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A Long-Term Glycyrrhizin Injection Therapy Reduces Hepatocellular Carcinogenesis Rate in Patients with Interferon-Resistant Active Chronic Hepatitis C: A Cohort Study of 1249 Patients

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To elucidate the influence of a glycyrrhizin therapy on hepatocarcinogenesis rate in interferon (IFN)-resistant hepatitis C, we retrospectively analyzed 1249 patients with chronic hepatitis with or without cirrhosis. Among 346 patients with high alanine transaminase value (twice or more of upper limit of normal), 244 patients received intravenous glycyrrhizin injection and 102 patients did not, after judgment of IFN resistance. Crude carcinogenesis rates in the treated and untreated group were 13.3%, 26.0% at the 5th year, and 21.5% and 35.5% at the 10th year, respectively ($P = .0210$). Proportional hazard analysis using time-dependent covariates disclosed that glycyrrhizin treatment significantly decreased the hepatocarcinogenesis rate (hazard ratio 0.49, 95% confidence interval 0.27–0.86, $P = .014$) after adjusting the background features with significant covariates. Glycyrrhizin injection therapy significantly decreased the incidence of hepatocellular carcinoma in patients with IFN-resistant active chronic hepatitis C, whose average aminotransferase value was twice or more of upper limit of normal after interferon.

KEY WORDS: chronic hepatitis; hepatitis C virus; glycyrrhizin; hepatocellular carcinogenesis; cancer prevention.

Until recently, hepatitis C virus (HCV) has been reported to be a causative agent of hepatocellular carcinoma (HCC) aside from hepatitis B virus (1–5). In our cohort studies of Japanese patients with HCV-related cirrhosis (5), the cumulative appearance rates of HCC at the 5, 10, and 15 years were 21.5%, 53.2%, and 75.2%, respectively. The carcinogenesis rate was higher in those patients with cirrhosis caused by HCV than in those with hepatitis B virus-related cirrhosis.

Interferon (IFN) is effective in eliminating HCV in some patients with chronic hepatitis C (6–8) and cirrhosis (9–11), and in reducing hepatocellular carcinogenesis rate through suppression of necro-inflammatory process and reduction of serum alanine transaminase (ALT). Kasahara *et al.* (6) reported that sustained normal ALT value after IFN therapy was significantly associated with a decreased hepatocellular carcinogenesis rate in patients with chronic hepatitis C. Our data (7) also demonstrated an anticarcinogenic activity of IFN in patients who attained normal ALT

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level after the therapy compared with IFN-treated patients without normalization of ALT.

Oka *et al.* (12) reported in a randomized controlled trial that a kind of medicinal herb, *Sho-saiko-to*, significantly decreased hepatic carcinogenesis rate in patients with HBsAg-negative cirrhosis. Tarao *et al.* (13) showed that HCC appearance rate was significantly higher in HCV-related cirrhotic patients with a high ALT value of 80 IU/mL or more than that of those with lower ALT value (<80 IU/mL), and also suggested that treatment of cirrhosis and prevention of HCC should be directed to suppress the necro-inflammation of HCV-related hepatitis. A glycyrrhizin-containing product, Stronger Neo-Minophagen C (SNMC; Minophagen Pharmaceutical Co. Ltd., Tokyo, Japan), is widely used in Japan for suppression of hepatitis activity and for prevention of disease progression in patients with hepatitis B virus and HCV-induced chronic hepatitis. Glycyrrhizin has been reported to suppress hepatic inflammation with an effect to improve the elevated ALT levels and histologic findings of the liver (14–17). We reported its favorable effect on hepatocellular carcinogenesis in those patients with chronic hepatitis C who received glycyrrhizin for more than 10 years (18).

To elucidate whether glycyrrhizin suppress the carcinogenesis rate in patients with IFN-resistant chronic hepatitis C, we retrospectively assessed a cohort of 1249 patients without sustained virologic response (SVR) after IFN therapy.

PATIENTS AND METHODS

Study Population. A total of 1249 consecutive Japanese patients with chronic hepatitis C with or without cirrhosis were examined, who did not show an SVR of HCV-RNA under IFN therapy. Sera of the patients showed positive anti-HCV (second-generation anti-HCV kit, enzyme-linked immunosorbent assay, Dainabot, Tokyo, Japan), positive HCV-RNA (nested PCR), and negative hepatitis B surface antigen (HBsAg; radioimmunoassay, Dainabot). Anti-HCV and HCV-RNA were assayed using stored frozen sera at –80°C. There were 778 men and 471 women aged 18–81 years, with a median age of 53 years in the study. They were diagnosed as having liver cirrhosis by peritoneoscopy, liver biopsy, or both between 1987–2002.

All the patients had a history of receiving once or more times of IFN therapy: 1179 patients underwent IFN monotherapy only and the other 70 patients had received an IFN plus ribavirin combination therapy before the entry of this study. A total of 347 patients showed a normal ALT for at least 6 months after cessation of IFN (biochemical responders), and the other 902 patients abnormal ALT at 6 months after the end of IFN therapy. A retrospective cohort study was performed using these 1249 consecutive patients with chronic hepatitis or cirrhosis who failed to show SVR.

Glycyrrhizin Treatment. Glycyrrhizin therapy was performed using intravenous injection of SNMC. The preparation contains 0.2% (4 mg) glycyrrhizinic acid as the main active constituent, 2% (40 mg) glycine, and 0.1% (2 mg) L-cysteine in 20-mL ampoules.

Of 1249 patients with IFN-resistant chronic liver disease, 453 patients underwent glycyrrhizin injection therapy and the remaining 796 patients did not receive the therapy until the end of observation. The purpose of the introduction of the glycyrrhizin injection therapy was to suppress elevated ALT and to prevent disease progression in all the patients. Of the 453 patients, 129 (28.5%) received a daily dose of 40–60 mL of SNMC (80–120 mg as glycyrrhizin) and 324 (71.5%) received 80–100 mL (160–200 mg as glycyrrhizin). A total of 110 patients received the treatment for less than 2 years and 107 patients continued the therapy for 2–4 years, 132 patients for 4–6 years, and the remaining 104 patients for 6 years or longer. When the treatment was regarded as effective from the viewpoint of ALT levels, treatment was usually continued for a period as long as possible. As a result, a median daily dose of 100 mL of SNMC was administered 3 times a week during a median period of 4.3 years (range, 0.1–14.5 years) in the treated group.

Two (0.44%) of 453 treated patients were withdrawn from the glycyrrhizin injection therapy because of side effects: 1 because of hypertension and 1 from skin rash.

Background and Laboratory Data of Patients With and Without Glycyrrhizin Therapy. Table 1 summarizes the profiles and data of the patients at the time of diagnosis of chronic hepatitis with or without cirrhosis. The male/female ratio was not different between the 2 groups. Median age was older by 2 years in the treated group than in the untreated group ($P < .001$). Results of histologic staging of liver disease were classified according to Desmet *et al.* (19). F1stage hepatitis was found significantly more often in the untreated group than in the glycyrrhizin group ($P < .001$, χ^2 test). Both AST and ALT median levels were significantly higher in the treated group than in the untreated group ($P < .001$). HCV subtype was analyzed by the immunoserologic typing method with a commercial kit (Kokusai Diagnostic Corporation, Kobe, Japan): serologic group 1 indicated genotypes 1a and 1b, and group 2 included 2a and 2b subtypes. The rate of HCV serologic group 1 was significantly higher in the glycyrrhizin group than in the untreated group ($P = .032$).

Follow Up. Follow-up of the patients was made monthly after the judgment of IFN-resistance by monitoring hematologic, biochemical, and virologic data. Imaging diagnosis with ultrasonography (US) and/or computed tomography (CT) was made 3 or more times per year in a majority of patients with cirrhosis and once a year in patients without cirrhosis. Angiographic study was performed only when HCC was strongly suspected on US or CT.

When angiography revealed a characteristic hypervascular nodule suggesting a specific finding for HCC, no histologic examination was made in a majority of these patients. An increasing trend of tumor markers was also taken into account in establishment of the diagnosis of HCC. Microscopic examination through a fine needle biopsy was also performed in patients whose angiogram did not show a typical image of HCC.

The number of cases lost to follow-up was 121 (9.7%): 28 patients (6.2%) in the glycyrrhizin group and 93 (11.7%) in the untreated group. Because the outcomes regarding appearance of HCC were not identified in these patients, they were dealt as censored data in the following statistics (20). Death unrelated to HCC was also classified as withdrawal and regarded as a censored case. The median observation period of the total number of patients was 5.7 years with a range of 0.1–16.1 years. Because

TABLE 1. PATIENT PROFILES AND LABORATORY DATA AT TIME OF JUDGMENT OF IFN RESISTANCE

	Glycyrrhizin Group (n = *453*)	*Untreated Group* (n = *796*)	P
Demographics			
Gender (M/F)	283/170	495/301	.92
Age (y)*	54 (25–81)	52 (18–77)	<.001
Observation period (y)*	8.3 (0.1–16.1)	5.1 (0.1–13.1)	<.001
Liver histology			
F1	146 (32.7%)	502 (64.0%)	<.001
F2	193 (43.3%)	192 (24.5%)	
F3	38 (8.5%)	52 (6.6%)	
F4	69 (15.5%)	38 (4.8%)	
Laboratory data*			
Aspartic transaminase (IU/L)*	81 (19–446)	54 (11–355)	<.001
ALT (IU/L)*	122 (12–630)	83 (10–822)	<.001
HCV serologic group 1 (1a or 1b)	360 (80.2%)	582 (73.7%)	.032
Group 2 (2a or 2b)	73 (16.3%)	165 (20.9%)	
Others	16 (3.6 %)	43 (5.4%)	

*Expressed as median (range).

many patients receiving glycyrrhizin therapy migrated from the untreated group to the treated group, observation period of the untreated group was significantly shorter than that of the treated group (see Table 1). The date of the last follow-up for this study was September 1, 2003.

Statistical Analysis. Nonparametric procedures were employed for the analysis of background characteristics of the patients, including Mann-Whitney U-test and χ^2 method. HCC appearance rates were calculated from the time period between the judgment of IFN ineffectiveness and appearance of HCC in each group, using Kaplan-Meier technique (20). The differences in carcinogenesis curves were tested using the log-rank test. Independent factors associated with the appearance rate of HCC were studied using time-dependent Cox regression analysis (21). An interaction term of IFN treatment and "waiting time" to the therapy was introduced in the analysis as a time-dependent covariate. The independence of treatment factor from "waiting time" was also confirmed by log-minus-log plot of proportional hazard model. Several variables were transformed into categorical data consisting of 2–3 simple ordinal numbers to estimate each hazard ratio. All factors found to be at least marginally associated with liver carcinogenesis ($P < .15$) were tested by the multivariate Cox proportional hazard model. A P-value of less than .05 was considered to be significant. All data analysis was performed using the computer program SPSS version 11 (22).

TABLE 2. INDEPENDENT RISK FACTORS AFFECTING HEPATOCELLULAR CARCINOGENESIS

Factors	*Category*	*Risk Ratio (95 % CI)*	P
Fibrotic stage			
	F1	1	
	F2–3	2.94 (1.20–7.21)	.018
	F4 (cirrhosis)	9.21 (3.73–22.8)	<.001
Gender			
	1: Female	1	
	2: Male	2.80 (1.35–5.81)	.006
Glycyrrhizin injection			
	1: No	1	
(SNMC)*	2: Yes	0.49 (0.27–0.86)	.014

Time-dependent Cox proportional hazard analysis. *SNMC, Stronger Neo-Minophagen C (herbal medicine containing glycyrrhizin).

RESULTS

Initial Aminotransferase and Carcinogenesis Rates

Patients with and without glycyrrhizin therapy were classified into 6 categories according to average ALT value during the first year after cessation of IFN therapy: group 1, normal ALT; group 2, <1.5 times of upper limit of normal (ULN); group 3, 1.5–2 times ULN; group 4, 2–3 times ULN; group 5, 3–4 times of ULN; and group 6, >4 times ULN. Hepatocellular carcinogenesis rates were 2.5%, 5.0%, 8.1%, 11.8%, 12.0%, and 12.7% at the end of 5 years and 6.6%, 7.2%, 19.6%, 15.1%, 21.0%, and 39.3% at 10 years, respectively (Figure 1). There was a significant statistical difference among the 6 subgroups (log-rank test, $P < .0001$). The higher the average ALT, the higher the carcinogenesis rate was.

Influence of Glycyrrhizin on Carcinogenesis in Patients With High Aminotransferase

Glycyrrhizin therapy was usually performed in patients with a high ALT value and high hepatitis activity. In this retrospective study, average ALT values were significantly different between the treated and untreated groups: group 1, normal average ALT was found in 38 among patients with glycyrrhizin therapy and in 188 among patients without therapy; in group 2, ALT <1.5 times of ULN was found in 42 and 331; in group 3, 1.5–2 times ULN in 84 and 138; in group 4, 2–3 times ULN in 143 and 92; in group 5, 3–4 times in 53 and 29; and in group 6, ALT

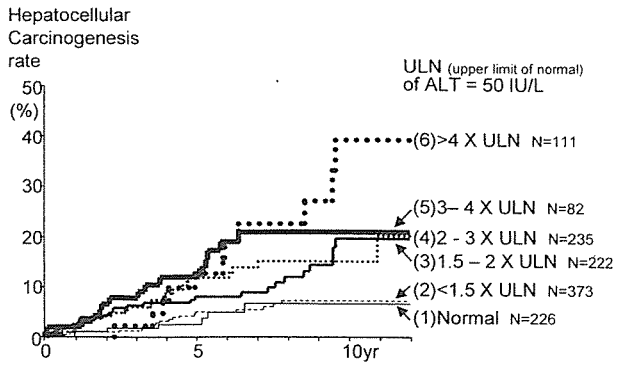

Fig 1. Carcinogenesis rates according to initial ALT values classified into six groups: (1) normal ALT, (2) <1.5 times ULN, (3) 1.5–2 times ULN, (4) 2–3 times ULN, (5) 3–4 times ULN, and (6) >4 times of ULN. The higher the average ALT, the higher the carcinogenesis rate was.

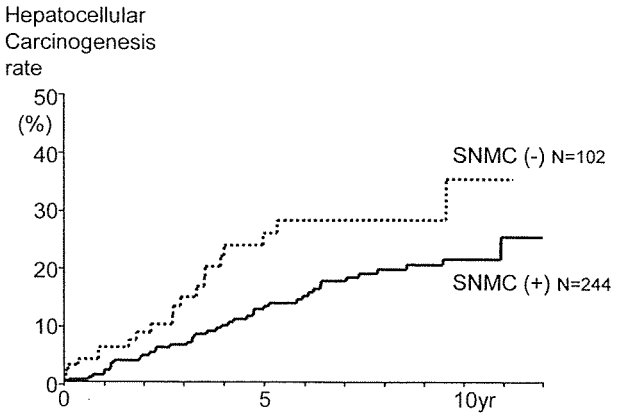

Fig 2. Carcinogenesis rates in patients with high average ALT values of twice or more of ULN, excluding those patients with biochemical responders who continued a normal ALT value at least 6 months just after IFN therapy. The carcinogenesis rate of glycyrrhizin-treated group was significantly lower than that of the untreated group (log-rank test, $P = .0210$).

>4 times ULN in 93 of the glycyrrhizin group and 18 of the untreated group. The rate of a high ALT value of twice or more of ULN in the glycyrrhizin treated group (64.2%, 289/453) was significantly higher than that of the untreated group (16.2%, 129/796).

Of the 418 patients with a high average ALT in both groups, 68 patients showed a normal ALT value for at least 6 months just after IFN therapy (biochemical response). Because biochemical response with normal ALT for a certain period after IFN was likely to affect carcinogenesis rates in those patients, biochemical responders were excluded in the following analyses about the influence of glycyrrhizin on carcinogenesis: after all, 244 patients with glycyrrhizin therapy and the 102 patients without therapy were assessed.

Cumulative hepatocellular carcinogenesis rates were calculated in these 346 patients with a high average ALT values, excluding biochemical responders from both groups. Carcinogenesis rates in the glycyrrhizin group and the untreated group were 6.5% and 13.3% at the end of year 3, 13.3% and 26.0% at the end of year 5, 17.7% and 28.3% at the end of year 7, and 21.5% and 35.5% at year 10, respectively (Figure 2). In the stratified and selected patient group, the carcinogenesis rate of glycyrrhizin-treated group was significantly lower than that of the untreated group (log-rank test, $P = .0210$).

Carcinogenesis Rates According to Hepatitis Staging

Crude carcinogenesis rates were compared between the groups, according to each hepatitis stage. In F1 stage chronic hepatitis, hepatocellular carcinogenesis rates in the glycyrrhizin group ($n = 82$) and the untreated group ($n = 32$) were 1.4% and 4.2% at year 5 and 7.0% and 12.1% at 10 years, respectively (Figure 3A). In F2–3 stage chronic hepatitis, hepatocellular carcinogenesis rates in the glycyrrhizin group ($n = 121$) and the untreated group ($n = 53$) were 14.8% and 28.4% at the end of year 5, and 21.5% and 38.6% at year 10, respectively (Figure 3B). In patients with F4 stage chronic hepatitis (cirrhosis), hepatocellular carcinogenesis rates in the glycyrrhizin group ($n = 38$) and the untreated group ($n = 15$) were 35.2% and 58.0% at the end of year 5, and 57.2% and 58.0% at year 10, respectively (Figure 3C).

In each fibrotic stage of hepatitis, carcinogenesis rates were lower in the glycyrrhizin group than in the untreated group, but statistical significance was not obtained owing to shortage of patient number in these stratified groups.

Aminotransferase Activity Before and After Glycyrrhizin Therapy

ALT values in the patients with glycyrrhizin treatment were serially assessed in those patients who began the therapy after they had shown a high average ALT value (Figure 4). Median value of ALT at the beginning of the glycyrrhizin therapy was 150 IU/L (25th percentile 120, 75th percentile 221), 72 IU/L at month 3, 70 IU/L at month 6, and 64 IU/L (25th percentile 48, 75th percentile 93) at month 12, respectively. ALT value significantly decreased after the initiation of glycyrrhizin injection therapy.

Factors Affecting Carcinogenesis Rates in Active Hepatitis and Cirrhosis

In the selected patients with active hepatitis with an average ALT value of twice ULN or higher, multivariate analysis was performed to explore associating factors with carcinogenesis, using time-dependent Cox proportional hazard model. Time between the judgment of IFN

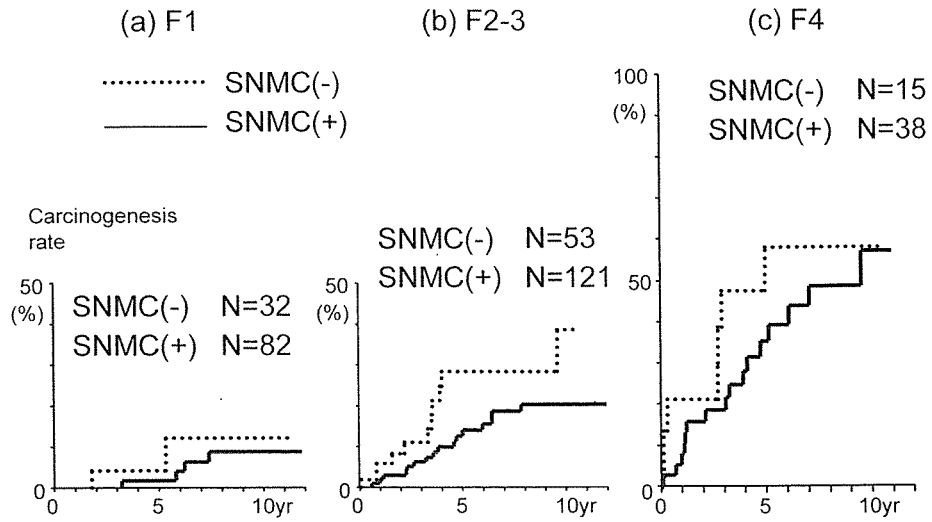

Fig 3. Carcinogenesis rates according to hepatitis staging: (a) F1 stage hepatitis, (b) F2–F3 stage hepatitis, and (c) F4 or cirrhotic stage. In each fibrotic stage of hepatitis, carcinogenesis rates were lower in the glycyrrhizin group than in the untreated group.

ineffectiveness and initiation of glycyrrhizin therapy was set as a time-dependent variable to clarify the significance of glycyrrhizin therapy in the clinical course of HCV-related chronic liver diseases. Patients with biochemical response with a normal ALT value sustained for at least 6 months after IFN therapy were also excluded from the analysis.

In multivariate analysis, following 3 factors influenced the carcinogenesis: fibrotic staging, gender ($P = .006$), and glycyrrhizin therapy ($P = .014$). When a hazard of F1 stage fibrosis for carcinogenesis was set as 1 in the model, hazard ratio of F2–F3 stage fibrosis was calculated as 2.94 ($P = .018$), and that of F4 (cirrhosis) was estimated as 9.21 ($P < .001$). Similarly, the hazard ratio for carcinogenesis of male gender was 2.80, and use of glycyrrhizin independently decreased the carcinogenesis rate in patients with active chronic hepatitis after IFN therapy. Following factors did not affect the HCC appearance rate significantly: age, association of diabetes mellitus, serologic grouping of HCV, HCV-RNA concentration, AST, ALT at the time before IFN therapy, and bilirubin.

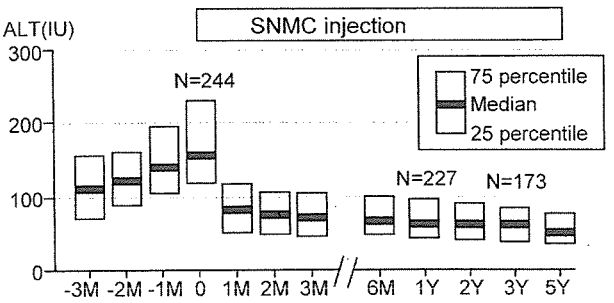

Fig 4. Aminotransferase activity before and after glycyrrhizin therapy. ALT value significantly decreased after the initiation of glycyrrhizin injection therapy.

DISCUSSION

IFN is effective in patients with chronic liver disease caused by HCV, from the viewpoints of anti-inflammatory effect and cancer prevention (6–11). Although the carcinogenesis rate is noticeably reduced when aminotransferase becomes normal with or without HCV-RNA eradication (6–8) after the therapy, the rate of normalization of ALT after IFN therapy is approximately half of patients with high viral load and group 1 HCV-subtype.

This retrospective study was undertaken to evaluate whether long-term glycyrrhizin injection therapy could decrease hepatocellular carcinogenesis rate in patients with IFN-resistant HCV-related chronic hepatitis and cirrhosis. Because it requires at least 5 years to show a statistical difference in carcinogenesis rate from hepatitis or cirrhosis between glycyrrhizin-treated and "untreated" groups, a prospective randomized trial using untreated control patients is difficult from both ethical and medical viewpoints in Japan, where glycyrrhizin injection therapy is covered by standard medical insurance and is already regarded as a usual choice of therapy as a salvaging procedure for IFN-ineffective patients. We, therefore, attempted to carry out this retrospective cohort study to prove an anticarcinogenic activity of glycyrrhizin, with a statistical adjustment using possible covariates explored in multivariate analysis.

Because glycyrrhizin injection therapy was chiefly performed for patients with a high ALT value and because cancer prevention was meaningful in just those patients with a high carcinogenesis risk with high hepatitis activity, we analyzed the role of a long-term glycyrrhizin injection therapy in the patients with a high ALT value. The treated group consisted of significantly more numbers of patients with a high ALT value of twice or more of ULN. When carcinogenesis rates were assessed only in those patients with a high ALT value of twice or more ULN excluding biochemical responders, the rate of the treated group became significantly higher than that of the untreated group ($P = .021$). The cancer preventive effect of glycyrrhizin in IFN-resistant patients was also confirmed by time-dependent Cox proportional analysis that adjusted the background features of the retrospective cohort (hazard ratio = 0.49, $P = .014$). We previously reported a study focused on the anticarcinogenic action of glycyrrhizin for patients with chronic hepatitis C, but the pilot study only demonstrated that 10 years or longer treatment with glycyrrhizin ($n = 84$) could suppress the carcinogenesis rate (18). Current study dealing with a large cohort ($n = 1249$) showed that glycyrrhizin injection therapy significantly decreased carcinogenesis rate irrespective of the length of treatment when comparison was made in a selected patient cohort with high hepatitis activity.

Although a statistically significant difference was not shown for a lack of sufficient patient number in subgroups of chronic hepatitis and cirrhosis, this study also demonstrated that glycyrrhizin was effective not only in chronic hepatitis but also in cirrhosis. Considering that liver cirrhosis generally shows a resistance to IFN treatment, our current study demonstrated encouraging results from the viewpoint of HCC prevention. When IFN therapy was attempted in 7 patients with decompensated cirrhosis by Nevens *et al.* (23), complications sometimes occurred in these patients, including variceal bleeding, aggravation of ascites or encephalopathy, development of pneumonia, and recurrence of spontaneous bacterial peritonitis or gastric ulcer bleeding. Because patients with cirrhosis usually showed lower platelet and leukocyte counts than those with chronic hepatitis and because cirrhotic patients tended to show deterioration with a large dose of IFN, glycyrrhizin therapy proved to be a useful alternative of therapy. Intermittent long-term glycyrrhizin therapy was well tolerated with withdrawal of only 2 patients (0.44%).

Because carcinogenesis is not a single-step event but a complex, multistep process, the exact mechanism of the glycyrrhizin activity in suppression of liver carcinogenesis remains unknown. One of the principal roles of long-term administration of glycyrrhizin in decreasing the carcinogenesis rate is considered to be anti-inflammatory, which blocks the active carcinogenic process of continuous hepatic necro-inflammation and cell damage. In the treated group, median ALT values markedly decreased after initiation of the glycyrrhizin injection, suggesting that pathologic process of hepatocyte necrosis or apoptosis was significantly suppressed by glycyrrhizinic acid. The importance of the action of amino acids, glycine and cysteine contained in SNMC has not been completely explained, but they have been demonstrated to suppress increased aldosterone levels that are induced by glycyrrhizinic acid. Tarao *et al.* (24) reported that high aminotransferase level resulted in an increase of an HCC recurrence rate in patients with HCC. From the viewpoint of these anti-inflammatory activities, SNMC may be considered to only postpone the time of HCC appearance in the clinical course of cirrhosis. Because the entire process of hepatocellular carcinogenesis from the initial transformation of a hepatocyte to a detectable growth of cancer is considered to take at least several years, the influence of glycyrrhizin on the carcinogenesis rate will not be evaluated in a short period. Although several reports suggested a relationship of anti-hepatitis B core antibody or hepatitis B surface antibody with carcinogenesis (25–27), we could not show the association because of insufficient available data.

Because current data were obtained from a retrospective cohort analysis, dose of glycyrrhizin per time, times of injection per week, and duration of therapy varied in each patient in the treated group. To elucidate the cancer preventive effect of glycyrrhizin therapy in active HCV-related liver disease, we should further stratify the treated patients or perform much more detailed statistical procedures. Future studies should, therefore, aim at defining the basic oncogenic mechanisms and roles of long-term administration of glycyrrhizin in carcinogenesis in patients with cirrhosis caused by HCV.

In conclusion, a long-term intermittent glycyrrhizin therapy for a few years or more successfully reduced hepatocellular carcinogenesis in patients with HCV-related chronic liver disease. A randomized control study with a larger number of cases, with or without glycyrrhizin therapy, is expected to confirm the effectiveness of this therapy.

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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

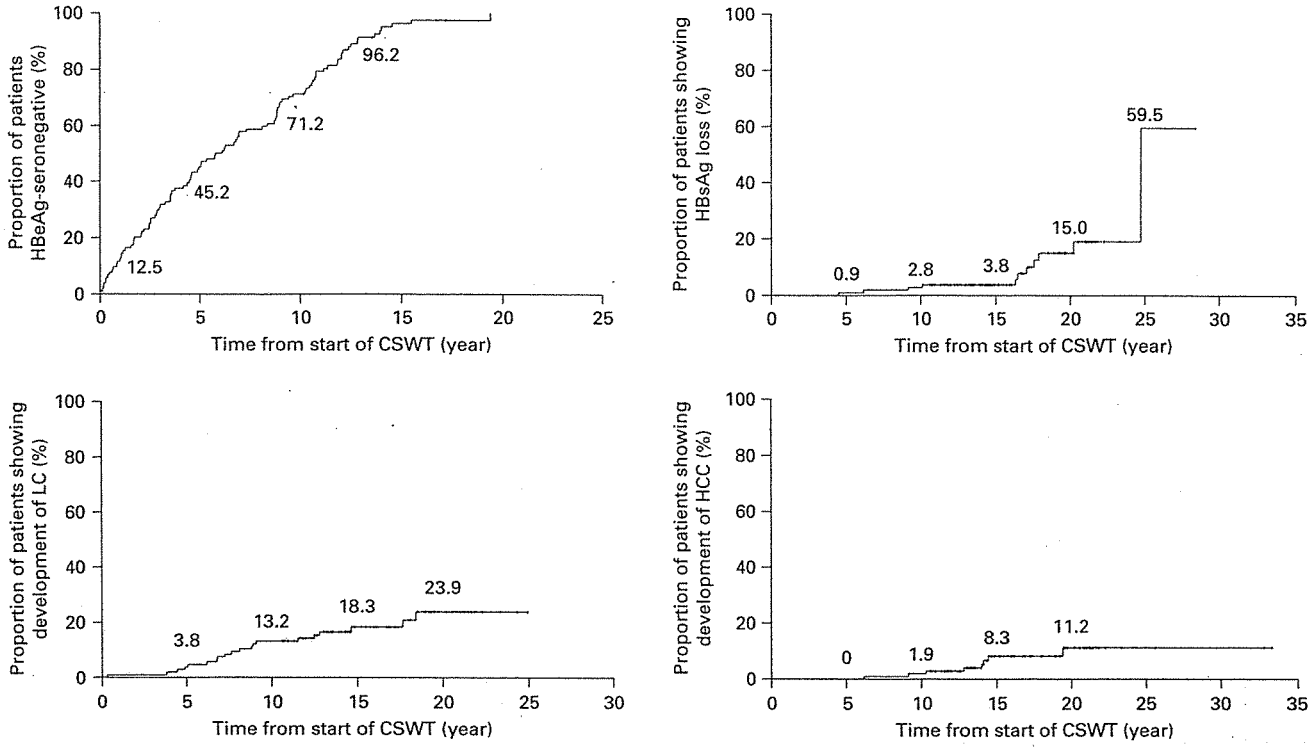

Fig. 1. Time to HBeAg seronegativity, HBsAg loss, development of liver cirrhosis (LC) and hepatocellular carcinoma (HCC) after corticosteroid withdrawal therapy (CSWT) calculated for all patients who participated in the study.

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).

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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

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

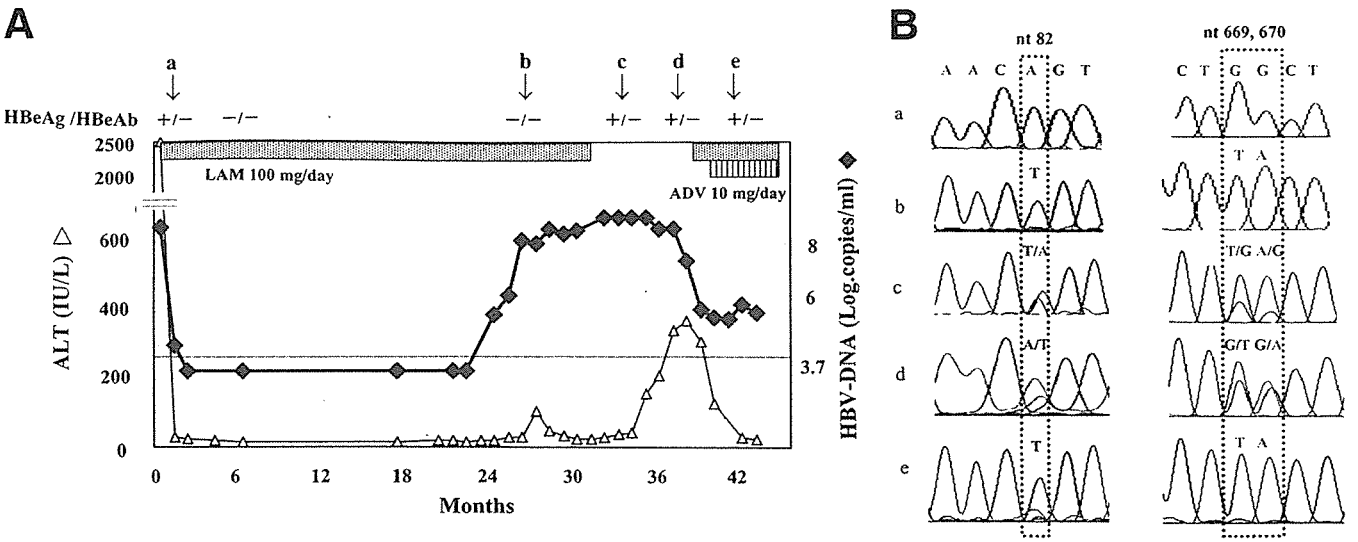

FIG. 1. (A) Clinical course of a patient who developed breakthrough without emergence of YMDD mutants during lamivudine therapy. Arrows a to e indicate time points of serum sampling for direct sequencing and RFLP PCR. (B) Nucleotide sequence analysis of the reverse transcriptase/polymerase gene of hepatitis 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.

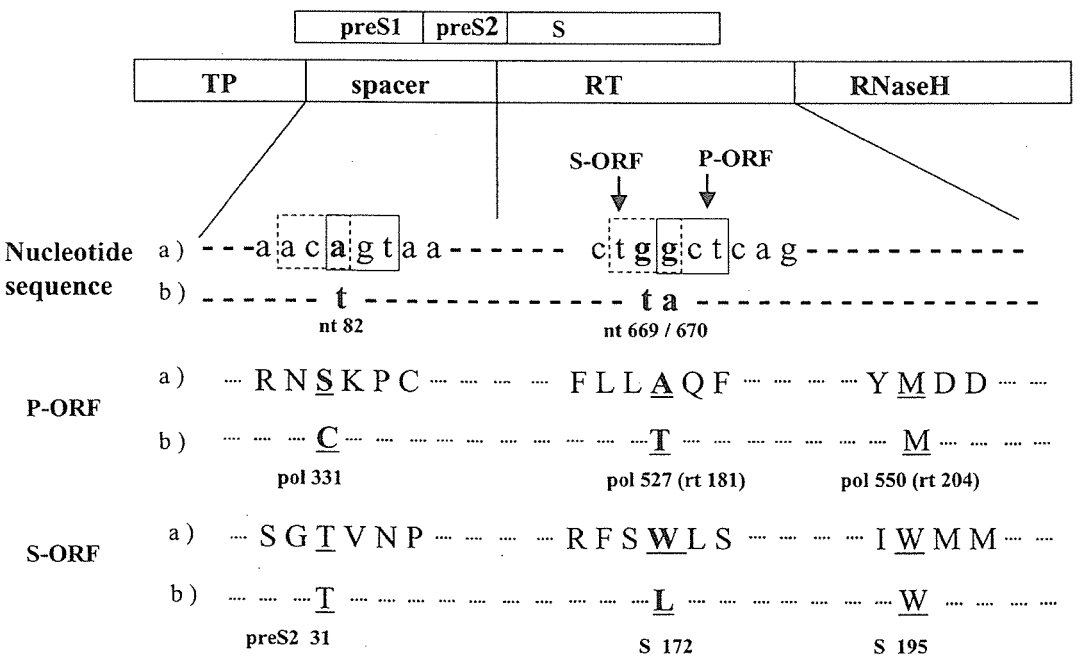

FIG. 2. Comparison of nucleotide sequences and amino acid sequences of two overlapping open reading frames, reverse transcriptase/polymerase and the HBs gene of the hepatitis B virus, before and after viral breakthrough. Sequences obtained from serum samples before (a) and after (b) breakthrough were 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.

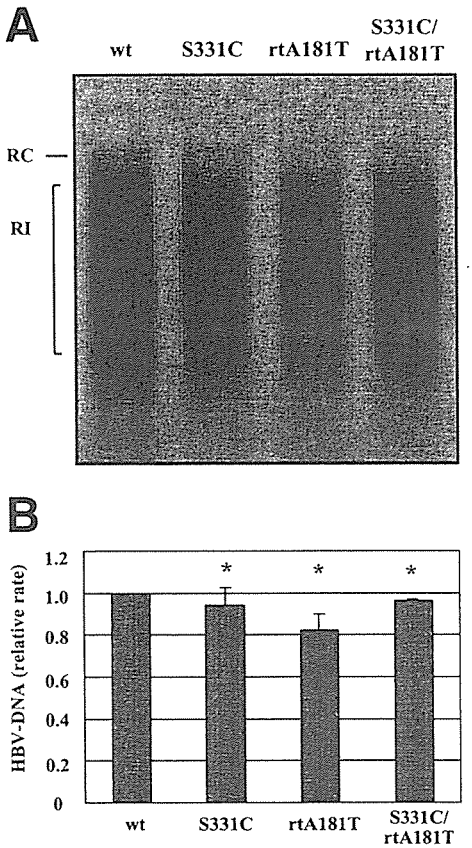

FIG. 3. Replication ability of wild-type HBV and three mutants (S331C, rtA181T, and S331C/rtA181T). Plasmids containing 1.4-genome-length HBV were transiently transfected into HepG2 cells. (A) The replicative intermediates were analyzed by Southern blot hybridization. Core-associated replicative intermediates 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

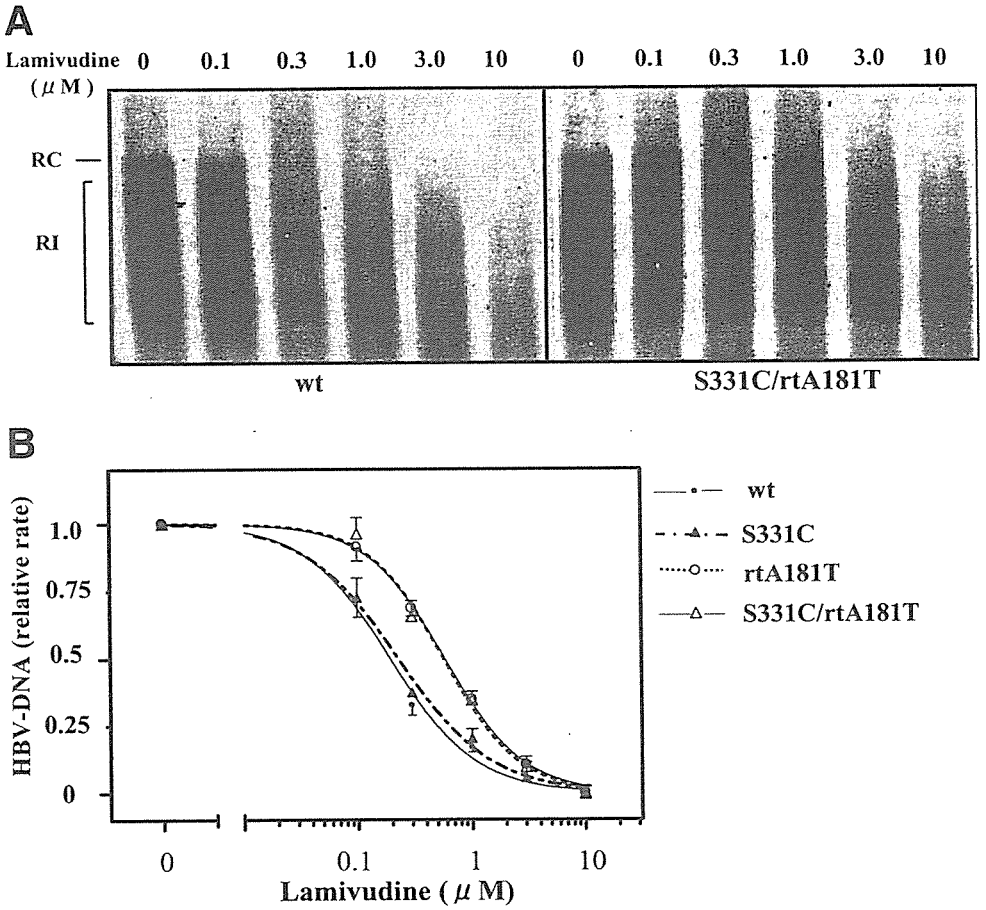

FIG. 4. In vitro analyses of susceptibility of wild-type HBV and three mutants (S331C, rtA181T, S331C/rtA181T) to lamivudine after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4-genome-length HBV and treated with the indicated amount of lamivudine. (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).

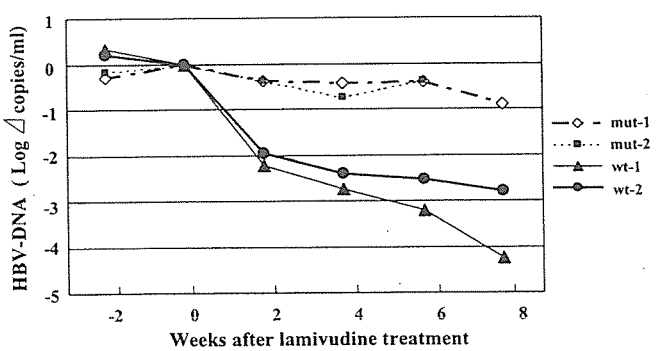

FIG. 5. In vivo analyses of the effect of lamivudine on wild-type and S331C/rtA181T mutant HBV. Four human hepatocyte-chimeric mice were inoculated with serum samples containing wild-type or mutant HBV. One of the animals fed with lamivudine died 6 weeks after the beginning of therapy.

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.

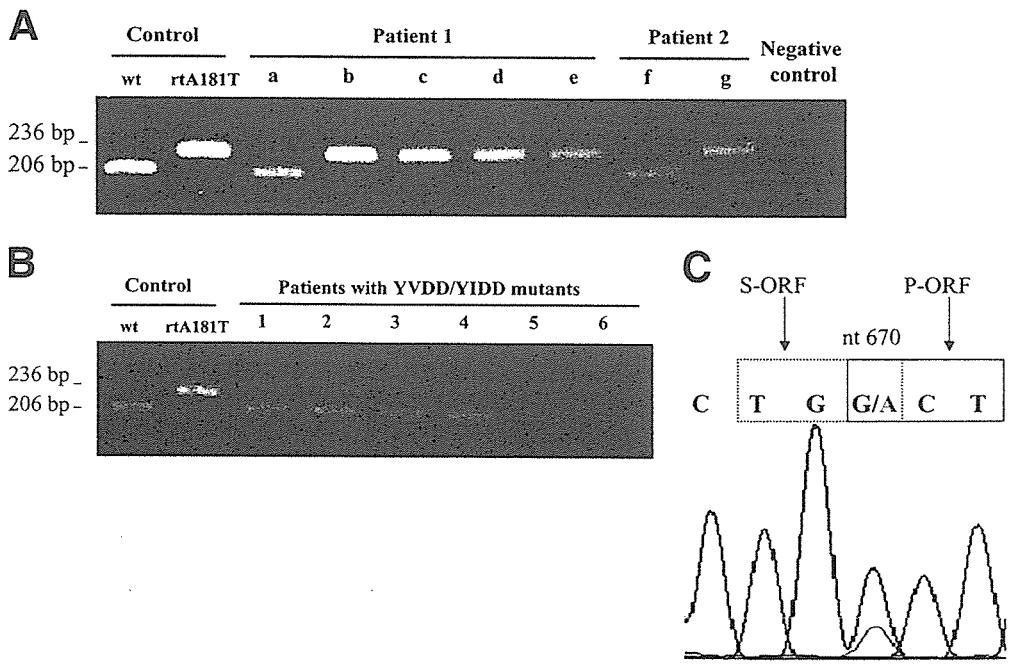

FIG. 6. Detection of the rtA181T mutant by RFLP PCR assay. PCR-amplified DNA fragments were treated with EspI, which digests only wild-type sequences, and separated in a 3.5% agarose gel. (A) Agarose gel electrophoresis of RFLP PCR products. Wild-type and rtA181T mutant plasmids were used as controls. 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).