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ORIGINAL ARTICLE

Quantification of hepatitis C amino acid substitutions 70 and 91 in the core coding region by real-time amplification refractory mutation system reverse transcription-polymerase chain reaction

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Abstract

Objective. The effects of hepatitis C virus (HCV) sequence variations on the success of antiviral therapy or the development of hepatocellular carcinoma (HCC) are complex for many reasons. Recently, there have been several reports on the effects of genotype 1b HCV core amino acid substitutions 70 and/or 91 on the outcome of antiviral therapies and the clinical course. The purpose of this study was to establish real-time amplification refractory mutation system (ARMS) reverse transcription (RT)-polymerase chain reaction (PCR) assays for easy detection of these HCV mutations. ***Material and methods.*** Plasmids p-core-W, including the wild-type HCV core coding region (70R and 91L), and p-core-M, including the mutant-type HCV core (70Q and 91M), were constructed by cloning and PCR-based mutagenesis for control vector of the wild-type core and that of the mutant core, respectively. Using serially diluted forms of these vectors, SyBr Green-based real-time ARMS RT-PCR detection with each of the specific primer pairs was performed. ***Results.*** Each primer could clearly distinguish the difference between p-core-W and p-core-M at the same copy numbers. Concerning substitution 70, the ratios 100:1, 10:1, 1:1, 1:10, and 1:100 of p-core-W versus p-core-M could be distinguished, while for substitution 91, the ratios 100:1, 10:1, 1:1, 1:10, 1:100, and 1:1000 could be distinguished, confirming the sensitivity and specificity of the assay. ***Conclusions.*** This method could be a useful alternative for the detection of genotype 1b HCV core amino acid substitutions 70 and 91 and be reliably applied for rapid screening.

Key Words: *ARMS, core, HCV, interferon response, real-time PCR*

Introduction

More than 170 million people world-wide are chronically infected with hepatitis C virus (HCV), which can lead to hepatic cirrhosis and hepatocellular carcinoma (HCC) [1]. Treatment with peginterferon and ribavirin for 24–48 weeks can result in a sustained loss of serum HCV-RNA (termed a sustained virological response (SVR)), with resolution of chronic hepatitis in approximately half of the patients [2]. Several new, potent HCV protease and polymerase inhibitors have been described recently, but none of them are available for therapeutic use.

The genomic region encoding the HCV core protein is located between amino acids 1 and 191 and is likely to be the first gene product synthesized due to its localization at the 5′ end of the HCV polyprotein transcript [3]. The core protein has an ability to interact with the viral genomic region to form nucleocapsids [4], and the presence of a putative DNA-binding motif, nuclear localization signals, phosphorylation sites, and a nucleocytoplasmic localization of the core protein suggest its possible function as a gene regulatory protein [3,5]. In many previous studies it has been suggested that the HCV core protein may be important in hepatocarcinogenesis and interferon signaling [3,6–8].

HCV genotype 1b is a major genotype (~70%) in Japan. HCV genotype 1 is one of the most refractory to interferon treatment with or without ribavirin. It has been reported that its response to interferon

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monotherapy is affected by HCV NS5A gene diversity [9]. Thus, sequence diversity may predict the response to the combination therapy of peginterferon and ribavirin. Furthermore, ribavirin has different antiviral effects from those of interferon [10]. An approach to the prediction of treatment against hepatitis C in patients who do not have SVR is urgently needed. Several reports suggest that HCV amino acid substitutions 70 and 91 in the core coding region affect the results of combination therapies of interferon and ribavirin [11–13], but most of these studies were retrospective, and we do not know whether these substitutions already existed before treatment or were selected by the treatment. A sensitive, real-time polymerase chain reaction (PCR)-based assay for the detection of these mutations in the presence of high levels of wild-type virus is described here. The method is based on the amplification refractory mutation system (ARMS) reverse transcription (RT)-PCR for detection of single base mutations [14,15].

Material and methods

Plasmid DNA controls

Plasmids carrying HCV genotype 1 b core wild-type and mutant clones were made as described previously [16,17] and are summarized in Table I. Plasmid DNA was purified using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Plasmids were serially diluted 1:10 in EASY dilution (for real-time PCR) (Takara, Ohtsu, Shiga, Japan) to give a dilution range of 1–1 $\times 10^9$ copies for controls of real-time PCR.

Extraction of HCV-RNA from serum

Serum samples (100 µl) were extracted using the high pure viral RNA kit (Roche Diagnostics, Indianapolis, Ind., USA) according to the manufacturer's protocol. The RNA was eluted in RNase-free water. Written informed consent was obtained from each patient included in this study.

cDNA synthesis and SyBr Green real-time PCR

Reverse transcription was carried out using random hexamers to make HCV cDNA by superscript cDNA synthesis kit (Invitrogen, Carlsbad, Calif., USA). ARMS primers were designed so that the 3′ base matched either the wild-type or mutant sequence [18] (Table II). Each 25-µl reaction contained 2× Power SYBR Green PCR Master Mix (Applied Biosystems, Tokyo, Japan), 2.5 pmol of each primer (Table II). Reactions were run on the Step One real-time PCR system (Applied Biosystems). Cycling conditions were: denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 s and 60°C for 1 min, followed by a melting curve analysis, confirming their specificity. A plasmid DNA standard was included in each run.

Cloning of clinical HCV sequences and site-directed mutagenesis

To make the plasmid p-core-mutant, PCR products were cloned into pCR-TOPO2.1 vector (Invitrogen). To make the plasmid p-core-wild, PCR-based *in vitro* site-directed mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, Calif., USA). DNA sequences of clones were confirmed by direct sequencing.

Results

Optimization of real-time PCR

For this study, real-time PCR using the SYBR Green I detection system (Applied Biosystems) was implemented to detect the HCV amplicon. ARMS PCR specificity is conferred by direct placement of the 3′ end of one of the primers (Figure 1). Cross-reactivity was tested to ensure that the primer sets specifically bound their targets.

When 10^8 copies of the CAA (codon c70) template were amplified using the primer with a base mismatch, approximately 15 cycles were required before the crossing threshold was reached. This compares with 8 cycles for the matching primer. On the other hand, when 10^8 copies of the CGA (codon c70) template were amplified using the primer with a base mismatch, approximately 16 cycles were required before the crossing threshold was reached. This compares with 6 cycles for the matching primer (Figure 1A and B).

When 10^8 copies of the ATG (codon c91) template were amplified using the primer with a base mismatch, approximately 25 cycles were required before the crossing threshold was reached. This compares

Table I. Plasmid DNA used as standard in this study.

Plasmid	Amino acid c70	Codon c70	Amino acid c91	Codon c91
p-core-wild	Arginine	CGA	Leucine	CTG
p-core-mutant	Glutamine	CAA	Methionine	ATG

Table II. Primers used for detection of substitutions at residues c70 (A) and c91 (B).

A.

Primers for detection of substitution at c70		
Primer common to all reactions		
c70 sense primers HCV-c-reverse: 5′-CGGGGTGACAGGAGCCATCC-3′	Codon	Amino acids
HCV 70W: 5′-TATCCCCAAGGCTCGCCG-3′	CGN	Arg
HCV 71M: 5′-TATCCCCAAGGCTCGCCA-3′	CAN	Gln, His

N = A, G, T, or C; Arg = arginine; Gln = glutamine; His = histidine.

B.

Primers for detection of substitution at c91		
Primer common to all reactions		
c91 reverse primers HCV-c-sense: 5′-TCGCAACCTCGTGGAAGGC-3′	Codon	Amino acids
HCV 91W: 5′-CATCCTGCCCACCCCAR-3′	TTG or CTG	Leu
HCV 91M: 5′-CATCCTGCCCACCCCAT-3′	ATG	Met

R = A, G; Met = methionine; Leu = leucine.
HCV sequences are identical to AJ238799. Ref. [11].

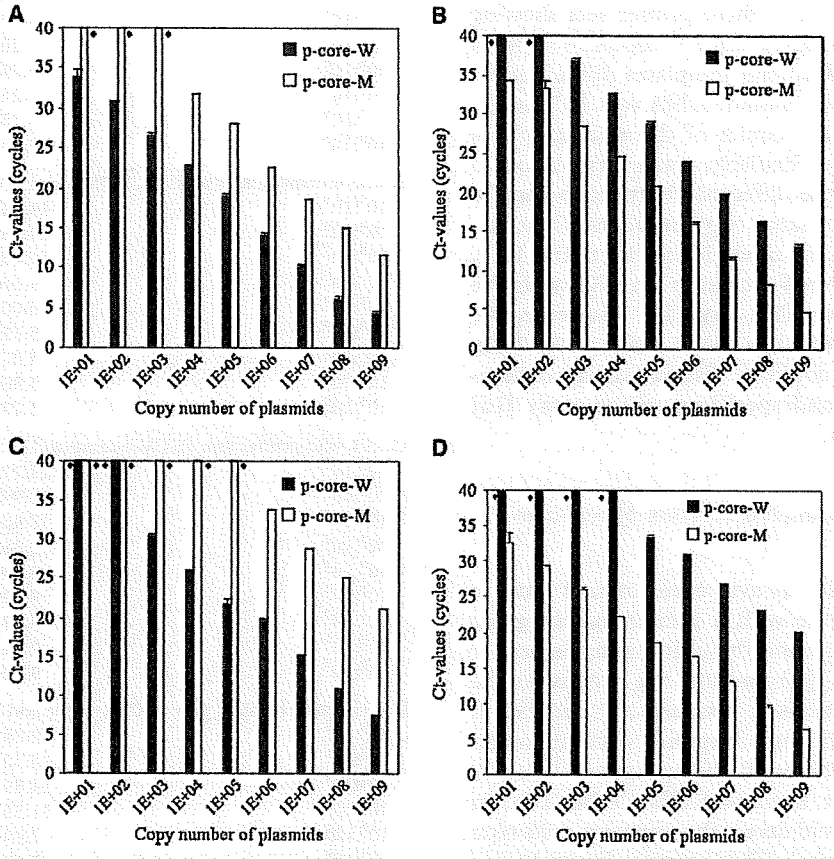

Figure 1. Quantitation of a 10-fold dilution of plasmid p-core-W or p-core-M with wild- or mutant-type primers. Cycle numbers were plotted against the logarithmic concentration of serial dilutions. A. c70-wild primer sets (HCV-70W and HCV-c-reverse). B. c70-mutant primer sets (HCV-70M and HCV-c-reverse). C. c91-wild primer sets (HCV-c-sense and HCV-91W). D. c91-mutant primer sets (HCV-c-sense and HCV-91M). *Unable to detect any signals by 40 cycles.

with 10 cycles for the matching primer. On the other hand, when 10^8 copies of the CTG (codon c91) template were amplified using the primer with a base mismatch, approximately 23 cycles were required before the crossing threshold was reached. This compares with 10 cycles for the matching primer (Figure 1C and D).

The detection limits of these methods were at least 10 copies, 10 copies, 1000 copies, and 10 copies of c70-wild primer sets (HCV-70W and HCV-c-reverse), c70-mutant primer sets (HCV-70M and HCV-c-reverse), c91-wild primer sets (HCV-c-sense and HCV-91W), and c91-mutant primer sets (HCV-c-sense and HCV-91M), respectively (Figure 1).

Selectivity of ARMS assay

Using the plasmid mixture containing the wild-type (p-core-W) and the mutant-type (p-core-M) as a template, real-time ARMS PCR was performed to establish the concentration at which the c70-wild primer sets (HCV-70W and HCV-c-reverse) would detect the wild-type DNA (codon c70). In Table IIIA we present the results of these primer sets showing that, when the wild DNA was 10^9 copies/tube, from 10^5 to 10^9 copies of mutant templates did not affect the results. When the mutant DNA was 10^9 copies/tube, from 10^9 to 10^7 copies of the wild templates could be detected. Similarly, each primer could clearly distinguish the difference between p-core-W and p-core-M at the same copy numbers. Concerning substitution 70, the ratios 100:1, 10:1, 1:1, 1:10, and 1:100 of p-core-W versus p-core-M could be distinguished (Table IIIA and B). However, for substitution 91, the ratios 100:1, 10:1, 1:1, 1:10, 1:100, and 1:1000 could be distinguished, confirming the sensitivity and specificity of the assay [16] (Table IIIC and D).

Hepatitis C core substitutions in serum by real-time ARMS RT-PCR

Quantitative ARMS assays were carried out in parallel reactions, one with a primer matching the variant at the 3′ end, and the other with the primer matching the wild-type variant. We measured the HCV core substitutions at residues c70 and c91 in two patients who did not respond to combination peginterferon and ribavirin therapy after 12 weeks and finally did not become SVRs (Table IV). In patient no. 1, we could detect the minority, wild-type at c70 (4% at 4 weeks). This became diminished at 12 weeks after treatment. In both patients, we could not detect any wild-type template at 12 weeks after treatment.

Comparison of real-time ARMS RT-PCR and conventional sequencing

The real-time ARMS RT-PCR method was compared to direct sequencing in patients treated with peginterferon and ribavirin. In patient no. 1, the minority, wild-type at c70 at 4 weeks could not be detected by direct sequencing (Table IV). In patient no. 2, there were some discrepancies between the results of direct sequencing and those of real-time ARMS RT-PCR (Table IV).

Table III. A mixture of the dilution series of mutants with fixed concentration of wild-type DNA or mutant-type DNA was assayed with each primer to establish the concentration at which the primers would detect each DNA by real-time ARMS PCR. Copy number: copies/tube; template W: p-core-W; template M: p-core-M.

Copy number of template (W:M)	Ct (cycle number)
A. c70-wild primer sets (HCV-70W and HCV-c-reverse).	
10^5:10^9	12.66±0.050
10^6:10^9	12.47±0.099
10^7:10^9	11.46±0.036
10^8:10^9	8.87±0.279
10^9:10^9	5.29±0.018
10^9:10^8	5.24±0.075
10^9:10^7	5.24±0.070
10^9:10^6	5.15±0.091
10^9:10^5	5.13±0.014
B. c70-mutant primer sets (HCV-70M and HCV-c-reverse).	
10^9:10^5	14.44±0.026
10^9:10^6	14.18±0.017
10^9:10^7	12.66±0.044
10^9:10^8	9.68±0.041
10^9:10^9	6.00±0.126
10^8:10^9	5.72±0.10
10^7:10^9	5.57±0.028
10^6:10^9	5.90±0.072
10^5:10^9	5.77±0.063
C. c91-wild primer sets (HCV-c-sense and HCV-91W).	
10^5:10^9	22.77±0.197
10^6:10^9	20.99±0.182
10^7:10^9	17.46±0.0457
10^8:10^9	13.36±0.10
10^9:10^9	9.30±0.053
10^9:10^8	9.29±0.12
10^9:10^7	9.19±0.043
10^9:10^6	9.14±0.060
10^9:10^5	9.23±0.0011
D. c91-mutant primer sets (HCV-c-sense and HCV-91M).	
10^9:10^5	20.89±0.056
10^9:10^6	18.52±0.351
10^9:10^7	14.89±0.016
10^9:10^8	11.53±0.033
10^9:10^9	7.99±0.023
10^8:10^9	7.82±0.0040
10^7:10^9	7.80±0.0098
10^6:10^9	7.86±0.044
10^5:10^9	7.82±0.0025

Table IV. HCV core substitutions at residues c70 and c91 detected by real-time ARMS RT-PCR and direct sequencing.

Patients No.	Study Week	ALT (IU/L)	HCV-RNA (log copies/ml)	c70 W:M	c91 W:M	Direct sequencing c-70/c-91
1.	0	31	6.6	0:100	0:100	M/M
	4	26	6.3	4:96	0:100	M/M
	12	24	5.8	0:100	0:100	M/M
2.	0	53	6.3	0:100	ND	Mix/M
	4	25	6.0	0:100	0:100	M/M
	12	14	5.3	0:100	0:100	M/M

Abbreviations: ARMS = amplification refractory mutation system; ALT = alanine aminotransferase; W = wild-type; M = mutant-type; Mix = mixed-type; ND = not determined.
"Study Week" = weeks after administration of peginterferon and ribavirin.

Discussion

In this article we describe a rapid and sensitive method for the quantitative detection and monitoring of the core amino acid substitutions of HCV genotype 1b. SyBr Green real-time PCR and specific ARMS primers were used to quantify viral RNAs carrying particular sequences, HCV amino acid substitutions 70 and 91 in the core coding region. The specificity of the ARMS primers results in large differences in PCR crossing thresholds being observed between matching and mismatched targets.

For the current standard treatment with peginterferon alpha and ribavirin in patients with chronic hepatitis C, infection with HCV genotypes 2 and 3, lower baseline viral load, Asian and Caucasian ethnicity, younger age, low γ-GTP levels, absence of advanced fibrosis/cirrhosis, and absence of steatosis in the liver have been identified as independent pretreatment predictors of SVR [19]. Early virological response (EVR), defined as a ≥2-log reduction in HCV-RNA or undetectable HCV-RNA at 12 weeks, is associated with a favorable virological response. EVR is reached in only ~70% of patients infected with genotype 1 treated with combination therapy [20,21].

Recently, it was reported that core residues Arg70 and Leu91 were associated with response therapy in Japanese genotype 1b patients [11,13]. Donlin et al. [12] reported a similar association of Arg70 with a marked response for genotype 1b but not 1a; however, Met91 was highly dominant in both the marked- and poor-responder sequences, but few other studies have examined the role of diversity in the core in the outcome of therapy. Concerning hepatocarcinogenesis associated with HCV genotype 1b, Akuta et al. [22] reported that cumulative hepatocarcinogenesis rates in double wild-type (Arg70 and Leu91) of the HCV core region were significantly lower than those in non-double wild-type. Direct sequencing [11,13] and nested-RT-PCR using ARMS primers with gel electrophoresis [12,22] were performed in these studies. Higher sensitivity assays may be more useful for predicting the outcomes of therapy and hepatocarcinogenesis [23]. The real-time ARMS RT-PCR described here does not require restriction enzyme digestion, gel-electrophoresis or sequence analysis of PCR products, and it can quantify the core substitution proportions more quickly.

Hepatitis C core substitutions in serum detected by real-time ARMS RT-PCR showed mutant c70 and mutant c91 at 12 weeks in two non-EVRs (Table IV). Most non-SVR rates result from non-EVR. It was reported that the 72-week regimen significantly improved the SVR rates in non-EVRs with Arg70 and/or Leu91 of core [24]. Peginterferon plus ribavirin treatment is costly and has several side effects, possibly reducing its attractiveness for patients. If we were able to identify these HCV core substitutions at 12 weeks, we would know whether to stop or continue treating patients. This could prevent patients from serious side effects or bring about a better treatment outcome by the resulting shorter regimens. Moreover, if direct viral enzyme inhibitors such as protease inhibitor and polymerase inhibitor, which potently suppress viral replication, could be used, the predictability of outcome would be even more important. Recently, it was also reported that maintenance or prolonged peginterferon did not reduce the incidence of HCC in advanced chronic hepatitis C patients [1,25]. We are now focusing on a larger study, and real-time ARMS RT-PCR is expected to be useful for the important prediction of peginterferon plus ribavirin treatment outcomes or that of hepatocarcinogenesis in hepatitis C patients.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The influence of hepatitis B DNA level and antiviral therapy on recurrence after initial curative treatment in patients with hepatocellular carcinoma

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Abstract
Background Prediction and prevention of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) recurrence is an important clinical issue. We investigated whether HBV DNA level and antiviral therapy are associated with HCC recurrence.
Methods This retrospective study involved 103 patients who underwent hepatic resection or radiofrequency ablation for initial HCC. Patients were divided into four groups. Thirty had high serum HBV DNA levels (>4 $\log_{10}$ copies/mL) and had not received antiviral therapy (high virus group; HVG). Thirty-four had low HBV DNA levels (≤4 $\log_{10}$ copies/mL) and had not received antiviral therapy (low virus group; LVG). Twenty received antiviral therapy after HCC developed (therapeutic group A, TG-A). Nineteen received antiviral therapy before HCC developed (therapeutic group B, TG-B).
Results Cumulative HCC recurrence rates at 3 years in the HVG, LVG, TG-B, and TG-A were 71.1%, 42.2%, 42.3%, and 52.0%, respectively. Recurrence rates differed significantly between the HVG and LVG ($P = 0.016$) and between the HVG and TG-B ($P = 0.008$). Recurrence rate in the TG-A was marginally lower than in the HVG ($P = 0.10$). On multivariate analysis, high serum hepatitis B virus DNA levels (hazard ratio: HR 2.67; 95% CI 1.31–5.47; $P = 0.007$) and absence of antiviral therapy (HR 2.57; 95% CI 1.34–4.94; $P = 0.005$) were independent risk factors for hepatocellular carcinoma recurrence.
Conclusion HBV DNA level and antiviral therapy are associated with HCC recurrence. For patients with high HBV DNA levels, antiviral therapy before the development of HCC is important for prevention of recurrence.

Keywords Hepatocellular carcinoma · Hepatitis B virus · Recurrence · Antiviral therapy

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third leading cause of death from cancer worldwide [1, 2]. Hepatic resection or transplantation can provide a complete cure [3, 4], and radiofrequency ablation (RFA) is also recognized as a curative treatment option [5, 6]. Recent remarkable advances in curative treatment have improved the prognosis of patients with HCC, but these techniques remain unsatisfactory because of a high posttreatment recurrence rate [7, 8]. Previous studies have noted that factors contributing to recurrence include: tumor size, number, and differentiation; vascular

invasion; levels of alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II); gender; alcohol consumption; hepatitis C virus (HCV) infection; hepatic reserve; and degree of liver fibrosis [8–12].

Only a few recent studies have evaluated hepatitis B virus (HBV) replication status as a predictor of HCC recurrence [13–15], and interpretation of their results was complicated by use of antiviral therapy. Since HBV DNA level is reduced by antiviral agents, HBV DNA level at the time of HCC treatment differs significantly between patients who have received antiviral therapy and those who have not. To determine whether HBV DNA level at the time of HCC treatment is associated with HCC recurrence, it is therefore necessary to exclude patients who have received antiviral therapy after the development of HCC.

Furthermore, the efficacy of antiviral therapy in reducing the risk of HBV-related HCC recurrence is far from clear. Three anti-HBV agents are predominantly used in Japan. Lamivudine is a nucleotide analog that inhibits reverse transcriptase, ameliorates hepatitis, and improves histologic findings in the liver during long-term treatment by inhibiting the replication of HBV [16, 17]. Furthermore, lamivudine is considered to slow the progression of severe liver disease to cirrhosis as well as to HCC [17–19]. Adefovir dipivoxil and entecavir are potent inhibitors of HBV DNA polymerase which have been shown to be safe and effective for the treatment of patients with chronic hepatitis B infection (CHB) that does not respond to lamivudine [20, 21]. With regard to lowering the risk of HCC recurrence, the literature contains only one report for lamivudine [22] and none for adefovir dipivoxil or entecavir.

In this study, by strict classification of patients into groups, comparison of cumulative HCC recurrence rates between the groups, and multivariate analysis, we aimed (1) to clarify the influence of HBV DNA level in the absence of antiviral therapy on recurrence of HCC, and (2) to clarify the influence of antiviral therapy on the risk of HCC recurrence.

Patients and methods

Patients

Between January 2001 and December 2007, a total of 196 patients who were diagnosed with HBV-related HCC at our liver unit underwent hepatic resection or RFA as a primary treatment. HCC was diagnosed based on the American Association for the Study of Liver Disease (AASLD) guidelines [23]. All patients were positive for serum hepatitis B surface antigen (HBsAg) for at least 6 months before their diagnosis of HCC and were negative for antibodies to hepatitis C and human immunodeficiency virus. In this retrospective study, of the 196 patients who were assessed initially, 103 fulfilled the following criteria and were enrolled (hepatic resection, 52 patients; RFA, 51 patients). Inclusion criteria were: (1) hepatic resection or RFA for initial HCC treatment; (2) three or fewer lesions, each 3 cm or less in diameter; (3) no extrahepatic metastasis or vascular invasion; (4) curative treatment and no local recurrence after treatment; (5) no recurrence 3 months after treatment; (6) liver function of Child-Pugh class A or B; (7) no excessive alcohol intake (>65 g/day); and (8) no evidence of any other active neoplastic site.

Since HBV DNA level reduced to ≤ 4 $\log_{10}$ in all cases after administration of antiviral agents (lamivudine, adefovir dipivoxil or entecavir), the patients were divided into four groups (Fig. 1).

1. Thirty patients had consistently high serum HBV DNA levels (>4 $\log_{10}$ copies/mL) during serial examinations from the time of HCC diagnosis to recurrence (high virus group; HVG). These patients did not receive antivirals.
2. Thirty-four patients had consistently low serum HBV DNA levels (≤ 4 $\log_{10}$ copies/mL) during the serial follow-up (low virus group; LVG). These patients did not receive antivirals.
3. Twenty patients had high serum HBV DNA levels (>4 $\log_{10}$ copies/mL) when HCC was diagnosed and received antiviral therapy after the development of HCC (therapeutic group A, TG-A). Seventeen patients received antiviral therapy within 1 month after HCC treatment. The remaining three patients received antiviral therapy from diagnosis of active viral hepatitis B; the intervals between HCC treatment and the commencement of nucleotide analogue in these three patients were 12, 15, and 22 months.
4. Nineteen patients received antiviral therapy before the development of HCC (therapeutic group B, TG-B). In these patients, HBV DNA level was high (>4 $\log_{10}$ copies/mL) at commencement of the antiviral agents but low (≤ 4 $\log_{10}$ copies/mL) at HCC diagnosis.

As shown in Fig. 1, first, to determine whether HBV DNA level was associated with HCC recurrence, we selected the patients who had not received antiviral therapy (HVG plus LVG) and then compared cumulative HCC recurrence rates between the HVG and LVG. We then performed univariate and multivariate analysis of the hazard ratios for the recurrence of HCC in these patients. Second, to determine whether antiviral therapy was associated with lower risk of HCC recurrence, we selected the patients who had high serum HBV DNA levels when they had not received antiviral therapy (HVG plus TG-A plus

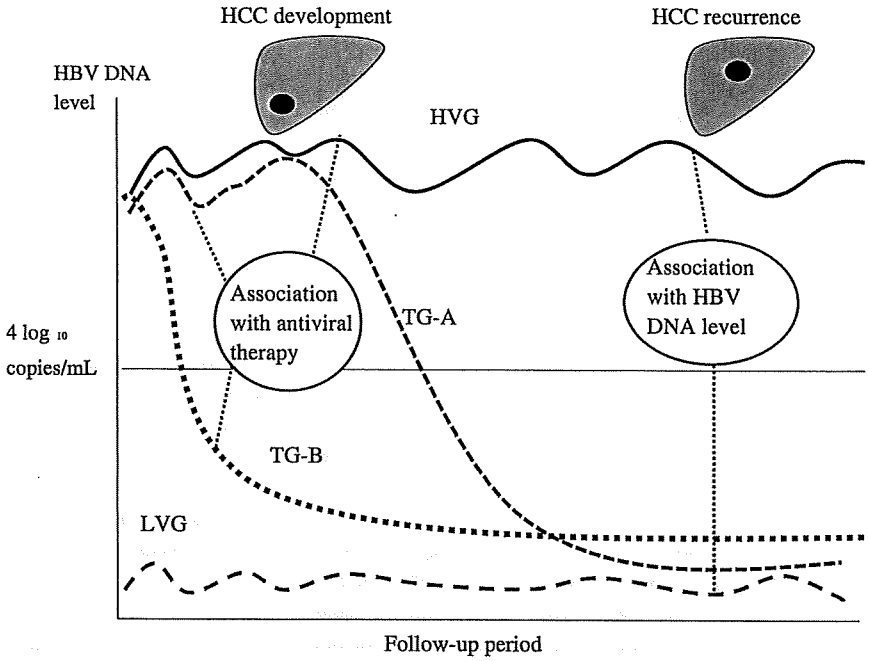

Fig. 1 Classification of patients according to HBV DNA level and use of antiviral therapy. Enrolled patients were divided into four groups according to HBV DNA level and use of antiviral therapy: (1) patients who did not have antiviral agents and who had consistently high serum HBV DNA levels during the time of HCC development to recurrence (high virus group; HVG), (2) patients who did not have antiviral agents and had consistently low serum HBV DNA levels (low virus group; LVG), (3) patients who had high serum HBV DNA levels at development of HCC and who received antiviral therapy after this (therapeutic group A; TG-A), and (4) patients who received antiviral therapy before HCC developed (therapeutic group B; TG-B)

TG-B) and then compared cumulative HCC recurrence rates between the HVG and TG-A and between the HVG and TG-B. We then performed univariate and multivariate analysis of the risk factors for recurrence of HCC in these patients.

Initial work-up and follow-up

The initial evaluation included a complete medical history and physical examination, focusing on the symptoms and signs often associated with HCC or chronic liver disease. All patients were tested at baseline for HBsAg, antibody to HBsAg, hepatitis B e antigen (HBeAg), antibody to HBeAg (anti-HBe), serum levels of alanine aminotransferase (ALT), albumin, bilirubin, AFP, and PIVKA-II, prothrombin time (PT), and complete blood cell counts. HBV DNA was quantified by polymerase chain reaction (PCR) assay (Amplicor HBV monitor assay, Roche Diagnostics, Manheim, Germany). The lower limit of detection of the assay was 2.6 log copies/mL.

During follow-up, clinical evaluations and biochemical tests were performed every 1–3 months. Patients underwent triphasic computed tomography of the liver every 3 months. The endpoint used in this study was the recurrence of HCC.

Antiviral therapy

In the TG-A, seven patients received lamivudine only (100 mg/day). Entecavir alone (0.5 mg/day) was used in five patients. Adefovir dipivoxil (10 mg/day) was used together with lamivudine to suppress lamivudine-resistant hepatitis B virus (HBV) in eight patients. In the TG-B, five patients received lamivudine only (100 mg/day). Entecavir (0.5 mg/day) was used in six patients; four of these patients were switched from lamivudine to entecavir. Adefovir dipivoxil (10 mg/day) was used together with lamivudine in eight patients.

Statistical analysis

Cumulative HCC recurrence rate was calculated by the Kaplan-Meier method and differences were compared by the log-rank test.

Univariate and multivariate analysis of the risk ratios for the recurrence of HCC were performed using Cox's proportional hazards regression analysis. The risk factors examined included gender, age, HBeAg status, ALT (>35 IU/L versus ≤35 IU/L), platelet count (>120 × 10^3/μL versus ≤120 × 10^3/μL), PT (>70 versus ≤70%), albumin (>3.5 mg/dL versus ≤3.5 mg/dL), bilirubin (>1.2 mg/dL versus ≤1.2 mg/dL), liver fibrosis (cirrhosis versus no cirrhosis), tumor differentiation (well and moderately differentiated versus poorly differentiated), AFP (>20 ng/mL versus ≤20 ng/mL), PIVKA-II (>40 mAU/mL versus ≤40 mAU/mL), tumor size (>2 cm versus ≤2 cm), tumor number (single versus multiple), and initial treatment (hepatic resection versus RFA). HBV DNA level (>4 $\log_{10}$ copies/mL versus ≤4 $\log_{10}$ copies/mL) was added to these factors when analyzing the influence of

HBV DNA level, and antiviral therapy (received versus not received) was added when analyzing the influence of antiviral therapy. Differences between the two groups were analyzed using the log-rank test. All P values were two-tailed, and those <0.05 were considered significant. Statistical analysis was performed using Stat View software (version 5.0; SAS Institute Inc., Cary, NC, USA).

Results

Baseline clinical characteristics

Baseline characteristics at the time of initial HCC treatment for the four groups are summarized in Table 1. The mean follow-up period for all patients was 40 (12–92) months. There were no significant differences among the four groups with regard to gender; age; HBeAg; levels of ALT, PT, albumin, PIVKA-II, AFP, or bilirubin; platelet count; Child-Pugh score; tumor size; tumor number; stage of HCC; initial HCC treatment; or follow-up period. However, there was a significant difference with respect to HBV DNA level among the four groups. Median HBV DNA levels in the HVG (5.9 log copies/mL, range 4.1–7.6) and TG-A (6.0 log copies/mL, range 4.1–8.1) were significantly higher than those in the LVG (<2.6 log copies/mL, range <2.6–3.6) and TG-B (<2.6 log copies/mL, range <2.6–4.0) ($P = 0.005$).

Overall recurrence rate

Overall, 56 of 103 patients (54.4%) had a recurrence of HCC, with the mean period until recurrence from initial treatment being 34.7 ± 22.7 months (range 7.0–67.0 months). The estimated recurrence rates at 1 and 3 years after curative treatment were 16.5% and 53.0%, respectively (Fig. 2).

Comparison of cumulative HCC recurrence rates between the HVG and LVG

To clarify the influence of HBV DNA level on HCC recurrence, we selected the patients who had not received

Table 1 Baseline characteristics of the four groups

Variables	HVG ($n = 30$)	LVG ($n = 34$)	TG-A ($n = 20$)	TG-B ($n = 19$)
Gender (men/women)	22/8	28/6	14/6	15/4
Age (years)[a]	55.6 ± 8.3	55.9 ± 8.3	55.7 ± 7.9	54.3 ± 9.2
HBeAg (+/−)	13/17	8/26	10/10	6/13
HBV DNA ($\log_{10}$ copies/ml)[b]	5.9 (4.1–7.6)	<2.6 (<2.6–3.6)	6.0 (4.1–8.1)	< 2.6 (<2.6–4.0)
Genotype (B/C)	1/17	0/11	0/10	0/7
ALT (IU/L)[a]	37.7 ± 16.8	23.5 ± 9.1	43.1 ± 19.6	27.9 ± 13.8
Platelet count ($\times 10^3/\mu L$)[a]	13.5 ± 6.2	14.3 ± 8.3	11.4 ± 4.9	12.0 ± 4.3
PT (%)[a]	83.7 ± 15.9	85.5 ± 15.7	84.6 ± 14.5	81.7 ± 12.4
Albumin (mg/dL)[a]	3.9 ± 0.4	3.9 ± 0.4	4.0 ± 0.6	4.1 ± 0.6
Bilirubin (mg/dL)[a]	0.9 ± 0.5	0.9 ± 0.4	0.9 ± 0.3	0.8 ± 0.3
Child-Pugh score (A/B)	27/3	31/3	17/3	15/4
Liver fibrosis (F1/F2/F3/F4)	3/2/4/16	1/2/2/15	1/0/2/11	1/0/3/7
Differentiation (well/mod./poor)	8/12/2	4/17/2	4/6/2	4/4/0
AFP (ng/mL)[b]	20.7 (1.0–3387.6)	15.1 (1.0–1124.0)	26.7 (2.8–2009.7)	26.0 (1.0–2870.3)
PIVKA-II (mAU/mL)[b]	40.0 (8.0–795.0)	33.0 (11.0–403.9)	36.5 (9.0–1651.0)	34.0 (12.0–1162.0)
Tumor size (cm)[a]	2.1 ± 0.7	1.9 ± 0.7	1.7 ± 0.5	1.8 ± 0.5
Multiple tumors (number, %)	7 (23.3%)	9 (26.5%)	5 (25.0%)	4 (21.0%)
TNM stage (I/II/III)	10/17/3	12/17/5	9/10/1	6/10/3
Treatment for HCC (OPE/RFA)	16/14	16/18	10/10	9/10
Follow-up period (months)	49.2 (12–89)	55.5 (15–92)	35.5 (12–67)	34.0 (12–58)

HVG high virus group (HBV DNA ≥ 4 log copies/mL), *LVG* low virus group (HBV DNA < 4 log copies/mL), *TG-A* antiviral therapy group after the development of HCC, *TG-B* antiviral therapy group before the development of HCC, *HBeAg* hepatitis B e antigen, *HBV* hepatitis B virus, *ALT* alanine aminotranferase, *PT* prothrombin time, *Differentiation* Tumor differentiation, *AFP* alpha-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonist II, *HCC* hepatocellular carcinoma, *OPE* hepatic resection, *RFA* radiofrequency ablation

[a] Mean ± SD

[b] Median (range)

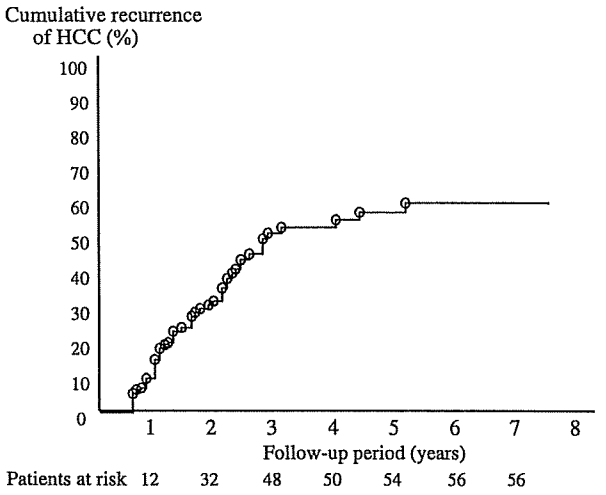

Fig. 2 Overall recurrence rate. The estimated hepatocellular carcinoma (*HCC*) recurrence rates at 1 and 3 years after curative treatment were 16.1 and 53.2%, respectively

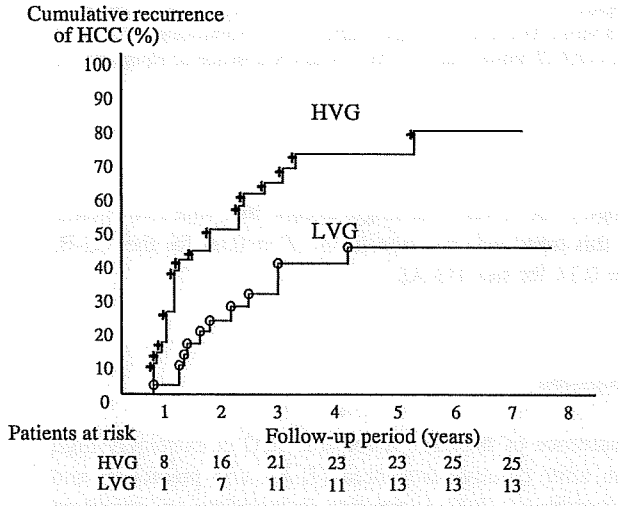

Fig. 3 Comparison of cumulative HCC recurrence rates between the high virus group (HVG) and low virus group (LVG). Cumulative HCC recurrence rates at 3 years were significantly higher in the HVG than in the LVG (71.1 versus 42.2%, $P = 0.016$)

antiviral therapy (the HVG and LVG), and compared cumulative HCC recurrence rates in these two groups. The cumulative HCC recurrence rates in the HVG and LVG were 26.7% and 9.4% at 1 year and 71.1% and 42.2% at 3 years, respectively. There were significant differences regarding the recurrence rates of HCC between the HVG and LVG ($P = 0.016$) (Fig. 3). The follow-up period for cases in which no recurrence was detected was 12–89 months in the HVG and 15–92 months in the LVG.

Multivariate analysis of risk factors for HCC recurrence in the absence of antiviral therapy

To evaluate the factors that affected recurrence after curative treatment, the 16 variables of interest shown in Table 2 were included in the analysis. In the multivariate analysis, only high serum HBV DNA level (hazard ratio 2.67; 95% CI 1.31–5.47; $P = 0.007$) was an independent risk factor for recurrence (Table 2).

Comparison of cumulative HCC recurrence rates between the TG-B and HVG, and between the TG-A and HVG

Next, to clarify the influence of antiviral therapy on the risk of HCC recurrence, we selected the patients who had a high HBV DNA level when they had not received antiviral therapy (the HVG plus TG-A plus TG-B), and compared cumulative HCC recurrence rates between the TG-A and HVG, and between the TG-B and HVG. The cumulative HCC recurrence rates in the TG-B and TG-A were 5.3% and 15.0% at 1 year, and 42.3% and 52.0% at 3 years, respectively. There were significant differences regarding the recurrence rates of HCC between the HVG and TG-B ($P = 0.008$). On the other hand, while recurrence rate was lower in the TG-A than in the HVG, this was not statistically significant ($P = 0.10$) (Fig. 4). The follow-up period for cases in which no recurrence was detected was 12–67 months in the TG-A and 12–58 months in the TG-B.

Analysis of risk factors including antiviral therapy for HCC recurrence

To evaluate the factors that affected recurrence after curative treatment, the 16 variables of interest shown in Table 3 were included in the analysis. In the multivariate analysis, multiple tumors (hazard ratio 2.81; 95% CI, 1.45–5.42; $P = 0.002$) and absence of antiviral therapy (hazard ratio 2.57; 95% CI 1.34–4.94; $P = 0.005$) were independent risk factors for recurrence (Table 3).

Cumulative HCC recurrence for each antiviral agent

Table 4 shows HCC recurrence rates for the antiviral agents used in the TG-A and TG-B. In the TG-A, cumulative HCC recurrence rate at 3 years was 47.9% in patients who were administered a single agent (lamivudine or entecavir) and 75.0% in patients who were administered two agents (lamivudine plus adefovir). In the TG-B, cumulative HCC recurrence rate at 3 years was 21.7% in patients administered a single agent and 63.5% in those given two agents. In both the TG-B and TG-A, HCC recurrence

Table 2 Factors affecting HCC recurrence in patients not given antiviral therapy (HVG plus LVG)

Characteristic	Univariate analysis	Multivariate analysis	Hazard ratio (95% CI)
Gender (male)	0.171	–	
Age (≥55 years)	0.204	–	
HBeAg status (positive)	0.433	–	
HBV DNA (≥4 log copies/mL)	0.015	0.007[a]	2.67 (1.31–5.47)
ALT (≥35 IU/L)	0.309	–	
Platelet count (<120 × 10^3/μL)	0.04	0.077	2.05 (0.93–4.51)
PT (<70%)	0.037	0.191	1.83 (0.74–4.54)
Albumin (<3.5 mg/dL)	0.382	–	
Bilirubin (≥1.2 mg/dL)	0.122	–	
Liver fibrosis (cirrhosis)	0.366	–	
Tumor differentiation (mod., poor)	0.703	–	
AFP (≥20 ng/mL)	0.336	–	
PIVKA-II (≥40 mAU/mL)	0.185	–	
Tumor size (≥2 cm)	0.072	–	
Tumor number (multiple)	0.155	–	
Initial treatment (resection versus RFA)	0.291	–	

HCC hepatocellular carcinoma, *HVG* high virus group (HBV DNA ≥ 4 log copies/mL), *LVG* low virus group (HBV DNA < 4 log copies/mL), *95% CI* 95% confidence interval, *HBeAg* hepatitis B e antigen, *HBV* hepatitis B virus, *ALT* alanine aminotranferase, *PT* prothrombin time, *mod.* moderately differentiated, *poor* poorly differentiated, *AFP* alpha-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonist II, *RFA* radiofrequency ablation

[a] Statistically significant

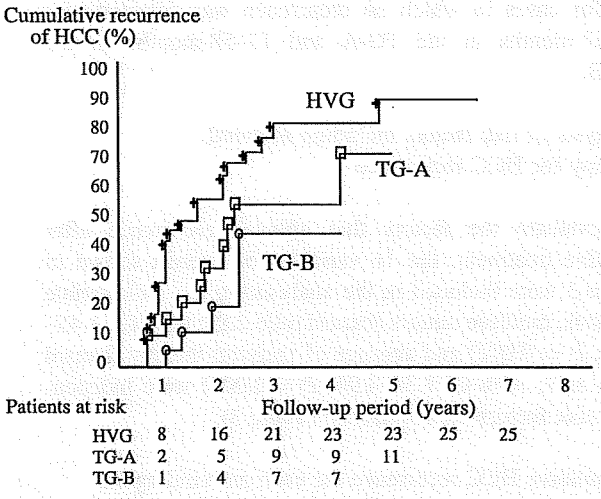

Fig. 4 Comparison of cumulative HCC recurrence rates between the antiviral therapy before HCC diagnosis group (TG-B) and the high virus group (HVG), and between the antiviral therapy after HCC diagnosis group (TG-A) and the HVG. The cumulative HCC recurrence rates at 3 years in the TG-B and TG-A were 42.3% and 52.0%, respectively. There were significant differences regarding HCC recurrence rates between HVG and TG-B ($P = 0.008$). On the other hand, although HCC recurrence rate was lower in the TG-A than in the HVG, this difference was not statistically significant ($P = 0.10$)

tended to be lower with single agents than with two agents, but this trend was not significant ($P = 0.07$ for the TG-B, $P = 0.14$ for the TG-A).

Discussion

Recurrence of hepatitis B-related HCC is extremely high even after curative treatment [7, 8], and prediction and prevention of HCC recurrence is therefore an important clinical issue. Several factors are reported to be associated with an increased risk of HCC recurrence after surgical resection or local ablation therapies, including tumor characteristics such as multiplicity, size, and portal invasion; AFP level; PIVKA-II level; and hepatic functional parameters such as albumin level, PT, and Child-Pugh class [8–12]. In addition, recent studies have suggested that a high viral load is another risk factor for recurrence [13–15]. However, these studies included patients who had received antiviral therapy and did not fully account for this. Furthermore, the efficacy of antiviral therapy in reducing the risk of HCC recurrence is far from clear. In our study, by clearly categorizing the patients and comparing cumulative HCC recurrence among the groups, we clarified (1)

Table 3 Factors affecting HCC recurrence in patients with high HBV DNA levels (HVG plus TG-A plus TG-B)

Characteristic	Univariate analysis	Multivariate analysis	Hazard ratio (95% CI)
Gender (male)	0.54	–	
Age (≥55 years)	0.661	–	
HBe Ag status (positive)	0.075	–	
Antiviral therapy (not received)	0.018	0.005[a]	2.57 (1.34–4.94)
ALT (≥35 IU/L)	0.902	–	
Platelet count (<120 × 10^3/μL)	0.36	–	
PT (<70%)	0.341	–	
Albumin (<3.5 mg/dL)	0.158	–	
Bilirubin (≥1.2 mg/dL)	0.392	–	
Liver fibrosis (cirrhosis)	0.49	–	
Tumor differentiation (mod., poor)	0.852	–	
AFP (≥20 ng/mL)	0.424	–	
PIVKA-II (≥40 mAU/mL)	0.229	–	
Tumor size (≥2 cm)	0.284	–	
Tumor number (multiple)	0.009	0.002[a]	2.81 (1.45–5.42)
Initial treatment (resection versus RFA)	0.851	–	

HCC hepatocellular carcinoma, *HBV* hepatitis B virus, *HVG* high virus group (HBV DNA ≥4 log copies/mL), *TG-A* antiviral therapy group after the development of HCC, *TG-B* antiviral therapy group before the development of HCC, *HBeAg* hepatitis B e antigen, *95% CI* 95% confidence interval, *ALT* alanine aminotranferase, *PT* prothrombin time, *mod.* moderately differentiated, *poor* poorly differentiated, *AFP* alpha-fetoprotein, *AFP* alpha-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonist II, *RFA* radiofrequency ablation

[a] Statistically significant

Table 4 Cumulative HCC recurrence according to antiviral agents

Antiviral agents	No. of patients	Recurrence rate (3 years, %)
TG-A ($n = 20$)		
Lamivudine	7	54.3
Entecavir	5	20.0
Single agent (lamivudine or entecavir)	12	47.9
Lamivudine plus adefovir dipivoxil	8	75.0
TG-B ($n = 19$)		
Lamivudine	5	0
Entecavir	2	0
Lamivudine then entecavir	4	50.0
Single agent (lamivudine or entecavir)	11	21.7
Lamivudine plus adefovir dipivoxil	8	63.5

TG-A antiviral therapy group after the development of HCC, *TG-B* antiviral therapy group before the development of HCC

the influence of HBV DNA level in the absence of antiviral therapy on the risk of HCC recurrence, and (2) the influence of antiviral therapy on recurrence of HCC. In patients who had not undergone antiviral treatment, multivariate analysis demonstrated that HBV DNA level >4 $\log_{10}$ copies/mL was an independent factor associated with higher cumulative risk of HCC recurrence after curative treatment.

The mechanism for recurrent carcinogenesis associated with HBV in the remaining liver in patients who have undergone curative treatment remains unclear. Both direct and indirect carcinogenic mechanisms are thought to be involved [24]. Active replication of HBV may initiate malignant transformation through a direct carcinogenic mechanism by increasing the probability of viral DNA insertion in or near proto-oncogenes, tumor-suppressor genes or regulatory elements of cellular DNA [25, 26]. The integration of viral DNA may increase the production of transactivator protein hepatitis B X antigen, which may promote neoplasia of hepatocytes, as well as bind to the p53 tumor-suppressor gene and disrupt its functions [27, 28]. Indirectly, continuing HBV replication can also induce chronic liver fibrosis and inflammation and mediate alteration in transforming growth factor-beta1 (TGF-β1) and alpha-M production, thereby leading to carcinogenesis [29, 30]. High HBV viral load can induce hepatocarcinogenesis via direct and indirect ways; hence, the risk of multicentric recurrent tumors in the liver remnant is thought to be increased.

Given the strong association between HBV DNA level and cancer recurrence, we next investigated and demonstrated that antiviral therapy is associated with lower risk of HCC recurrence. Multivariate analysis showed that absence of antiviral therapy and number of tumors were the

two independent factors associated with higher cumulative risk of HCC recurrence in patients with high serum HBV DNA level after curative treatment. The number of tumors has previously been associated with HCC recurrence. Recently, the efficacy of lamivudine in preventing hepatocellular carcinoma in chronic hepatitis B has been described [19], and one study demonstrated that lamivudine therapy reduced the recurrence of HCC in patients with chronic hepatitis B [22]. The authors stated that remission of active hepatitis in response to lamivudine therapy may decrease HCC development and metastatic potential. Taken together, these findings suggest that, although antiviral therapy itself does not have anticancer effects, it may suppress HCC recurrence directly and indirectly by decreasing HBV viral load. We further showed that, while recurrence rate of HCC was significantly lower in the TG-B than in the HVG, it was only marginally lower in the TG-A than in the HVG. This suggests that, for patients with high serum HBV DNA levels, it is important to give antiviral therapy before HCC develops to prevent HCC recurrence. In addition, we showed that the rate of HCC recurrence was marginally lower for single antiviral agent therapy than for therapy using two agents. The patients who received two agents were unresponsive to lamivudine and had high serum HBV DNA level in the lamivudine-refractory period, despite receiving antiviral therapy. The difference in HBV DNA level between these two modes of therapy may be associated with the difference in rate of HCC recurrence (data not shown). However, we were unable to further evaluate such relationships, as there were few patients in each therapeutic group. Further analysis needs to be performed in a larger population of patients with HBV-related HCC and with a longer follow-up period in order to clarify our findings.

Conclusion

Both HBV DNA level and absence of antiviral therapy appear to be associated with HCC recurrence. To prevent HCC recurrence for patients with high serum HBV DNA levels, it seems important to commence antiviral therapy before HCC develops. Large-scale prospective trials are necessary to elucidate the effects of HBV DNA viral load on recurrence after curative treatment and the protective roles of antiviral therapy.

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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 distinct 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/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 ± SD)	51.4 ± 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/mm^3)	21.4 ± 30.2
ALT (IU/liter)	59.8 ± 103.0
ALP (IU/liter)	270.4 ± 136.0
γ−GTP (IU/liter)	47.4 ± 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 ± 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 pivoxil, 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 (n = 1,271)	2000–2001[a] (n = 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

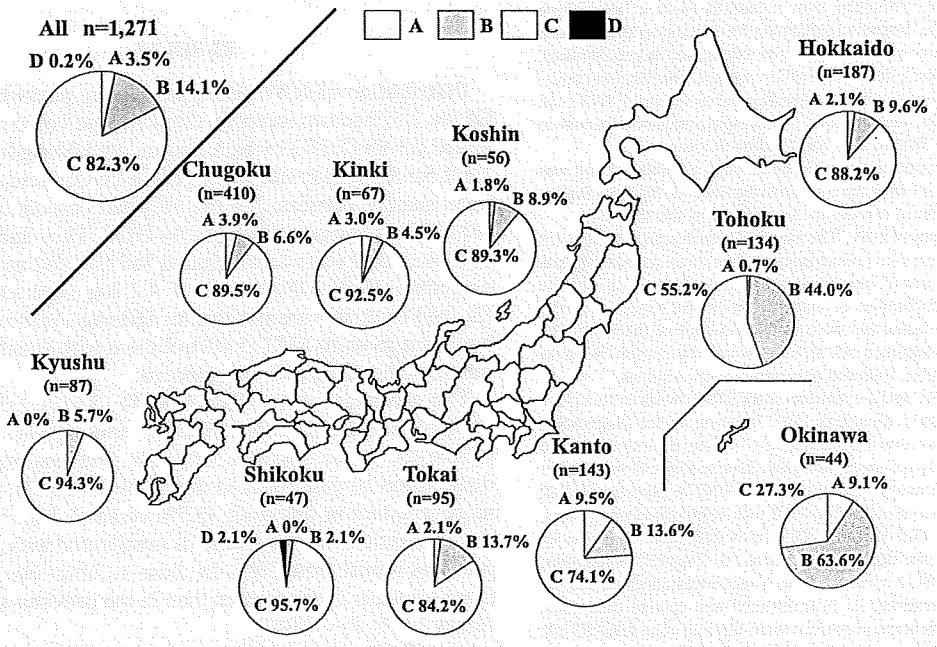

FIG. 1. Geographic distribution of HBV genotypes in patients with chronic HBV infection in Japan during 2005 and 2006.

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 ± SD])	41.3 ± 14.9[a]	55.8 ± 13.7[b]	48.8 ± 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 ± 21.9	25.9 ± 35.9[e]	20.6 ± 29.5
ALT (IU/liter)	56.2 ± 83.8	42.2 ± 104.2[e]	63.0 ± 103.3
ALP (U/liter)	247.1 ± 123.0	255.5 ± 97.9	273.9 ± 141.9
γ-GTP (U/liter)	39.6 ± 34.6	49.3 ± 63.4	47.5 ± 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 versusC.
[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.

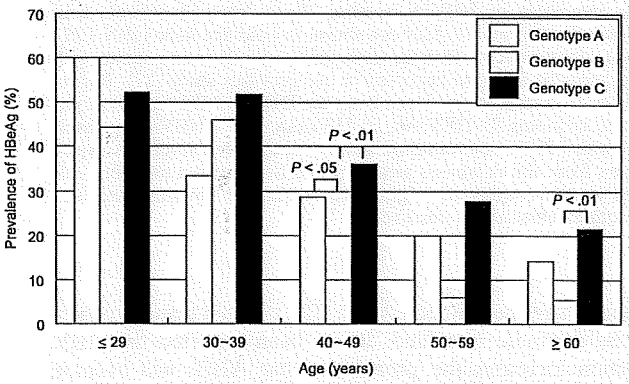

FIG. 2. Prevalence of HBeAg among patients infected with HBV of different genotypes stratified by the age.

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 coinfected 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%

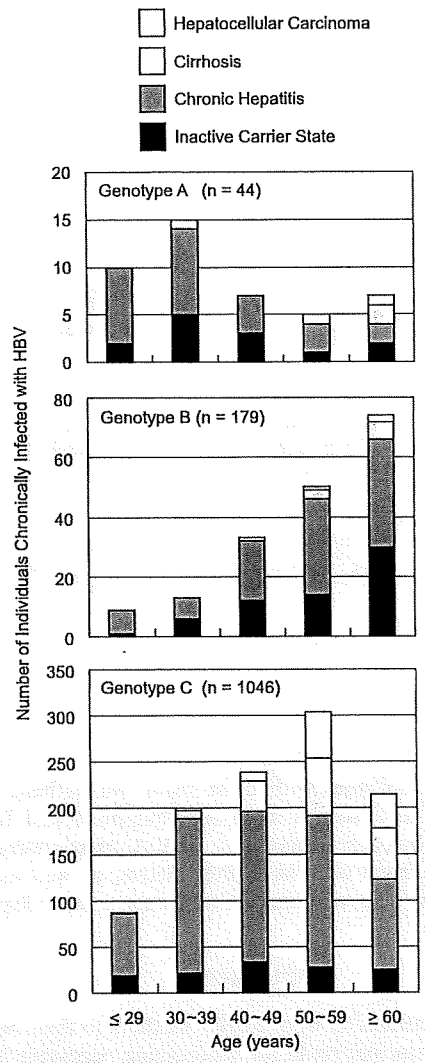

FIG. 3. Distribution of HCC, cirrhosis, chronic hepatitis, and inactive carrier state among the 1,271 patients infected with HBV of different genotypes stratified by the age.

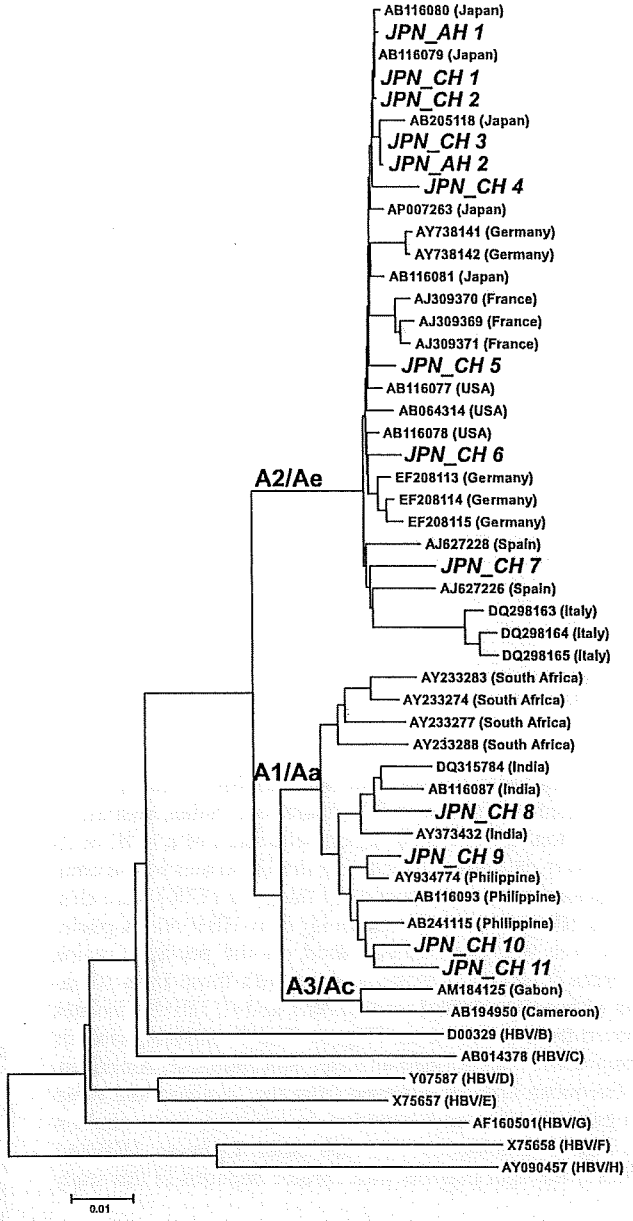

FIG. 4. Phylogenetic tree constructed based on the complete genome sequences of HBV/A isolates. Those from 11 patients with chronic infection in this study are shown in boldface italic (JPN_CH1 to -11), along with two isolates (JPN_AH1 and -2) from patients with acute hepatitis in Japan reported in our 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.

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 coinfected with HIV-1. They were both MSM, and they were suspected 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