B were evaluated by EIA and/or PCR-INVADER assay, and HBV genotype A strains, commonly isolated in Africa, Europe and India, were found in 9.4% of the patients; genotype A strains were isolated from 50.0% of patients with acute liver diseases and 3.1% of patients with chronic liver diseases. These values were almost in line with those reported from other institutions in Japan.[11–13] HBV genotype A strains are known to be frequently isolated from patients with

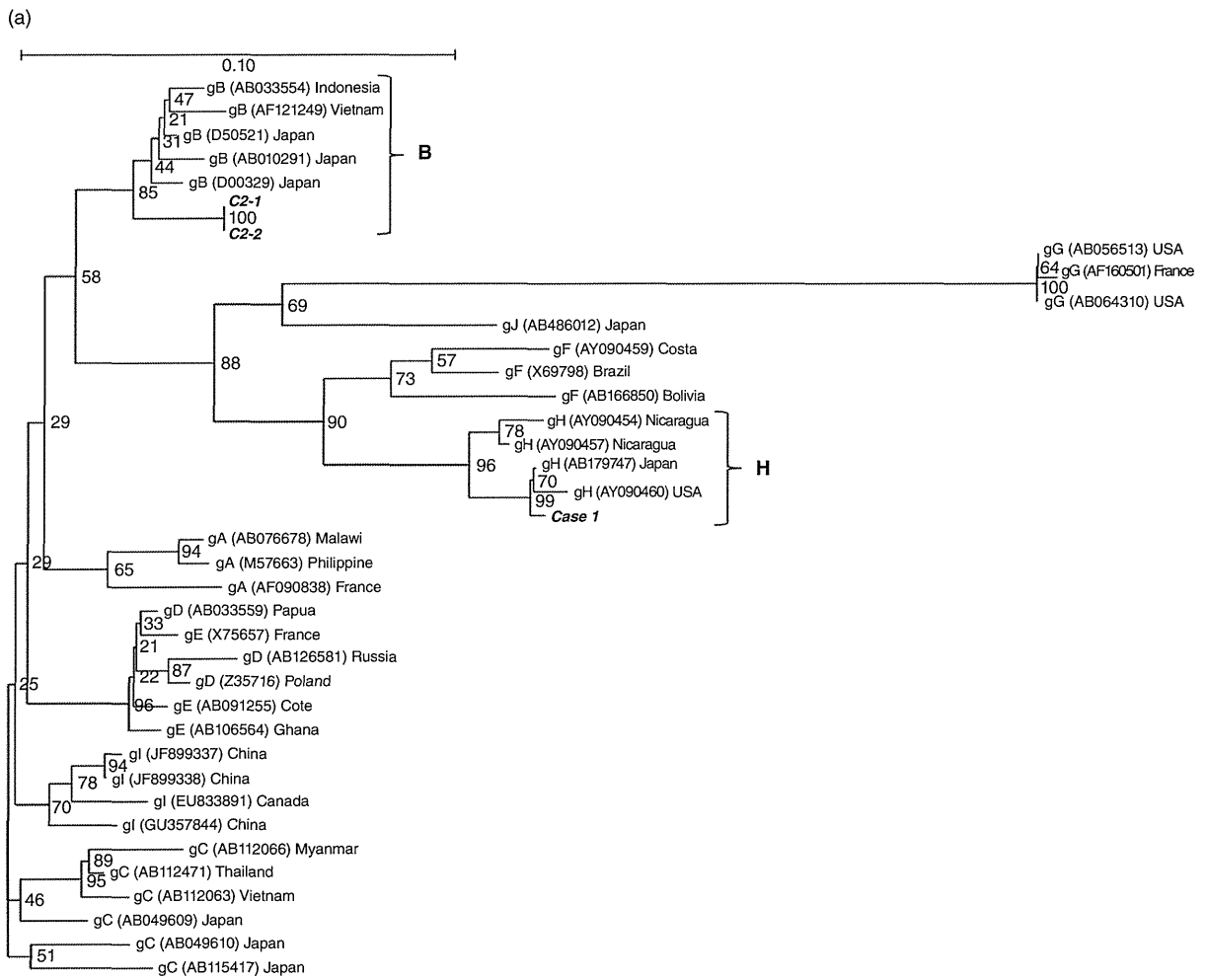

Figure 4 A phylogenetic tree constructed based on the sequence of the hepatitis B virus (HBV) strain isolated from case 2 in comparison with that of 35 reference strains. The bootstrap values are indicated at each tree root and the genotypes are on the right. The horizontal bar provides a genetic distance. The regions included in the analysis were: (a) nucleotide sequence between DR2 (1590 nt) and DR1 (1834 nt) in the X region, (b) between 2023 and 2262 nt in the precore/core region.

acute liver diseases caused by HBV, especially in urban areas as compared to the countryside,[29] suggesting that globalization and diversification of the sex industry may change the distribution pattern of the HBV genotypes in Japan, including in Saitama Prefecture, the area around our institution.

To our surprise, HBV genotype H strains, which are mainly prevalent in Central America, were isolated from two patients, one each with chronic and acute liver diseases. The HBV strain isolated from the patient with acute liver disease (case 1) showed a nucleotide sequence with 99.8% identity to the Thailand strain (EU498228), which has recently been reported to be isolated from Japan as well as Central America.[30] Considering that case 1 was a bisexual male with HIV co-infection contracted as a result of sexual activities with a number of unspecified Japanese partners, the HBV strain isolated from this patient may be resident in Japanese persons engaging in unusual sexual activities. On the other hand, HBV genotype A strains, especially the genotype A2/Ae strain, have been isolated increasingly frequently from patients with HBV and HIV co-infection.[31] These observations prompted us to postulate that HBV genotype H strains as well as genotype A

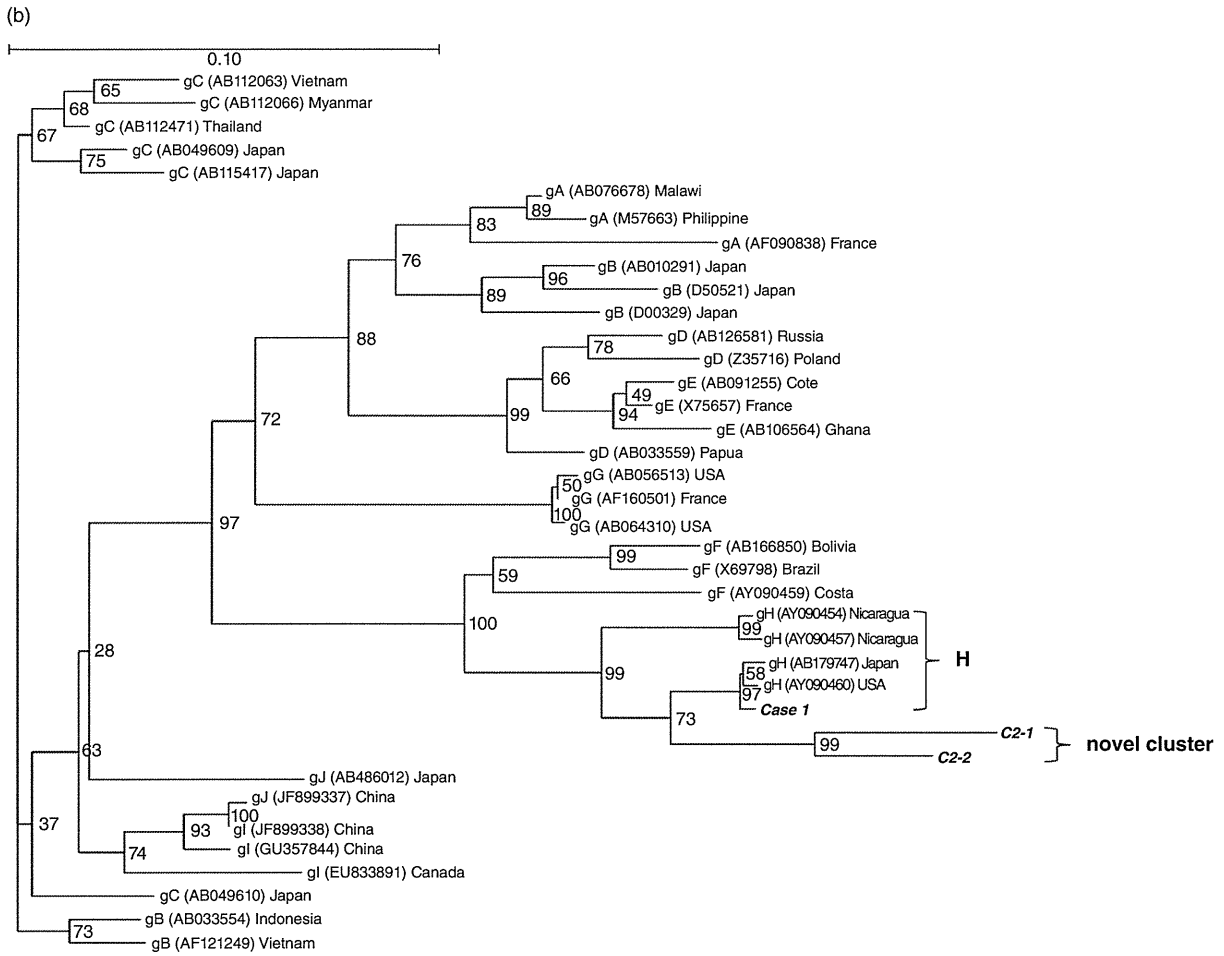

Figure 4 *Continued*

strains seem to spread among Japanese persons with unusual sexual habits. Previously, Tanaka *et al.* reported a HIV-infected patient in whom co-infection of both HBV genotype H and G strains was observed.[32] In case 1, however, co-infection of HBV genotype G strain was not detected.

It is noteworthy that HBV genotype H strains were isolated even from a Japanese patient with chronic liver disease (case 2), which showed recombination with a genotype B strain. The recombination breakpoint was estimated at positions 1590 and 1834 nt, located between DR2 and DR1 in the X region (Fig. 5): the nucleotide sequence in the X region of this strain showed an identity of 97.2% to that of genotype B strains in Malaysia (JQ027316) and Indonesia (JQ429079) despite the full-length nucleotide sequence showing 97.1% identity to a genotype H strain isolated from Mexico (AB375164). In the present study, nucleotide sequences were analyzed using two fragments (WA2 and gN2), suggesting that the possible recombination points exist in the overlapping regions of both fragments. However, the possibility that both genotypes B and H HBV strains existed as quasispecies in case 2 was neglected, because the sequences of the overlapping regions (1702–1780 and 1908–2081 nt) showed 100% identity between WA2 and gN2 fragments. It is well known that a HBV genotype B2/Ba strain, widely prevalent in Asian countries, shows nucleotide sequences identical to genotype C strains in the precore/core region due to the inter-genotype recombination

Table 1 Percentages of differences in the nucleotide and amino acid sequences of hepatitis B virus (HBV) strains isolated from case 2 (C2-1 and C2-2) and representative strains of genotypes A–J HBV

		Percentages of differences to representative HBV strains of genotypes								
		A (3)	B (5)	C (6)	D (3)	E (3)	F (3)	G (3)	H (4)	I (4)	J (1)
C1-1	Nucleotide	25.9–30.0	25.6–28.6	24.4–26.9	26.9–29.6	28.5–29.8	17.6–17.9	26.2–26.7	9.6–13.0	24.8–26.5	26.1
	Amino Acid	18.6–25.7	21.3–25.1	23.8–27.9	22.8–25.5	24.2–25.7	18.1–18.2	22.8–24.2	18.1–19.4	22.7–27.3	24.6
C2-2	Nucleotide	24.4–28.5	24.1–27.1	22.9–25.4	25.4–28.1	27.0–28.3	16.1–16.4	24.7–25.2	8.1–11.5	23.3–25.0	24.6
	Amino Acid	17.6–24.7	20.3–24.1	22.8–26.9	21.8–24.5	23.3–24.7	17.1–17.2	21.8–23.3	17.1–18.4	21.7–26.3	23.6

Values in parenthesis indicate the number of HBV strains.

between B and C strains.[33] Also, HBV strains developing as a consequence of the inter-genotype recombination between A and D, A and E, A and C, C and D, and C and G have been reported from Africa, Vietnam, Tibet and Thailand.[34–37] Moreover, recombination among HBV strains of the same genotype, the so-called intra-genotype recombination, has been proposed to occur especially in HBV genotype A, D, F and H strains.[38] However, HBV genotype H strains showing recombination with other genotype strains have not ever been reported. Considering the fact that the father of case 2 had lived in Brazil in his youth, the sequences of genotype H in case 2 strains might have originated in Brazilian strains. In Brazil, genotypes A and D HBV strains are predominantly distributed with frequencies of 49.5% and 24.3%, respectively, while genotype B HBV strains are only 2.9%.[39] Thus, the recombination event with the genotype B HBV strain might have developed following the emigration of his father to Japan. To clarify the area and era in which the recombination developed, the full-length nucleotide sequence of the HBV strain isolated from the elder brother of case 2 needs to be evaluated, but, unfortunately, the brother, receiving medical examination at another institution, rejected further viral genome analysis.

Although the mechanisms involved in the development of inter-genotype and intra-genotype recombination of the HBV genomes remains unclear, several observations reported in previous publications prompted us to postulate the "non-random pathway"; DR1 (1830 nt) in the X gene, a possible origin of viral replication, is considered to be a hot spot that may be responsible for recombination of HBV genomes among different strains.[40,41] Hino *et al.* reported, based on *in vitro* recombination assay, that HBV DNA fragments containing the region spanning DR1 increased the recombination events reproducibly in the presence of extracts from actively dividing HCC cells.[40] Also, Pineau *et al.* revealed that the integration sites of covalently closed circular HBV DNA were usually located in the nucleotide sequence between 1600 and 2000 nt, when the HBV genomes chromosomally integrated in the host genomes were evaluated in human HCC tissues.[41] These *in vitro* and *in vivo* observations were consistent with the results obtained from the analysis of the HBV strains isolated from case 2, showing that the genome of the HBV genotype B strains were integrated in that of the HBV genotype H strain between DR2 and DR1.

Hepatitis B virus strains isolated from case 2 were classified as quasispecies in accordance with the nucleotide sequence between 2023 and 2262 nt in the precore/

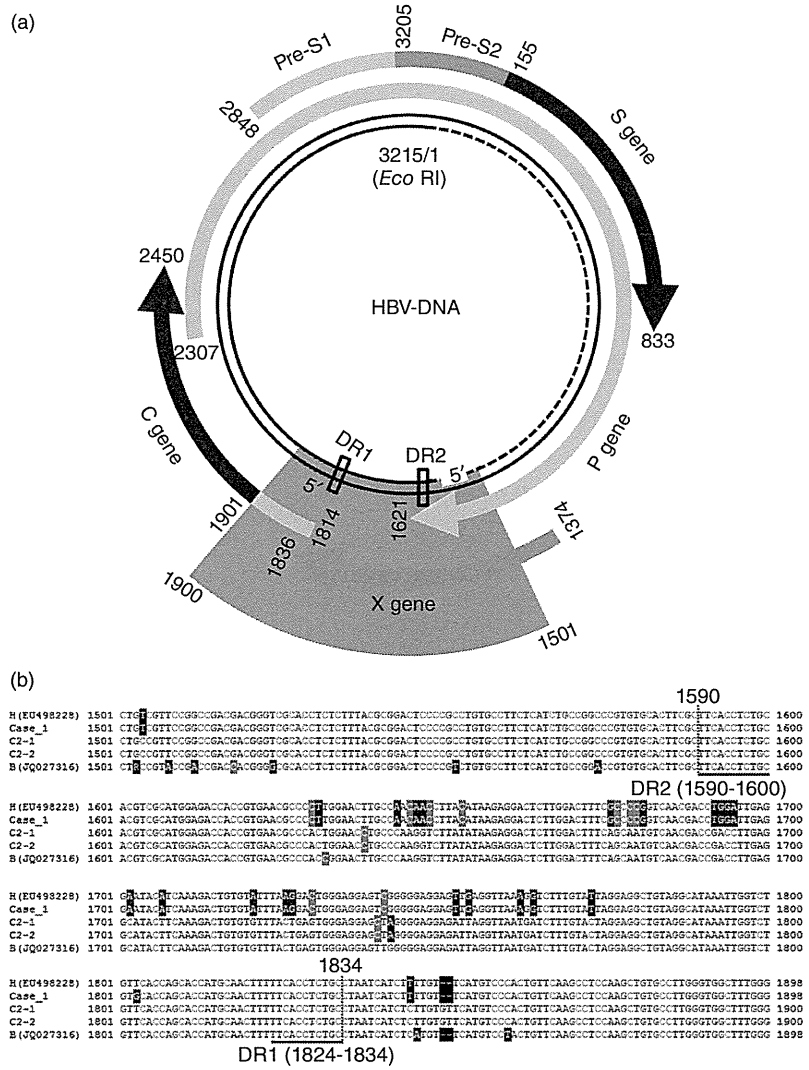

Figure 5 Hepatitis B virus (HBV) genome and the open reading frame. (a) The sequence region (shaded in red) includes the recombination breakpoint at position 1590 and 1834 nt, located between DR2 and DR1 in X region. (b) Nucleotide alignments over the sequences spanning 1501–1900 nt in case 1, C2-1, C2-2 and reference strains of HBV genotype H (accession no. EU498228) and B (JQ027316). Dashed lines at 1590 and 1834 nt represent the recombination breakpoint.

core regions. Thus, the nucleotide sequences were analyzed following cloning of the HBV genome, and two major clones, C2-1 and C2-2, were isolated. Neither clone showed any similarity to any of the previously reported strains in the precore/core regions, and a phylogenetic tree constructed based on these regions revealed that these strains may be classified into the novel cluster of HBV; sequence divergences of nucleotides in the range of 8.1–30.0% and of amino acid in the range of 17.1–27.9% as compared to previously reported genotype A–J strains. The possibility that intergenotype recombination of the HBV genome between H and B strains may provoke mutation of the nucleotide sequence in the precore/core regions leading to development of a possible novel genotype HBV strain needs to be evaluated in the future.

In conclusion, HBV genotype H strains, which are prevalent in Central American countries, were isolated from Japanese patients with chronic as well as acute liver diseases. HBV strains isolated from the chronic liver disease patient showed recombination of the genome between genotype H and B strains, and no similarity was found in the nucleotide sequences of the precore/core regions in comparison with those of the previously reported HBV strains. Thus, globalization may promote development of a possible novel genotype of HBV through recombination between Central American and East Asian strains.