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Long-Term Follow-Up of HBeAg-Positive Young Adult Japanese Patients Treated with Corticosteroid Withdrawal Therapy for Chronic Hepatitis B

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Key Words
Chronic hepatitis B · Corticosteroid withdrawal therapy · HBeAg seronegative rate

Abstract
Objectives: To evaluate the long-term effects of corticosteroid withdrawal therapy (CSWT) in young adult Japanese patients with chronic hepatitis B (CH-B) virus infection. ***Methods:*** The subjects were 106 patients with CH-B who received CSWT, were less than 35 years of age and had been followed for more 10 years after CSWT. ***Results:*** Retreatment was not required in 41 patients (38.7%; retreatment(–) group) while 65 (61.3%) received treatment after the initial CSWT (retreatment(+) group). Larger proportions of patients of the retreatment(–) group were females, had liver histology stage F2/F3, high ICG R15, and genotypes A/B/D/E, compared with the retreatment(+) group. At the last follow-up examination, the HBeAg seronegative rate was 90.2% in the retreatment(–) group and 98.5% in retreatment(+) group. In the retreatment(–) group, the rate of liver cirrhosis (LC; 7.3%, 3 patients) was lower, but the rate of hepatocellular carcinoma (HCC; 12.2%, 5 patients) was higher than in the retreatment(+) group (20%, 13 patients, and 4.6%, 3 patients, respectively). At the 10-year period, the overall HBsAg loss, LC and HCC rates were 2.8, 13.2 and 1.9%, respectively. ***Conclusions:*** Our results suggest that CSWT is good short-term therapy and has possible long-term effects in young adult Japanese patients with CH-B.

Introduction

Chronic hepatitis B (CH-B) is associated with high morbidity and mortality. It is estimated that 2 billion people worldwide have been infected with the hepatitis B virus (HBV), among whom more than 350 million have CH-B. Approximately 25–40% of them will develop hepatocellular carcinoma (HCC) and liver cirrhosis (LC) [1]. With regard to treatment for CH-B, we have used corticosteroid withdrawal therapy (CSWT) and interferon (IFN)-α, and recently nucleoside analogs such as lamivudine. The aims of any treatment are to inactivate liver disease as indicated by hepatitis B e antigen (HBeAg) seroconversion and disappearance of serum HBV DNA and to impede the progression of the pathological process and the development of LC/HCC.

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Previous studies reported the disappearance of HBV DNA, loss of HBeAg with or without seroconversion to anti-HBe, normalization of serum transaminase levels during the natural course of the disease [1, 2], CSWT [3–8] and IFN therapy [9–12] in patients with CH-B infection. For IFN therapy, the results of long-term follow-up studies have already been reported [10–12]. However, there is little or no information on the long-term effects of CSWT on disease progression and mortality in patients with CH-B infection.

To evaluate the long-term effects of CSWT on disease progression and mortality in chronic HBV-infected patients, we performed a retrospective study on HBeAg-positive CH-B patients, especially young adults who were less than 35 years old at the start of therapy, who received CSWT and were followed up for more than 10 years in our hospital.

Patients and Methods

From 1971 to 2002, a total of 193 CH-B patients who were less than 35 years of age received CSWT for the first time at Toranomon Hospital, Tokyo, Japan. The diagnosis of CH-B was based on the presence of hepatitis B surface antigen (HBsAg) for more than 6 months, liver biopsy and HBeAg positivity. The median follow-up period was 11.6 (range 0.2–32.9) years. To evaluate the long-term effects of CSWT in these patients, we selected only patients with a more than 10-year follow-up from the commencement of CSWT. Accordingly, 106 patients were enrolled in this study. They included 84 males and 22 females, aged 12–34 years, with a median age of 29 years. All patients were negative for anti-HCV antibody.

Treatment Protocol

Patients were treated with oral corticosteroid in a single dose of 40 mg/day for the 1st week, 30 mg/day for the 2nd week, 20 mg/day for the 3rd week, and then 10 mg/day for the last week. Then, 25 (23.6%) of them received IFN therapy within 4 weeks when a clinical rebound following CSWT and a tendency to increasing alanine aminotransferase (ALT) levels were observed within 3–5 weeks after discontinuation of CSWT. Clinical rebound after CSWT represented an increase above 5-fold the upper limit of normal ALT levels.

Patients were divided into 2 groups based on the need for retreatment: a group without retreatment (retreatment(–)) and a group with retreatment (retreatment(+)). Retreatment included CSWT, IFN therapy and nucleoside analog therapy. We regarded initiation of some kind of therapy more than 5 weeks after discontinuation of CSWT or administration of IFN for more than 4 weeks, even if within 4 weeks after discontinuation of CSWT, as retreatment.

Blood Tests

Routine biochemical and hematological tests were performed at each visit to our outpatient clinic during and after the first CSWT. The remaining serum samples were divided and stored at –80°C until the virological tests were performed. HBsAg was determined by hemagglutination, using commercially available kits (MyCell, Institute of Immunology, Tokyo, Japan), and HBeAg and antibody to HBeAg (anti-HBe) were measured using an enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (Abbot Diagnostics, Chicago, Ill., USA). HBV-DNA was assessed by a transcription-mediated amplification and hybridization protect assay (TMA; Chugai Diagnostics Science Co., Tokyo) [13] and Cobas Amplicor HBV Monitor Test (Amplicor; Roche Diagnostics, Branchburg, N.J., USA). The lower limit of the TMA assay was 3.7 LGE/ml and the lower limit of the Amplicor assay was 2.6 log copies/ml. Genotyping of HBV was performed by an ELISA kit (HBV Genotype ELISA, Institute of Immunology, Tokyo) using monoclonal antibodies for the genotype-specific epitopes in the pre-S2 region product [14].

Liver Histopathological Examination

Histopathological staging of the liver biopsy specimens was performed according to the classification of Desmet et al. [15].

Follow-Up

Data were collected by reviewing patient clinical records, survival, development of LC (defined as histopathological findings or the presence of ascites, encephalopathy and gastroesophageal varices), and HCC. Follow-up time was calculated from the start of CSWT until the last visit or death.

Statistical Analysis

Nonparametric tests, including the χ^2, Fisher exact probability and Mann-Whitney U tests, were used to analyze the background characteristics of patients. A p value of <0.05 was considered statistically significant. The Kaplan-Meier method was used to estimate the time to HBeAg seronegativity, HBsAg loss and development of LC and HCC. All analyses were performed using SPSS version 10.1 (SPSS Inc., Chicago, Ill., USA).

Results

Baseline Characteristics

The baseline characteristics of the patients at commencement of CSWT are shown in table 1. Of 106 patients, 41 (38.7%) did not receive any retreatment (retreatment(–) group) while the remaining 65 (61.3%) patients received some kind of retreatment (retreatment(+) group). There were no differences between the 2 groups with respect to age, serum ALT, total bilirubin, platelet count and HBV DNA levels. The ICG R15 level in the retreatment(–) group was higher than in the other group (table 1). The proportion of females in the retreatment(–) group was significantly higher than in the other group (39 vs. 9%, respectively; p = 0.000). Furthermore, the proportion of the retreatment(–) group with a pretreatment liver histopathology grade of F2/F3 was significantly higher than those of the retreatment(+) group (34 vs. 31%, respective-

Table 1. Baseline characteristics at the start of first CSWT

	Total (n = 106)	Retreatment(–) (n = 41)	Retreatment(+) (n = 65)	p value
Follow-up, years[a]	15.6 (10.2–32.9)	14.7 (10.2–32.9)	16.3 (10.5–25.6)	
Age, years	29 (12–34)	28 (12–34)	29 (13–34)	
Sex, male/female	84/22	25/16	59/6	0.000
Family history of liver disease	76 (71.0%)	30 (73.1%)	46 (69.7%)	
Histology, F1/2/3	64/26/8	23/10/4	41/16/4	0.002[b]
ALT, IU/l	380 (48–835)	384 (64–746)	370 (48–835)	
T-Bil, mg/dl	0.7 (0.2–2.0)	0.7 (0.4–2.0)	0.7 (0.2–1.9)	
Platelets, $\times 10^3/\mu l$	19.6 (9.9–51.5)	18.8 (9.9–30.0)	20.3 (11.8–51.5)	
ICG R15, %	13 (2–29)	16 (4–29)	12 (2–27)	0.007
HBV DNA, LGE/ml	8.2 (<3.7–8.7<)	8.1 (<3.7–8.7<)	8.4 (6.6–8.7<)	
HBV genotype, A/B/C/D(E)/unknown	4/5/93/1/3	3/2/34/1/1	1/3/59/0/2	0.000[c]

ALT = Alanine aminotransferase; T-Bil = total bilirubin; ICG R15 = indocyanine green retention rate at 15 min.

[a] Data are presented as median (range).

[b] p value was compared F1 with except F1.

[c] p value was compared C with except C.

Table 2. Comparison of HBeAg seronegative rate in patients with or without retreatment

	Retreatment(–) (n = 41)	Retreatment(+) (n = 65)
HBeAg seronegative rate after first CSWT	38 (92.7%)	34 (52.3%)[a]
Period until HBeAg seronegative, years[b]	1.0 (0.0–15.5)	1.1 (0.1–10.4)
HBeAg positive re-conversion rate	19/38 (50.0%)	20/34 (58.8%)
HBeAg re-seronegative rate	18/19 (94.7%)	6/20 (30.0%)
HBeAg seronegative rate within a year after CSWT	19 (46.3%)	15 (23.1%)
HBeAg seronegative rate at last observation	37 (90.2%)	64 (98.5%)
Period until last HBeAg seronegative, years	5.4 (0.0–15.5)	5.8 (0.1–19.4)

[a] Retreatment cases were assessed before retreatment.

[b] Median value (range).

ly; p = 0.002). The proportion of the retreatment(–) group with genotype A, B, D, E or unknown (17%) was significantly higher than that of the retreatment(+) group (9%, p = 0.000). The median follow-up period for the whole group was 15.6 (range 10.2–32.9) years, and was not different between the 2 groups (table 1).

Comparison of HBeAg Seronegative Rates

We also examined HBeAg seronegative conversion rates in the 2 groups (table 2). HBeAg seronegative rate after the first CSWT was higher in the retreatment(–) than the retreatment(+) group. In particular, the proportion of the retreatment(–) group with an early HBeAg seronegative rate (within 1 year after CSWT) was higher than that of the retreatment(+) group (table 2). Although about half of both groups became positive again for HBeAg, 94.7% of the retreatment(–) group later spontaneously converted to seronegativity. In comparison, only 30.0% of the retreatment(+) group converted spontaneously to seronegativity (which explains why they needed retreatment). However, the HBeAg seronegative rates were high in both groups at the last observation (table 2).

Table 3. Comparison of prognosis in patients with or without retreatment after first CSWT

	Retreatment(–) (n = 41)	Retreatment(+), final treatment (n = 65)			Total (n = 106)
		CSWT (n = 9)	IFN (n = 34)	lamivudine (n = 22)	
ALT normalization	34 (82.9%)	7 (77.8%)	29 (85.3%)	19 (86.4%)	90 (84.1%)
HBeAg seronegative	37 (90.2%)	9 (100%)	33 (97.1%)	22 (100%)	101 (95.2%)
HBV DNA negative[a]	10 (24.4%)	1 (12.5%)	14 (46.7%)	12 (54.5%)	37 (34.9%)
HBsAg loss	4 (9.8%)	2 (22.2%)	5 (14.7%)	0	11 (10.4%)
Development of LC	3 (7:3%)	1 (11.1%)	7 (20.6%)	5 (22.7%)	16 (15.1%)
Development of HCC	5 (12.2%)	0	3 (8.8%)	0	8 (7.5%)
Death	5 (12.2%)	0	0	0	5 (4.7%)

[a] In cases measured by Amplicor assay, <2.6 log copies/ml was considered as less than sensibility.

Prognosis after First CSWT

Table 3 summarizes the effect of retreatment or no retreatment at the last observation. For the whole group, the ALT normalization rate and HBeAg seronegative rate were high. In particular, the HBeAg seronegative rate and HBV DNA negative (less than sensitivity, <2.6 log copies/ml) rate in the lamivudine therapy group were the highest among the groups. On the other hand, the HBsAg loss rate was highest in patients with final retreatment by CSWT, followed by patients with final retreatment by IFN, retreatment(–) group, while none of the patients with final retreatment by lamivudine showed HBsAg loss. Furthermore, the proportion of patients in the retreatment(–) group who developed LC (7.3%) was lower than that of patients in the retreatment(+) group (13 of 65 patients, 20%). On the other hand, the proportion of patients in the retreatment(–) group who developed HCC (12.2%) was higher than that of the retreatment(+) group (3 of 65 patients, 4.6%). Only 5 deaths were recorded during the follow-up and all of them were in the retreatment(–) group (table 3). Death was due to HCC in 4 cases and other illness in the remaining patient.

Time to HBeAg Seronegativity, HBsAg Loss, Development of LC and HCC

Figure 1 shows the time to HBeAg seronegativity, HBsAg loss, development of LC and HCC in all patients aged less than 35 years and who received CSWT. The HBeAg seronegative rate increased progressively from 1 to 15 years. However, the increase in the rate of HBsAg loss was less each year up to 20 years of follow-up. The rates of development of LC and HCC during the 20-year follow-up period were also low (fig. 1).

Discussion

The main goals of treatment for patients with CH-B are loss of HBeAg and normalization of aminotransferase. Various treatments such as CSWT, IFN, nucleoside analogs have been used, and their effects have been discussed [3–12]. Previous studies reported the short-term effects of CSWT [3–5, 7]. We also reported previously the short-term effects of CSWT; the HBeAg seronegative rate was 70% within 1 year after CSWT [3]. In that report, the age of the subjects was 39.0 ± 9.9 years. In the present study, we evaluated the effect of therapy in younger adult patients (<35 years) with CH-B. In addition, the study was designed to evaluate the long-term effects and prognosis of CSWT, and accordingly only patients who were followed up for more than 10 years after CSWT were selected.

In the present study, the proportion of patients who did not need retreatment after the first CSWT was 38.7%, and 38 of them (92.7%) became HBeAg seronegative. The HBeAg seronegative rate in patients who needed retreatment after the first CSWT was obviously lower (34/65, 52.3%). However, in both groups the median period until HBeAg seronegativity was approximately 1.0 year; and thus, the HBeAg seronegative rate within 1 year after CSWT was almost the same in the 2 groups; 46.3 (19/38) and 44.1% (15/34) in the HBeAg seronegative cases of the retreatment(–) and retreatment(+) groups, respectively. Of 72 cases who became HBeAg seronegative once, 39 cases (54%) converted to HBeAg positivity, and this rate was almost similar in both groups. During the later part of the follow-up period, though HBeAg changed spontaneously to become negative again at a high rate (94.7%) in the retreatment(–) group, the HBeAg re-seronegative

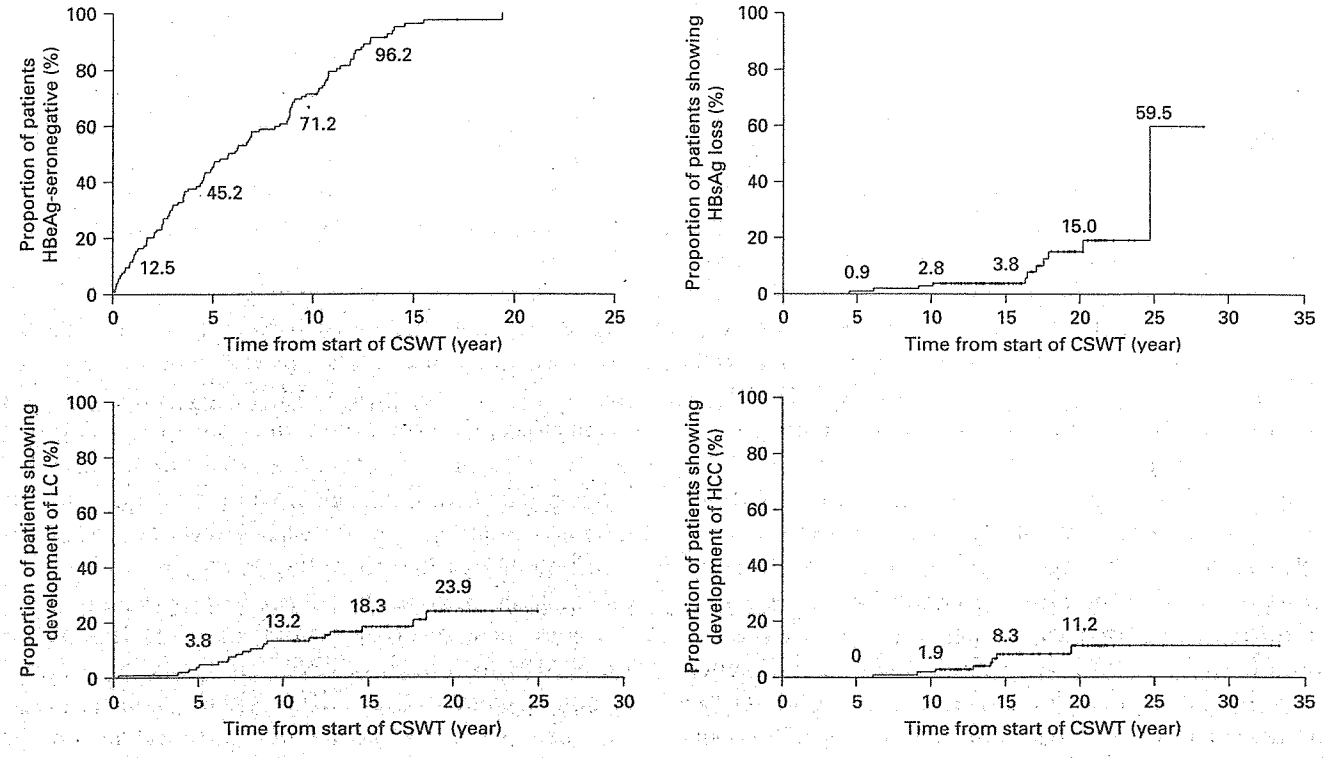

Fig. 1. Time to HBeAg seronegativity, HBsAg loss, development of liver cirrhosis (LC) and hepatocellular carcinoma (HCC) after corticosteroid withdrawal therapy (CSWT) calculated for all patients who participated in the study.

rate was low in the retreatment(+) group (30.0%). Considering the difference in the clinical course, it is suggested to be different according to whether patients achieve HBeAg seronegative easily. The retreatment(–) group contained more females and more patients of genotypes A/B/D/E/unknown than the other group. One reason for the high female ratio in the retreatment(–) group is possibly pregnancy, continuation of therapy was difficult in these patients. In addition, as reported previously [16], the clinical course in female patients with CH-B is often better than in their male counterparts. On the other hand, in terms of HBV genotypes, Kao et al. [17, 18] reported that HBV genotype C is associated with a lower response rate to IFN-α therapy compared with genotype B. In the present study, although there were only a few patients with genotype B, we speculate that genotype C, compared with others except for genotypes C, may be associated with greater resistance to CSWT. However, the HBeAg seronegative rate was 90.2% in the retreatment(–) group at the last observation. On the other hand, in the retreatment(+) group, although their spontaneous HBeAg seroconversion rate was also low, repeating administration of CSWT, IFN, or lamivudine in fact markedly increased the rate to 98.5%, which was even higher than that of the retreatment(–) group (table 2). In addition, as shown in table 3, the ALT normalization rate and HBV DNA negative rate in the retreatment(+) group were also equal or higher than those of the retreatment(–) group. Considered together, the present results suggest that even if the response to the first CSWT is not satisfactory, good virological effects can be provided by repeating or providing alternative treatments.

Although there are no studies that evaluated the long-term effects of CSWT in CH-B, several studies evaluated the long-term effects of IFN therapy. Niederau et al. [10], Yuen et al. [11] and van Zonneveld et al. [12] reported the long-term effect of IFN therapy, although the follow-up period was shorter than ours, including evaluation of HBeAg seroconversion rate, HBsAg loss rate and development rate of LC and HCC. Therefore, we compared the results of these studies with ours. In the study by Niederau et al. [10], the HBeAg seroconversion rate and

HBsAg loss rate were higher than those of our CSWT patients (56 vs. 45.2% at 5 years and 11.6 vs. 0.9%, respectively). The reasons for the differences are possibly race-related (they evaluated Caucasian patients) and the genotypes of their cases were different from ours. The study by van Zonneveld et al. [12] also included predominantly Caucasian patients and reported that the HBsAg loss rate was 21.8% and the rate of HCC development was 4.8% (we grouped responders and non-responders together), which are considerably good results in comparison with our CSWT results. On the other hand, Yuen et al. [11] studied many Asian patients whose ages were also similar to ours. The long-term effects at 10-year IFN therapy in their study included an HBeAg seroconversion rate of 43.8%, HBsAg loss rate of 2.4% and rate of HCC development of 2.4%. Their results were equal or better than those reported here in our study, 71.2, 2.8 and 1.9%, respectively (fig. 1). Although they considered that the long-term effects of IFN therapy were not different compared with untreated controls, we suggest that because the untreated group showed a good clinical course and hence did not need treatment for CH-B, it is possible that the clinical course of both groups was not different. We compared the long-term effects in the retreatment(–) group and retreatment(+) group after first CSWT in our patients. The ALT normalization rate, HBeAg seronegative rate, HBV DNA negative rate and HBsAg loss rate were not different between the 2 groups. These results suggest that because the retreatment(–) group did not need retreatment based on the good clinical course and the retreatment(+) group required repeated treatments for CH-B, the virological effects could become almost equal at the final observation.

The rates of development of LC and HCC were not similar in the 2 groups. With regard to the development of LC, although the progression of fibrosis after the first CSWT was more severe in the retreatment(–) group, the rate of development of LC was lower than in the retreatment(+) group. The reason for this finding is probably related to the good clinical course after CSWT as they did not need retreatment. On the other hand, the rate of development of HCC in the retreatment(–) group was higher than in the retreatment(+) group. Although the incidence of complications was low most likely due to the young age of our patients, we found disaggregation between the LC development rate and carcinogenic rate. Ikeda et al. [19] used multivariate analysis and showed that the severity of fibrosis in HBV-related chronic hepatitis was not associated with the development of HCC. Another clinicopathologic study of HCCs in chronic HBV carriers revealed that about 20–50% of such patients do not have accompanying cirrhosis [20]. As we previously stated in patients with HBV infection at a young age who later developed HCC without LC [21] and previous studies that showed HBV DNA integration into the cellular genomic DNA in HCC cases [22–25], we speculate that the development of HCC in our cases without LC might also be associated with HBV DNA integration into the cellular genomic DNA.

The choice of therapy for young patients with CH-B is difficult. The aim of therapy is HBeAg to anti-HBe seroconversion and inactivation of the disease process. However, despite administration of the same therapy, some patients show good response to one course of CSWT, while others repeatedly require other treatments for CH-B. However, because of the age of young patients with HBV infection in patients like ours, it is important to provide a good quality of life, including cessation of all medications at some stage of their lives. Comparison of our results with those of other studies that used IFN therapy showed that the effects of CSWT in long-term follow-up was almost equal to that of IFN. Although nucleoside analogs such as lamivudine are good antiviral agents, their long-term effects are still unclear especially when treatment can be finished in the short-term and the problem associated with long-term induced lamivudine-resistant mutation and breakthrough hepatitis. Therefore, it is important to examine the long-term effects and safety of IFN therapy and nucleoside analogs such as lamivudine.

In conclusion, we evaluated the long-term effects of CSWT in young adult Japanese HBeAg-positive patients. In patients less than 35 years of age who received CSWT for the first time, 38.7% did not need retreatment with good virological effects. The main results are: (1) the retreatment(–) group consisted of more females and more patients infected with hepatitis B virus than another genotype except for C who showed good response to CSWT and HBeAg might easily become seronegative; (2) the overall long-term effects of CSWT on HBeAg seroconversion rate, HBsAg loss rate and rate of HCC development were equal or better compared with previous reports of IFN therapy [11], and (3) the rate of HCC development was high in the retreatment(+) group compared with the rate of LC development. We speculate that the high rate is probably due to HBV DNA integration into the cellular genomic DNA. Our results suggest that CSWT is a good short-term therapy with possible long-term effects for young adult Japanese patients with CH-B. Other studies should also evaluate the long-term effects of IFN and nucleoside analogs such as lamivudine therapy in Japan.

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Emergence of a Novel Lamivudine-Resistant Hepatitis B Virus Variant with a Substitution Outside the YMDD Motif▽

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Lamivudine is a major drug approved for treatment of chronic hepatitis B virus (HBV) infection. Emergence of drug-resistant mutants with amino acid substitutions in the YMDD motif is a well-documented problem during long-term lamivudine therapy. Here we report a novel lamivudine-resistant strain of HBV with an intact YMDD motif, which included an amino acid substitution, rtA181T, in the reverse transcriptase (RT) domain of HBV polymerase. The substitution also induced a unique amino acid substitution (W172L) in the overlapping hepatitis B surface (HBs) protein. The YMDD mutant strains were not detected even by using the sensitive peptide nucleic acid-mediated PCR clamping method. The detected nucleotide substitution was accompanied by the emergence of an additional nucleotide substitution that induced amino acid change (S331C) in the spacer domain. The rtA181T mutant strain displayed a threefold decrease in susceptibility to lamivudine in in vitro experiments in comparison with the wild type. In vivo analysis using human hepatocyte-chimeric mice confirmed the resistance of this mutant strain to lamivudine. We developed a method to detect this novel rtA181T mutation and a previously reported rtA181T mutation with the HBs stop codon using restriction fragment length polymorphism PCR and identified one patient with the latter pattern among 40 patients with lamivudine resistance. In conclusion, although the incidence is not high, we have to be careful regarding the emergence of lamivudine-resistant mutant strains with intact YMDD motif.

Hepatitis B virus (HBV) is a small, enveloped DNA virus that causes chronic hepatitis and often leads to cirrhosis and hepatocellular carcinoma (4, 12, 33). To date, interferon and three nucleoside and nucleotide analogs (lamivudine, adefovir dipivoxil, and entecavir) have been approved by the United States Food and Drug Administration for the treatment of chronic HBV infection. Lamivudine, an oral cytosine nucleoside analogue, potently inhibits HBV replication by interfering with RNA-dependent DNA polymerase (10, 16, 22). Lamivudine therapy suppresses HBV replication in most patients and improves transaminase levels and liver histology (16, 22, 25, 30). However, prolonged therapy results in the emergence of drug-resistant mutants in 24% and 70% of patients after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels (18).

Most lamivudine-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed (1, 9). In vitro analyses have confirmed that the rtL180M mutation augments the level of lamivudine resistance and enhances viral replication, while the rtV173L mutation enhances only viral replication (9, 23). On the other hand, only a few uncommon mutations associated with lamivudine resistance have been reported so far (3, 7, 24, 34). In the C domain of HBV polymerase, rtM204S and rtD205N were detected in patients with lamivudine resistance (3, 7). In the B domain, rtL180C and rtA181T were associated with lamivudine resistance (7, 24, 34). Yeh et al. (34) reported the emergence of rtA181T mutants in 4 of 23 patients who received long-term lamivudine therapy. The mutant appeared concomitantly with or after emergence of YMDD motif mutants and persisted thereafter. The nucleotide substitution in the FLLA motif resulted in early termination of the overlapping HBs gene transcription by creating a stop codon (TGG to TGA). Yeh et al. (34) demonstrated that the mutation reduced the

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susceptibility to lamivudine in vitro. They also detected such mutations in virus from a patient with leukemia and speculated that truncated HBs gene might be related to the development of leukemia (7).

Analyzing nucleotide and amino acid sequences of HBV in patients who developed a breakthrough, we identified a novel mutant that showed nucleotide substitutions in the B domain of the reverse transcriptase. The G residues of nucleotides 669 and 670 were mutated to T and A, respectively, and associated with the amino acid substitution rtA181T. The substitutions also induced the amino acid substitution W172L in the overlapping HBs protein. Since the nucleotide substitution was associated with nucleotide and amino acid substitutions in the putative spacer region of the polymerase, we checked the importance of these substitutions for resistance to lamivudine in vitro. We also analyzed the resistance of this new strain in vivo using a human hepatocyte-chimeric mouse (27, 31). Furthermore, we analyzed the susceptibility of the mutant strain to adefovir and entecavir. When used alone or in combination with lamivudine, these drugs are known to be effective against wild-type as well as lamivudine-resistant HBV (2, 5, 14, 17, 32). Infrequent emergence of resistance compared with lamivudine resistance has been reported for both of these two drugs (2, 5). We also developed a detection system to identify the novel and previously reported (7, 34) nucleotide substitutions to study the incidence of such mutations.

MATERIALS AND METHODS

Antiviral compounds. Lamivudine [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, United Kingdom). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and entecavir {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingford, CT).

Analysis of virological markers. Hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), and antibody against HBeAg (anti-HBe) were quantified by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using a Light Cycler (Roche, Mannheim, Germany). The primers used for amplification were 5′-TTTGGGCATGGACA TTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s, and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Cloning of HBV DNA and plasmid construction. HBV DNA was extracted from 100 μl of each serum sample by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H_2O. Full-length HBV DNA was amplified using the above HBV DNA samples by the method of Gunther et al. (13). Nucleotide sequence positions were numbered from the unique EcoRI site. The 1.4-genome-length HBV DNA amplified from the serum of a patient who showed lamivudine resistance was cloned into plasmid vector pTRE (Takara Bio, Tokyo, Japan) (patient strain). In brief, the PCR product amplified using serum from the patient was cleaved with BamHI and ApaI (HBV positions 1400 to 2600) and cloned into pcDNA3 (Invitrogen, San Diego, CA), and the resulting construct was named pcDNA3-1. Similarly, the PCR product was cleaved with ApaI and BamHI (HBV positions 2600 to 3215 and 1 to 1400) and cloned into pBlueScript SK+ (Stratagene, La Jolla, CA), and the resulting construct was named pB-1. The KpnI-BamHI fragment from pB-1 and the KpnI-ApaI fragment from pcDNA3-1 were cloned into pcDNA3-1. Finally, the plasmids were cleaved with HindIII and NotI within the multicloning site and cloned into plasmid vector pTRE. As a laboratory strain, we employed a plasmid containing a 1.4-genome-length wild-type genotype C HBV (wild-type strain; GenBank accession number AB206816) (31). To introduce the nucleotide substitutions into the S331C/rtA181T patient and wild-type strains, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene).

TABLE 1. In vitro susceptibility of the S331/rtA181 mutant to lamivudine[a]

Strain		S331/rtA181 mutation	Lamivudine IC_{50} (μM)	Resistance (fold)
Source	Type			
Patient	WT	−/−	0.19 ± 0.01	1
	S331C	C/−	0.23 ± 0.01	1.2*
	rtA181T	−/T	0.58 ± 0.08	3**
	S331C/rtA181T	C/T	0.57 ± 0.06	3**
Laboratory	WT	−/−	0.23 ± 0.04	1
	S331C	C/−	0.3 ± 0.05	1.3*
	rtA181T	−/T	0.88 ± 0.2	3.9**
	S331C/rtA181T	C/T	0.98 ± 0.12	4.3**

[a] Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** $P < 0.001$ compared to the wild type.

The eight plasmids with and without amino acid substitutions in the spacer and reverse transcriptase domain are listed in Table 1.

Cell culture, transfection, and determination of IC_{50}. HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C in 5% CO_2. Cells were seeded to semiconfluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 cells was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC_{50}s) for each antiviral drug, various concentrations of lamivudine, adefovir, and entecavir were added after 24 h to the culture plate containing the cells, and cells were harvested after 5 days. The medium containing the drugs was changed on days 1, 3, and 4. A plasmid encoding β-galactosidase (β-Gal) was cotransfected to adjust the transfection efficiency. The β-Gal enzyme assay was performed with a β-Gal enzyme assay system (Promega, Madison, WI). All experiments were performed in triplicate. GraphPad Prism software (GraphPad Software, Inc.) was used to determine the best-fit values for individual dose-response equations.

Analysis of replicative intermediate of HBV by Southern blot hybridization and quantitation. The cells were harvested at 3 or 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, and 0.5% [vol/vol] NP-40) followed by centrifugation for 2 min at 15,000 × *g*. The core-associated HBV genome was immunoprecipitated by mouse anticore monoclonal antibody 2A21 (Institute of Immunology, Tokyo, Japan) and subjected to Southern blot analysis after sodium dodecyl sulfate–proteinase K digestion followed by phenol extraction and ethanol precipitation. The DNA was detected with a full-length HBV-DNA probe labeled by the DIG DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions provided by the manufacturer. Quantitative analysis was performed by real-time PCR with SYBR green using a Light Cycler. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGT GAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Evaluation of effects of antiviral drugs on mutant strains using human hepatocyte-chimeric mice. Human hepatocyte-chimeric mice were generated and used in the drug evaluation studies as described previously (27, 31). Briefly, human hepatocytes were transplanted into urokinase-type plasminogen activator-transgenic SCID mice, which are immunodeficient and develop liver failure. The transplanted cells were characterized in terms of in vivo growth potential and function. The human hepatocytes progressively repopulated the murine host liver and were susceptible to cultured-cell-line-produced HBV. All animal protocols were performed in accordance with the guidelines of the local committee for animal experimentation. The mice were inoculated with 50 μl of serum samples containing wild-type and newly identified drug-resistant strains. Serum samples obtained from mice were stored at −80°C before further analyses. After stable high-level HBV viremia was confirmed, the mice were administered food containing 30 mg of lamivudine/kg of body weight/day. The nucleotide sequences of wild-type and mutant strains were confirmed by sequencing analysis.

Detection of rtA181T mutants by PCR with restriction fragment length polymorphism (RFLP). HBV DNA extracted from serum samples were amplified by

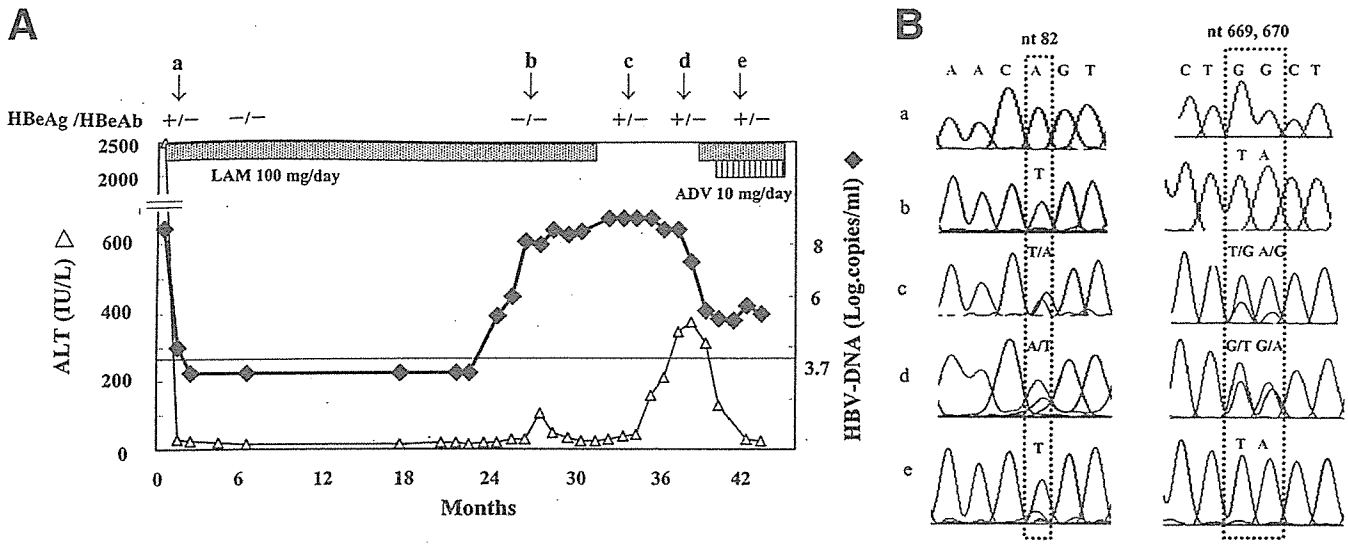

FIG. 1. (A) Clinical course of a patient who developed breakthrough without emergence of YMDD mutants during lamivudine therapy. Arrows a to e indicate time points of serum sampling for direct sequencing and RFLP PCR. (B) Nucleotide sequence analysis of the reverse transcriptase/polymerase gene of hepatitis B virus by direct sequencing. Time points of serum sampling (see panel A) were as follows: (a) just before lamivudine treatment, (b) after breakthrough, (c) after cessation of lamivudine treatment, (d) just before readministration of lamivudine, and (e) during adefovir and lamivudine therapy. Note that the wild type reappeared during the cessation of therapy (c and d), but it disappeared after readministration of the drug (e).

PCR using the primers 5′-GCCCGTTTGTCCTCTACTTCCA-3′ and 5′-ACCA CTGAACAAATGGCACTAGTAAGCTGA-3′. The reverse primer was designed to introduce an EspI site (GCTCAGC) into only wild-type sequences. The PCR was performed in a total volume of 25 μl, consisting of a reaction buffer (100 mmol/liter Tris-HCl [pH 8.3], 50 mmol/liter KCl, and 15 mmol/liter $MgCl_2$), 0.2 mmol/liter of each deoxynucleoside triphosphate, 1 μl of the DNA solution, 10 pmol of each primer and 1 U of *Taq* DNA polymerase (Gene Taq; Wako Pure Chemicals, Tokyo, Japan) with 0.2 μg of anti-*Taq* high (Toyobo Co., Osaka, Japan). The amplification conditions included an initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 1 min, annealing of primer at 58°C for 1 min, extension at 72°C for 2 min), and final extension at 72°C for 7 min. Two μl of PCR products was digested with 5 U of EspI and subjected to electrophoresis in a 3.5% agarose gel.

Statistical analysis. Data are expressed as means ± standard deviations (SD). Group comparisons were performed using the Student *t* test. A *P* value of less than 0.05 was considered statistically significant.

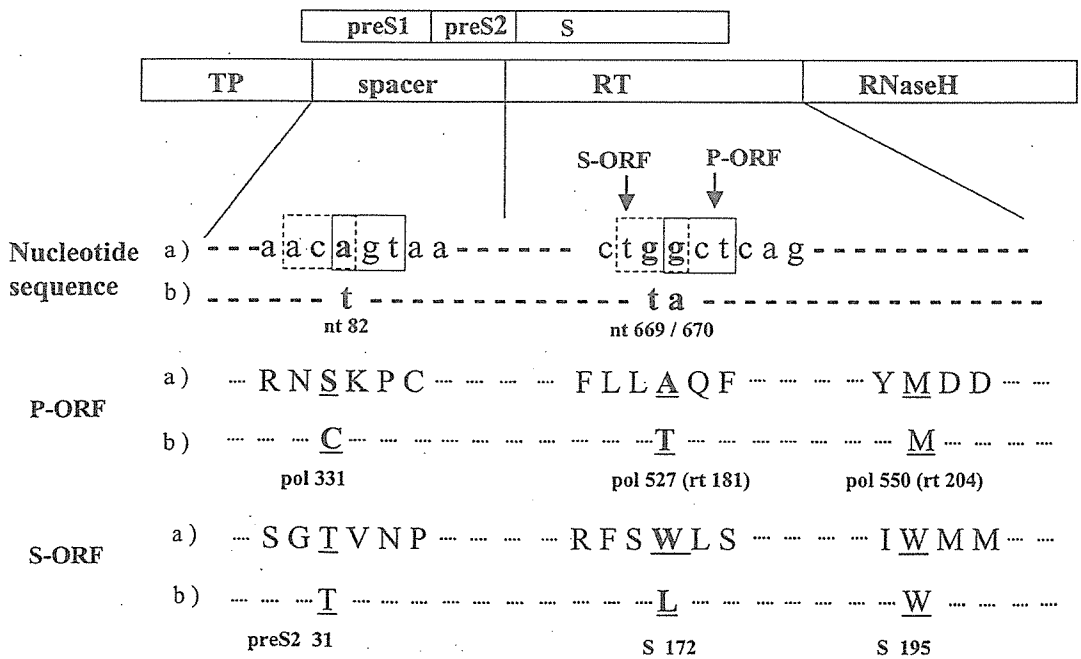

FIG. 2. Comparison of nucleotide sequences and amino acid sequences of two overlapping open reading frames, reverse transcriptase/polymerase and the HBs gene of the hepatitis B virus, before and after viral breakthrough. Sequences obtained from serum samples before (a) and after (b) breakthrough were compared. See Fig. 1A for time points of serum sampling. Nucleotide sequence numbers are those of typical HBV (e.g., accession no. AB206816 [31]), which starts from a unique EcoRI site.

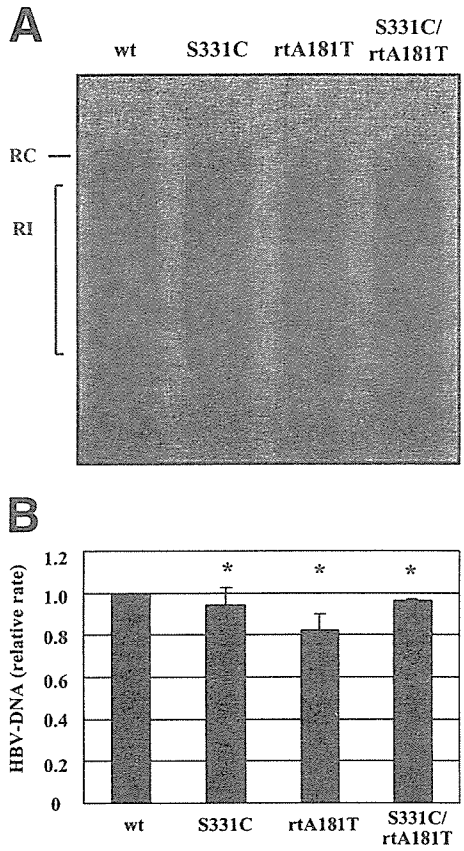

FIG. 3. Replication ability of wild-type HBV and three mutants (S331C, rtA181T, and S331C/rtA181T). Plasmids containing 1.4-genome-length HBV were transiently transfected into HepG2 cells. (A) The replicative intermediates were analyzed by Southern blot hybridization. Core-associated replicative intermediates of HBV DNA were isolated from HepG2 cells at 3 days after transfection. The positions of relaxed circular DNA (RC) and replication intermediates (RI) are indicated. (B) Quantitative analyses of core-associated intermediates of HBV. Experiments were performed in triplicate. Values are relative to those of the wild type and are expressed as means ± SD. *, not significant compared to the wild type.

RESULTS

Isolation of a novel lamivudine-resistant strain with an intact YMDD motif. The novel lamivudine-resistant strain of HBV was isolated from a 44-year-old Japanese man with chronic HBV infection (Fig. 1A). In this patient, lamivudine successfully reduced the HBV level at the initial stage of treatment, but viral breakthrough was observed at 24 months after the beginning of therapy. The patient was very punctual and confirmed that he took lamivudine with perfect compliance. The HBV viral load reached up to 8.5 log copies/ml, but nucleotide sequence analysis showed no YMDD mutation. The YIDD and YVDD mutants were not detected even with a peptide nucleic acid-mediated PCR clamping method sensitive for detection of YMDD mutants (6). The analysis also showed that this isolate belonged to genotype C of HBV. Comparison by the direct sequence method of nucleotide sequences obtained before and after the viral breakthrough showed three nucleotide substitutions that induced two amino acid substitutions in both spacer (polS331C) and reverse transcriptase (polA527T or rtA181T) domains of the polymerase (Fig. 1B and 2). The latter nucleotide substitutions induced an amino acid change in the overlapping HBs protein (W172L) (Fig. 2). Twelve HBV genomes were cloned from the serum of this patient after viral breakthrough, and eleven of them showed the above amino acid substitutions. Only one clone showed the wild-type sequence. The new strain of HBV became undetectable when lamivudine therapy was discontinued, and this strain outcompeted the wild-type strain upon administration of the drug (Fig. 1B). These results prompted us to study the significance of each of these mutations.

Effect of substitutions on HBV replication. To assess the effect of nucleotide substitutions on HBV replication, four plasmids containing 1.4-genome-length patient-specific HBV genome (Table 1) were generated and transfected into HepG2 cells. In comparison with the patient's wild-type strain, the replication capacities of the S331C, rtA181T, and S331C/rtA181T mutants were not different (94%, 82%, and 96%, respectively), suggesting that these mutants can replicate at almost the same rate as the wild-type strain (Fig. 3).

Susceptibility of mutants to lamivudine in vitro. To analyze the role of the polS331C and rtA181T mutations in lamivudine resistance, four patient-specific strains and four laboratory strains were transfected into HepG2 cells (Fig. 4; Table 1). A single amino acid substitution in the spacer region did not contribute to resistance in either patient or laboratory strains. In contrast, an amino acid substitution in the polymerase (rtA181T) induced resistance that was 3.0 and 3.9 times greater than that of patient and laboratory strains ($P < 0.001$), respectively. The presence of both of these amino acid changes induced 3.0 and 4.3 times greater resistance in each of the above strains. Thus, the spacer mutation had little effect on the susceptibility to lamivudine (Table 1).

We also compared the rtA181T mutant identified in this study with the rtA181T mutant reported previously, which had premature termination in the HBs protein (7, 34), for replication ability and susceptibility to lamivudine. Although the HBs antigen produced to culture supernatant was different between the two strains (52.5 ± 8.2 and 4.4 ± 0.6 IU/ml, respectively), there was no noticeable difference in replication ability and lamivudine sensitivity between the two mutants (data not shown).

Assessment of drug resistance of novel mutations in vivo using human hepatocyte-chimeric mice. To confirm the lamivudine resistance of the novel mutant strain, two human hepatocyte-chimeric mice were each inoculated with a serum sample obtained from the patient who developed breakthrough without mutations in the YMDD motif (Fig. 1A). The serum was obtained during breakthrough while the patient was still taking the drug. Twelve weeks after the inoculation of the serum samples, both mice developed high-level viremia (7.8 and 6.6 log copies/ml, respectively). Direct sequence analysis showed that the nucleotide sequence of the virus that replicated in the chimeric mice was in accordance with the mutant strain. Cloning and sequencing analysis showed that only 1 of 12 clones obtained from the inoculum was wild type, while the remaining 11 clones were rtA181T mutants with an intact YMDD motif. We also analyzed the serum of the two infected mice before and after lamivudine therapy. All 11 and 15 clones before and all 11 and 12 clones during therapy had the

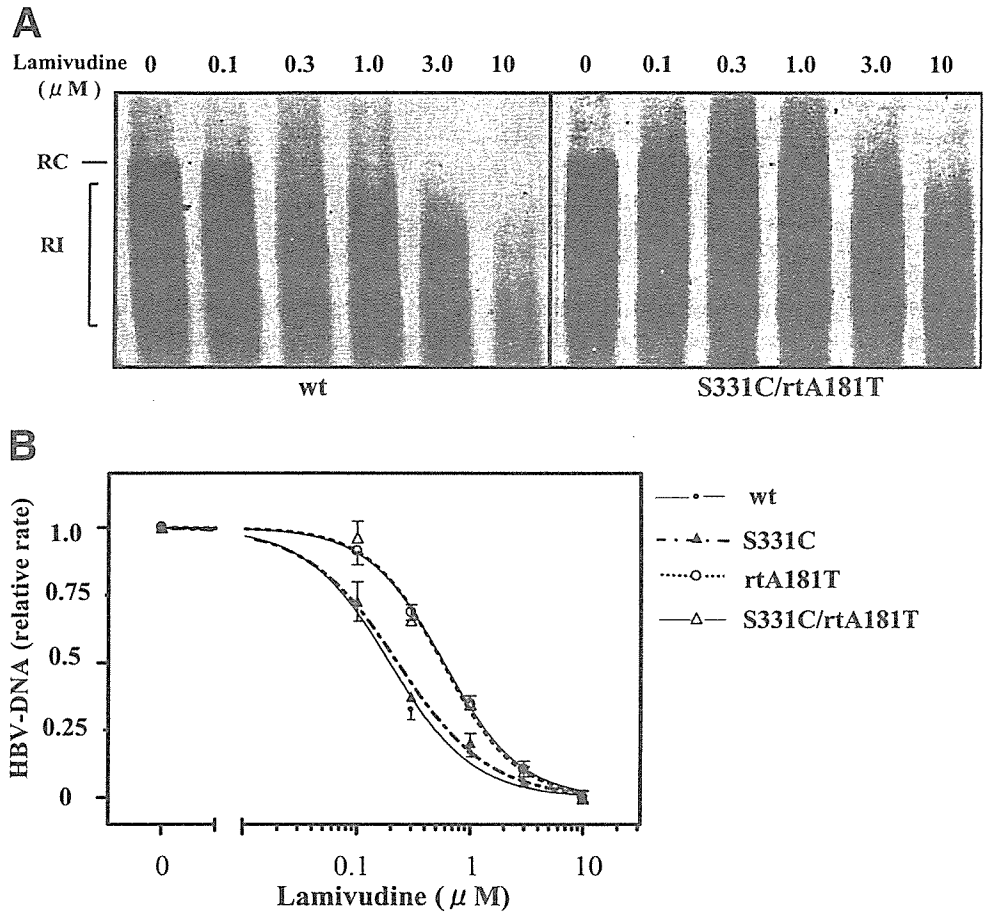

FIG. 4. In vitro analyses of susceptibility of wild-type HBV and three mutants (S331C, rtA181T, S331C/rtA181T) to lamivudine after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4-genome-length HBV and treated with the indicated amount of lamivudine. (A) Southern blot analysis of replicative intermediate. Representative results for the wild type (wt) and the S331C/rtA181T mutant are shown. The positions of relaxed circular (RC) and replication intermediate (RI) forms of HBV DNA are indicated. (B) Dose-response curves of the four HBV strains against lamivudine. The curves were used to estimate the lamivudine IC_{50}s for each HBV strains. Values are relative to no-lamivudine controls for each strain. Experiments were performed in triplicate. Values are expressed as means ± SD.

rtA181T mutation (data not shown). Two other mice were inoculated with wild-type HBV obtained from a patient not treated with lamivudine as a control, and both mice also developed high-level viremia (8.3 and 9.3 log copies/ml, respectively). Thirteen weeks later, the viremia reached plateau and the mice were fed food containing lamivudine. After 6 weeks of treatment, the mean viral load decreased by 2.8 log copies/ml in the wild type, whereas it decreased by only 0.39 log copy/ml in the mutant ($P < 0.001$) (Fig. 5).

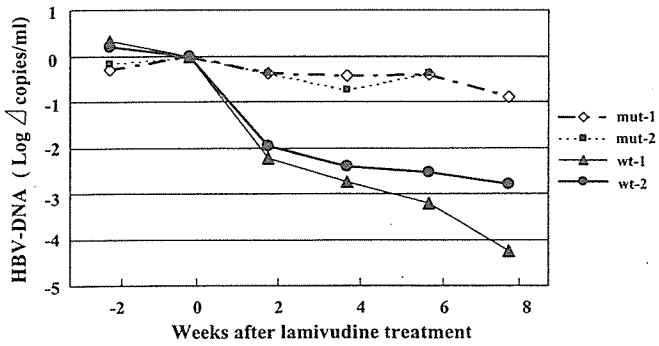

FIG. 5. In vivo analyses of the effect of lamivudine on wild-type and S331C/rtA181T mutant HBV. Four human hepatocyte-chimeric mice were inoculated with serum samples containing wild-type or mutant HBV. One of the animals fed with lamivudine died 6 weeks after the beginning of therapy.

Susceptibility of mutants to adefovir and entecavir in vitro. We also analyzed the effects of adefovir and entecavir against the S331C/rtA181T mutant using a transient-transfection assay with HepG2 cells. The IC_{50}s of these drugs for the mutant strain and wild type were almost the same (Table 2).

Detection of rtA181T mutant in patients treated with lamivudine. In this study, we developed a RFLP PCR method to detect the rtA181T mutants, by which we were able to detect mutant strains even when they were mixed with the wild type (Fig. 6). The system also detected the rtA181T (HBs stop) mutant reported by Chien et al. (7) and Yeh et al. (34). Using this method, we analyzed 40 patients who showed viral breakthrough (increase in viral load equal to or more than 1 log) during lamivudine therapy. We found that only one of these patients was positive (Fig. 6A). Nucleotide sequence analysis of serum samples obtained from this patient showed that the

TABLE 2. In vitro susceptibility of the S331/rtA181 mutant to lamivudine, adefovir, and entecavir[a]

Patient strain	S331/rtA181	Lamivudine		Adefovir		Entecavir	
		IC_{50} (μM)	Resistance (fold)	IC_{50} (μM)	Resistance (fold)	IC_{50} (nM)	Resistance (fold)
WT	−/−	0.19 ± 0.01	1	0.37 ± 0.1	1	0.19 ± 0.02	1
S331C/rtA181T	C/T	0.57 ± 0.06	3**	0.36 ± 0.08	0.98*	0.23 ± 0.05	1.2*

[a] Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** $P < 0.001$ compared to the wild type.

mutant strain had the rtA181T mutation with a truncated HBs antigen, as reported previously (7, 34). The YMDD motif of HBV detected in this patient was of the wild type. All 39 remaining patients with viral breakthrough were positive for YIDD and/or YVDD mutants. The RFLP PCR analysis of these 39 samples showed that four contained a small amount of rtA181T mutants (Fig. 6B). Nucleotide sequence analyses of these samples showed that they contained only a small amount of rtA181T mutants with a truncated HBs antigen (Fig. 6C).

Finally, we examined the presence of YMDD or rtA181T mutants in eight patients who showed a poor response with lamivudine treatment (HBV viral load above 6.0 log copies/ml after 6 months of treatment). None of these patients tested positive for both of these mutations (data not shown).

DISCUSSION

In this study, we identified a novel lamivudine-resistant strain of HBV with an intact YMDD motif in a patient who received long-term lamivudine therapy. YMDD mutants were not detected even by a sensitivity-enhanced detection method, which was reported previously by our group (6). The double nucleotide substitutions (GG to TA) induced amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L). One might assume that the compliance of the patient was poor. However, the patient was very punctual and confirmed that he took lamivudine with perfect compliance.

Our study demonstrated that the rtA181T mutation reduced the susceptibility to lamivudine 3.0- to 3.9-fold in vitro (Table 1). Furthermore, we also confirmed lamivudine resistance of this mutant strain in vivo using human hepatocyte-chimeric mice. The amino acid substitution in the reverse transcriptase (RT) domain is similar to that reported previously (7, 34). However, in contrast to our results, the mutant strains in the latter reports emerged with or after those with the mutation in the YMDD motif (YIDD or YVDD) and took over them (34). There are two additional differences between the substitutions we identified and those described by Yeh et al. (34), as detailed below.

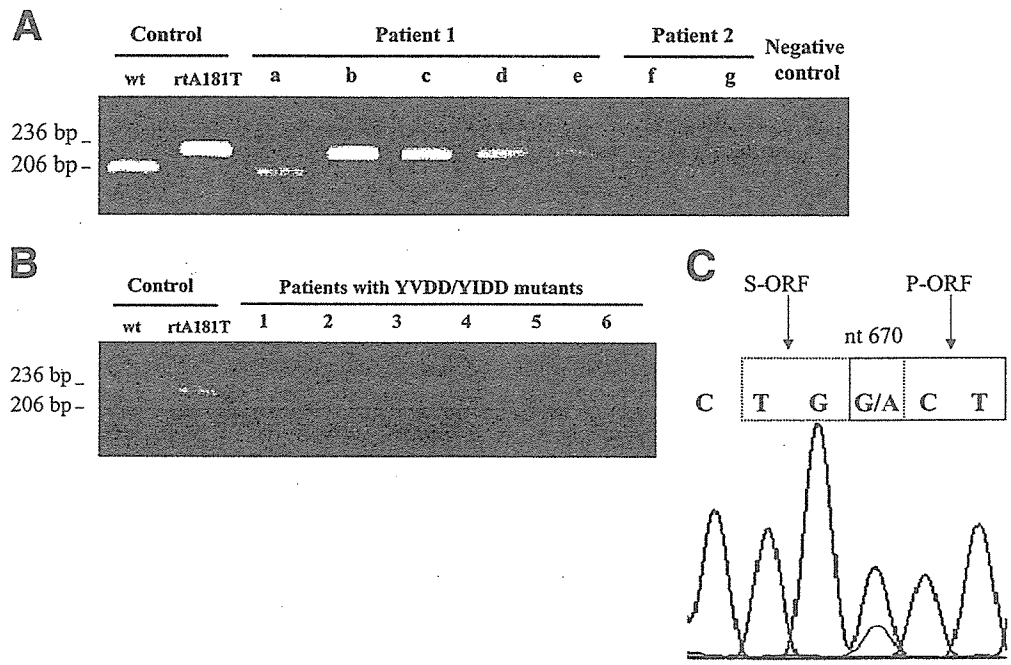

FIG. 6. Detection of the rtA181T mutant by RFLP PCR assay. PCR-amplified DNA fragments were treated with EspI, which digests only wild-type sequences, and separated in a 3.5% agarose gel. (A) Agarose gel electrophoresis of RFLP PCR products. Wild-type and rtA181T mutant plasmids were used as controls. See Fig. 1A for the time points of serum sampling (a to e) for patient 1 and see Fig. 1B for a comparison with nucleotide sequence analyses, f and g indicate the time points before and after viral breakthrough for patient 2. (B) Agarose gel electrophoresis of RFLP PCR products using HBV DNA samples obtained from 39 patients who showed lamivudine breakthrough. Of the 39 samples, 35 were wild type (lanes 1 and 2). The remaining four samples (lanes 3 to 7) showed partial digestion, suggesting a mixture of wild-type and mutant strains. (C) Nucleotide sequence analysis of a sample by RFLP PCR suggested the presence of a wild-type–mutant mixture (lane 5 of panel B).

Firstly, the HBs antigen was prematurely terminated in the mutant strain reported by Yeh et al. (34). In this regard, a similar amino acid substitution of the B domain of the polymerase FLLA motif in woodchuck hepatitis virus (WHV) treated with lamivudine was reported (15, 28). The HBs antigen in these WHV mutant strains also had premature stop codons. These findings suggest that the mutant strains of HBV and WHV cannot replicate and spread by themselves because of the lack of HBs antigen. Such strains are thought to replicate by using in vivo-supplied HBs antigen from wild-type strains as helper antigens. In contrast, the novel strain identified in this study had no premature termination of the HBs gene. The in vitro study suggested that the strain had a replication ability similar to that of the wild type. Furthermore, we also showed that the strain infected and reached a high viral load in human hepatocyte-chimeric mice. Although the inoculum contained only a small amount of wild-type strain (one of 12 clones), all clones obtained from mouse serum were mutant strains (rtA181T). Considering these results and the fact that the index patient showed high viral titers after breakthrough (more than 7.6 log copies/ml), this mutant strain can spread and replicate by itself and has strong replicative ability.

Secondly, the substitutions identified in this study appeared with nucleotide and amino acid substitutions in the spacer region of the polymerase (S331C). There are only a few studies that reported the function of the spacer domain (19–21, 28), leaving the biological significance of this region unknown. The substitution in the spacer region reappeared with the A181T mutation in the RT domain in the index patient after the patient restarted lamivudine therapy. Although our study showed no significant contribution of this mutation to drug resistance (Fig. 3 and 4; Table 1), the significance of the mutation in this region (fingers in the HBV polymerase homology model [8]) should further be investigated.

Recently, the amino acid substitutions rtA181T and rtA181V were reported to emerge with resistance against adefovir (11, 32). Tillmann et al. (29) reported one case in which the virus developed the rtA181T mutation during famciclovir breakthrough. The A556T mutation of WHV, analogous to the rtA181T mutation of HBV, has been reported to be associated with lamivudine resistance (15, 28). These results indicate that the amino acid substitutions at position 181 may associate with resistance against many nucleoside analogues, including lamivudine, famciclovir, and adefovir. Although our in vitro study indicated that the rtA181T mutant had no resistance against adefovir and the animal study showed that combination therapy with lamivudine and adefovir effectively reduced the virus load in woodchucks (15), such combination therapy did not produce sufficient suppression of HBV in the index patient (Fig. 1A). The amino acid substitution at position 181 has to be further analyzed with regard to resistance to anti-HBV drugs.

The rtA181T mutation detection system using RFLP PCR developed in this study is a useful tool, as we were able to distinguish the wild type from all mutants with nucleotide substitutions in a given region. The system also enabled us to monitor the fluctuation of the wild-type/mutant ratio during therapy against HBV (Fig. 1 and 6). The incidence of rtA181T mutants with an intact YMDD motif is rare in Japanese patients with chronic HBV infection treated with lamivudine. Interestingly, 4 of the 39 (10%) patients who developed lamivudine breakthrough and were positive for YMDD mutants were found to have small amounts of rtA181T mutant strains. Different from the previous report (34), the mutants did not take over another strain and were not preceded by exacerbation. We have to monitor these patients carefully for further population change of mutants and for exacerbation of hepatitis.

A recent study reported that the prevalence of genotype A HBV infection is increasing in Japan and that the incidence of disease chronicity is higher than for other genotypes (26). It is thus expected that an increasing number of the sexually active population will receive nucleoside analogue therapy against HBV and multiple mutant strains can potentially emerge and spread along with long-term treatment. There is an increasing possibility of emergence of novel mutants resistant to multiple anti-HBV drugs. The importance and significance of the rtA181 mutations, including the novel mutant strain identified in this study, should be investigated further to develop more useful treatment strategies.

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CLINICAL RESEARCH STUDY

AJM Theme Issue: Infectious Disease

Long-term Outcome of HBV Carriers with Negative HBe Antigen and Normal Aminotransferase

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ABSTRACT

PURPOSES: To elucidate the incidence of hepatitis activation and hepatocellular carcinogenesis in patients with negative HBe antigen and normal aminotransferase, long-term observation was performed in a retrospective cohort.

METHODS: Among 116 consecutive patients with normal aminotransferase and negative HBe antigen at the time of liver biopsy, sequential frozen sera for initial 5 years were available for 95 patients. Hepatitis B virus (HBV) DNA assay (sensitivity >400 copy/mL) and aminotransferase were annually examined in the initial 5 years after biopsy.

RESULTS: Liver biopsy showed minimal hepatitis (F0) in 9, F1 in 53, F2 in 21, F3 in 6, and F4 or cirrhosis in 6. Initial HBV DNA concentration was low (less than 10^4 copies/mL) in 33, intermediate (10^4-10^6) in 53, and high (10^6 or more) in 9. Hepatitis activation rates with twice as high as normal aminotransferase at the end of the third year were 12.1% in the low DNA group, 43.4% in the intermediate group, and 66.7% in the high DNA group. Initial HBV DNA values were significantly associated with future increase in aminotransferase ($P < .0001$). Initial DNA, history of aminotransferase elevation, and histological staging independently affected future hepatitis activation. Carcinogenesis rate in patients with and without high DNA of 10^6 copies/mL during the initial 3 years were 6.9% and 0%, respectively, at the end of the 5th year, and 11.5% and 1.8%, respectively, at the 10th year, ($P = .021$).

CONCLUSION: Advanced stages of hepatitis were sometimes found in HBe antigen-negative, aminotransferase-normal HBV carriers. Serial HBV DNA assessment in early 3 years predicted future hepatitis activation and carcinogenesis.

KEYWORDS: Hepatitis B virus; DNA; HBe-antigen; Carcinogenesis; Prognosis; Chronic hepatitis

HBe antigen loss and appearance of anti-HBe antibody are usually associated with normalization of aminotransferase in patients with chronic hepatitis,[1-3] and these changes have long been considered as a reliable clinical indicator of inactive and innocent state of chronic hepatitis B. However, the level of aminotransferase sometimes fluctuates under the state of negative HBe antigen in chronic hepatitis B, and there is occasional histological evidence of disease progression.[4-6] Because hepatitis B virus (HBV) DNA is usually detected in the sera of these patients, mutant HBV has been confirmed to cause the hepatitis activity.[7,8] Moreover, hepatocellular carcinoma (HCC) sometimes occurs in patients with mild hepatitis and normal aminotransferase levels.[9,10] It is an important problem from the viewpoint of early detection of HCC that general practitioners and even hepatologists are sometimes unaware of those patients with negative HBe antigen and normal aminotransferase as high-risk subjects for HCC.

There is little information about how many patients with both normal alanine aminotransferase (ALT) and negative HBe antigen have active or advanced histologically confirmed hepatitis,[11,12] and how many patients in

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whom ALT level increases over long-term follow-up. Recently, for evaluation of the activity of chronic hepatitis B, simple and sensitive HBV DNA assays using polymerase chain reaction (PCR) have replaced serologic examination of HBe antigen-antibody. The high sensitivity of DNA assays has contributed to our understanding of the pathogenetic role of the virus and provided useful information about hepatitis activity in individual patients.[12-18] An arbitrary serum DNA level of 10^5 copies/mL was proposed by the National Institute of Health Workshop for the differentiation of chronic hepatitis B from an inactive carrier state (HBe antigen-negative, persistently normal ALT).[19] Martinot-Peignoux et al[20] also reported that a cutoff DNA value of 10^5 copies/mL effectively discriminated inactive hepatitis in a follow-up study of 1 to 6 years. On the other hand, Manesis et al[21] suggested that a cutoff DNA level of 3×10^4 copies/mL was more appropriate for differentiating inactive carriers from patients with HBeAg-negative chronic hepatitis B. Furthermore, Chu et al[22] concluded that a single cutoff DNA value could not differentiate inactive carriers from patients with HBeAg-negative chronic hepatitis after retrospective analysis of 165 Chinese patients. Previous studies indicated that the above data still did not provide sufficient information as to the longitudinal outcome of hepatitis activation and carcinogenesis, considering the fluctuating course of HBeAg-negative chronic hepatitis.[19,22] Here we report the results of our long-term study (median follow-up of 10.2 years) in patients with negative HBe antigen and normal alanine aminotransferase (ALT).

The purposes of this retrospective study were to elucidate the incidence of advanced liver disease in patients with negative HBe antigen and normal ALT value, and to estimate the future activation of hepatitis and carcinogenesis rate in those patients, using life-table method with multivariate analysis. We also analyzed whether the initial few years of biochemical and virological observation (annual measurements of ALT and HBV DNA concentration) could predict future outcome of the patients.

CLINICAL SIGNIFICANCE

- Advanced stages of hepatitis should not be overlooked in patients with HBe antigen-negative, aminotransferase-normal HBV carriers.
- Serial DNA assessment in the early few years predicted future hepatitis activation and the risk of carcinogenesis.

PATIENTS AND METHODS

Patients

Among 1048 patients who were diagnosed with HBV-related chronic hepatitis by peritoneoscopy and/or biopsy from 1974 to 2002 in our hospital, 116 patients were negative HBe antigen and had normal aminotransferase at the time of liver biopsy. Of these, 95 (81.9%) were thoroughly observed for 5 years or longer, and their data were available for analysis of HBV DNA concentration. The initial sera of all these 95 patients were positive for HBs antigen, negative HBe antigen, and negative anti-HCV antibody. ALT concentration was also normal for 2 months or longer before admission to the hospital for examination of liver biopsy. The enrolled patients included 75 men and 20 women aged 21 to 67 years, with a median of 39 years. None of the patients received antiviral therapy before liver biopsy. All the clinical practices were performed in accordance with the ethical committee of the hospital and with general agreement of domestic medical insurance.

Table 1 shows demographic and laboratory data of the 95 patients. Family history of liver disease or HBV infection has been closely investigated and assessed to a relative in the fourth degree using each family line. Because all the patients had been negative for HBe antigen and normal ALT since the initial visit to our hospital, the time of seroconversion from HBe antigen to HBe antibody was not clear in most of the patients. HBV subtyping showed that genotype C was the most prevalent type (74.7%) in the cohort, the rate of which was slightly lower than that of Japanese patients with chronic hepatitis type B.[23,24] Two authors (K.I. and H.K.) assessed the pathology and translated the biopsy findings to the classification of Desmet et al.[25]

Table 1 Demography and Laboratory Data of 95 Patients with Positive HBs Antigen who Showed Normal Aminotransferase and Negative HBe Antigen at the Time of Biopsy

Data	
Demographic data	
Men:Women	75:20
Age (years - median, range)	39 (18-67)
HBe antigen positive	0 (0%)
HBV genotype A	3 (3.2%)
B	18 (18.9%)
C	71 (74.7%)
Others/undetectable	3 (3.3%)
Anti-HCV antibody positive	0 (0%)
Past alcohol intake of 500 kg or more	15 (15.8%)
History of blood transfusion	4 (4.2%)
Laboratory data*	
Aspartic aminotransferase (normal 6-38 IU)	26 (10-37)
Alanine aminotransferase (normal 6-50 IU)	28 (6-50)
Platelet count (normal 149-315×10^3/mm^3)	185 (86-311)
ICG_{R15}** (normal, ≤10%)	11 (3-33)
Albumin (normal, 3.9-5.2 g/dL)	4.3 (3.6-5.4)
Bilirubin (normal, ≤1.1 mg/dL)	0.9 (0.2-1.9)
Prothrombin time (normal, ≥70%)	97.5 (67-111)

*Data are median (range).
**ICG_{R15}: Indocyanine green retention rate at 15 minutes.

Follow-up Studies

After liver biopsy, follow-up examinations were conducted on a monthly to tri-monthly basis by monitoring biochemical and virological data. Although 27 (28.4%) patients started to receive interferon (IFN) therapy because of ALT elevation, none underwent lamivudine therapy during the course of the study. "Activation of hepatitis" or "flare-up of ALT" was defined as an increase of ALT value of twice or more of the upper limit of normal.

Imaging studies were performed annually in each patient and included computed tomography (CT) or ultrasound (US) after 1985. At least 2 imaging procedures were performed in those patients with cirrhosis. HCC was diagnosed by typical hypervascular characteristics on angiography in addition to certain features of CT and US. Pathological confirmation of surgically resected specimens or autopsy was made in 3 (60.0%) of 5 patients who developed HCC during the study.

A total of 17 patients were lost to follow-up after 5 years. The observation period ranged from 5.1 to 24.7 years, with a median of 10.2 years.

Assays of HBV Markers

Serum HBsAg was measured by radioimmunoassay (Dainabot, Tokyo, Japan), and HBeAg and anti-HBe were determined by enzyme-linked immunosorbent assay (ELISA, Institute of Immunology) using commercially available kits. Anti-HCV antibody was analyzed using second-generation anti-HCV kits (enzyme-linked immunosorbent assay, Dainabot). HBV DNA was assayed using frozen sera stored at −80°C and measured with Amplicor HBV Monitor kits (Roche Diagnostics Japan Co., Tokyo). The effective measurement of the concentration ranged from $10^{2.6}$ (400) copies/mL to $10^{7.6}$ (40 000 000) copies/mL. All measurements of HBV DNA were performed at the same time using the same assay kits. Quantitative HBV DNA was conducted at the time of liver biopsy, at the end of 6 months, and at the end of the 1st, 2nd, 3rd, and 5th years.

Statistical Analysis

The chi-square test, Fisher's exact test, and Kruskal-Wallis test were used to analyze the relationship between HBV markers and the clinical course. Cumulative flare-up rate of hepatitis and carcinogenesis rate were calculated with the Kaplan-Meier method,[26] and differences in the rates were analyzed by the log-rank test. Multivariate Cox proportional hazard model[27] was adopted to analyze the prediction of future hepatitis activation and carcinogenesis. A *P*-value of less than .05 with 2-tailed test was considered significant. Data analysis was performed using the SPSS software version 11.[28]

RESULTS

Histological Findings

According to the classification of Desmet et al,[25] liver biopsy showed minimal hepatitis (F0) in 9 (9.5%), F1 in 53 (55.8%), F2 in 21(22.1%), F3 in 6 (6.3%), and F4 or cirrhosis in 6 (6.3%). Occult cirrhosis with normal ALT and negative HBe antigen was found in 6.3%. Inflammatory activity was minimal (A0) in 18 (18.9%), A1 in 72 (75.8%), and A2 in 5 (5.3%).

Reasons for Liver Biopsy

The reasons for liver biopsy in spite of "stable hepatitis" included a history of ALT elevation in the past (group A, n = 41), patient's apprehension based on family history of advanced liver diseases (group B, n = 25), possible advanced liver disease on ultrasonography (US) or liver function tests (group C, n = 8), or simple desire for thorough examination including biopsy (group D, n = 21). We also assessed the relationship between background features and reasons for undergoing liver biopsy (Table 2).

Advanced liver disease of F3 or F4 was present in 4 of 41 (9.8%) patients in group A, 4 of 25 (16.0%) in group B, 3 of 8 (37.5%) in group C, and 1 of 18 (5.6%) in group D. The median platelet count was the lowest in group C, which suggested that the group included more numbers of patients with advanced stages of the disease. The median HBV DNA concentration at the beginning was the highest in group A. HBV genotype A was exclusively found in group D and the incidence of genotype B was higher in groups A and D.

Initial and Serial Assays of HBV DNA Concentration

The median DNA concentration at the time of liver biopsy was $10^{4.4}$ copies/mL, with 25th percentile of $10^{3.2}$, and 75th percentile of $10^{5.2}$ (range, $<10^{2.6}$ to $>10^{7.6}$).

The relationship between the initial HBV DNA values and serial measurements of DNA during the observation were assessed, classifying the initial DNA concentration into 4 groups: low DNA group ($<10^4$ copies/mL), DNA concentration of 10^4-$10^{4.9}$ copies/mL, DNA concentration of 10^5-$10^{5.9}$ copies/mL, and high DNA of $\geq 10^6$ copies (Figure 1). Among 33 patients with initial HBV DNA concentration of $<10^4$ copies/mL, 29 (87.9%) showed a continuously low DNA of less than 10^6 copies/mL, and 4 (12.1%) showed an intermittent rise of the DNA to $>10^6$ copies/mL. Among 34 patients with initial HBV DNA of 10^4 to $10^{4.9}$ copies/mL, 25 (73.5%) showed persistently low DNA, and 9 (26.5%) an intermittent rise of the DNA. Of 18 patients with initial DNA of 10^5 to $10^{5.9}$ copies/mL, 10 (55.5%) showed persistently low DNA course, and 8 (44.4%) an intermittent elevation of the DNA. Among 10 patients with initial DNA of $\geq 10^6$ copies/mL, an intermittent rise of the DNA was found in 9 patients (90.0%) and persistently high DNA in one (10.0%). The initial HBV DNA concentration was significantly associated with the subsequent serial HBV DNA course ($P < .0001$).

Table 2 Demographic, Histological, Biochemical and Virological Findings According to the Reasons for Examination Including Liver Biopsy

	Reasons for Liver Biopsy			
	Group A	Group B	Group C	Group D
Number of patients	41	25	8	21
Male/Female	36/5	15/10	5/3	19/2
Age (years – median, range)	39 (18-61)	38 (30-67)	41 (30-57)	41 (21-58)
Family history of liver disease or HBV infection	18 (37.5%)	38 (100%)	5 (62.5%)	6 (28.6%)
Liver histology				
F0 (minimal)	1	5	0	3
F1	21	15	2	15
F2	15	1	3	2
F3	2	2	1	1
F4 (cirrhosis)	2	2	2	0
AST (IU/L) (median, range)	27 (17-37)	22 (10-37)	24 (16-28)	21 (14-34)
ALT (IU/L)	34 (18-50)	20 (6-48)	21 (12-48)	20 (8-50)
Platelet ($\times 1000/mm^3$)	169 (94-274)	185 (96-311)	156 (86-216)	196 (128-296)
ICG_{R15} (%)	12 (6-24)	9.5 (3-33)	9 (7-13)	12 (5-72)
Albumin (g/dL)	4.3 (3.6-5.1)	4.4 (3.9-5.4)	4.15 (3.9-5.4)	4.4 (3.7-4.9)
Bilirubin (mg/dL)	1.0 (0.2-1.5)	1.1 (0.4-1.8)	0.75 (0.3-1.1)	0.8 (0.3-1.9)
Prothrombin (%)	89 (67-111)	100 (70-108)	100 (76-101)	100 (73-101)
HBV DNA (Log_{10} copy/ml) (median, 25-75 percentiles)	4.6 (4.2-5.3)	4.2 (3.1-5.4)	3.45 (<2.6-4.8)	3.9 (<2.6-4.8)
HBV genotype				
A	0	0	0	3
B	11	0	0	7
C	29	25	7	10
Others/unidentified	1	0	1	1

Group A: History of ALT elevation in the past.
Group B: Advanced liver disease in family members.
Group C: Possible advanced disease on ultrasonography or blood examination.
Group D: Simple desire for thorough examination

Activation of Hepatitis and Its Prediction

Twenty-five patients showed twice as high as normal ALT concentration, and the other 27 patients showed activation of hepatitis with ALT elevation followed by IFN therapy. Consequently, a total of 52 patients (54.7%) showed an abnormal ALT concentration during a median observation period of 10.2 years. The cumulative rate of hepatitis activation was 34.7% at the end of the 3rd year, 45.4% at the 5th year, and 55.7% at the 10th year.

The incidence of hepatitis activation was assessed in the following 4 groups according to the initial HBV DNA concentration: $<10^4$ copies/mL (n = 33), 10^4 to $10^{4.9}$ (n = 34), 10^5 to $10^{5.9}$ (n = 18), and $\geq 10^6$ (n = 10). Cumulative hepatitis flare-up rates in each group were 12.0%, 41.2%, 44.4%, and 70.0%, respectively, at the end of the 3rd year; 12.0%, 61.8%, 55.5%, and 80.0%, respectively, at the 5th year; and 18.6%, 70.3%, 80.0%, and 80.0%, respectively, at the 10th year (Figure 2). The initial DNA load correlated significantly with future hepatitis activation rate ($P < .0001$). Average DNA concentration during the initial 5 years was closely associated with hepatitis activation for the period: 52 patients with ALT elevation had a high average DNA value of $10^{3.3}$ (median, range: $<10^{2.6}$-$10^{5.3}$), and 43 patients without ALT elevation showed $10^{4.8}$ (median, range: $10^{2.9}$->$10^{7.6}$).

The hepatitis activation rates in groups A, B, C, and D were 70.7%, 36.0%, 25.0%, and 14.3%, respectively, at the end of the 5th year; and 81.7%, 45.1%, 25.0%, and 26.7%, respectively, at the end of the 10th year (Figure 3). The ALT flare-up rate in patients of group A was the highest ($P < .0001$).

Multivariate proportional hazard analysis disclosed that initial HBV DNA concentration, a past history of ALT elevation, and histological staging were significantly associated with reactivation of hepatitis in the follow-up (Table 3). Patients with HBV DNA of 10^4 to $10^{5.9}$ copy/mL had a high hazard ratio of 5.73, and those with 10^6 copy/mL or higher showed a hazard ratio of 10.43, compared with those with lower DNA concentration $<10^4$. Past history of ALT elevation also was associated with hepatitis activation with a hazard ratio of 3.62. Histological staging of F2 or higher also showed a high risk for carcinogenesis with a hazard ratio of 3.06.

Relationship between HBV DNA in First 2 Years and Hepatitis Activation

The prediction of hepatitis activation was analyzed using HBV DNA level in the first 2 years (DNA measurements at the time of observation, 6th, 12, and 24th month). Using various cutoff values of DNA, patients were classified into