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Inhibitory effect of branched-chain amino acid granules on progression of compensated liver cirrhosis due to hepatitis C virus

Masahiro Kobayashi, Kenji Ikeda, Yasuji Arase, Yoshiyuki Suzuki, Fumitaka Suzuki, Norio Akuta, Tetsuya Hosaka, Naoya Murashima[1], Satoshi Saitoh, Takashi Someya, Akihito Tsubota[2], and Hiromitsu Kumada

Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan
[1]Present address: Department of Gastroenterology, Mishuku Hospital, Tokyo, Japan
[2]Present address: Institute of Clinical Medicine and Research, Jikei University School of Medicine, Kashiwa, Chiba, Japan

Background. A phase II randomized controlled trial was conducted in patients with compensated liver cirrhosis to investigate the inhibitory effect of branched-chain amino acid (BCAA) granules for oral use (TK-98) on disease progression. ***Methods.*** Patients who had compensated liver cirrhosis due to hepatitis C virus with baseline serum albumin levels between 3.6 and 4.5 g/dl were assigned to the TK-98 group, which was treated with BCAA granules (TK-98) for 168 weeks, or to a control group (no treatment). ***Results.*** No symptoms indicating decompensated cirrhosis, including ascites, edema, and hepatic encephalopathy were reported in either the TK-98 or control group during the study observation period. Hepatocellular carcinoma (HCC) was noted in eight of the 39 patients studied, and of these three received TK-98 (15.8%) and five were untreated (25.0%). A time-to-event analysis for the effect of BCAA therapy on development of HCC revealed no statistically significant differences between the two groups. However, an additional analysis of data from a subgroup with a baseline serum albumin level of <4.0 g/dl showed that the incidence of HCC was likely to be lower in BCAA-treated patients. ***Conclusions.*** BCAA may inhibit hepatic carcinogenesis in patients with compensated cirrhosis with a serum albumin level of <4.0 g/dl.

Key words: BCAA, HCV, compensated liver cirrhosis, hepatocellular carcinoma

Introduction

Liver cirrhosis is classified into two types according to the progression phase of the disease: compensated cirrhosis and decompensated cirrhosis. For improved prognosis and quality of life of patients with liver cirrhosis, it is important to delay progression of the disease from the asymptomatic compensated phase to the decompensated phase, which is accompanied by symptoms such as ascites, edema, and hepatic encephalopathy. The use of branched-chain amino acid (BCAA) granules has been shown to improve hypoalbuminemia in decompensated patients with cirrhosis and hypoalbuminemia despite adequate dietary intake. In addition, several studies have reported that BCAA granules improve the above symptoms of decompensated cirrhosis as well as delay development of serious complications that affect the prognosis for survival.[1–5] Therefore, the drug has now been extensively used for the purpose of improving serum albumin levels and ameliorating the disease state in patients with cirrhosis.

Serum albumin levels have been reported to serve as an important indicator of the severity of liver cirrhosis, and the maintenance or improvement of these levels is vital for improving the prognosis of liver cirrhosis.[3] We conducted a phase II randomized controlled trial to investigate whether supplementation with BCAA granules increased lowered serum albumin levels and delayed progression of the disease in patients with compensated cirrhosis. Furthermore, we also explored the inhibitory effect of BCAA therapy on development of hepatocellular carcinoma (HCC), based on results of a study showing that the development of HCC has a substantial impact on prognosis of patients with cirrhosis and that the lower the serum albumin level, the greater the risk of HCC.[6]

Materials and methods

Study design

This study was conducted in accordance with Japanese Good Clinical Practice, after review and approval by the

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Reprint requests to: M. Kobayashi

Institutional Review Board of Toranomon Hospital. Subjects were fully informed of the nature of the study, and informed consent to participation in the study was obtained in writing from each subject. Patients enrolled were randomized to receive either BCAA granules (TK-98) or no treatment (control).

The inclusion criteria were as follows: (1) presence of compensated cirrhosis due to hepatitis C virus; (2) no prior or concurrent ascites, edema, or hepatic encephalopathy; (3) serum albumin level between 3.6 and 4.5 g/dl within 2 months prior to the study; (4) male sex and age between 50 and 70 years inclusive. Excluded from the study were patients who had or were considered to have HCC or cancer other than HCC, those with concurrent alcoholic cirrhosis and alcohol dependence, and those receiving nutritional supplements for the management of hepatic failure.

As the present study was intended to evaluate the effect of BCAA, study subjects were those with hepatitis C virus (HCV)-related cirrhosis. Such patients account for more than 60% of Japanese patients with liver cirrhosis.[7] The study had as an additional objective the exploration of the inhibitory effect of BCAA on HCC; therefore, the inclusion criteria included male sex and age between 50 and 70 years, because men in that age range are generally considered to have a propensity to develop HCC.[8]

The following drugs were prohibited during the study: high-BCAA agents for treatment of hepatic disorders, because these may alter plasma albumin and malotilate levels. There were no other restrictions on the concomitant use of drugs.

The primary end point was time to onset of ascites, edema, or hepatic encephalopathy, which are considered to be an indication of disease progression to decompensated cirrhosis. Transition to the decompensated phase of cirrhosis was defined to the time point at which one of the following manifestations was noted for the first time: (a) ascites found on palpation, (b) slight edema in the lower extremities, and (c) grade I or higher hepatic encephalopathy. The secondary variables were serum albumin level, blood Fischer's ratio (BCAA/aromatic amino acids, molar ratio), development of jaundice, performance status (PS), and development of HCC.

It has been reported that the serum albumin level decreases at a rate of 0.15 g/dl per year in patients with liver cirrhosis.[9] We assumed that a serum albumin level above an approximate threshold of 3.5 g/dl might indicate transition to decompensated cirrhosis.[10] Therefore, patients enrolled in the study were expected to have a baseline serum albumin level between 3.6 and 4.5 g/dl. We made the assumption that 15% of the control group would progress to decompensated cirrhosis annually and that treatment with TK-98 would reduce the progression rate to 5% with a hazard ratio of around 3.2. An observation period of 168 weeks was chosen on the presumption that compensated cirrhosis might progress into the decompensated phase in around 3.5 years in half of the patients. For a statistical significance level set at two-sided 20% and a statistical power at 60%, the sample size needed for the analysis was calculated to be 17 patients per group. Estimating a dropout rate of 15%, we set the target number of study patients at 20 patients per group, that is, a total of 40 patients.

Study checkups were carried out at 8-week intervals for the presence or absence of ascites, edema, hepatic encephalopathy, or jaundice; PS; subjective and/or objective symptoms; and laboratory parameters. In addition, each study subject was assessed for development of HCC with diagnostic imaging at intervals of 24 weeks. When any abnormal changes were noted in serum α-fetoprotein or protein induced by vitamin K absence or antagonist II levels, examination for HCC was additionally undertaken as appropriate.

The TK-98 group and control group each consisted of 20 subjects. Patients were dropped from the study if any symptoms of ascites, edema, hepatic encephalopathy, or jaundice appeared, indicating the decompensated phase of cirrhosis, or if HCC was found to have developed during the study period.

Study drug

BCAA granules (TK-98) containing 952 mg of L-isoleucine, 1904 mg of L-leucine and 1144 mg of L-valine per packet were orally administered to subjects at doses of one packet three times daily after meals. The control patients received no treatment.

Statistical analysis

Statistical analysis was performed with SAS Release 9.1.3 Service Pack 2. A time-to-event analysis was carried out to determine the transition to the decompensated phase of cirrhosis using the time point of event onset at which any of symptoms such as ascites, edema, or hepatic encephalopathy were noted for the first time. Survival functions were estimated by the Kaplan-Meier method, and the survival functions were compared between the two groups by using the log-rank test. Cox's proportional hazards models were used to examine the effect of the treatment and prognostic variables. Serum albumin levels and Fischer's ratio data were analyzed by using a mixed-effects model in terms of respective time-course patterns.

Results

Disposition of patients

Study subjects were selected from patients with compensated cirrhosis who visited the Department of Hepatology, Toranomon Hospital between January 1999 and March 2003. A total of 40 patients who met the inclusion criteria and gave written informed consent were enrolled in this study. Flow chart of patients through the trial is shown in Fig. 1. Of these 40 patients, one was dropped from the study prior to study commencement because he withdrew his consent, and nine patients were dropped from the study during the study period because of the development of HCC in eight patients and for a visit-related reason in the case of the remaining patient. All 39 patients who began the study were judged to be eligible and were included in the full analysis set and the per protocol set, as well as in the safety analysis.

Patient demographic and baseline characteristics are shown in Table 1. No significant differences were noted between the two groups with respect to age, concurrent esophageal varices or diabetes mellitus, history of alcohol drinking, serum albumin levels, blood Fischer's ratio, total bilirubin, platelet count, serum aspartate aminotransferase levels, or serum alanine aminotransferase levels.

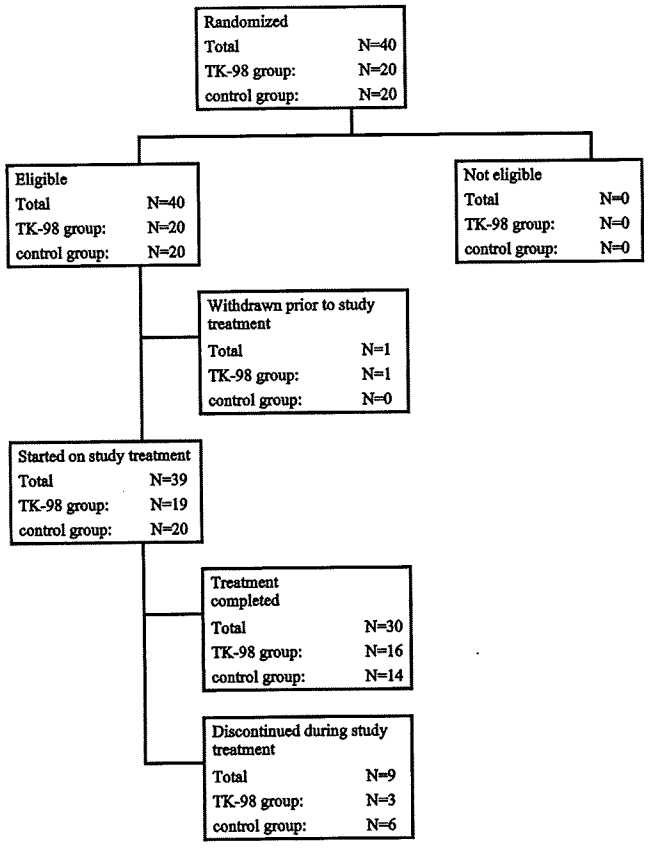

Fig. 1. Flow chart of patients. A total of 39 subjects who initiated study treatment were included in the full analysis set (FAS) and the per protocol set (PPS), as well as a safety analysis

One patient in the control group was positive for anti-hepatitis B surface antigen, but negative for anti-hepatitis B e antigen and with a low anti-hepatitis B core (HBc) antibody titer. The patient's serum hepatitis B virus (HBV) DNA level remained at <2.6 log copies/ml; therefore, the hepatic disorder in this patient was considered to be due mainly to HCV. All patients were negative for antinuclear antibodies and antimitochondrial-M2 antibodies, indicating no concurrent autoimmune hepatitis or primary biliary cirrhosis. A positive anti-HBc antibody result was reported in 12 patients (63.2%) in the TK-98 group (n = 19) and in 11 patients (55.0%) in the control group (n = 20). Of these patients, HCC developed in three patients in each group. High serum anti-HBc antibody titers were observed in four patients (21.1%) of the TK-98 group four (20.0%) of the control group, among whom only one patient of the TK-98 group contracted HCC.

Ursodeoxycholic acid (UDCA) was used in 13 patients (68.4%) in the TK-98 group and in 17 patients (85.0%) in the control group, and parenteral glycyrrhizinate was administered to 14 patients (73.7%) of the TK-98 group and 12 patients (60.0%) of the control group. Of the eight patients with HCC, seven received both UDCA and parenteral glycyrrhizinate. Interferon was used in one patient (5.0%) of the control group.

Primary end point

During the 168-week observation period, no patients had symptoms of ascites, edema, or hepatic encephalopathy indicating decompensated cirrhosis in either the TK-98 group or the control group. Therefore, analysis for primary end-point assessment was not performed.

Secondary variables

No remarkable findings were noted regarding jaundice or PS in the two groups. The time courses of the serum albumin level and Fischer's ratio are presented in Figs. 2 and 3, respectively. The serum albumin levels (mean ± SD) at baseline and at weeks 56, 112, and 168 of study observation were 3.86 ± 0.26, 3.82 ± 0.24, 3.81 ± 0.19, and 3.73 ± 0.29, respectively, in the TK-98 group, and 3.90 ± 0.33, 3.91 ± 0.29, 3.91 ± 0.28, and 4.03 ± 0.30, respectively, in the control group (Table 2). A group-effect analysis of the serum albumin levels revealed no significant differences between the two groups (P = 0.8488). A mixed effect model was used to analyze changes in serum albumin levels over time during the 168-week period, using the study group and the assessment time point as

Table 1. Baseline characteristics of two groups

	TK-98 group ($n = 19$)	Control group ($n = 20$)
Age (years)	62.9 ± 5.7	59.5 ± 7.2
Height (cm)	165.07 ± 6.46	166.94 ± 4.48
Body weight (kg)	62.81 ± 9.41	68.39 ± 10.64
BMI (kg/m^2)	23.03 ± 3.03	24.51 ± 3.50
Time since contraction of disease (years)	4.86 ± 4.64	4.29 ± 3.86
History of alcohol consumption (yes/no)	6/13	6/14
Ascites	0	0
Edema	0	0
Hepatic encephalopathy	0	0
Gastric and esophagus varices	10	10
Concurrent of diabetes mellitus	3	4
Concurrent hypertension	7	6
Concurrent gallstone	4	3
Platelet count (×10^4/mm^3)	12.23 ± 6.48	11.59 ± 4.33
Total protein (g/dl)	7.73 ± 0.47	7.64 ± 0.37
Serum albumin (g/dl)	3.86 ± 0.26	3.90 ± 0.33
Total bilirubin (mg/dl)	0.77 ± 0.23	0.75 ± 0.22
AFP (mAU/ml)	11.0 ± 12.9	10.9 ± 10.9
PIVKA-II (ng/ml)	21.5 ± 11.6	19.5 ± 7.1
Fischer's ratio	3.047 ± 0.637	2.734 ± 0.647
AST (GOT) (IU/l)	42.9 ± 16.3	41.8 ± 14.6
ALT (GPT) (IU/l)	48.0 ± 24.2	47.7 ± 23.3
HBsAg (+)	0	1
HBcAb (+)	12	11
HBcAb (+) (high titer)[a]	4	4
ANA (+)	0	0
AMA-M2 (+)	0	0

Data are expressed as number of patients or mean ± standard deviation
BMI, body mass index; AFP, a-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist II; AST, aspartate aminotransferase; GOT, glutamyl oxaloacetic transaminase; ALT, alanine aminotransferase; GPT, glutamyl pyruvic transaminase; HBsAg, hepatitis B surface antigen; HBcAb, hepatitis B core antibody; ANA, antinuclear antibody; AMA-M2, anti-mitochondrial antibody-M2; S/CO, sample/cut off
[a] S/CO score ≥ 10.00 (CLIA method)

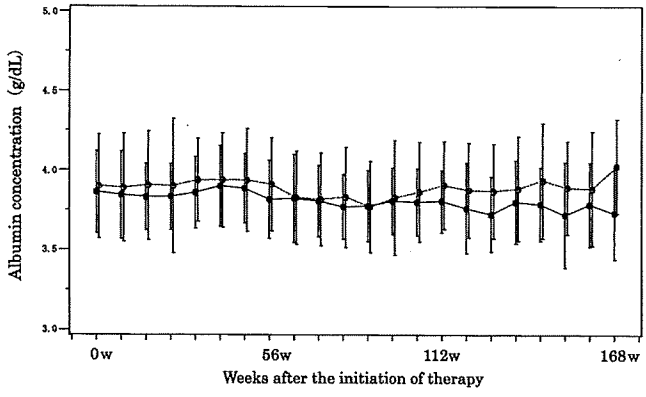

Fig. 2. Serum albumin concentration in TK-98 group (*black dots*) and control group (*white dots*). Data are means; bars show the standard deviation

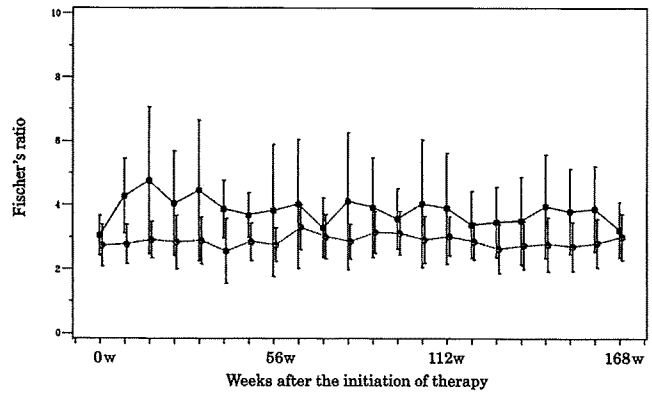

Fig. 3. Fischer's ratio in TK-98 group (*black dots*) and control group (*white dots*). Data are means; bars show the standard deviation

interaction terms, and resulted in an estimate of –0.005, $P = 0.0288$. These findings implied that the intergroup difference in serum albumin levels widened progressively by –0.005 g/dl every 8 weeks. However, these changes were negligible with respect to the time course of serum albumin levels over the 168 weeks.

A group-effect analysis revealed that Fischer's ratio was significantly higher in TK-98 treated patients ($P =$

Table 2. Mixed-effects model analysis of the pattern of changes in serum albumin levels and Fischer's ratio

Group	Baseline	Week 56	Week 112	Week 168	Group effect			Time point × group interaction		
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Estimate	*t*	*P*	Estimate	*t*	*P*
Serum albumin levels										
TK-98 group	3.86 ± 0.26	3.82 ± 0.24	3.81 ± 0.19	3.73 ± 0.29	0.01157	0.19	0.8488	−0.00497	−2.19	0.0288
Control group	3.90 ± 0.33	3.91 ± 0.29	3.91 ± 0.28	4.03 ± 0.30						
Fischer's ratio										
TK-98 group	3.05 ± 0.64	3.83 ± 2.06	3.91 ± 1.74	3.22 ± 0.86	0.3054	4.10	0.0001	−0.00883	−2.46	0.0143
Control group	2.73 ± 0.65	2.75 ± 0.52	3.02 ± 0.61	3.01 ± 0.72						

Table 3. Cox proportional hazards model analysis of the event of hepatocellular carcinoma

	Hazard ratio	95% confidence interval	χ^2	Two-sided *P* value
Analysis with treatment group as an independent variable				
Independent variable				
Treatment group	0.606	0.145–2.539	0.4690	0.4935
Analysis with treatment group as an independent variable and serum albumin level as a explanatory variable				
Independent variable				
Treatment group	0.546	0.130–2.299	0.6808	0.4093
Explanatory variable				
Albumin	0.452	0.058–3.522	0.5755	0.4481

0.0001). Fischer's ratio (mean ± SD) at baseline and at weeks 56, 112 and 168 of study observation was 3.047 ± 0.637, 3.831 ± 2.056, 3.905 ± 1.735, and 3.221 ± 0.862, respectively, in the TK-98 group, and 2.734 ± 0.647, 2.754 ± 0.521, 3.021 ± 0.614, and 3.012 ± 0.715 in the control group (Table 2).

HCC developed in three of 19 patients in the TK-98 group and in five of 20 in the control group. Cox's proportional hazards model analyses were performed to determine the effect of BCAA treatment and serum albumin levels on development of HCC. The results showed that the hazard ratio of the BCAA treatment relative to no treatment was 0.606 (95% confidence interval, 0.145–2.539; Table 3). A time-to-event analysis was performed with the development of HCC. The result was $P = 0.4889$ (log-rank test, Fig. 4). Furthermore, another time-to-event analysis for subgroups with baseline body mass index (BMI) of 25 and higher or those with a baseline serum albumin level of ≤4.0 g/dl yielded $P = 0.2473$ and $P = 0.0930$ (log-rank test), respectively, in these two subgroups (Fig. 5).

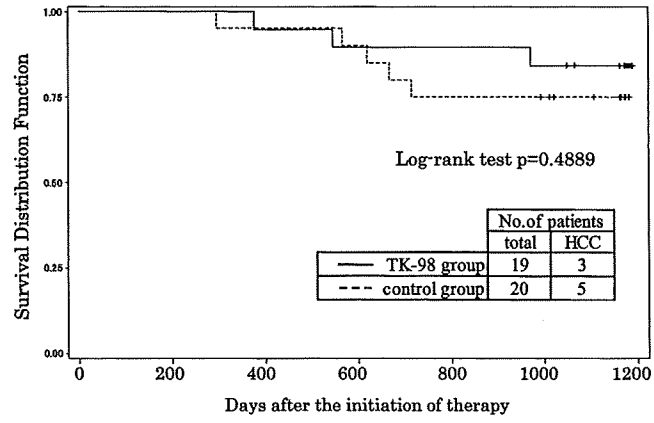

Fig. 4. Kaplan-Meier estimates of event-free survival for hepatocellular carcinoma (*HCC*) in patients with compensated liver cirrhosis caused by hepatitis C virus (HCV) infection

Safety

During the study, adverse events were reported in 17 (89.5%) of 19 patients treated with TK-98 (75 events) and in 19 (95.0%) of 20 untreated patients (85 events). No significant difference was found in the incidence of adverse events between the two groups. Two adverse reactions were reported in TK-98 treated patients: constrictive pericarditis in one patient, and a gastrointestinal symptom in another.

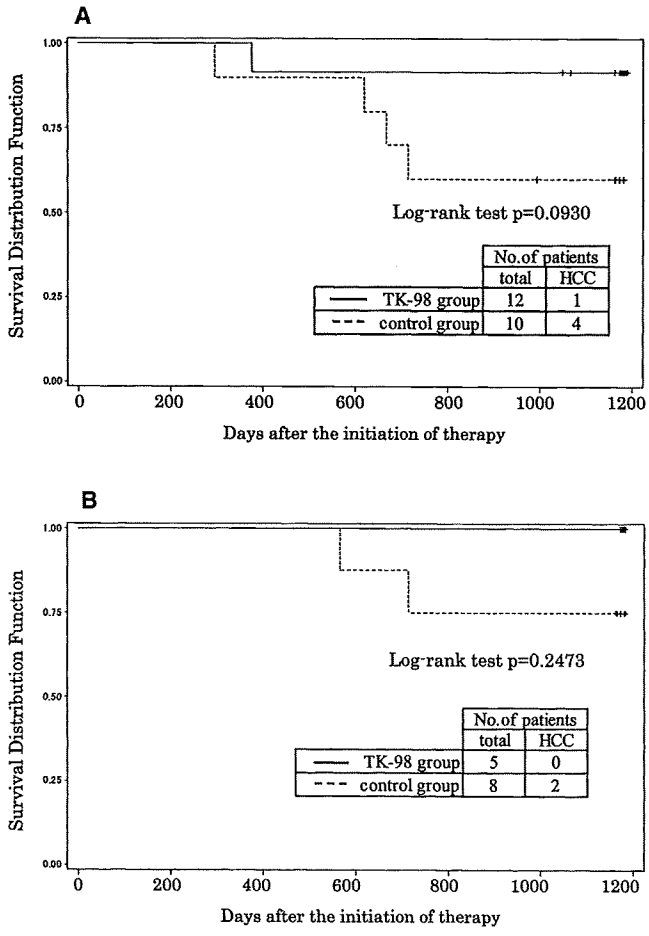

Fig. 5A,B. Kaplan-Meier estimates of event-free survival for HCC in patients with compensated liver cirrhosis caused by HCV infection. **A** Subgroup with a baseline serum albumin level of <4.0 g/dl; **B** subgroup with baseline body mass index (*BMI*) ≥ 25

Discussion

In Japan, no effective treatment has been established for compensated cirrhosis, whereas an effect of BCAA therapy has been confirmed in patients with decompensated cirrhosis and a serum albumin level of <3.5 g/dl. Several studies have shown an effect of BCAA in patients with compensated cirrhosis by investigating influence of the therapy on serum albumin levels,[11,12] but no studies have been performed to investigate the effect of BCAA on the entire disease state of liver cirrhosis. Therefore, we conducted a randomized controlled trial on the presumption that treatment with BCAA in patients with compensated cirrhosis might possibly delay disease progression.

In the present study, we assumed that the disease phase might shift to decompensated cirrhosis in several of the patients randomized to the control group. In the course of the 168-week observation period, however, no appreciable changes in serum albumin levels or Fischer's ratio were found in this group. Also, no symptoms of ascites, edema, or hepatic encephalopathy, indicating decompensated cirrhosis, developed. The results therefore failed to demonstrate any inhibitory effect of BCAA on progression from compensated to decompensated cirrhosis. A slightly extended observation period and a larger sample size would be necessary to identify such an effect of BCAA.

The mechanism whereby BCAA can improve hypoalbuminemia has been considered to consist in the supply of substrates for protein synthesis from a nutritional standpoint. Later, it was clarified that BCAA, especially L-leucine, acts to facilitate protein synthesis by stimulating initiation of albumin mRNA translation via activation of the intracellular signal transduction system, primarily pertaining to mammalian target of rapamycin (mTOR).[13,14] A study assessing albumin synthesis in primary cultures of rat hepatocytes with BCAA showed that the albumin level increased in the presence of BCAA from 0.1 to 0.5 mM in a dose-dependent fashion, whereas there was no such elevation in the albumin concentration at higher levels of BCAA.[14]

Habu and colleagues reported the effect of BCAA on serum albumin levels in relation to the BCAA/tyrosine molar ratio (BTR) in studies in which they administered BCAA granules for 2 years to patients with compensated cirrhosis with serum albumin levels between 3.5 and 3.9 g/dl. They showed that the BCAA treatment increased serum albumin levels in patients with cirrhosis and with BTR < 4, whereas there was no appreciable elevation in serum albumin levels in patients with BTR ≥ 4.[11,12] The BTR has been reported to correlate well with Fischer's ratio,[15] and a BTR value of 4 corresponds to a Fischer's ratio of 2.[11] In the present study, nearly all patients had a baseline Fischer's ratio of 2 or greater, and the BTR value was maintained without any decrease during the study. Our results revealed that an albumin-increasing effect of BCAA treatment was unclear in patients with compensated cirrhosis and a Fischer's ratio of 2 or higher, which is consistent with the findings of Habu and colleagues. We thus inferred that no appreciable elevation in serum albumin level occurs in response to treatment with BCAA of patients with cirrhosis but without an amino acid imbalance.

HCC developed in three (15.8%) of the 19 TK-98 treated patients and in five (25.0%) of the 20 untreated patients (control). There was no evidence of an inhibitory effect of BCAA treatment on the development of HCC (Fig. 4). A previous study indicated that, in patients with cirrhosis due to HCV infection, the lower the serum albumin level, the greater the risk for hepatic carcinogenesis, and that the hazard ratio in this respect was 1.92-fold higher in patients with cirrhosis and a serum albumin level of <4.0 g/dl than in those with a serum albumin level of 4.0 g/dl or higher.[6] Another study dem-

onstrated that BCAA suppressed cancer development in patients with decompensated cirrhosis and a BMI of ≥25.[16] In the present study, we also performed a time-to-event analysis of pertinent data from a subset of patients with BMI ≥ 25 or those with a baseline serum albumin level of <4.0 g/dl to explore for any suppressive effect of BCAA on hepatic carcinogenesis, using the development of HCC as the event. The analysis revealed a tendency toward suppression of hepatic cancer development in the subgroup with a baseline serum albumin level of <4.0 g/dl ($P = 0.0930$, log-rank test), but the P value was 0.2473 (log-rank test) for the subgroup with BMI ≥ 25 (Fig. 5).

It is generally recognized that abnormal carbohydrate metabolism occurs frequently in patients with cirrhosis due to HCV infection,[17] and the incidence is higher in patients presenting with more advanced symptoms. Hyperinsulinemia and insulin resistance have been identified as major factors contributing to the development of abnormal carbohydrate metabolism, and recent studies have implicated hyperinsulinemia and obesity as risk factors in the genesis of HCC.[18–22] Furthermore, another study has documented acceleration of HCC proliferation in the presence of postprandial hyperinsulinemia.[23]

Recent studies using a CCl_4-induced rat cirrhosis model have demonstrated that L-leucine and L-isoleucine improve abnormal carbohydrate metabolism by facilitating non-insulin-mediated glucose uptake in skeletal muscles and by stimulating m-TOR signaling-mediated glycogen synthesis.[24–26] We thus infer that in patients with cirrhosis and abnormal glucose tolerance, BCAA treatment provides correction of hyperinsulinemia via improvement of abnormal carbohydrate metabolism. Therefore, our results showing that hepatic cancer development tended to be suppressed following treatment with BCAA may indicate an effect of BCAA in ameliorating abnormal carbohydrate metabolism. In fact, the large-scale LOTUS study conducted in patients with decompensated cirrhosis demonstrated that long-term dietary supplementation with BCAA inhibited liver carcinogenesis in patients with cirrhosis and BMI ≥ 25, who are often considered to have hyperinsulinemia or insulin resistance.[16] However, blood glucose and insulin were not determined in this study, so assessment of the effect of BCAA on carbohydrate metabolism is left for future studies.

The present study, though of a small scale, represents the first clinical trial ever undertaken to explore the inhibitory effect of BCAA on disease progression in patients with compensated cirrhosis. No symptoms indicating progression of cirrhosis from the compensated to decompensated phase were noted in either the TK-98 group or the control group during this study, and we could not evaluate any inhibitory effect of BCAA therapy on progression of cirrhosis. However, the results suggested that BCAA may inhibit hepatic carcinogenesis in patients with compensated cirrhosis and a serum albumin level of <4 g/dl. Long-term therapy with BCAA granules is not considered to entail any safety concerns because there was no statistically significant difference between the two groups in the incidence of adverse events, nor was there any adverse event of clinical concern.

BCAA has a variety of pharmacologic effects, among which the effect of improving abnormal carbohydrate metabolism is considered to have an inhibitory effect on liver carcinogenesis. The underlying mechanism of this action, nevertheless, has yet to be further clarified. It is important to explore whether BCAA therapy inhibits development of hepatic or other types of cancer in larger clinical trials with patients with compensated cirrhosis.

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The Efficacy of Short-term Interferon-beta Therapy for Chronic Hepatitis C Patients with Low Virus Load

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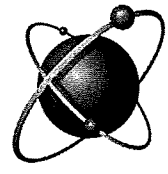

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□ ORIGINAL ARTICLE □

The Efficacy of Short-term Interferon-beta Therapy for Chronic Hepatitis C Patients with Low Virus Load

Yusuke Kawamura[1], Yasuji Arase[1], Kenji Ikeda[1], Fumitaka Suzuki[1], Yoshiyuki Suzuki[1], Masahiro Kobayashi[1], Norio Akuta[1], Tetsuya Hosaka[1], Hitomi Sezaki[1], Hiromi Yatsuji[1], Mariko Kobayashi[2] and Hiromitsu Kumada[1]

Abstract

Objective The aim of this study was to elucidate the efficacy of short-term interferon (IFN) therapy for chronic hepatitis C patients with low virus load.

Methods The present study was a retrospective cohort study. Inclusion criteria were biopsy-proven chronic hepatitis, the serum hepatitis C virus (HCV) RNA level of less than 100 KIU/ml, IFN period of 8weeks or less. One hundred and eleven consecutive patients satisfied above criteria were treated with IFN-beta (dose: 6 MU, daily for 4, 6, or 8 weeks).

Results Background of clinical profiles were as follows: median (range) age=56 (20-73) years, male/female=64/47, genotype 1b/2a/2b=40/68/3, and median (range) HCV-RNA= 34 (4.5-81) KIU/ml. Out of 111, 64 patients (57.7%) had sustained viral response (SVR). Based on the difference of HCV genotype, the SVR rate was 47.5% (19/40) in genotype 1 and 63.3% (45/71) in genotype 2. In genotype 1, the SVR rate in patients treated with the 8-week-regimen was significantly higher than that in patients treated with the 4- or 6-week regimen. In contrast, in genotype 2, the SVR in patients treated with the 8-week regimen was not significantly different from that in patients treated with the 6-week regimen. None of the patients had severe IFN-related side effects.

Conclusions The 6 or 8-week regiment of IFN-beta therapy is one selection of therapy for chronic hepatitis C patients who have tended to have a SVR and who show IFN-related adverse events.

Key words: chronic hepatitis C, low virus load, interferon, sustained viral response

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Introduction

Current interferon (IFN) therapy for patients with chronic hepatitis C viral infection has been directed at viral clearance. Recent studies reported improvement of therapeutic efficacy when IFN is combined with ribavirin (1-5). Moreover, novel long-acting formulations of IFN known as pegylated IFN induced higher eradication rate of hepatitis C virus (HCV) (6-8). However, IFN is expensive and has a number of serious side effects. Therefore, if the treatment period becomes shorter, it could be preferable.

Several predictive factors of sustained viral response (SVR) to IFN have been identified, and these include a short duration of disease, young age, absence of liver cirrhosis, low HCV-RNA levels, HCV genotype 2a and mutant type of nonstructural 5A region (9-15). Low dose IFN tends to eradicate HCV RNA in patients who had a low serum level of HCV-RNA. However, there is also controversy over how long the IFN therapy should be continued to eradicate HCV RNA in patients. Thus, in this study we evaluated the duration of IFN therapy in order to eradicate HCV RNA in patients who had low serum levels of HCV-RNA.

Abbreviations: HCV: hepatitis C virus, IFN: interferon, SVR: sustained viral response

[1]Department of Hepatology, Toranomon Hospital, Tokyo and [2]Department of Hepatic Research Unit, Toranomon Hospital, Tokyo
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Correspondence to Dr. Yusuke Kawamura, k-yusuke@toranomon.gr.jp

Table 1. Clinical Characteristics before Interferon Therapy in Chronic Hepatitis C Patients*

Characteristics	(n=111)
Age (years old) †	56(20-73)
Male/female ‡	64/47
Liver histology (fibrosis, 1/2/3) ‡	60/25/26
HCV genotype(1b/2a/2b) ‡	40/68/3
HCV load (KIU/ml) †	34 (4.5-81)
AST (IU/L) †	56 (14-226)
ALT (IU/L) †	76 (15-434)

***ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; and HCV, hepatitis C virus.**

† Data are expressed as median(range).

‡ Data are number of patients.

Materials and Methods

Patients

A total of 111 consecutive chronic hepatitis C patients treated with IFN-beta for HCV RNA clearance at Toranomon Hospital in Tokyo, Japan between 1997 and 2006 were enrolled in this study. This study was a retrospective cohort study. Enrollment criteria were: repeated alanine aminotransferase (ALT) elevation greater than the upper normal limits (ALT normal range: 12-50 IU/L) for more than six months; histological evidence of chronic hepatitis within one year of entry into the trial; positive serum HCVRNA; serum HCV RNA level of less than 100 KIU/ml or 1 Meq/ml; genotype 1b, 2a and 2b. We excluded from the study all of the patients: 1) with concurrent hepatitis B virus (HBV); 2) with a history of IFN therapy; 3) Leukocytes <3,000/mm^3, platelets <80,000/mm^3 and bilirubin >1.5 mg/ml before IFN therapy.

One hundred eleven patients received IFN at a dose of 6 million units (MU) of natural IFN-beta (Toray Industries or Daiichi Pharmaceutical Co., Tokyo, Japan) daily for 4, 6 or 8 weeks. In general, patients were treated with IFN for 8 weeks. Eleven patients treated for 4 weeks and thirty patients treated for 6 weeks were assigned by randomized controlled trial. We regarded sustained viral response (SVR) to therapy as clearance of HCV RNA by RT-nested PCR (16) or amplicor method (17) for more than 6 months after cessation of therapy. Our study was approved by the institutional ethics review board of our hospital. The physician in charge explained the purpose and method of this clinical trial as well as the potential adverse reactions to each patient, who later gave his/her informed consent for participation.

Blood testing

Blood samples were obtained just before IFN therapy and stored at -80℃. Using these blood samples, HCV-RNA levels before IFN therapy were analyzed by quantitative PCR assay (Amplicor GT-HCV Monitor Version 2.0, Roche Molecular Systems) (18).

On the other hand, serum HCV-RNA at 6 months after the termination of IFN therapy was analyzed by the qualitative PCR assay or RT-nested PCR. The lower detection limit of the qualitative assay is 100 copies/ ml. HCV genotype was examined by the PCR assay, using a mixture of primers for the six subtypes known to exist in Japan, as reported previously (19).

Liver histology

Liver biopsy specimens were obtained percutaneously or by peritoneoscopy using a modified Vim Silverman needle

Table 2. Predictive Factors for SVR in Patients with HCV Genotype 1*

Factor	Category	Odds ratio	95% CI†	p value
Period of IFN therapy (week)	4 or 6/ 8	1/8.93	2.14-37.03	0.003
AST (IU/L)	<76/≥76	1/2.17	0.85-5.55	0.102
Sex	Man / Woman	1/0.56	0.16-2.00	0.367
ALT (IU/L)	<100/≥100	1/1.67	0.47-5.93	0.430
Liver histology (fibrosis)	1 /2,3,4	1/0.79	0.39-1.60	0.507
Age (years)	<50/ ≥50	1/0.80	0.23-2.79	0.726

***ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IFN, interferon and CI; confidence interval.**

with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin-eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The size of specimens for examination included more than six portal areas. Histopathological interpretations of these 3-to 4-μm thick sections were made independently by experienced liver pathologists (YA and HK) who had no clinical information or knowledge of chronological order of the biopsies in each pair. The biopsy specimens were scored according to the system of Desmet et al (20).

Statistical analysis

Independent factors that might have influenced SVR were studied using the logistic regression analysis, and the following variables were evaluated as prognostic factors: sex, age, liver histology, biochemical factors (aspartate aminotransferase (AST), ALT before IFN therapy, and period of IFN therapy. Significance of trends in SVR based on periods of IFN therapy was determined Cochran-Armitage trend test. The SPSS software package (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. A p value of <0.05 was considered to indicate a significant difference.

Abbreviations: ALT: alanine aminotransferase, AST: aspartate aminotransferase

Results

Patients' characteristics

Table 1 shows the characteristics of the 111 patients who received IFN therapy. A total of 40 patients showed HCV genotype 1 and the remaining 71 patients showed HCV genotype 2.

Efficacy of treatment

Out of 111, 64 patients (57.7%) had SVR. Based on the difference of HCV genotype, the SVR rate was 47.5% (19/40) in genotype 1 and 63.3% (45/71) in genotype 2. We then investigated the factors associated with SVR after termination of IFN. Univariate analysis in patients with genotype 1 identified the following one factor that influenced SVR when the period of IFN treatment was 8weeks (Table 2). As one factor was associated with SVR, we did not evaluate the multivariate analysis.

On the other hand, univariate analysis in patients with genotype 2 did not identify the factor that influenced SVR (Table 3). In genotype 2, the SVR in patients treated with

Table 3. Predictive Factors for SVR in Patients with HCV Genotype 2 *

Factor	Category	Odds ratio	95% CI†	p value
AST (IU/L)	<76 /≥76	1/2.21	0.80-6.14	0.126
Sex	Man / Woman	1/0.61	0.22-1.64	0.324
Period of IFN therapy (week)	4 or 6/ 8	1/1.63	0.57-4.69	0.361
ALT (IU/L)	<100/≥100	1/1.22	0.41-3.57	0.721
Age (years)	<50/ ≥50	1/0.80	0.23-2.79	0.726
Liver histology (fibrosis)	1 /2,3	1/0.88	0.54-1.70	0.876

***ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; and IFN, interferon and CI; confidence interval.**

Table 4. SVR Based on HCV Genotype and Administration Period of Interferon

HCV genotype	Administration period (week)		
	4W	6W	8W
Genotype 1†	0% (0/6)	33.3% (5/15)	73.7% (14/19)
Genotype 2†‡	40% (2/5)	60% (9/15)	66.7% (34/51)

***HCV indicates hepatitis C virus; and SVR, sustained virological response.**
†p <0.001 in genotype 1, p =0.32 in genotype 2 by Cochran-Armitage method
‡ Three patients had HCV genotype 2b. These three patients were treated for 8 weeks and all the patients showed SVR. Remaining patients had genotype 2a.

the 8-week regimen was similar statistically to that in patients treated with the 4- or 6-week regimen.

Table 4 shows the SVR based on the HCV genotype and period of IFN therapy. According to Cochran-Armitage method, the 8-week IFN therapy regimen was the best in order to eradicate HCV RNA in genotype 1. On the other hand, in genotype 2, the 6-week regimen was almost the same as the 8-week regimen.

Adverse events

Within one week after the initiation of treatment, flu-like symptoms appeared in all the patients. Pain in the joints or muscle occurred in 50 cases. However, none of the patients withdrew from this treatment due to IFN-related side effects.

Discussion

We have described the efficacy of short-term IFN-beta therapy for chronic hepatitis C patients with low virus load. The present study was limited by a retrospective cohort trial. However, several findings from the present study have direct implications for the short-term IFN treatment of CH patients with low virus load. First, HCV RNA was cleared in more than 50% patients. Second, no patients withdrew from the treatment due to IFN-related side effects. Okanoue et al reported that side effects occurred when the daily IFN dose was increased (21). However, in the 8-week study period, there were no serious side effects. Third, the 8-week regimen of IFN therapy was preferable to eradicate HCV RNA compared to the 4 or 6-week regimen in genotype 1. On the other hand, in genotype 2, SVR by the 6-week regimen of IFN therapy was not significantly different from SVR by the 8-week regimen. These results indicate that 1) in patients with genotype 1 and low virus load, the 8-week regimen of IFN was recommended as the first treatment, 2) in patients with genotype 2 and low virus load, the 6-week regimen of IFN was recommended as the first treatment. This result is likely in agreement with several previous clinical trials (22-26).

In patients with genotype 1b and a high load of HCV-RNA, the clearance rate of HCV-RNA is less than 10% by the usual 6-month course of IFN monotherapy. In these IFN-resistant patients, the eradication rate of HCV-RNA level is at most 20-50% by the latest prolonged IFN therapy, combination therapy of IFN/ribavirin or pegylated IFN administration.

At present, combined IFN and ribavirin therapy is the standard therapy for chronic hepatitis C patients with genotype 1b and a high load of HCV-RNA. Next, in our hospital SVR of the 24-week IFN regimen in patients with a low load of HCV-RNA was 50.9% (220/432) in genotype 1b, 79.9% (279/349) in genotype 2a, and 71.4% (45/63) in genotype 2b. These results indicate that SVR of the 24-week regimen was higher than that of the short term regimen in genotype 2. However, prolonged IFN therapy is often associated with various side effects. A lower total dose and shorter administration period of IFN would be preferable in terms of both cost and safety.

Fortunately, patients with low HCV-RNA levels tend to eradicate HCV RNA with a low dose of IFN. The present study indicates that short-term IFN-beta therapy has no severe side effects. Thus, short-term IFN therapy is recommended for the patients who tend to have a SVR and have IFN- induced adverse events.

Conclusion

We think that the 6 or 8-week regimen of IFN-beta therapy is one therapy selection for chronic hepatitis C patients who tend to have a SVR and have IFN-induced adverse events.

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Original Article

Changes in viral loads of lamivudine-resistant mutants during entecavir therapy

Fumitaka Suzuki,[1] Yoshiyuki Suzuki,[1] Norio Akuta,[1] Hiromi Yatsuji,[1] Hitomi Sezaki,[1] Yasuji Arase,[1] Yusuke Kawamura,[1] Tetsuya Hosaka,[1] Masahiro Kobayashi,[1] Kenji Ikeda,[1] Mariko Kobayashi,[2] Sachiyo Watahiki[2] and Hiromitsu Kumada[1]

[1]*Department of Hepatology, Toranomon Hospital, Tokyo and* [2]*Research Institute for Hepatology, Toranomon Branch Hospital, Kawasaki, Japan*

Aim: Entecavir therapy is effective against lamivudine-resistant virus in patients with hepatitis B virus infection. We investigated viral load changes of YMDD mutant virus (rtM204I [YIDD sequence], rtM204V [YVDD]) in serial serum samples during entecavir treatment for lamivudine-resistant virus and determined changes in viral precore and core promoter mutants.

Methods: Nineteen patients were treated in randomized, double-blind phase II clinical trials of entecavir at 0.5 or 1.0 mg for breakthrough hepatitis due to lamivudine-resistant virus. Viral changes in YMDD mutants (rtM204I, rtM204V), amino acid changes in the polymerase reverse transcriptase region and precore/core promoter mutations at 52 weeks were determined in 18 patients.

Results: Changes in viral loads of rtM204I and rtM204V were similar. No differences in load changes were seen between the 0.5 and 1.0 mg groups. However, load changes for rtM204I alone were greater than those for the rtM204I + rtM204V mixed-type ($P = 0.042$, at both 40 and 52 weeks). Load changes in rtM204I and rtM204V with *G1896A* tended to be greater than those without. Moreover, *G1896A* was replaced by wild-type virus in two patients at 52 weeks.

Conclusion: RtM204I only or the existence of precore mutation was more sensitive to entecavir therapy against lamivudine-resistant virus.

Key words: entecavir, hepatitis B virus, lamivudine, precore, YMDD mutant

INTRODUCTION

THERAPY IN PATIENTS with hepatitis B virus (HBV) aims to limit or reverse progression of the disease through the sustained suppression of HBV replication.[1] Approved therapies for chronic HBV infection involving treatment with interferon (IFN) have a low sustained response rate, undesirable side-effects, and high cost.[2,3] Several studies have reported that lamivudine is more effective and less costly than IFN in suppressing HBV replication, and also improves transaminase levels and liver histology, and enhances the rate of loss of hepatitis B e antigen (HBeAg).[4–7] On long-term use, however, lamivudine has the potential to induce viral resistance, with associated increases in HBV-DNA and serum transaminases.[8–10]

Entecavir (ETV), a deoxyguanosine analog, is a potent and selective inhibitor of HBV replication, with *in vitro* potency 100- to 1,000-fold greater than that of lamivudine.[11,12] Human clinical trials have demonstrated the efficacy of ETV in the treatment of chronic HBV infections.[13,14] The potential for additional therapeutic benefits with ETV was indicated by a reduced frequency of hepatocellular carcinoma in the woodchuck model and a prolongation of life span in chronically infected animals.[15] Data on the *in vitro* efficacy of ETV against lamivudine-resistant HBV are limited,[12] but several clinical studies have demonstrated *in vivo* efficacy.[16,17]

A recent report described a rapid, highly sensitive and reproducible method for quantifying mutant HBV virus in lamivudine-treated patients.[18] Using a real-time polymerase chain reaction (PCR; LightCycler; Roche

Correspondence: Fumitaka Suzuki, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan. Email: fumitakas@toranomon.gr.jp
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Diagnostics, Mannheim, Germany) with a ResonSense probe, this method detects as little as 0.01% of YMDD mutant DNA among 10^5–10^9 copies of wild-type DNA. However, few reports have appeared on viral load changes in YMDD mutant virus (rtM204I [YIDD sequence] and rtM204V [YVDD]) during treatment with ETV against lamivudine-resistant HBV infection.

Among these, one recent study reported that two patients for whom previous therapies (lamivudine or famciclovir, ganciclovir, foscarnet and lamivudine) had failed exhibited virological breakthrough while on ETV.[19] The efficacy of ETV in these cases was decreased and viral load changes of YMDD mutant virus were increased, specifically via new substitutions plus lamivudine substitution (rtL180M and rtM204V) in the reverse transcriptase (rt) domain. We were therefore interested to analyze mutations of the rt domain of HBV polymerase in patients who had received long-term (52 weeks) ETV therapy against lamivudine-resistant HBV infection.

During chronic HBV infection, natural seroconversion to antibody to HBeAg (anti-HBe) usually correlates with the resolution of viremia and clinical recovery. Mutation in the precore region (nucleotide [nt] 1896) is related to the absence of HBeAg secretion[20] and may enhance the stability of the secondary structure of pregenome encapsidation signals, ensuring perpetuation of viral replication and thus contributing to viral persistence.[21] Buckwold *et al.* showed that the HBV genome carrying core promoter mutations (nt G1762A and A1764T) influenced viral replication.[22] Cho *et al.*[23] and our group[24] reported that lamivudine therapy resulted in reversion from precore and core promoter mutants to wild-type, but that these mutants reappeared during prolonged therapy. However, it is unclear how ETV influences precore and core promoter mutants of lamivudine-resistant virus.

In this prospective study, we investigated viral load changes in YMDD mutant virus (rtM204I, rtM204V) during ETV therapy against lamivudine-resistant HBV infection. Furthermore, we also analyzed serial serum samples from patients with lamivudine resistance to determine viral precore and core promoter mutants during treatment with ETV.

PATIENTS AND METHODS

Patients

THE PATIENTS WERE 19 consecutive adult Japanese patients treated in phase II between June 2003 and December 2004 at the Department of Gastroenterology, Toranomon Hospital. At entry, all patients were being treated with lamivudine (100 mg/day) for chronic hepatitis due to HBV infection when the emergence of YMDD motif mutations indicated the development of breakthrough hepatitis. They had not received other nucleoside analog drugs before lamivudine. The study was a phase II randomized (1:1), double-blind trial of ETV by repeat oral administration at 0.5 mg or 1.0 mg for 12 months. They were switched from lamivudine directly to ETV without any break in administration. All patients were negative for hepatitis C serological markers. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki, and written informed consent to participate was obtained from all patients.

Blood tests and serum viral markers

Routine biochemical tests were performed using standard procedures before and at least once monthly during therapy. Hepatitis B surface antigen (HBsAg), HBeAg and anti-HBe were determined with radioimmunoassay kits (Abbot Diagnostics, Chicago, IL, USA) according to the manufacturer's instructions. Serum HBV-DNA level and DNA sequence samples were stored at −80°C until assay. Serum HBV-DNA was quantified using the Roche Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN, USA), a PCR-based assay with a lower limit of detection of 400 copies of HBV-DNA/mL (2.6 log copy/mL).

Quantitation of lamivudine-resistant mutants by real-time amplification refractory mutation system PCR

DNA was extracted from 100 μL serum. The assay was performed using a sensitive, real-time PCR-based assay for the detection of lamivudine resistance-associated mutations in the presence of high levels of wild-type virus, as reported recently.[18,25] Briefly, this method is based on the amplification refractory mutation system (ARMS) PCR for the detection of single-base mutations[26] and uses the same ARMS primers, reactions and cycling conditions on the LightCycler. To prepare the standards (rt204M, rtM204I and rtM204V), the first PCR product amplified using the primers P1 and P2[27] was cloned into the plasmid vector pBluescript (Stratagene, La Jolla, CA, USA) as reported previously. The concentration of purified plasmids was based on absorbance at 260 nm (GeneQuant II; Amersham Pharmacia Biotech, Tokyo, Japan). The standards for real-time PCR were prepared by serial dilution of a plasmid of known concentration. DNA values of these mutants below the

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lower limit of detection were expressed as 2.0 log copy, and those over the upper limit as 9.0 log copy. The selectivity of this assay was tested as described previously[18,25] using reactions containing 10^9 copies of wild-type DNA (rt204M) template and from 0 to 10^9 copies of mutant virus (rtM204I or rtM204V) template. Under these conditions, the mutant primers (for rtM204I and rtM204V) detected the number of copies of mutant template present within the range of 10^4–10^9 copies. Moreover, one primer (for rt204I or rt204V) detected the number of copies within the range of 10^4–10^9 copies (mixed with 10^9 copies of the other mutant virus [rtM204V DNA or rtM204I DNA], respectively). Total HBV-DNA levels were measured by real-time PCR as described previously.[18] Serum samples were assayed at 11 time points, namely before (baseline) and at 2, 4, 8, 12, 16, 20, 24, 32, 40 and 52 weeks after the start of ETV. Data for the time-dependent decline in viral load relative to baseline were log-transformed, and thus all results for quantitative HBV level are expressed as $\log_{10}$ copy.

Determination of nucleotide sequences of HBV-DNA

DNA was extracted from 100 μL serum. PCR reactions for detection of the rt region (nt 130–1161) of HBV-DNA were performed in two parts. The first and second PCR reactions for detection of the first part of the rt region were performed using primers BGF1 (sense; 5′-CTGTGGAAGGCTGGCATTCT-3′) and BGR2 (antisense; 5′- GGCAGGATAGCCGCATTGTG-3′), and PreS-BamH1 (sense; 5′-CTTGGGATCCAGAGCTACAGCAT GG-3′) and BR112 (antisense; 5′-TTCCGTCGACATA TCCCATGAAGTTAAGGGA-3′), respectively, under conditions of initial denaturation for 4 min, 35 cycles of amplification at 94°C for 1 min, 55°C for 2 min, 72°C for 3 min and final extension at 72°C for 7 min. The first and second PCR reactions for detection of the second part of the same region were performed using primer pairs B11F (sense; 5′-GGCCAAGTCTGTA CAACATC-3′) and B12R (antisense; 5′-TGCAGAGGTG AAGCGAAGTG-3′), and B11F and B14R (antisense; 5′-GATCCAGTTGGCAGCACACC-3′), respectively, under the same conditions. The amplified PCR products were used for direct sequencing. Measurement of sequences in the rt region was performed at three time points, namely at the start of lamivudine, start of ETV, and 1 year after the start of ETV therapy. Nucleotide sequences of the core promoter and precore regions were determined as described previously,[24] with measurements taken at the same three time points.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were examined for statistical significance using the χ^2 test and Mann–Whitney *U*-test where appropriate. A two-tailed *P*-value less than 0.05 was considered significant.

RESULTS

Viral load changes in lamivudine-resistant mutants during ETV therapy

OF THE 19 patients participating in the present study, 10 received ETV at 0.5 mg and nine at 1.0 mg. However, serum samples for one patient without HBeAg receiving 0.5 mg were not available, and this patient was excluded. Baseline characteristics of the remaining 18 patients in Table 1 show no significant differences between the groups.

Changes in viral loads of rtM204I and rtM204V were measured in 18 patients. At the start of ETV, the number of patients with detectable rtM204I alone, rtM204V alone and mixed-type (rtM204I and rtM204V) was seven, 0 and 11, respectively. RtL180M was detected in all but one patient (no. 18) at ETV baseline. Figure 1 shows mean log changes in the viral loads of rtM204I ($n = 18$) and rtM204V ($n = 11$) from baseline during the initial 52 weeks of ETV, with no differences seen in viral load changes for rtM204I and rtM204V in the two ETV groups. The low rate of decrease in changes in the viral loads of rtM204V in the 1.0 mg group was due to a lower viral load of baseline.

Two patient types were recognized, rtM204I alone and rtM204I + rtM204V mixed. Table 2 shows that there were no differences except for HBeAg status between these two groups at the start of ETV therapy. The rate of HBeAg positivity in the rtM204I + rtM204V mixed group was high. Moreover, there were no differences in the rates of histological improvement, ALT normalization and loss of HBeAg until 52 weeks of treatment. ALT flare (ALT levels > twofold of baseline levels and > 10-fold of upper limit for the normal range) was found in one patient (no. 18 in Table 3) in the rtM204I alone group and in one patient (no. 1) in the rtM204I + rtM204V mixed group until 52 weeks of treatment. However, in both patients, the ALT flare was transient and was associated with declining HBV-DNA.

Compared with baseline for ETV, one or two new amino acid substitutions (except for ETV resistance substitutions) in the rt region were shown in six patients (one in the rtM204I alone group and five in the

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Table 1 Patient characteristics at the start of entacavir therapy for lamivudine-breakthrough hepatitis

	0.5 mg	1.0 mg
Total number	9	9
Sex (female/male)	1/8	1/8
Age (years)	37 (29–65)	39 (30–49)
Alanine aminotransferase (IU/L)	124 (64–347)	119 (52–251)
Liver histology (F1/F2/F3/F4/N)‡	6/0/3/0/0	6/1/1/0/1
Serum HBV-DNA§ (Amplicor; log copy/mL)†	7.5 (6.2–>7.6)	> 7.6 (7.2–>7.6)
HBeAg (positive/negative)	6/3	7/2
HBV genotype (A/C)	1/8	0/9
YMDD mutant type (I/V/Mix)¶	4/0/5	3/0/6

†Data are median (range).
‡Liver histology, as liver fibrosis assessed on a four-point scale: F0, no fibrosis; F1, periportal expansion; F2, portoportal septa; F3, portocentral linkage or bridging fibrosis; F4, cirrhosis; N, liver biopsy not performed.
§HBV-DNA levels were measured by Amplicor HBV Monitor assay. HBV-DNA values below the lower limit of detection are listed as 2.6 log copy/mL and those over the upper limit of detection as 7.6 log copy/mL.
¶YMDD mutant type: I, rtM204I; V, rtM204V; Mix, rtM204I + rtM204V.
HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

rtM204I + rtM204V mixed group) at 52 weeks. However, four patients in the rtM204I group were PCR negative at 52 weeks and it was difficult to compare the difference of amino acid substitutions between both groups. Figure 2 shows that changes in the viral load of mutants were greater for rtM204I alone than for rtM204I in the mixed-type patients (3.00 ± 0.91 *vs* 2.21 ± 0.63, $P = 0.042$, at 40 weeks; 2.99 ± 0.87 *vs* 2.23 ± 0.78, $P = 0.042$, at 52 weeks). However, changes in the viral load of mutants were greater for rtM204I alone than for rtM204V in the mixed-type patients, although the difference was not statistically significant (2.99 ± 0.87 *vs* 1.90 ± 1.51, $P = 0.070$, at 52 weeks). Changes in the viral load of rtM204I and rtM204V in patients with the rtM204I + rtM204V mixed type were similar.

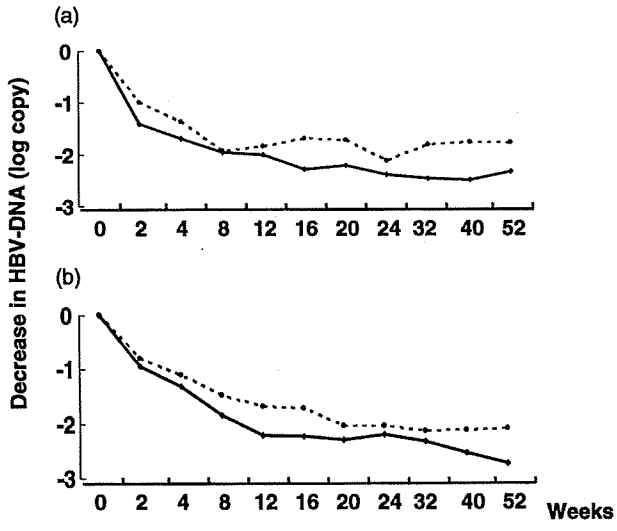

Figure 1 Mean log changes in viral loads of rtM204I and rtM204V from baseline during the initial 52-week treatment with entacavir at (a) 1.0 mg and (b) 0.5 mg. HBV-DNA levels of rtM204I (——) and rtM204V (-----) were measured by real-time polymerase chain reaction. HBV, hepatitis B virus.

Moreover, Table 3 shows precore sequences (nt 1896) at ETV baseline. Analysis of serum samples obtained at this time revealed a precore stop codon mutation (G1896A) in nine of 18 patients, among whom G1896A occurred as a mixed population with wild-type virus (G1896) in two and as a pure population in seven. Based on these findings, four groups were established by type of YMDD mutant and the presence of G1896A (rtM204I with G1896A [$n = 9$] and without G1896A [$n = 9$], and rtM204V with G1896A [$n = 4$] and without G1896A [$n = 7$]). Changes in the viral loads of rtM204I and rtM204V in these groups is shown in Figure 3; although patient numbers were small, changes tended to be greater in rtM204I and rtM204V with G1896A than in those without. Moreover, HBV-DNA levels in four patients (nos. 3, 16, 17 and 18) by Amplicor HBV Monitor assay were negative after 1 year of ETV therapy. YMDD motif in these patients was rtM204I only in all. Further, HBV-DNA levels in two additional patients (nos. 1 and 5) by Amplicor HBV Monitor assay were negative at 76 weeks of ETV therapy. These two patients had G1896A in the precore gene, although the YMDD

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Table 2 Patient characteristics at the start of entacavir therapy in rtM204I alone and mix groups

	rtM204I	Mix (rtM204I + rtM204V)
Total number	7	11
Sex (female/male)	1/6	1/10
Age (years)†	37 (34–65)	39 (29–55)
Alanine aminotransferase (IU/L)†	119 (54–347)	112 (52–251)
Serum HBV-DNA‡ (Amplicor; log copy/mL)†	> 7.6 (6.2–>7.6)	> 7.6 (7.2–>7.6)
HBeAg (positive/negative)	3/4	10/1
HBV genotype (A/C)	0/7	1/9
Duration of lamivudine therapy (month)†	31 (19–47)	36 (10–48)

†Data are median (range).
‡HBV-DNA levels were measured by Amplicor HBV Monitor assay. HBV-DNA values below the lower limit of detection are listed as 2.6 log copy/mL and those over the upper limit of detection as 7.6 log copy/mL.
HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

motif type was mixed. Therefore, none of the seven patients with both the rtM204I + rtM204V mixed-type and precore wild-type showed a negative result on the Amplicor HBV Monitor assay at 76 weeks.

Entecavir-resistant mutant during therapy

Analysis of the rt region sequences (amino acids 1–344) of HBV polymerase in one patient (no. 11) at 52 weeks showed a new substitution of rtS202G in addition to the lamivudine substitutions (rtL180M and rtM204V), which may indicate introduced ETV resistance.[19] Virological rebounds of rtM204V viral load of this patient were observed at 40 and 52 weeks (increase of 1.27 and 1.08 log copies from nadir by real-time PCR). There were no patients with virological rebounds except this patient (no. 11).

Changes in precore and core promoter sequences before and during therapy

Precore and core promoter sequences in 18 patients were analyzed during 1 year of treatment with ETV for lamivudine-breakthrough hepatitis. Precore sequences at baseline for lamivudine were the same as those at baseline for ETV in 10 of 18 patients (excluding one lacking lamivudine baseline data; Table 3). Analysis of serum samples obtained at ETV baseline revealed a precore stop codon mutation (*G1896A*) in nine of 18 patients. After the start of ETV, *G1896A* was replaced by wild-type virus in two patients (nos. 1 and 5) at 1 year. However, G1896 was replaced by *G1896A* in one patient (no. 11) with ETV resistance. Thus, *G1896A* was observed in five of 14 patients, excluding four PCR-negative patients, at 1 year.

Core promoter sequences at baseline for lamivudine therapy were the same as those at baseline for ETV in 15 of 18 patients (Table 3). Among the 18, 15 had core promoter mutations (A1762T and G1764A) in samples collected at ETV baseline. During treatment, core promoter mutations at baseline were similar to those at 1 year.

YMDD mutant type was changed after 1 year of ETV treatment in three patients (nos. 1, 7 and 13), with the baseline rtM204I + rtM204V mixed types replaced by the respective major YMDD mutant.

DISCUSSION

PATIENTS WHO RECEIVE lamivudine therapy may be treated for an extended period, increasing the probability of viral resistance and loss of clinical efficacy, involving an associated risk of increased viral replication, flares of ALT levels, and progression of liver disease.[5,8–10,28,29] ETV has previously been shown to be superior to lamivudine in the treatment of chronic HBV in nucleoside-naïve patients infected with wild-type HBV.[14] Moreover, a recent report showed that treatment with ETV at 0.5 or 1.0 mg daily was well tolerated, and resulted in significant reductions in HBV-DNA levels as well as normalization of ALT levels in HBeAg-positive and -negative lamivudine-refractory patients.[17] Although ETV in the recent report[17] was more effective at 1.0 than 0.5 mg, no differences between the two groups were seen in changes in viral load in the present study. Moreover, results for viral load changes by Amplicor HBV Monitor assay were the same in the present study (data not shown). This difference between these studies

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