Table 2 (*Continued*)

Parameter	Category	Group	Total number of patients (number)	No. of patients with HCC (number)	Average follow-up period (year)	Adjusted incidence of HCC (%/year)
Stage of fibrosis	F0	Lamivudine group	12	0	7.2	0.00
		Control group	49	3	5.7	1.07
	F1	Lamivudine group	201	6	6.0	0.50
		Control group	721	29	6.7	0.60
	F2	Lamivudine group	167	8	4.7	1.02
		Control group	524	38	5.8	1.25
	F3	Lamivudine group	171	11	4.0	1.61
		Control group	491	61	6.0	2.07
	F4	Lamivudine group	98	6	3.6	1.70
		Control group	331	99	6.2	4.82
	Unknown	Lamivudine group	8	0	6.7	0.00
		Control group	22	9	8.3	4.93
HBeAg	–	Lamivudine group	280	10	4.2	0.85
		Control group	723	83	6.4	1.79
	+	Lamivudine group	355	19	5.3	1.01
		Control group	1272	134	6.0	1.76
	Unknown	Lamivudine group	22	2	6.2	1.47
		Control group	143	22	7.4	2.08
HBeAb	–	Lamivudine group	418	19	4.9	0.93
		Control group	1330	137	6.0	1.72
	+	Lamivudine group	215	10	4.7	0.99
		Control group	642	75	6.3	1.85
	Unknown	Lamivudine group	24	2	6.1	1.37
		Control group	166	27	7.4	2.20
Albumin (g/dL)	<4.0	Lamivudine group	257	19	4.5	1.64
		Control group	619	113	5.7	3.20
	4.0≦	Lamivudine group	372	9	4.9	0.49
		Control group	1322	90	6.1	1.12
AST (IU/L)	<50	Lamivudine group	187	7	5.7	0.66
		Control group	905	82	6.1	1.49
	50≦ and <100	Lamivudine group	200	14	4.7	1.49
		Control group	572	81	5.9	2.40
	100≦ and <200	Lamivudine group	142	7	5.1	0.97
		Control group	367	31	6.2	1.36
	200≦	Lamivudine group	64	2	4.4	0.71
		Control group	179	15	6.0	1.40
ALT (IU/L)	<50	Lamivudine group	117	5	4.7	0.91
		Control group	570	69	6.1	1.98
	50≦ and <100	Lamivudine group	155	7	4.9	0.92
		Control group	506	60	5.8	2.04
	100≦ and <150	Lamivudine group	109	9	4.7	1.76
		Control group	297	36	5.9	2.05
	150≦	Lamivudine group	260	9	4.8	0.72
		Control group	649	44	6.2	1.09
Platelet count ($\times 1000/mm^3$)	<150	Lamivudine group	254	18	3.8	1.86
		Control group	629	125	5.8	3.43
	150≦	Lamivudine group	375	11	5.3	0.55
		Control group	1302	67	6.1	0.84

Table 3
Estimation of effects of covariates following selection of regressor in Cox regression model

Category	Hazard ratio	95% Confidence interval (CI)	*p*-Value
Lamivudine therapy			
No	1		
Yes	0.49	0.31–0.77	0.002
Gender			
Male	1		
Female	0.42	0.28–0.62	<0.001
Family clustering of hepatitis B			
No	1		
Yes	1.44	1.08–1.94	0.015
Age at liver biopsy			
<40 y.o.	1		
≧40 y.o.	2.09	1.77–2.48	<0.001
Stage of liver fibrosis			
F0 or F1	1		
F2, F3, or F4	1.43	1.24–1.64	<0.001
Serum albumin level			
<4.0 g/dL	1		
≧4.0 g/dL	0.58	0.43–0.79	0.001
Platelet count			
<150 × 1000/μL	1		
≧150 × 1000/μL	0.53	0.38–0.73	<0.001

In the analysis of retrospective studies, great precautions are required in order to eliminate any bias between lamivudine-treated and non-treated groups. To minimize inter-group bias, we conducted with the cooperation of multiple medical institutions and a large number of patients ($n = 2795$). The effect of lamivudine on HCC was ultimately analyzed in a matched case-controlled study. Because the time of liver biopsy was used as the starting point in our analysis, the analytical results were not expected to appropriately reflect lamivudine's effect if the therapy was started a long time after the biopsy. Therefore, from among the 657 patients who received lamivudine therapy, we selected 377 patients who started lamivudine therapy within 2 years after biopsy. For a control group, the same number of patients ($n = 377$) without lamivudine therapy was selected from the 2138 subjects.

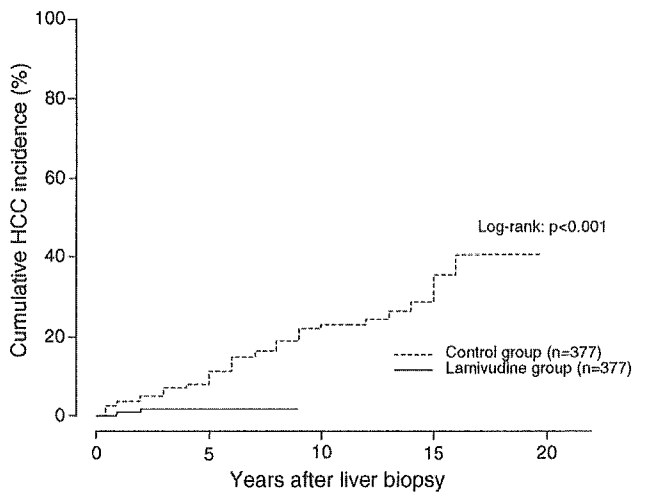

Fig. 1. Comparison of the cumulative HCC incidence between the lamivudine group (solid line) and the control group (broken line) by the Kaplan–Meier method in a case-matched control study. A significant difference was seen between the two groups ($p < 0.001$, log-rank test).

The regimen was not the same in all patients who have been treated by lamivudine. It was transiently discontinued before being recommenced later in some patients, whereas it was uninterrupted throughout the follow-up period in the majority (63%) of subjects in the matched case-controlled study. The duration of lamivudine regimen was not taken into account in the design of our study. Some patients received lamivudine for relatively short periods to improve acute exacerbation of their clinical course in chronic hepatitis B. On the other hand, some patients received lamivudine for the long-term to suppress the development of HCC. In the analysis by a multivariate Cox regression model in all unmatched patients, lamivudine therapy was selected as one of the factors inhibiting the occurrence of HCC. In the matched case-controlled study, the annual occurrence rate of HCC was significantly lower (0.4%/(patient/year)) in the lamivudine group than in the control group (1.8%/(patient/year)), suggesting that lamivudine treatment is effective for inhibiting the occurrence of HCC.

Recently, Liaw et al. conducted a multicenter, centrally randomized, double-blind, placebo-controlled, parallel group study to evaluate the effects of lamivudine on the progression of chronic hepatitis B to hepatic cancer [21]. They randomized 651 patients with histologically confirmed (F3 and F4), compensated hepatic cirrhosis to receive either lamivudine or a placebo at a ratio of 2:1 and continued the treatment for up to 5 years. The study was terminated after a median treatment duration of 32.4 months (range 0–42) owing to a significant difference between the groups in the number of end points reached. The end points were reached by 7.8% of the patients receiving lamivudine and 17.7% of those receiving placebo (hazard ratio for disease progression, 0.45; $p = 0.001$). The Child–Pugh score increased in 3.4% of the patients receiving lamivudine and in 8.8% of those receiving placebo (hazard ratio, 0.45; $p = 0.02$), whereas HCC occurred in 3.9% of those in the lamivudine group and in 7.4% of those in the placebo group (hazard ratio, 0.49: $p = 0.047$). The results of our analysis, which included patients with F0 through F2 hepatic fibrosis, were similar to those of Liaw et al. [21]. Thus, two studies demonstrated that the use of potent anti-viral agents such as lamivudine represents a major advance in the treatment of chronic hepatitis B and slows the progression of severe liver disease to liver cirrhosis as well as HCC.

Both hepatitis B and C are caused by persistent infection with hepatitis viruses, and both have a high probability of resulting in HCC. For this reason, these two diseases have a number of common traits, but some differences have been noted in their relationships with HCC. Among both

Table 4
Comparison of background factors between lamivudine group and control group assessed at the time of liver biopsy (matched case-controlled study)

Parameter	Lamivudine group ($n=377$)	Control group ($n=377$)	p-Value
Gender[a]			
Male	276 (73.2%)	273 (72.4%)	0.806
Female	101 (26.8%)	104 (27.6%)	
Age (years)[b]	41.5 ± 12.0	41.4 ± 12.2	0.950
Follow-up period (years)[b]	2.7 ± 2.1	5.3 ± 4.7	<0.001
Family clustering of hepatitis B[a]			
Yes	238 (63.1%)	242 (64.2%)	0.762
No	139 (36.9%)	135 (35.8%)	
Drinking during the course of the study (>ethanol 80 g/day)[a]			
Yes	38 (10.1%)	62 (16.4%)	0.007
No	333 (88.3%)	314 (83.3%)	
Unknown	6 (1.6%)	1 (0.3%)	
IFN therapy[a]			
Yes	129 (34.2%)	143 (37.9%)	0.046
No	236 (62.6%)	231 (61.3%)	
Unknown	12 (3.2%)	3 (0.8%)	
Liver histology			
Grade of inflammation[a]			
A0	6 (1.6%)	18 (4.8%)	0.001
A1	110 (29.2%)	101 (26.8%)	
A2	157 (41.6%)	186 (49.3%)	
A3	98 (26.0%)	72 (19.1%)	
Unknown	6 (1.6%)	0 (0.0%)	
Stage of fibrosis[a]			
F0	7 (1.9%)	6 (1.6%)	0.647
F1	103 (27.3%)	117 (31.0%)	
F2	95 (25.2%)	97 (25.7%)	
F3	107 (28.4%)	90 (23.9%)	
F4	65 (17.2%)	67 (17.8%)	
HBeAg[a]			
+	193 (51.2%)	220 (58.4%)	0.005
−	178 (47.2%)	141 (37.4%)	
Unknown	6 (1.6%)	16 (4.2%)	
HBeAb[a]			
+	126 (33.4%)	121 (32.1%)	0.030
−	245 (65.0%)	237 (62.9%)	
Unknown	6 (1.6%)	19 (5.0%)	
Albumin (g/dL)[b]	4.00 ± 0.51	4.00 ± 0.52	0.989
AST (IU/L)[b]	118.5 ± 155.4	95.5 ± 126.4	0.031
ALT (IU/L)[b]	191.7 ± 234.8	151.5 ± 180.5	0.009
Platelet count ($\times 1000/mm^3$)[b]	161.7 ± 52.7	164.3 ± 59.5	0.523

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.

hepatitis B patients and hepatitis C patients, HCC occurs mainly in those with advanced hepatic fibrosis, but the incidence of liver cirrhosis as a background of liver disease is lower in patients with B than in those with C. Furthermore, among hepatitis C patients HCC occurs mainly in those 60 years or older, while among hepatitis B patients it occurs mainly in those under 60 [22–24]. Studies on the cumulative incidence of HCC in hepatitis B patients showed that the HCC incidence increases linearly during the initial 12 years, plateaus, and then increases again in the 17th or 18th year [24,25]. In hepatitis C patients, on the other hand, the HCC incidence shows a continuous, linear increase [26,27]. Various findings obtained to date suggest that these clinical differences are related not only to differences in the hepatitis viral infection route and the timing of infection but also to differences in the mechanisms underlying cancer associated with hepatitis B and C. HCV is an RNA virus, and viral genes are not integrated into the host's genes, whereas HBV is a DNA virus with reverse-transcriptase activity. Thus, HBV genes are often integrated into the host's chromosomes

Table 5
Comparison of distribution of background factors between patients who developed HCC and those who did not in the lamivudine group (matched case-controlled study)

Parameter	Patients with HCC ($n=4$)	Patients without HCC ($n=373$)	p-Value
Gender[a]			
Male	3 (75.0%)	273 (73.2%)	1.000[c]
Female	1 (25.0%)	100 (26.8%)	
Age (years)[b]	55.0 ± 19.5 ($n=4$)	41.3 ± 11.9 ($n=373$)	0.024
Follow-up period (years)[b]	1.5 ± 0.6 ($n=4$)	2.7 ± 2.1 ($n=373$)	0.236
Family clustering of hepatitis B[a]			
Yes	2 (50.0%)	236 (63.3%)	0.628[c]
No	2 (50.0%)	137 (36.7%)	
Drinking during the course of the study (>ethanol 80 g/day)[a]			
Yes	1 (25.0%)	37 (9.9%)	0.393[c]
No	3 (75.0%)	330 (88.5%)	
Unknown	0 (0.0%)	6 (1.6%)	
IFN therapy[a]			
Yes	0 (0.0%)	129 (34.6%)	0.387[c]
No	4 (100.0%)	232 (62.2%)	
Unknown	0 (0.0%)	12 (3.2%)	
Liver histology			
Grade of inflammation[a]			
A0	0 (0.0%)	6 (1.6%)	0.458[c]
A1	0 (0.0%)	110 (29.5%)	
A2	3 (75.0%)	154 (41.3%)	
A3	1 (25.0%)	97 (26.0%)	
Unknown	0 (0.0%)	6 (1.6%)	
Stage of fibrosis[a]			
F0	0 (0.0%)	7 (1.9%)	0.918[c]
F1	1 (25.0%)	102 (27.3%)	
F2	1 (25.0%)	94 (25.2%)	
F3	2 (50.0%)	105 (28.2%)	
F4	0 (0.0%)	65 (17.4%)	
HBeAg[a]			
+	3 (75.0%)	190 (50.9%)	0.648[c]
−	1 (25.0%)	177 (47.5%)	
Unknown	0 (0.0%)	6 (1.6%)	
HBeAb[a]			
+	2 (50.0%)	124 (33.2%)	0.632[c]
−	2 (50.0%)	243 (65.1%)	
Unknown	0 (0.0%)	6 (1.6%)	
Albumin (g/dL)[b]	4.23 ± 0.45 ($n=4$)	4.00 ± 0.51 ($n=373$)	0.384
AST (IU/L)[b]	47.0 ± 22.8 ($n=4$)	119.4 ± 156.2 ($n=326$)	0.356
ALT (IU/L)[b]	46.3 ± 24.2 ($n=4$)	193.2 ± 235.5 ($n=372$)	0.213
Platelet count ($\times 1000/mm^3$)[b]	141.0 ± 27.0 ($n=4$)	161.9 ± 52.9 ($n=373$)	0.431

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.
[c] Fisher's exact test.

and play an important role in hepatic carcinogenesis [28,29]. It is known that the repeat of necrosis and regeneration of liver might accelerate the mutation of oncogenes. In addition, de novo carcinogenesis is thought to be promoted in hepatitis B patients as a result of the increased genetic instability caused by the integration of the HBV genome into the host's chromosomes. When administered to patients with hepatitis B, lamivudine decreases the blood HBV-DNA concentration and markedly improves ALT levels, with consequent improvement of liver histological findings [7,11,13,14]. An early in vitro study showed that lamivudine decreases the amount of free HBV-DNA in hepatocytes but does not affect integrated HBV genes [30]. Therefore, lamivudine is thought to inhibit HCC by abating hepatitis and not by inhibiting viral gene integration. In fact, as shown in the matched case control study, all four patients who developed HCC in the lamivudine group had non-cirrhotic liver disease, whereas 23 (46%) of 50 patients who developed HCC had liver cirrhosis. Due to the small number of patients included, however, further studies are necessary to confirm this finding.

Table 6
Comparison of distribution of background factors between patients who developed HCC and those who did not in the control group (matched case-controlled study)

Parameter	Patients with HCC ($n = 50$)	Patients without HCC ($n = 327$)	p-Value
Gender[a]			
Male	40 (80.0%)	233 (71.3%)	0.236[c]
Female	10 (20.0%)	94 (28.7%)	
Age (years)[b]	50.6 ± 10.1	40.0 ± 11.9	<0.001
Follow-up period (years)[b]	5.3 ± 4.3	5.2 ± 4.8	0.951
Family clustering of hepatitis B[a]			
Yes	29 (58.0%)	213 (65.1%)	0.345[c]
No	21 (42.0%)	114 (34.9%)	
Drinking during the course of the study (>ethanol 80 g/day)[a]			
Yes	14 (28.0%)	48 (14.7%)	0.050[c]
No	36 (72.0%)	278 (85.0%)	
Unknown	0 (0.0%)	1 (0.3%)	
IFN therapy[a]			
Yes	16 (32.0%)	127 (38.8%)	0.578[c]
No	34 (68.0%)	197 (60.2%)	
Unknown	0 (0.0%)	3 (0.9%)	
Liver histology			
Grade of inflammation[a]			
A0	2 (4.0%)	16 (4.9%)	0.026[c]
A1	6 (12.0%)	95 (29.1%)	
A2	27 (54.0%)	159 (48.6%)	
A3	15 (30.0%)	57 (17.4%)	
Stage of fibrosis[a]			
F0	0 (0.0%)	6 (1.8%)	<0.001[c]
F1	7 (14.0%)	110 (33.6%)	
F2	8 (16.0%)	89 (27.2%)	
F3	12 (24.0%)	78 (23.9%)	
F4	23 (46.0%)	44 (13.5%)	
HBeAg[a]			
+	26 (52.0%)	194 (59.3%)	0.564[c]
–	22 (44.0%)	119 (36.4%)	
Unknown	2 (4.0%)	14 (4.3%)	
HBeAb[a]			
+	20 (40.0%)	101 (30.9%)	0.319[c]
–	27 (54.0%)	210 (64.2%)	
Unknown	3 (6.0%)	16 (4.9%)	
Albumin (g/dL)[b]	3.63 ± 0.59	4.06 ± 0.49	<0.001
AST (IU/L)[b]	96.9 ± 100.8	95.3 ± 130.0	0.934
ALT (IU/L)[b]	132.8 ± 165.5	154.4 ± 182.7	0.431
Platelet count ($\times 1000/mm^3$)[b]	126.8 ± 50.7	170.0 ± 58.7	<0.001

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.
[c] Fisher's exact test.

Seven HBV genotypes (A–G) have been identified to date, and their distribution shows regional variations [31–36]. In Japan, genotypes C, B, and the other five account for 85, 12, and 3% of hepatitis B patients [36]. The virological differences between HBV genotype B and genotype C might influence not only on the natural course of hepatitis B but also the efficacy by lamivudine. The patients with HBV genotype B are frequently negative for HBeAg, have lower ALT levels and a better prognosis. In contrast, the patients with HBV genotype C tend to remain HBeAg-positive for a longer duration and tend to have elevated ALT levels and more advanced liver disease, such as liver cirrhosis and HCC. This indicates that the analysis of HBV genotypes will be needed in this study.

In conclusion, our multicenter, retrospective, matched case study indicated that lamivudine treatment might suppress the risk of HCC in patients with chronic hepatitis B. However, the study has several limitations, such as the relatively short duration of treatment and the lack of virological analyses (HBV genotype, YMDD mutation, and HBV-DNA volume). To relief these limitations, further long-term observation should be continued to clarify the conclusion.

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Appendix A

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Nucleotide Mutations Associated With Hepatitis B e Antigen Negativity

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One hundrded and forty four patients with chronic hepatitis B were tested to identify new mutations associated with hepatitis B e antigen (HBeAg) negativity, using a full genome sequence analysis. All the patients were Chinese and had hepatitis B virus infection of genotype C. Patients with none of the pre-core or core promoter mutations were significantly ($P < 0.001$) less common in the group with anti-HBe (13%) than in the group with HBeAg (56%). The complete nucleotide sequence was determined in four anti-HBe-positive patients who had neither pre-core nor core promoter mutations and in five HBeAg-positive patients who also had neither of these mutations (the groups were matched for age and sex). Six mutations were found to be significantly more common in the former group than in the latter: G529A (3/4 vs. 0/5), C934A (4/4 vs. 1/5), A1053G (4/4 vs. 1/5), G1915T/A (4/4 vs. 0/5), T2005C/A (4/4 vs. 0/5), and C3026T (3/4 vs. 0/5). Three of the six mutations were significantly more common in the four anti-HBe-positive patients who had neither pre-core nor core promoter mutations, compared to 11 HBeAg-positive patients who had pre-core and core promoter mutations, and also compared to 15 anti-HBe-positive patients who had pre-core and core promoter mutations, suggesting further the specificity of these mutations. Of the six mutations, two resulted in amino acid substitution in the polymerase protein, and one is located near the enhancer I region. The results suggest that the six newly discovered mutations are associated with HBeAg negativity. ***J. Med. Virol. 76:170–175, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: hepatitis B e antigen (HBeAg); genotype; nucleotide mutation

INTRODUCTION

Approximately 350 million people are chronic carriers of hepatitis B virus (HBV) worldwide [Maynard, 1990; Maddrey, 2000]. Chronic HBV infection is the cause of up to 50% of cirrhosis and 70–90% of hepatocellular carcinomas (HCC) in China, South-East Asia, and Africa [Lok, 1992; Fattovich, 1998], and in Asian countries, almost all patients with chronic HBV infection have been infected perinatally from hepatitis B e antigen (HBeAg)-positive mothers [Okada et al., 1976]. HBeAg is considered to be a marker for viral replication, but some HBeAg-negative patients remain viremic and continue to have active liver disease [Hadziyannis et al., 1983; Lok et al., 1984; Bonino et al., 1986]. Many of these patients are found to have a G to A change at nucleotide 1896, which creates a stop codon (TAG) in the precore (Pre-C) open reading frame, which in turn prevents translation of the Pre-C protein and aborts HBeAg production [Carman et al., 1989]. Other patients have mutations in the core promoter (CP) region, including an A to T mutation at nucleotide 1762 and a G to A mutation at nucleotide 1764 [Okamoto et al., 1994]. In vitro studies of this double mutation show decreased transcription of Pre-C messenger RNA and hence a resultant decrease in HBeAg production by 70% [Buckwold et al., 1996; Chan et al., 1999]. A recent follow-up study on Pre-C and CP mutations has also

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shown that the presence of these mutations is useful for predicting seroconversion [Yamaura et al., 2003].

Besides the G1896A mutation and the A1762T/G1764A mutation, a number of point mutations, as well as deletions and insertions of nucleotides, have been detected in the Pre-C region and CP region that could correlate with seroconversion [Okamoto et al., 1990; De Castro et al., 2001]. In the present study, the complete HBV genome was examined for other nucleotide mutations associated with HBeAg negativity, in addition to mutations in the Pre-C and CP regions.

MATERIALS AND METHODS

Patients

A cohort of 193 Chinese patients with chronic HBV infection who visited the Liver Disease Clinic of the Second Hospital of HeBei Medical University in Shijiazhuang city, North China, between June and August 2001 were enrolled in the study. These patients comprised 124 men and 69 women and had a median age of 29.1 years old (range: 5–73 years old). Patients who were co-infected with hepatitis C or D virus or with the human immunodeficiency virus and patients with other concomitant causes of chronic liver disease were excluded. According to the consensus diagnostic criteria for HBV infection, 182 patients were diagnosed with chronic hepatitis B. The remaining 11 patients had persistently normal alanine aminotransferase (ALT: normal range 10–21 IU/L) levels, suggesting an inactive HBV carrier stage. None of the 193 patients were treated with antiviral agents such as interferon or lamivudine. Of the 193 patients, 169 (87.6%) were of genotype C, 21 (10.9%) of genotype B, and 3 (1.5%) of genotype A. For the mutation analysis, 144 patients who were positive for either HBeAg or anti-HBe were selected from the 169 genotype C patients. Informed consent was obtained from each patient.

Conventional HBV Markers and Genotyping of HBV

Hepatitis B surface antigen (HBsAg), HBeAg and anti-HBe were measured using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan). Serum concentration of HBV DNA was measured using the AMPLICOR HBV Monitor test (Roche Diagnostics K.K., Tokyo, Japan), which has a quantitative range of 2.6–7.6 log copies/ml. When the concentration to be tested was beyond this range, the actual concentration was determined using a serum sample diluted 100-fold with normal human serum. The HBV genotype was determined using the restriction fragment length polymorphism (RFLP) method on an S-gene sequence amplified by polymerase chain reaction (PCR) with nested primers [Mizokami et al., 1999].

Determination of Pre-C and CP Mutations

The 1,896th nucleotide in the Pre-C region of G or A was detected with an enzyme-linked mini-sequence assay kit (Roche Diagnostics), and the results were expressed as the percentage mutation rate, as defined by Aritomi et al. [1998]. If the mutation rate was 0%, the strain was considered to be Pre-C mutation-negative, while a Pre-C mutation-positive strain was recorded when the mutation rate exceeded 0%. The double mutation in the CP region (A1762/T1764) was detected using an HBV CP mutation detection kit (Smitest: Genome Science Laboratories, Tokyo, Japan), and the results were classified into three categories: wild, mixed, and mutant types. A wild type strain was considered to be CP mutation-negative, while mixed and mutant types were recorded as CP mutation-positive strains. The detection limits of the pre-C and the CP mutation detection kits are both 1,000 copies/ml.

Determination of Nucleotide Sequence

The complete genome sequence was determined according to the method described by Rokuhara et al. [2000]. Briefly, nucleic acids were extracted from a serum sample of 100 μl with a DNA/RNA extraction kit (Smitest EX-R&D: Genome Science Laboratories Co., Ltd.). Two microliters of each DNA solution were used for amplification by PCR. The reaction was carried out in 25 μl of PCR-mixture containing 250 μmol/L of each dNTP, 1× PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 0.001% gelatin], 0.25 U EX-Taq DNA polymerase (TaKaRa, Tokyo), and 0.25 μM of a primer pair. The PCR was initiated using the hot-start technique.

To determine the full-length nucleotide sequence of HBV, two fragments (fragments A and B) were amplified by PCR, using the primers shown in Table I. Fragment A (1,498 bases in length; nt 457–nt 1954) was amplified with nested pairs of outer (SB1 and CB2) and inner primers (SB3 and CB4), while fragment B (2162 bases in length; nt 1611–nt 557), was amplified with nested pairs of outer primers (es2 and PS4) and inner primers (is2 and PS3). The first round of PCR was performed with an outer primer set for 40 cycles (94°C for 1.5 min, 55°C for 1 min, and 72°C for 2 min), and was followed by an extension reaction at 72°C for 7 min. The second round was undertaken with an inner primer set for 30 cycles, and was also followed by an extension reaction. PCR products were subjected to electrophoresis on a 1.0% agarose gel with ethidium bromide staining and visualization with an UV transilluminator. The band containing the target sequence was removed and DNA was isolated using GFX™ PCR DNA and a Gel Band Purification kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The nucleotide sequence was directly determined by the dideoxy method, using the sequencing primers shown in Table I. The accuracy of the sequence was ensured by comparison of the sequence data for the complete genome obtained with sense-sequencing primers and that obtained with anti-sense-sequencing primers.

Statistical Analysis

Mann–Whitney's *U* test was utilized for quantitative data, and Fisher's exact test and a Chi-square test were

TABLE I. Primers Used for PCR and Sequencing of HBV DNA

Primer		Sequence	nt position
Primers for PCR of fragment A			
SB1	Sense	5-TGCTGCTATGCCTCATCTTC	(414–433)
CB2	Anti-sense	5-GGAAAGAAGTCAGAAGGCAA	(1974–1955)
SB3	Sense	5-AGGTATGTTGCCCGTTTCTC	(457–476)
CB4	Anti-sense	5-AAAAGAGAGTAACTCCACAG	(1954–1935)
Primers for PCR of fragment B			
es2	Sense	5-ACGTCGCATGGAGACCACCG	(1601–1620)
PS4	Anti-sense	5-CAGTTTCCGTCCGAAGGTTTTG	(594–573)
is2	Sense	5-GAGACCACCGTGAACGCCCA	(1611–1630)
PS3	Anti-sense	5-GAAACATAGAGGTGCCTTGAGCAG	(557–534)
Primers for sequencing			
SB3	Sense	5-AGGTATGTTGCCCGTTTCTC	(457–476)
as1	Anti-sense	5-TGCGAAAGCCCAGGATGATG	(631–612)
s2	Sense	5-TGCGAAAGCCCAGGATGATG	(760–783)
as2	Anti-sense	5-AGTTGGCGAGAAAGTGAAAGCCTG	(1107–1084)
s3	Sense	5-CTCTGCCGATCCATACTGCGGAA	(1256–1278)
as3	Anti-sense	5-CGGGACGTAGACAAAGGACGT	(1434–1414)
is2	Sense	5-GAGACCACCGTGAACGCCCA	(1611–1630)
ea1	Anti-sense	5-TGAAAAAGTTGCATGGTGCTGGTG	(1827–1804)
s4	Sense	5-TATCGGGAGGCCTTAGAGTCTCCG	(2012–2035)
as4	Anti-sense	5-ATAGGGGCATTGGTCT	(2314–2298)
s5	Sense	5-CGCAGAAGATCTCAATCTCGG	(2417–2437)
as5	Anti-sense	5-GGATAGAACCTAGCAGGCAT	(2654–2635)
s6	Sense	5-GGGTCACCATATTCTTGGGAA	(2814–2834)
as6	Anti-sense	5-GGGTTGAAGTCCCAATCTGGATT	(2987–2965)
is1	Sense	5-AAGCTCTGCTAGATCCCAGAGT	(18–39)
ea2	Anti-sense	5-TAGAAAATTGAGAGAAGTCCACCA	(280–257)
s1	Sense	5-CATCCTGCTGCTATGCCTCATC	(409–430)
as1	Anti-sense	5-TGCGAAAGCCCAGGATGATG	(631–612)

Nucleotides are numbered from the unique *Eco*RI site of HBV.

used for qualitative data. P values less than 0.05 were considered significant. Analyses were carried out using SPSS version 10.0J (SPSS Inc., Chicago, IL).

RESULTS

Of the 144 patients selected for mutation analysis, 90 (62.5%) were HBeAg-positive and the remaining 54 (37.5%) were anti-HBe-positive. The clinical and virological backgrounds of the two groups of patients are compared in Table II. The 90 HBeAg-positive patients tended to be younger and have a higher concentration of HBV DNA than the 54 anti-HBe patients. Patients with none of the Pre-C and CP mutations were significantly ($P < 0.001$) more common in the HBeAg-positive patients (56%) than in the anti-HBe-positive patients (13%).

A comparison of the clinical background of seven anti-HBe-positive patients who had neither Pre-C nor CP mutations and 47 anti-HBe-positive patients who had at least one of the mutations is shown in Table III. Distributions of age, gender, ALT level, and HBV DNA concentration did not differ between the two groups.

Nucleotide sequences of the complete genome were determined in four out of seven anti-HBe-positive patients who had neither Pre-C nor CP mutations and in 5 out of 50 HBeAg-positive patients who also had neither mutation. All nine of the genome sequences

TABLE II. Comparison of Clinical and Virological Backgrounds of Patients With HBeAg and Those With Anti-HBe

	HBeAg-positive n = 90	Anti-HBe-positive n = 54	P
Age[a]	25 (5–53)	36 (11–73)	<0.001[b]
Gender (M:F)	58:32	30:24	>0.2[c]
ALT[a]	89 (11–2100)	62 (13–458)	>0.2[b]
HBV DNA (log copies/mL)[a]	8.3 (4.4–7.9)	5.0 (3.2–8.8)	<0.001[b]
Pre-C/CP mutations			
Both negative	50 (56%)	7 (13%)	<0.001[c]
Pre-C mutation only	13 (14%)	20 (37%)	
CP mutation only	12 (13%)	5 (9%)	
Both positive	15 (17%)	22 (41%)	

[a]Data are expressed as median values (range).
[b]Mann–Whitney test.
[c]Chi-square test.

TABLE III. Comparison of Clinical and Virological Backgrounds of Anti-HBe-Positive Patients With Neither Pre-C nor CP Mutations and Anti-HBe Patients With at Least one of These Mutations

	Pre-C and CP mutation-negative n = 7	Pre-C and/or CP mutation-positive n = 47	*P*
Age[a]	37 (18–60)	36 (11–73)	>0.2[b]
Gender (M:F)	4:3	26:21	>0.2[c]
ALT[a]	44 (18–86)	65 (13-458)	0.17[b]
HBV DNA (log copies/ml)*	4.7 (3.3–5.5)	5.0 (3.2–8.8)	>0.2[b]

[a]Data are expressed as median values (range).
[b]Mann–Whitney test.
[c]Chi-square test.

determined had nucleotide lengths of 3,215 bases, and thus there were no insertions or deletions. When the full genome sequences were compared, the six mutations shown in Table IV were significantly more common in the four anti-HBe-positive patients than in the five HBeAg-positive patients. The positions of the six mutations in the HBV genome are shown in Figure 1. Of the four mutations located in the polymerase gene, the G529A and C934A mutations cause amino acid substitutions in the polymerase protein. The C3026T mutation does not cause an amino acid substitution in the polymerase, but rather in the pre-S1 protein, while the A1053G mutation does not lead to an amino acid substitution, but the mutation is located near the enhancer I region. The G1915T/A and T2005C/A mutations are located in the core gene, but do not result in an amino acid substitution. Patients with at least one of the three mutations (G529A, C934A, and A1053G) which might affect HBV replication had a significantly ($P = 0.029$) lower level of HBV DNA (n = 22, median 5.3 copies/ml, range 3.8–8.9) than those patients who had no mutations (n = 13, median 8.5 copies/ml, range 3.8–8.9).

To examine further the specificity of the six mutations, these mutations were also determined in 11 HBeAg-positive patients who were positive for Pre-C and CP mutations and in 15 anti-HBe-positive patients who were also positive for Pre-C and CP mutations. The frequencies of the six mutations were compared between groups of patients classified according to their HBeAg/anti-HBe and Pre-C/CP mutation status. Three (G1915T/A, T2005C/A, and C3026T) of the six mutations were found to be significantly more common in anti-HBe-positive patients who had neither a Pre-C nor a CP mutation than in the two groups of patients with Pre-C and CP mutations, as shown in Table V.

The nucleotide sequence data reported in this paper have been registered in the DDBJ/EMBL/GenBank nucleotide sequence databases, with the accession numbers AB198076-84.

DISCUSSION

Studies to date have shown that the stop codon mutation in the Pre-C region (G1896A) and the double mutation in the CP region (A1762T/G1764A) are independently associated with the seroconversion of HBeAg, and that the Pre-C mutation is more directly associated with seroconversion than the core promoter mutation [Okamoto et al., 1994; Yamaura et al., 2003]. Only a small number of anti-HBe-positive patients (13%) were both negative for the Pre-C and CP mutations, and in the present study this rate was significantly lower than that (56%) in HBeAg-positive patients. These results are consistent with previous reports, suggesting that the two mutations are the main causes of seroconversion. However, there are also patients in whom HBeAg secretion discontinues without

TABLE IV. Comparison of Full Nucleotide Sequences of HBV With Neither Pre-C nor CP Mutations for HBeAg-Positive and Anti-HBe-Positive Patients

Nucleotide mutation	Amino acid substitution (viral protein)	HBeAg Pre-C and CP mutation-negative n = 5	Anti-HBe Pre-C and CP mutation-negative n = 4	*P*
G529A	D480N (P) None (S)	0	3	0.048
C934A	L615I (P)	1	4	0.040
A1053G	None (P)	1	4	0.040
G1915T/A	None (C)	0	4	0.008
T2005C/A	None (C)	0	4	0.008
C3026T	A60V (Pre-S1) None (P)	0	3	0.048

Six mutation sites with significant differences are shown. Data are expressed as the number of positives. Statistical analysis was performed with a chi-square test. P, polymerase protein; S, surface protein; C, core protein; Pre-S1, pre-surface 1 protein.

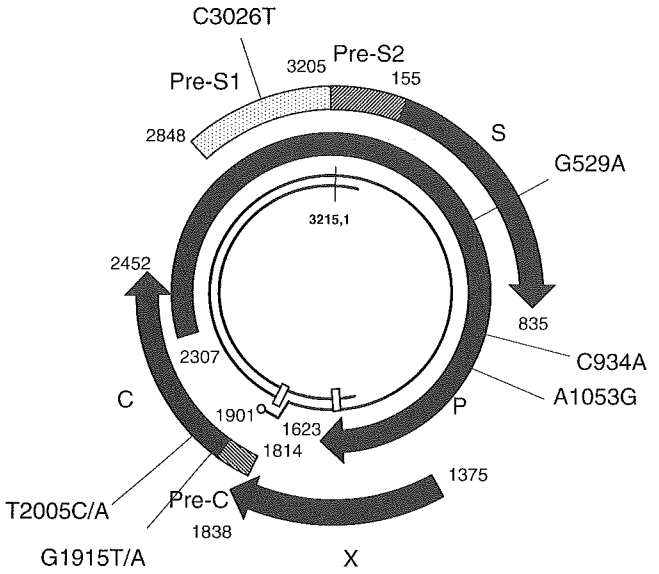

Fig. 1. Organization of the hepatitis B virus genome (genotype C) and the approximate positions of the six nucleotide mutations in the HBV genome. The inner circles represent the minus and plus DNA strands of the viral genome. The different open reading frames encoded by the genome, designated as S, C, P, and X, are indicated by the arrows. Abbreviations: S, surface antigen; C, core; P, polymerase; Pre-C, precore.

appearance of Pre-C and/or CP mutations. Thus, we speculated that some other mutations might be associated with HBeAg seroconversion. A variety of other mutations in the CP and Pre-C regions have been detected in previous studies [Carman et al., 1989; Tillmann et al., 1995; Baumert et al., 1996; Laras et al., 1998; Chan et al., 2000; De Castro et al., 2001; Yoo et al., 2003], but other regions of the HBV genome have not been analyzed sufficiently for mutations associated with HBeAg seroconversion.

When the full nucleotide sequences of HBV genomes of HBeAg-positive and anti-HBe-positive patients with neither Pre-C nor CP mutations were compared, six mutations (G529A, C934A, A1053G, G1915T/A, T2005C/A, C3026T) were found to be significantly more common in the anti-HBe-positive patients. The six mutations were also more common in anti-HBe-positive patients who had neither Pre-C nor CP mutations than in HBeAg-positive patients or in anti-HBe-positive patients who had Pre-C and CP mutations, with the results being statistically significant for three (G1915T/A, T2005C/A, C3026T) of the six mutations. These results suggest that the six mutations are associated with HBeAg negativity.

The mechanisms through which the six mutations facilitate HBeAg negativity were not investigated in the present study. However, some possible mechanisms can be considered, based on the locations of these mutations in the HBV genome. The G529A and C934A mutations cause amino acid substitutions in the polymerase protein. Thus, these two mutations may attenuate HBV replication through changes in the enzymatic activity of the polymerase. The A1053G mutation is located near the enhancer I region, which may affect the replication of HBV [Bock et al., 2000]. Patients who had at least one of the three mutations associated with HBV replication tended to have a lower level of HBV DNA than those who had none of these mutations, providing further support for a replication-associated mechanism. It has been reported that amino acid substitutions in immunogenic epitopes in the core protein are found most frequently during or after seroconversion from HBeAg to anti-HBe [Akarca and Lok, 1995; Carman et al., 1995]. We found two mutations in the core gene, but these mutations did not cause amino acid substitutions. Thus, the mechanisms through which the G1915T/A and T2005C/A mutations exert their effects remains unclear.

In anti-HBe-positive patients, the clinical background, including the mean age, gender distribution, ALT level and HBV DNA level, were similar in patients with and without Pre-C and/or CP mutations. Although these comparisons were cross-sectional, the results suggest that mutations other than those in the Pre-C and CP regions have a similar impact in patients in whom seroconversion occurs, compared to Pre-C and CP mutations.

The six mutations identified in the present study have not been described previously. These mutations

TABLE V. Comparison of Six Mutations Among Three Groups Classified According to Their HBeAg/anti-HBe and Pre-C/CP Mutation Status

Mutation site	Anti-HBe Pre-C and CP mutation-negative n = 4	HBeAg Pre-C and/or CP mutation-positive n = 11	Anti-HBe Pre-C and/or CP mutation-positive n = 15
G529A	3	3	3
C934A	4	6	10
A1053G	4	4	9
G1915T/A	4[a]	3	1
T2005C/A	4[b]	3	4
C3026T	3[c]	0	1

Data are expressed as the number of positives. Statistical analysis was performed with Fisher's exact test. Other comparisons were not statistically significant.
[a]$P = 0.026$ versus 11 patients with HBeAg, and $P = 0.001$ versus 15 patients with anti-HBe.
[b]$P = 0.026$ versus 11 patients with HBeAg, and $P = 0.018$ versus 15 patients with anti-HBe.
[c]$P = 0.009$ versus 11 patients with HBeAg, and $P = 0.016$ versus 15 patients with anti-HBe.

are thought to be associated with HBeAg negativity because they were found specifically in anti-HBe-positive patients with neither a Pre-C nor a CP mutation. However, several issues remain to be resolved to clarify the real significance of the six mutations, including the mechanisms through which they facilitate HBeAg negativity, their universality in genotypes other than genotype C, and their clinical relevance. Furthermore, it is possible that immune-based selection pressures that cause loss of HBeAg are responsible for the selection of the mutations identified in the present study [Locarnini, 2004]. Therefore, it is not possible to conclude that the new mutations are definitely associated with seroconversion, but they do provide new clues regarding the nature of seroconversion.

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Impact of daily high-dose IFNα-2b plus ribavirin combination therapy on reduction of ALT levels in patients with chronic hepatitis C with genotype 1 and high HCV RNA levels

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Abstract

The possibility of delaying progression to hepatocellular carcinoma in chronic hepatitis C patients with genotype 1 and high viral titers with baseline ALT levels of ≥50 IU/L was examined by administration of IFN plus ribavirin combination therapy using ALT normalization as index and IFN monotherapy as control. The rate of sustained ALT normalization (ALT normal at 24 weeks after the end of treatment) was 28.1% with combination therapy and 10.5% with IFN monotherapy ($P=0.001$). Furthermore, the number of patients with sustained viral response (SVR) and with sustained ALT normalization in non-SVR patients was also significantly higher in the combination therapy versus monotherapy group. Mean ALT values during treatment and for 6 months after the end of treatment were significantly lower with combination therapy versus monotherapy even in virological nonresponders, as well as significantly lower during the post-treatment observation period in patients who relapsed after the end of treatment. Since increase in the rate of sustained ALT normalization and SVR were successfully achieved, inhibition of progression to hepatocellular carcinoma should be studied with long-term IFN and ribavirin combination therapy.

Keywords: Chronic hepatitis C; IFNα-2b; Ribavirin; ALT; Hepatocellular carcinoma prevention

1. Introduction

With the aging of the chronic hepatitis C patient population in Japan, a rapid increase in the incidence of hepatocellular carcinoma (HCC) is being observed [1]. Deaths due to HCC number over 30,000 per year [1], and prevention of progression to HCC is now an urgent issue. Many reports on the efficacy of interferon (IFN) in preventing progression to HCC in patients with chronic hepatitis C have been published by Japanese researchers [2–8]. At first, normalization

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of serum alanine aminotransferase (ALT) levels while on IFN therapy was thought to inhibit progression to HCC [3]. However, the results of long-term follow-up studies clearly indicate that sustained viral response (SVR) and/or sustained normalization of ALT after the end of treatment are necessary for the long-term inhibition of progression to HCC [4,6–8]. Results indicating that such inhibition is possible if ALT levels are maintained long-term within about twice the upper limit of normal (80 IU/L) have also been reported [9,10].

The standard of treatment of chronic hepatitis C worldwide is pegylated-IFN (PEG-IFN) in combination with ribavirin. The addition of ribavirin has been shown radically to increase the rate of eradication of HCV [11–13], and this is thought to result in increased inhibition of progression to HCC in patients with chronic hepatitis C. The efficacy of PEG-IFN plus ribavirin in the prevention of histologic progression using fibrosis as index has already been reported although these studies did not directly examine the effect on inhibition of progression to HCC [14,15]. Based on an average follow-up period of 20 months, one-stage improvement in METAVIR score was observed in 73% of patients on 1-year administration of PEG-Intron 1.5 μg plus ribavirin. One-stage exacerbation was observed only in 8%. Ribavirin alone has almost no effect on reducing HCV levels, but is reported to normalize ALT levels during treatment [16,17], and similar effects may be expected with combination therapy. The focus worldwide is on antiviral efficacy, and there are very few reports of detailed examination of the effect of combination therapy on liver function [18].

Recently, the efficacy of combination therapy with ribavirin in the context of inhibition of progression to HCC has started to be investigated although the number of patients involved is small compared with the numbers enrolled in clinical studies of IFN in Japan. Yang et al. [19] reported that the 7-year cumulative HCC rate is 1.4% in patients receiving IFN plus ribavirin, which is much lower than the 10.2% reported in patients receiving IFN alone, although the difference was not statistically significant due to the small sample size. It has been reported elsewhere that factors contributing to inhibition of progression to HCC include absence of liver cirrhosis before the start of treatment and sustained viral response although the data include both combination therapy and monotherapy cases [20]. The above Japanese data indicate that prevention of progression to HCC can be expected with sustained normalization of ALT, although the presence of this factor does not necessarily indicate that liver histology is normal [18]. Hence we tested the hypothesis that combination therapy consisting of IFNα-2b plus ribavirin for 24 weeks in difficult-to-treat HCV genotype 1 patients leads not only to the eradication of HCV but ultimately to the prevention of histological progression with increased normalization of liver function.

2. Materials and methods

2.1. Patient selection

Two randomized comparative clinical studies of IFNα-2b plus ribavirin combination therapy versus IFNα-2b alone were initiated in Japan in 1998 in chronic hepatitis C patients; one in difficult-to-treat genotype 1 and high viral titer (> 100 kcopies/mL) patients [21] and the other in nonresponders and relapsers to previous IFN therapy [22]. From these two studies, data on patients with ALT levels ≥50 IU/L (i.e. about 1.5 times the mean upper limit of normal) at the start of treatment were extracted and the effects of treatment on ALT improvement were retrospectively analyzed. In both studies, IFN α-2b (Intron A; Schering Plough, Kenilworth, NJ) was administered at doses of 6 or 10 MIU six times per week for 2 weeks followed by 6 MIU three times per week for 22 weeks. Patients in the combination treatment groups additionally received ribavirin (Rebetol, Schering Plough, Kenilworth, NJ) at a dose of 600 mg/day (three capsules) and 800 mg/day (four capsules) in those weighing <60 kg and ≥60 kg, respectively, for 24 weeks. Patients in the control groups took ribavirin placebo capsules. In both studies, patients were randomized to either treatment.

The studies were approved by the institutional review boards of each study site and all patients provided written informed consent to participate. Inclusion criteria were as follow: (1) HCV RNA-positive and ALT abnormal in tests conducted within 12 weeks prior to the start of treatment; (2) HCV genotype 1 or if genotype 2 nonresponder or relapser to previous IFN treatment; (3) age between 20 and 64 years; (4) hemoglobin ≥ 12 g/dL and platelets ≥ 100,000 mm^{-3} in the most recent test conducted within 12 weeks prior to the start of treatment; (5) available for hospitalization for 4 weeks after the start of treatment; and (6) contraception possible both during and for 6 months after the end of treatment. Exclusion criteria were as previously reported [23]. The database for this retrospective study included information on sex, age, body weight, histological stage and activity index, IFN treatment history, HCV RNA levels, aspartate aminotransferase, ALT, hemoglobin, white blood cells (WBC), red blood cells, platelets, and creatinine.

2.2. Study design

Pretreatment ALT levels were classified into three grades: 50 to <100 IU/L, 100 to <150 IU/L, and ≥150 IU/L. The effect of timing of initial ALT normalization on sustained ALT normalization was examined as well as the association between virological efficacy and improvement in liver function. ALT was measured before and 1–4, 6, 8, 12, 16, 20, and 24 weeks after the start and 2, 4, 8, 12, 16, 20, and 24 weeks after the end of treatment. The judgment of ALT normalization and less than two times of upper normal ALT levels were made based on the normal values at each study site (median 37.5 IU/L, range 21–50 IU/L), and the timing of

initial ALT normalization was recorded as the day when the judgment of normal ALT was made for the first time during treatment. HCV RNA was measured before and 4, 12, and 24 weeks after the start and 24 weeks after the end of treatment. HCV RNA was measured by qualitative Amplicor assay (Mitsubishi Kagaku BCL, Tokyo, Japan), and genotype determined before the start of treatment by reverse transcriptase polymerase chain reaction (Mitsubishi). Evaluation of liver histology was conducted by a single evaluator based on liver tissue samples taken within 48 weeks prior to the start of treatment.

2.3. Definition of response

ALT normalization at 24 weeks after the end of treatment was considered "effective" and was the primary endpoint of this examination. Separately, the association with virological efficacy was also examined. HCV RNA negativity by qualitative assay at 24 weeks after the end of treatment was defined as sustained viral response. Virological relapsers were patients who were HCV RNA-negative by qualitative assay at the end of treatment but who became HCV RNA-positive after the end of treatment. Nonresponders were patients who were never HCV RNA-negative during or after treatment.

2.4. Statistical analysis

After confirming the absence of interaction in efficacy by the Breslow–Day test, comparison of sustained ALT normalization rate by pretreatment ALT levels was conducted using the Mantel–Haenszel test. The log-rank test was used to analyze the timing of initial ALT normalization, and *t*-test or Wilcoxon test was used for mean ALT values during the treatment and posttreatment observation periods. Significance level was two-sided 5%. All calculations were performed by SAS program version 6.12 (SAS Institute, Cary, NC).

3. Results

3.1. Patient characteristics

The study included 167 patients given combination therapy and 105 assigned monotherapy. Main patient characteristics are shown in Table 1. Mean age was 48–49 years and the majority of patients had extremely high HCV RNA levels exceeding the upper limit of quantitation of 850 kcopies/mL. No imbalance in patient background was observed between the two treatment groups.

3.2. ALT normalization rate

Sustained ALT normalization rate was 28.1% (47/167) in the combination therapy group and 10.5% (11/105) in the monotherapy group; combination therapy was significantly superior to monotherapy ($P=0.001$; Fisher's direct probability test). Sustained ALT normalization rate taking into account baseline ALT levels is shown in Fig. 1. In this respect, combination therapy was again significantly superior to monotherapy ($P=0.001$; Mantel–Haenszel test). Sustained ALT normalization rate was also significantly superior in the combination therapy group in patients whose pretreatment ALT was 100 to <150 IU/L ($P=0.001$; Fisher's direct probability test). In the combination group, the frequency of patients with ALT levels sustained within twice the upper limit of normal (i.e. an index of inhibition of progression to HCC [9,10]) was 34.1% (29/85), 44.7% (17/38), and 25.0% (9/36) in those whose pretreatment ALT was 50 to <100 IU/L, 100 to <150 IU/L, and ≥150 IU/L, respectively; in the monotherapy group the frequency was 22.9% (11/48), 0% (0/23), and 14.3% (4/28), respectively (Fig. 2). Combination therapy was hence significantly superior to monotherapy ($P=0.001$; Mantel–Haenszel test).

Table 1
Patient demographics at baseline

	IFN + ribavirin	IFN alone	*P*-value
n	167	105	–
Sex (M/F)	135/32	81/24	0.538 (F)
Age (years), mean (S.D.)	47.9 (10.1)	49.1 (9.3)	0.391 (T)
HCV levels (kcopies/mL)			
<500	37	25	0.677 (MH)
500 to <850	46	21	
≥850	84	59	
ALT levels (IU/mL)			
50 to <100	87	48	0.228 (MH)
100 to <150	41	26	
≥150	39	31	
IFN treatment history			
Naïve	39	17	0.060 (MH)
Relapser	82	53	
Nonresponder	40	35	

F, Fisher's exact test; T, *t*-test; MH, Mantel–Haenszel test.

3.3. Association between virological efficacy and sustained ALT normalization

The patients judged to have sustained ALT normalization were divided into SVR and non-SVR patients based on virological efficacy and the effect of the addition of ribavirin to IFN assessed (see Table 2). The sustained ALT normalization

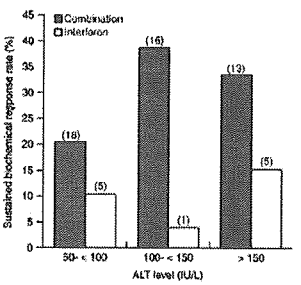

Fig. 1. Rate of sustained biochemical response to treatment by baseline ALT levels. The rate of sustained ALT normalization was significantly better with combination therapy than with interferon monotherapy (Mantel–Haenszel test: $P<0.01$). Numbers of patients in the parenthesis.

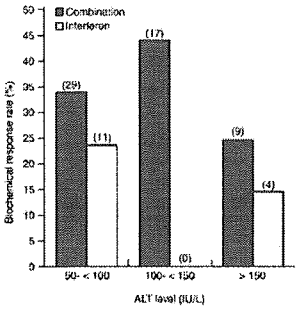

Fig. 2. Rate of patients with ALT levels sustained at less than twice the upper limit of normal (Mantel–Haenszel test: $P < 0.01$). ALT levels were measured ≥4 times during the post-treatment follow-up period. Numbers of patients in the parenthesis.

Table 2
Rate of sustained ALT normalization by virological response at end of follow-up and baseline ALT levels

ALT level (IU/L)	IFN + ribavirin		IFN alone	
	SVR	Non-SVR	SVR	Non-SVR
50 to <100	80% (8/10)	13% (10/77)	–	10% (5/48)
100 to <150	75% (6/8)	30% (10/33)	–	4% (1/26)
≥150	90% (9/10)	14% (4/29)	100% (2/2)	10% (3/29)
Total	82% (23/28)	17% (24/139)	100% (2/2)	9% (9/103)

rate in non-SVR patients was 17.3% (24/139) in the combination therapy group and 8.7% (9/103) in the monotherapy group. The results of the Mantel–Haenszel test taking ALT levels into account showed that combination therapy was significantly superior to IFN alone ($P = 0.034$). Logistic regression analysis determined the factors for sustained ALT normalization in both treatment groups, and are shown in Table 3. The risk for not achieving sustained ALT normalization in nonresponders to previous IFN treatment was four times higher than in IFN-treatment-naïve patients and relapsers. Among non-SVR patients with sustained ALT normalization, low pretreatment WBC count was the only significant influencing factor (data not shown).

3.4. Effect of timing of initial ALT normalization

The timing of initial ALT normalization with respect to pretreatment ALT levels is shown in Fig. 3. Log-rank test

Table 3
Multiple logistic regression analysis of factors associated with sustained ALT normalization

Variables	Odds ratio (adjusted)	95% CI	*P*-value
IFN nonresponder vs. relapser/naïve	0.250	0.093–0.669	0.0148
ALT	1.005	1.001–1.009	0.0116
WBC	1.000	0.999–1.000	0.0416
Serum creatinine	7.959	0.915–69.213	0.0598
IFN relapser vs. nonresponder/naïve	0.558	0.261–1.193	0.1277
Platelet	1.056	0.972–1.148	0.1931

indicated that the timing of normalization was significantly earlier in the combination group of patients with ALT levels ≥100 IU/L. ALT normalization rate at the end of treatment was directly correlated to pretreatment ALT levels: 83.9% (73/87), 80.5% (33/41), and 61.5% (24/39) of those whose baseline ALT was 50 to <100 IU/L, 100 to <150 IU/L, ≥150 IU/L, respectively, in the combination group and 79.2% (38/48), 53.8% (14/26), and 45.2% (14/31), respectively, in the monotherapy group showed normalization. ALT normalization rate at the end of treatment was significantly lower in the monotherapy group versus the combination group ($P = 0.015$; Mantel–Haenszel test).

3.5. Change in ALT levels by virological efficacy

Figs. 4 and 5 show mean ALT values during and after treatment in relapsers and nonresponders, respectively. No significant difference was observed between the combination and monotherapy groups in pretreatment ALT levels in either relapsers or nonresponders. When change in ALT levels during treatment in the monotherapy and combination therapy groups was compared, no difference in effect of HCV RNA-negativity during treatment was observed among relapsers. However, when the two treatment groups were compared at all time points after the end of treatment, ALT values were significantly lower in the combination therapy group ($P < 0.001$; Wilcoxon test). Furthermore, the mean value for all measurement time points was <80 IU/L in this treatment group. Moreover, in virological nonresponders all-time point ALT values were significantly lower in the combination group compared

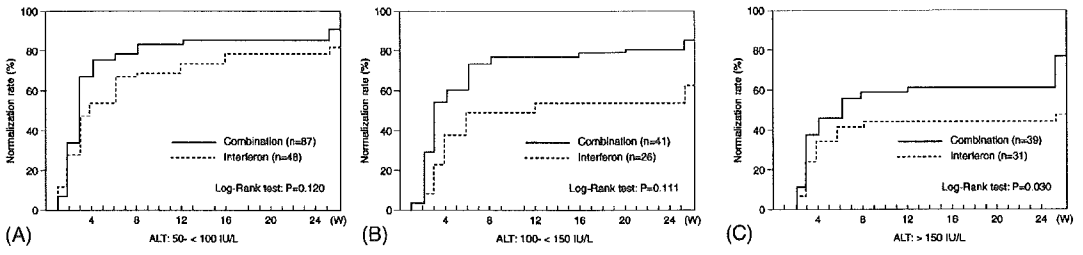

Fig. 3. Initial timing of ALT normalization by pretreatment ALT levels. No significant difference was observed between the combination and monotherapy groups in the timing of initial ALT normalization in patients with low pretreatment ALT levels (50–100 IU/L). In patients with pretreatment ALT levels of ≥100 IU/L, timing of initial ALT normalization was significantly earlier in patients receiving combination therapy than in patients receiving monotherapy.

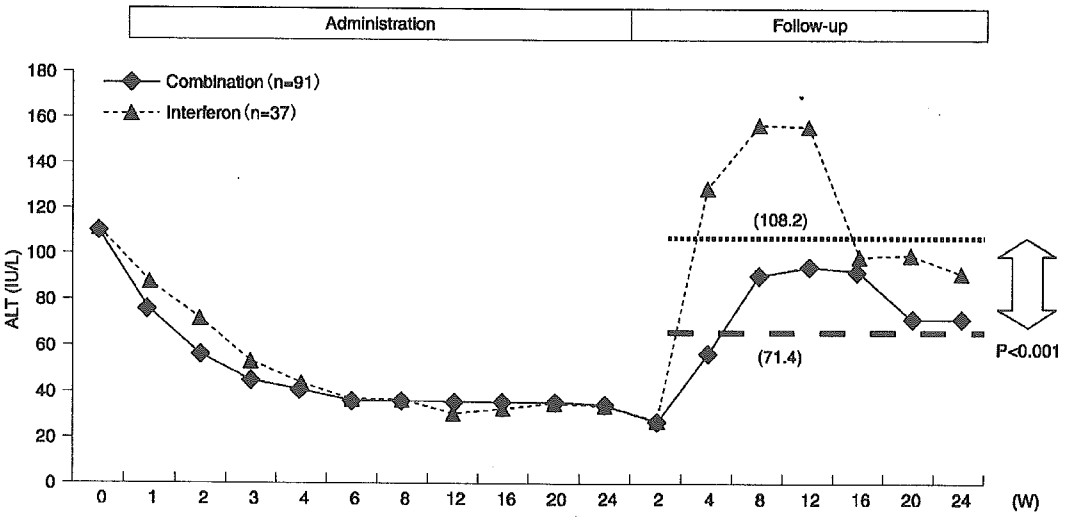

Fig. 4. Changes in ALT levels by viral response: relapsers. No difference was observed between patients receiving combination therapy and monotherapy during the treatment period. After the end of treatment, whereas ALT levels averaged within twice the upper limit of normal in patients receiving combination therapy, a period of marked increase in ALT levels was observed in patients receiving monotherapy. Mean ALT level over the entire period was significantly lower in the combination vs. monotherapy group.

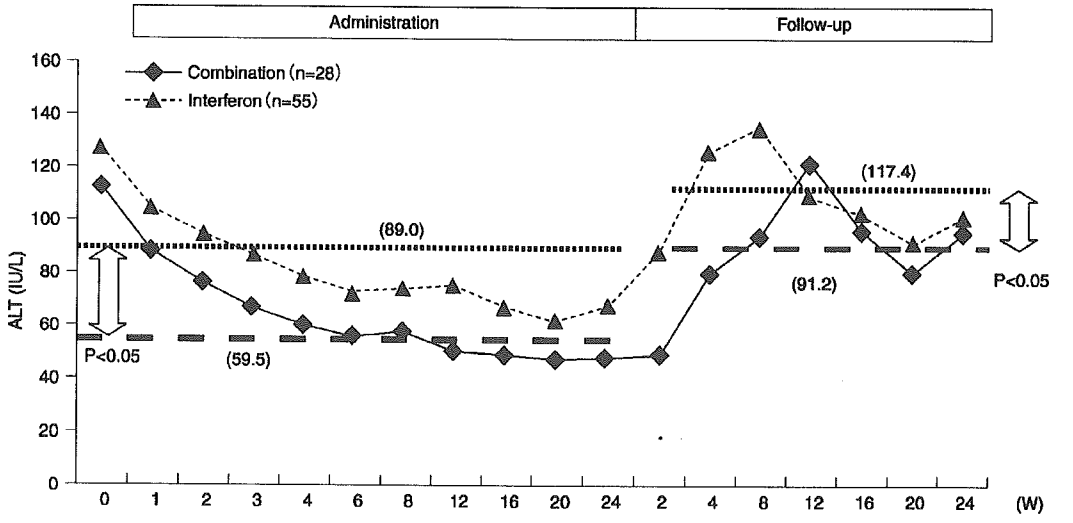

Fig. 5. Changes in ALT levels by viral response: nonresponders. Mean ALT level over the entire period was significantly lower in nonresponder patients receiving monotherapy than in patients receiving combination therapy.

with in the monotherapy group ($P=0.036$; Wilcoxon test). The mean value for all measurement time points during treatment was <80 IU/L.

4. Discussion

Pretreatment liver function affects progression to liver cirrhosis [24] and gender, alcohol consumption, ALT levels, and histological activity index are factors influencing progression to this condition in HCV-infected patients. This is perhaps related to the observation by Takimoto et al. [8] that achieving SVR in patients with high ALT levels is relatively easy. In the current study, SVR rate was observed to increase in correlation with higher pretreatment ALT levels in both the combination and monotherapy groups (data not shown). On the other hand, Yabuuchi et al. [7] have shown that ALT concentrations in patients with sustained ALT normalization, even in those who become HCV RNA-positive, are significantly lower than in SVR patients and virological nonresponders. In the present study, ALT normalization during treatment occurred at a higher rate in correlation with lower baseline ALT levels, but earlier timing of ALT normalization was not necessarily associated with sustained ALT normalization. However, sustained ALT normalization was achieved more easily in patients with high pretreatment ALT levels regardless of

treatment group. In contrast to non-Japanese reports [25,26], we found that achieving sustained ALT normalization was difficult in nonresponders and relapsers. Older age, longer disease duration, and difference in IFN dose may have been causative factors regarding this result; the reason could not be clarified in this study.

When IFN was first introduced for the treatment of chronic hepatitis C in Japan, the ALT normalization rates reported in genotype 1 patients ranged at about 18–32% [2,3,6]. Many of the patients included in the present study were nonresponders and relapsers to previous treatment, but nevertheless the observed sustained ALT normalization rate (15%) was not very different from those reported previously. In large-scale Japanese clinical studies, the incidence of sustained ALT normalization in non-SVR patients with genotype 1 was about 7–16% [2,4,6,7]. The incidence is estimated about 10% at maximum with IFN monotherapy. The present study not only indicates that addition of ribavirin improves SVR rate but also increases the incidence of sustained ALT normalization including in non-SVR patients compared with IFN monotherapy. Hence sustained liver function normalization was improved by about 30%. Furthermore, ribavirin add-on therapy significantly boosted the number of patients whose ALT was maintained within twice the upper limit of normal, which may reduce the risk of progression to HCC.

An issue that is gaining increasing importance for the future is the method of prevention of progression to HCC in virological nonresponders [27]. In Japan, the long-term rate of hepatocarcinogenesis is considered not greatly different between virological nonresponders to IFN therapy and untreated patients [5,8]. The 5-year incidence of cancer in virological nonresponders has been reported variously at 5–14% [6,7]. Yearly incidence rates of about 1–2% [4,8,28] including a high 5.1% [5] have also been reported. In studies conducted in the USA and Europe, the 5-year incidence rate of liver cirrhosis and HCC in virological nonresponders was 27.8% and 27%, respectively [29,30]. Furthermore, the incidence of progression to HCC in patients with advanced histology followed for 5–7 years was 9.6% [30]. We observed significantly lower on-treatment ALT levels in combination therapy patients versus those on monotherapy, which remained significantly lower after the end of treatment. The timing of ALT flare-up was also delayed in the combination group. This result cannot be explained by differences in change of HCV levels in the two groups since these were not significant (data not shown). However, whether the sustained low ALT levels associated with IFN plus ribavirin combination therapy will lead to less HCC will only be revealed with longer follow-up. Since improved liver function by continued ribavirin monotherapy in virological nonresponders to IFN plus ribavirin has been reported [31], long-term residual effects of ribavirin even after the end of treatment are a possibility. A large-scale clinical study of the effect of long-term treatment with PEG-IFN on prevention of progression to HCC in virological nonresponders to PEG-IFN plus ribavirin combination therapy is ongoing [32]. Long-term IFN monotherapy was reported to enhance ALT normalization in patients without HCV-RNA negative after previous IFN therapy [33].

On the other hand, in patients who relapsed after the end of treatment, mean ALT after the end of treatment was significantly lower in combination versus monotherapy patients. Relapse was observed delayed at 4 weeks after the end of treatment in the combination group (data not shown), suggesting a contribution of HCV to difference in the pattern of change in ALT levels. Viral relapse rate is known to be lowered by long-term combination therapy [12,13,34], and there is much expectation for increased efficacy by this regimen. The results of studies designed to test the hypothesis that time to onset of HCC is prolonged by combination therapy are awaited.

The present study was limited in that while it conclusively demonstrates that combination therapy with ribavirin increases the rate of sustained ALT normalization compared with IFN monotherapy, it was not powered to show prevention of progression to HCC in the long term. Large-scale clinical trials are necessary to examine this postulate. In particular, it is important to determine whether the period of prevention to progression to HCC is extended in virological nonresponders to combination therapy.

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