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Short Communication

Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b

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ABSTRACT

Background: Hepatitis C virus (HCV) of genotype 1b is the most prevalent worldwide, and the least responsive to interferon-based treatments. A combination therapy with two direct-acting antivirals has shown promising results in patients with HCV-1b, but the prevalence of drug-resistant variants before treatment is not known in the Japanese population.
Objectives: To detect HCV variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients infected with HCV-1b.
Study design: Drug-resistant mutations were determined in the 362 hepatitis patients infected with HCV-1b who had not received direct-acting antivirals before.
Results: Amino-acid substitutions resistant to NS3 inhibitors (V36A, T54S, Q80H and D168E) were detected in 15 of the 307 (4.9%) patients, who had been examined, and T54S (3.3%) predominated over V36A (0.3%), Q80R (0.7%) and D168E (0.7%) in them. Amino-acid substitutions resistant to BMS-790052 (L31M and/or Y93H) were detected in 33 of the 294 (11.2%) patients, and Y93H (8.2%) predominated over L31M (2.7%). One of the 239 (0.4%) patients, who had been examined for amino-acid substitutions in both NS3 and NS5A regions, possessed HCV-1b variants resistant to NS3 inhibitors (T54S) and BMS-790052 (L31M).
Conclusions: Mutations conferring resistance to NS3 inhibitors or BMS-790052 were frequent in our treatment-naive study population, but double mutants with possible resistance to both drugs were rare. Since single mutations did not result in treatment failure in a previous pilot trial combining BMS-790052 and an NS3 inhibitor, larger trials of this drug regimen appear warranted in the Japanese population.

1. Background

Worldwide, an estimated 170 million people are infected with hepatitis C virus (HCV) persistently,[1] and approximately one-third of them develop life-threatening liver diseases, such as decompensated cirrhosis and hepatocellular carcinoma.[2] The triple therapy with an NS3 protease inhibitor, telaprevir or boceprevir, in combination with pegylated (PEG)-interferon (IFN) and ribavirin (RBV), has increased sustained virological response (SVR) to about 70% in the patients with HCV of genotype 1b (HCV-1b).[3–7] Still, approximately 30% of them fail to clear HCV by the triple therapy, and, in addition, many more cannot receive it because of contraindications, such as advanced ages, anaemia and co-morbid conditions.

Recently, a combination therapy with two direct-acting antivirals (DAAs), which are targeted to different regions in the viral genome, was introduced to treatment of patients with HCV-1b, and has gained promising results. Thus, a second-generation NS3 protease inhibitor (BMS-650032 [asunaprevir]) combined with an NS5A inhibitor (BMS-790052 [daclatasvir]) for 24 weeks induced SVR in two of the two,[8] as well as in 10 of the 10,[9] patients with HCV-1b with excellent safety profiles.

Abbreviations: HCV, hepatitis C virus; IFN, interferon; SOC, standard-of-care; PEG, pegylated; RBV, ribavirin; SVR, sustained virological response; DAA, direct-acting antiviral.

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2. Objectives

For extending the combination treatment with BMS-650032 and BMS-790052 to many more patients with HCV-1b, it is necessary to examine how frequently viral variants, which have resistance to NS3 protease inhibitors or NS5A inhibitors,[10–13] occur in patients with HCV-1b.

3. Study design

3.1. Patients

During 2000 through 2010, sera were obtained from the 362 patients with HCV-1b at the Department of Hepatology in Toranomon Hospital in Tokyo, and had been stored frozen at −80 °C. They all were treatment-naive to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052); 134 of them (37.0%) had received IFN-based treatments previously. The nucleotide sequence of the NS3 region in HCV RNA was determined in 307 patients and that of NS5A region in 294, and sequences of both NS3 and NS5A were determined in 239.

3.2. Sequencing NS3 and NS5A regions

HCV RNA was amplified by polymerase chain reaction with appropriate nested primers in NS3[14] or NS5A[15] region, and sequences of the N-terminal 609 nucleotides in the NS3 region and those of the N-terminal 600 nucleotides in the NS5A region were determined by the direct sequencing method. The major sequences were adopted, which would represent the consensus sequence. They have been deposited in the Genbank under the accession numbers AB693834–AB693872 and AB709241–AB709802.

3.3. Amino-acid substitutions for the resistance to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052)

V36A/M/L/G, T54A/S, V55A, Q80K/R/H/G, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, D168A/V/E/G/N/T/Y/H/I and V170A have been identified as amino-acid substitutions resistant to NS3 protease inhibitors, including linear ketoamids (telaprevir, boceprevir, SCH900518 and BI201335) and macrocyclic compounds (MK7009, TMC435350, ITMN191, GS-9256, ABT450 and BMS-791325).[16,17] L31M and Y93H have been recognised as the most powerful substitutions in HCV-1b for the resistance to BMS-790052.[18–21]

4. Results

4.1. Baseline characteristics of patients with HCV-1b who were naive to DAAs

Table 1 lists the baseline characteristics of the 362 patients infected with HCV-1b. Of them, 134 (37.0%) had received IFN-based treatments previously, including 78 (21.6%) with IFN monotherapy and 56 (15.4%) given combination therapy with IFN or PEG-IFN and RBV. Liver biopsies had been performed on 201 of the 362 (55.5%) patients. The majority of them (47.5%) had fibrosis stages ≤F2, by the classification of Desmet et al.,[22] and none had cirrhosis.

4.2. Amino-acid substitutions for the resistance to NS3 inhibitors or the NS5 inhibitor (BMS-790052)

Table 2 shows frequencies of amino-acid substitutions for the resistance to NS3 inhibitors in 307 patients. Of them, 15 (4.9%) were infected with HCV-1b variants having V36A, T54S, Q80R or D168E, and T54S predominated over Q80R, V36A and D168E. Resistance profiles are comparable between Japanese patients in this study and 366 European and 34 American patients (total: 400 patients) retrieved from the Genbank.

Table 3 shows frequencies of amino-acid substitutions for the resistance to the NS5 inhibitor (BMS-790052) in the 294 patients. Y93H predominated over L31M, and one patient had both Y93H and L31M. Overall, 33 (11.2%) of them were infected with HCV-1b variants with L31M or Y93H, or both. One of the 239 (0.4%) patients, for whom both NS3 and NS5A sequences had been examined, was infected with HCV-1b variants with resistance to NS3 inhibitors (T54S) and NS5A inhibitor (L31M).

Table 1
Baseline characteristic of the patients infected with HCV of genotype 1b who were naive to direct-acting antivirals.

Demographic data	(n = 362)
Male (%)	213 (58.8%)
Age (years)	55 (18–75)
IFN-based treatments	
Treatment-naive	228 (63.0%)
IFN monotherapy	78 (21.6%)
IFN (or PEG-IFN) plus ribavirin	56 (15.4%)
Laboratory data	
Alanine aminotransferase (IU/L)	54 (12–348)
Asparatate aninotransferase (IU/L)	41 (17–350)
Platelets ($\times 10^3/mm^3/\mu L$)	174 (64–366)
HCV RNA (log IU/mL)	6.7 (<1.2 to >7.6)
Stage of liver fibrosis[a]	(n = 201)
F1	117 (58.2%)
F2	55 (27.4%)
F3	29 (14.4%)
F4	0

Values are the number with percentage in parentheses or the mean with range in parentheses.
[a] Classified by the criteria of Desmet et al.[22]

Table 2
Substitutions of amino acids in the NS3 protease region for the resistance to NS3 inhibitors in Japanese patients in the present study and in European or American patients with HCV-1b retrieved from the Genbank.

Substitutions	This study (n = 307) n (%)	Database[a] (n = 400) n (%)
V36A	1 (0.3%)	1 (0.3%)
T54A	0	1 (0.3%)
T54S	10 (3.3%)	5 (1.2%)
V55A	0	1 (0.3%)
Q80R	2 (0.7%)	16 (4.0%)
A156T	0	1 (0.3%)
D168E	2 (0.7%)	2 (0.5%)
V170A	0	2 (0.5%)
Total	15 (4.9%)	29 (7.3%)

[a] HCV-1b sequences were retrieved from the Genbank. There were 400 sequences in total, exclusive of repetitive sequences, including 307 from France, 53 from Spain, 6 from Germany and 34 from USA.

Table 3
Substitutions of amino acids in the NS5A region for the resistance to BMS-790052 in Japanese patients in the present study and in patients with HCV-1b retrieved from the European HCV database.

Substitutions	This study (n = 294) n (%)	Database[a] (n = 1796) n (%)
L31M	8 (2.7%)	68 (3.8%)
L31V	0	38 (2.1%)
Y93H	24 (8.2%)	149 (8.3%)
Y93H/L31M	1 (0.3%)	Unknown
Total	33 (11.2%)	255 (14.2%)

[a] The sequences of HCV-1b were retrieved from the European HCV database and reported by Fridell et al.[18]

Factors influencing HCV-1b variants resistant to NS3 inhibitors or BMS-790052 were evaluated by univariate analysis with use of the Statistical Package for Social Sciences (SPSSII v.11.0, IBM Co., Chicago, IL, USA). None of age, sex, transaminase levels, platelet counts, HCV RNA loads and histological stages increased the prevalence of HCV-1b variants resistant to either of these two kinds of DAAs.

5. Discussion

DAAs have different antiviral targets and distinct resistance profiles that are dependent on HCV genotypes/subtypes.[16,21,23] For treatment of patients with HCV-1b, a combination of a second-generation NS3 protease inhibitor (BMS-650032) and an NS5A inhibitor (BMS-790052) has gained SVR in two of the two, as well as 10 of the 10, patients with HCV-1b.[8,9] By contrast, the combination therapy was less effective in the nine patients with HCV-1a, and viral breakthroughs occurred in six (67%) of them.[8] In HCV-1a, only one nucleotide mutation gives rise to amino-acid substitutions resistant to NS3 protease inhibitors (R155K/T/S/M/I), instead of two required in HCV-1b,[23] which would be responsible, at least in part, for poor responses to the combination therapy in patients with HCV-1a.

There is a possibility that HCV-1b variants resistant to both BMS-650032 and BMS-790052 may be selected during the combination therapy, and result in viral breakthroughs during treatment. Of the 307 patients, who had been examined, 15 (4.9%) were infected with HCV-1b with amino-acid substitutions for the resistance to NS3 protease inhibitors. Of the NS3 resistance mutations detected, only D168E is relevant to the second-generation protease inhibitors,[16,17] and, therefore, only 0.7% of the treatment-naive patients carried relevant resistance mutations when focussing on a possible combination of BMS-650032 with other DAAs. It needs to be pointed out that a possibility remains for the presence of minor HCV populations with resistance to DAAs that might have escaped the detection by direct sequencing.

HCV-1b variants with L31M or Y93H, which confers strong resistance to the NS5A inhibitor (BMS-790052),[20] were detected in 33 of the 294 (11.2%) patients with HCV-1b; one of them was infected with variants with both L31M and Y93H. Such a frequency is comparable to those in 1796 patients from the European HCV database (L31M, 5.9%; Y93C/H, 8.4%).[18] Variants with Y93H were detected in 3 of the 10 (30%) patients receiving the combination therapy with BMS-650032 and BMS-790052.[9] Since they all gained SVR, variants with Y93H alone, in the absence of those resistant to macrocyclic NS3 protease inhibitors, would not cause treatment failure in the patients who receive the combination therapy. Co-occurrence of variants resistant to NS3 protease inhibitors and those to the NS5A inhibitor was observed in only one of the 239 (0.4%) patients for whom both of them were examined. They may or may not exist on the same virion, because they were detected by direct sequencing. Therefore, results suggest that most patients with HCV-1b in our geographic area can be good candidates to succeed in resolving infection after combination therapy with NS3 inhibitors and BMS-790052.

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Competing interest: Dr. Kumada reports having received investigator, lecture and consulting fees from Bristol-Myers KK. No other potential conflicts of interest relevant to this article were reported.

Ethical approval: Informed consent was obtained from each patient.

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Research Article

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Long-term continuous entecavir therapy in nucleos(t)ide-naïve chronic hepatitis B patients

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Background & Aims: We determined the antiviral potency and viral resistance rate after 4 years of continuous entecavir treatment in patients with chronic hepatitis B (CHB) infection.

Methods: The cumulative rates of undetectable hepatitis B virus DNA (HBV DNA; <2.6 $\log_{10}$ copies/ml), hepatitis B e antigen (HBeAg) seronegativity, seroconversion, alanine aminotransferase (ALT) normalization, and entecavir signature mutations were calculated in 474 nucleos(t)ide-naïve CHB patients (HBeAg-positive: 47%) on continuous entecavir treatment for 4 years.

Results: Median age was 47 years and follow-up period was 2.4 years, with 403, 281, 165, and 73 patients followed-up for at least 1, 2, 3, and 4 years, respectively. Incremental increases were observed in the rates of undetectable HBV DNA, HBeAg seroclearance and seroconversion, and ALT normalization, reaching 96%, 42%, 38% and 93%, respectively, by the fourth year. In all, 100% and 93% of patients negative and positive for HBeAg, respectively, had undetectable HBV DNA at year 4. Of 165 patients, HBV DNA was detectable in nine patients after 3 years. Multivariate analysis identified HBV DNA level ($\leqslant$7.6 $\log_{10}$ copies/ml, OR = 15.8; 95% CI = 43.1–79.9, P = 0.001) as an independent predictor of undetectable HBV DNA at year 3. Five patients experienced virological breakthrough including two (0.4%) who developed entecavir-resistance mutations.

Conclusions: Continuous treatment of nucleos(t)ide-naïve CHB patients with entecavir over 4 years was associated with 96% chance of undetectable HBV DNA and only 0.4% chance of emerging entecavir-resistant mutations.

Keywords: Hepatitis B virus; Entecavir; Resistance; Virological breakthrough.
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Abbreviations: AFP, α fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; CHB, chronic hepatitis B; CIs, confidence intervals; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HBV DNA, hepatitis B virus DNA; ORs, odds ratios; PCR, polymerase chain reaction; ULN, upper limit of normal; VBT, virologic breakthrough.

Introduction

Approximately 350–400 million people worldwide have chronic hepatitis B (CHB) infection, the majority of whom live in the Asia–Pacific region [1,2]. CHB patients with elevated viral load are at risk of developing cirrhosis, liver failure, and hepatocellular carcinoma.

Recent investigations have shown that entecavir suppressed HBV DNA replication to undetectable levels and normalized alanine aminotransferase (ALT) levels in nucleos(t)ide-naive CHB patients in Japan and other countries [3–10]. In addition, genotypic resistance to long-term entecavir monotherapy remained rare [5,6,9,10]. To date, there are two 5-year studies [6,8] and two 3-year studies [7,9] of entecavir therapy for nucleos(t)ide-naïve patients. Both studies stemmed from extension studies with the original cohorts from two large-scale phase III trials of treatment-naïve patients [3,4]. In these trials, patients were administered 0.5 mg entecavir for 1 year and later divided into three categories: (i) complete responders, defined as patients with HBV DNA $<7 \times 10^5$ copies/ml and ALT level <1.25 times the upper limit of normal (ULN) for hepatitis B e antigen (HBeAg)-negative patients and an additional loss of HBeAg for HBeAg-positive patients; (ii) non-responders, defined as HBV DNA $\geqslant 7 \times 10^5$ copies/ml; and, (iii) virological responders, defined as HBV DNA $<7 \times 10^5$copies/ml and ALT >1.25× ULN regardless of HBeAg status or persistent HBeAg for HBeAg-positive patients. Treatment was terminated in the complete responders but continued in virological responders. Non-responders were provided additional therapy in a rollover study in which some patients were initially treated with a combination of 1 mg entecavir and lamivudine for several months before receiving 1 mg entecavir as monotherapy. Furthermore, a substantial proportion of complete responders relapsed after various intervals following cessation of therapy and they were also assigned to a rollover study receiving 1 mg entecavir monotherapy. Because of these strict protocols, the precise viral-suppression and drug-resistance data for treatment-naïve patients who were treated continuously with 0.5 mg entecavir daily (the recommended dosage) remain unavailable.

The aims of this cohort study were (1) to investigate the efficacy of entecavir in clinical practice beyond 4 years for nucleos(t)ide-naïve CHB and cirrhosis patients, (2) to explore baseline factors associated with virological response to entecavir,

and (3) to investigate virological breakthrough during long-term entecavir treatment.

Patients and methods

Study population

We performed a retrospective analysis of 474 CHB and cirrhosis patients who received entecavir treatment at the Department of Hepatology, Toranomon Hospital, Tokyo, from March 2004 to May 2011, and adhered to the treatment for more than 6 months (Table 1). All patients were negative for hepatitis C serological markers, but all had detectable HBV surface antigen (HBsAg) for at least 6 months prior to the start of entecavir therapy. Two patients received 0.01 mg entecavir and one patient received 0.1 mg entecavir for 24 weeks, prior to 0.5 mg/day from a phase II study ETV-047 in Japan [11]. The other patients received 0.5 mg entecavir. None had received other nucleos(t)ide analogs. The diagnosis of chronic hepatitis and cirrhosis was established by needle biopsy, peritoneoscopy, or clinically before treatment. The clinical criteria for chronic hepatitis included elevated ALT levels over 6 months and absence of clinical evidence of portal hypertension, such as esophageal varices, ascites, hepatic encephalopathy, and features suggestive of cirrhosis on ultrasonography. Chronic hepatitis and cirrhosis were diagnosed in 374 and 102 patients, respectively. Twenty-eight patients were lost to follow-up, including 10 patients who moved to other locations, seven who never visited the hospital again, two who became pregnant, four who died, four who had virological breakthrough (VBT), and one who showed disappearance of HBsAg. Moreover, 18 patients developed HCC during treatment and their data until loss to follow-up or diagnosis of HCC were analyzed. Informed consent was obtained from each patient enrolled in the study and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical Committee.

Analysis of treatment efficacy

The clinical efficacy of entecavir was assessed as the proportion of patients who achieved HBV DNA suppression to undetectable levels (<2.6$\log_{10}$ copies/ml), and those who achieved ALT normalization (<1 × ULN). HBV DNA was measured using the polymerase chain reaction (PCR)-based Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN, lower limit of detection of 2.6$\log_{10}$ copies/ml) [12]. HBeAg seroclearance and seroconversion were also analyzed. Measurements were made on stored samples taken at baseline and every year after that since entecavir treatment initiation.

Statistical analysis

Differences between groups were examined for statistical significance using the χ^2 test where appropriate. Spearman correlation coefficient (two-tailed) was used to evaluate the correlation between albumin and other factors. Independent predictive factors associated with response to entecavir treatment were determined using multivariate multiple logistic regression. The following 12 potential predictors of response to entecavir treatment were assessed in this study: age, sex, severity of liver disease (CH or cirrhosis), HBV genotype, as well as levels of aspartate transaminase (AST), ALT, bilirubin, albumin, platelets, α fetoprotein (AFP), HBeAg, and HBV DNA. All factors found to be at least marginally associated with undetectable levels of HBV DNA after 1–4 years (p <0.10) were entered into the multivariate multiple logistic regression analysis. The above calculations were performed using The Statistical Package for Social Sciences version 11.0.1J (SPSS Inc., Chicago, IL).

The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the relative risk confidence. Independent risk factors predicting achievement of HBeAg seroclearance and seroconversion were analyzed using stepwise Cox regression analysis. Potential factors that could predict achievement of HBeAg seroclearance assessed here were the above 11 variables, each transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with HBeAg seroclearance and seroconversion (p <0.10) were tested in the multivariate Cox proportional hazard model. A Kaplan–Meier estimate was performed using the SPSS software, and p values were calculated using the Cox-Mantel log-rank test. The Mann–Whitney U test was used for comparison of HBV DNA levels in patients with seroconversion to those with seroclearance. A two-tailed p value <0.05 was considered statistically significant.

Table 1. Characteristics of patients at the start of entecavir therapy. Table data are number of patients or median (range).

Demography	
n	474
Sex, male/female	321/153
Age, yr	47 (17-82)
Family history of HBV	291 (61%)
Cirrhosis	102 (22%)
Median duration of treatment, yr (range)	2.37 (0.5-7.2)
Laboratory data	
AST, IU/L	52 (14-1595)
ALT, IU/L	70 (8-2121)
Bilirubin, mg/dl	0.7 (0.2-3.9)
γ-GTP, IU/L	38 (9-679)
Albumin, g/dl	3.9 (1.9-5.1)
Alpha fetoprotein, ng/ml	5 (1-379)
Viral load, $\log_{10}$ copies/ml	6.7 (<2.6->9.0)
HBeAg-positive	222 (47%)
HBV genotypes, A/B/C/H/unknown	12/67/336/2/57

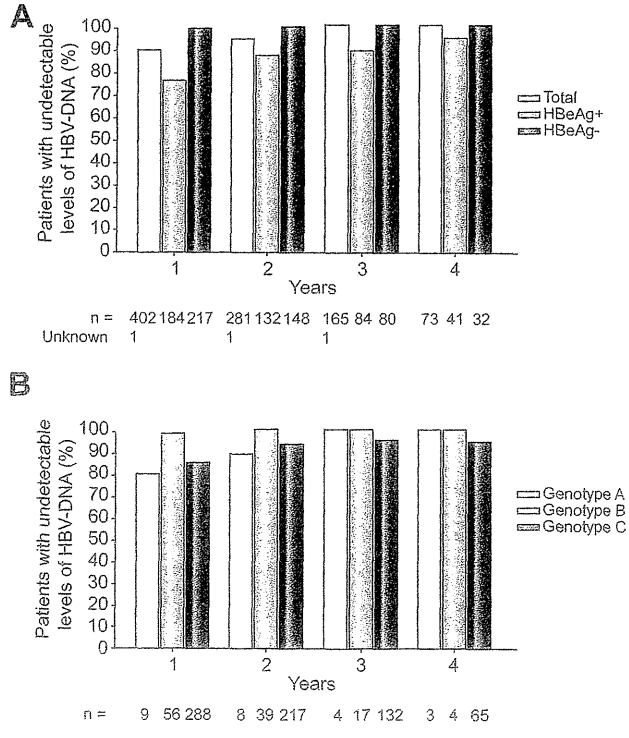

Fig. 1. Percentages of patients who had undetectable levels of HBV DNA between years 1 through 4. (A) HBeAg-positive and negative patients and (B) patients with genotype A, B, or C.

Table 2. Univariate and multivariate analyses of host and viral factors associated with undetectable levels of HBV DNA at year 1.

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Sex (female)	1.06 (0.56-2.02)	0.842		
Age (>40 yr)	1.85 (1.0-3.4)	0.047		
Cirrhosis (present)	2.39 (0.98-5.81)	0.048		
Albumin (>4 g/dl)	1.28 (0.63-2.62)	0.494		
Bilirubin (>1.2 g/dl)	1.63 (0.56-4.76)	0.366		
ALT (>5 x IU/L)	4.57 (1.38-15.1)	0.007	11.9 (3.3-41.7)	<0.001
AST (>5 x IU/L)	2.25 (0.67-7.53)	0.178		
γ-GTP (≤20 IU/L)	1.75 (0.60-5.08)	0.300		
AFP (>10 ng/ml)	1.63 (0.61-4.37)	0.328		
Platelets (≤10/mm^3)	2.39 (0.56-10.3)	0.288		
Genotype (B)	9.57 (1.29-70.92)	0.007		
HBeAg (negative)	23.78 (7.25-77.95)	<0.001	8.5 (2.3-31.2)	0.001
HBV DNA (≤7.6 $\log_{10}$ copies/ml)	16.5 (8.0-34.2)	<0.001	10.0 (4.3-23.1)	<0.001

Table 3. Univariate and multivariate analyses of host and viral factors associated with undetectable levels of HBV DNA at year 2.

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Sex (male)	0.524 (0.169-1.627)	0.257		
Age (>40 yr)	2.825 (1.1-7.25)	0.025		
Cirrhosis (present)	3.06 (0.69-13.5)	0.173		
Albumin (≤3.5 g/dl)	4.64 (0.603-35.73)	0.134		
Bilirubin (≤0.5 g/dl)	2.80 (0.79-9.93)	0.126		
ALT (>5 x IU/L)	5.35 (0.7-40.9)	0.054	16.7 (2.0-136.8)	0.009
AST (>5 x IU/L)	2.62 (0.34-20.3)	0.298		
γ-GTP (≤100 IU/L)	1.79 (0.557-5.73)	0.304		
AFP (>15 ng/ml)	2.12 (0.27-16.95)	0.699		
Platelets (≤12/mm^3)	4.74 (0.619-36.31)	0.136		
Genotype (B)	1.082 (1.042-1.123)	0.076		
HBeAg (negative)	23.21 (3.05-176.46)	<0.001		
HBV DNA (≤7.6 $\log_{10}$ copies/ml)	39.91 (8.912-178.76)	<0.001	121.7 (15.3-965.9)	<0.001

Results

Study population

Of the 474 subjects in this study, 68% were males, and the mean age was 47 years. The mean HBV DNA level was 6.7 $\log_{10}$ copies/ml, mean ALT level was 70 IU/L, and 47% of patients were HBeAg-positive. At baseline, there were 12, 67, and 336 patients of genotype A, B, and C, respectively, and among the patients belonging to these genotypes, 4, 11, and 188, respectively, were HBeAg-positive.

Virological response

Undetectable levels of HBV DNA were identified at years 1 through 4 in 88% (353/402), 93% (262/281), 95% (156/165), and 96% (70/73) of patients, respectively (Fig. 1A). Among the HBeAg-positive patients at baseline, 75% (138/184), 86% (114/132), 89% (75/84), and 93% (38/41), and among the HBeAg-negative patients at baseline, 99% (214/217), 99% (147/148), 100% (80/80), and 100% (32/32) had undetectable levels of HBV DNA at years 1 through 4, respectively.

Among the patients with genotype A, 78% (7/9), 88% (7/8), 100% (4/4), and 100% (3/3) of patients had undetectable levels of HBV DNA at years 1 through 4, respectively (Fig. 1B). Among the HBeAg-positive patients with genotype A at baseline, 50% (2/4), 67% (2/3), 100% (2/2), and 100% (2/2) had undetectable levels of HBV DNA at years 1 through 4, respectively. Among patients with genotype B, 98% (55/56), 100% (39/39), 100% (17/17), and 100% (4/4) had undetectable levels at years 1 through 4, respectively (Fig. 1B). Among the HBeAg-positive patients with genotype B at baseline, 88% (7/8), 100% (5/5), and 100% (3/3) had

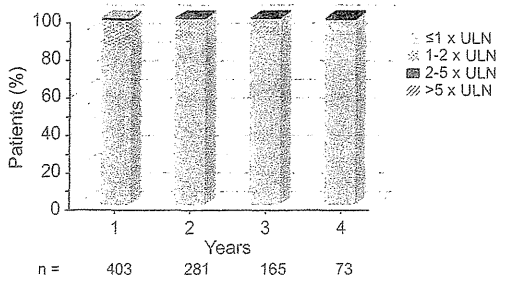

Fig. 2. Percentages of patients with ALT level <1× upper limit of normal level (ULN), <1–2× ULN, 2–5× ULN, and 5× ULN.

undetectable levels of HBV DNA at years 1 through 3, respectively. None of the patients with genotype B at baseline were HBeAg-positive at year 4. Among the patients with genotype C, 85% (246/288), 93% (201/217), 95% (125/132), and 95% (62/65) had undetectable levels of HBV DNA at years 1 through 4, respectively (Fig. 1B), and of these, 74% (116/156), 87% (102/117), 91% (67/74), and 92% (35/38), respectively, were HBeAg-positive.

Factors associated with detectable levels of HBV DNA at years 1, 2, and 3

Of the 402 patients, 353 had no detectable HBV DNA after 1 year. At the start of treatment, factors associated with undetectable levels of HBV DNA in the first year were age (>40 years, $p = 0.047$), cirrhosis (present, $p = 0.048$), ALT (>5× ULN, $p = 0.007$), genotype (B, $p = 0.007$), HBeAg (negative, $p < 0.001$), and HBV DNA level (<7.6 $\log_{10}$copies/ml, $p < 0.001$), by univariate analysis (Table 2). Multivariate analysis identified three parameters, namely ALT (>5× ULN, OR = 11.9; 95% CI = 3.3–41.7, $p < 0.001$), HBeAg (negative, OR = 8.5; 95% CI = 2.3–31.2, $p = 0.001$), and HBV DNA level (<7.6 $\log_{10}$ copies/ml, OR = 10.0; 95% CI = 4.3–23.1, $p < 0.001$).

Of 281 patients, HBV DNA was undetectable in 262 patients in the second year, with univariate analysis identifying the following associated factors: age (>40 years, $p = 0.025$), ALT (>5× ULN, $p = 0.054$), HBeAg (negative, $p < 0.001$), and HBV DNA level (≤7.6 log copies/ml, $p < 0.001$). Of these, multivariate analysis identified ALT (>5× ULN, OR = 16.7; 95% CI = 2.0–136.8, $p = 0.009$) and HBV DNA level (≤7.6 $\log_{10}$ copies/ml, OR = 121.7; 95% CI = 15.3–965.9, $p < 0.001$) as significant factors (Table 3).

Of 165 patients, HBV DNA was undetectable in 156 patients in the third year, with univariate analysis identifying the following associated factors at the start of treatment: Gender (male, $p = 0.04$), HBeAg (negative, $p = 0.002$) and HBV DNA level (≤7.6 log copies/ml, $p < 0.001$). Multivariate analysis identified only HBV DNA level as significant (≤7.6 $\log_{10}$ copies/ml, OR = 15.8; 95% CI = 43.1–79.9, $p = 0.001$).

Biochemical response

The percentages of patients with normal ALT levels (<1x ULN) at years 1, 2, 3, 4 were 83% (336/403), 89% (251/281), 92% (151/165), and 93% (68/73), respectively (Fig. 2). In HBeAg-positive patients at baseline, those who achieved normal ALT levels at years 1, 2, 3, 4 were 81% (148/183), 88% (116/132), 90% (76/84), and 95% (39/41), respectively. The respective data for HBeAg-negative patients at baseline were 85% (187/219), 91% (134/148), 93% (74/80), and 91% (29/32).

HBeAg seroclearance and seroconversion

HBeAg positivity at baseline was detected in 222 patients (47%) (Table 1), and Fig. 3A shows the cumulative clearance of HBeAg calculated with the Kaplan–Meier method. The percentages of patients with seroclearance were 16%, 24%, 37%, and 42% at years 1 through 4, respectively. Univariate analysis identified the following HBeAg seroclearance-associated factors at the start of treatment: age (>40 years, $p = 0.052$), platelet count (<12 × 10^4/mm^3, $p = 0.028$), and HBV DNA (<7.0 log copies/ml, $p = 0.006$). Multivariate analysis identified HBV DNA (<7 $\log_{10}$ copies/ml, RR = 1.9; 95% CI = 1.2–3.1, $p = 0.007$) as the only significant determinant of seroclearance. Of 70 patients who achieved anti-HBe seroclearance, 52 patients achieved anti-HBe seroconversion. Fig. 3B shows the cumulative seroconversion rate of HBeAg calculated by the Kaplan–Meier test. The proportions of patients who showed seroconversion were 12%, 18%, 29%, and 38% at years 1 through 4, respectively. Univariate analysis demonstrated that age (>40 years, $p = 0.020$), albumin (<3.5 g/dl, $p = 0.021$) and platelet count (<20 × 10^4/mm^3, $p = 0.067$) correlated with HBeAg seroconversion at the start of treatment. Multivariate analysis that included the above factors identified serum albumin as the only significant determinant of seroconversion (<3.5 g/dl, RR = 2.0; 95% CI = 1.1–3.6, $p = 0.019$). One patient achieved anti-HBe seroconversion at 25 months but became positive again at 28 months. Other patients who achieved anti-HBe seroconversion did not show HBeAg reversion. One patient achieved anti-HBe seroconversion but remained HBV DNA positive (Table 4, Patient 5). Another patient remained positive for HBV

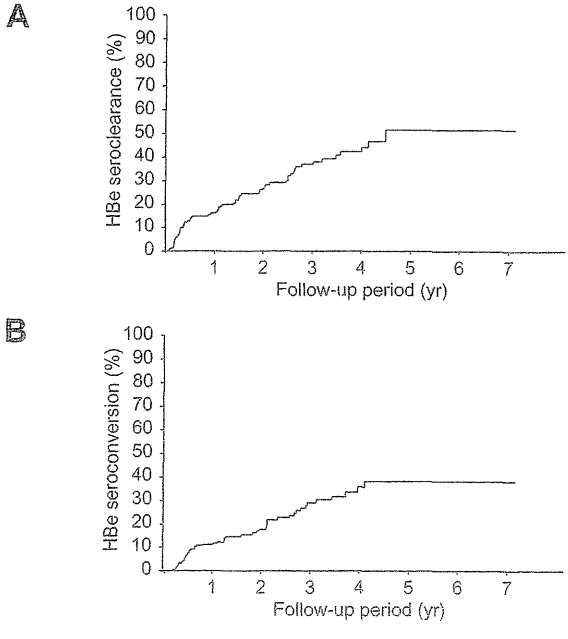

Fig. 3. Change of the HBeAg status during follow-up. Cumulative rates of (A) HBe seroclearance and (B) HBe seroconversion in HBeAg-positive patients, analyzed with the Kaplan–Meier test.

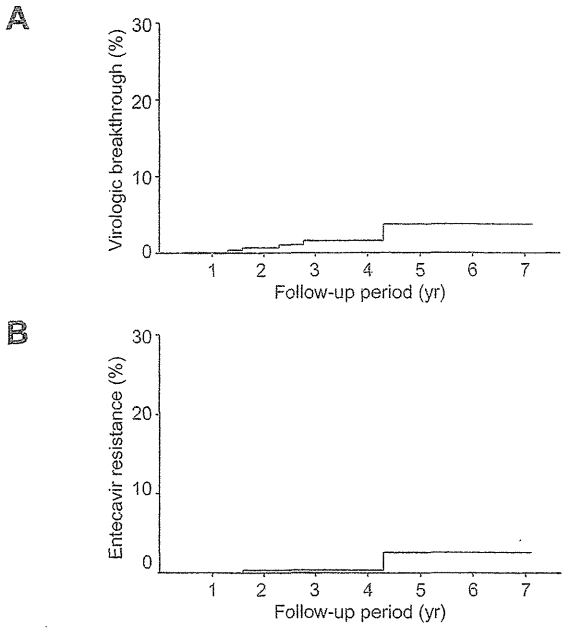

Fig. 4. Cumulative rates of patients who showed resistance to therapy analyzed with the Kaplan–Meier test. (A) Virologic breakthrough (VBT) and (B) entecavir-resistance.

DNA after anti-HBe seroconversion. One patient became negative for HBsAg at week 28.

Monitoring resistance to treatment

Five patients showed VBT during the treatment period, including two patients (Patient 1 had been reported previously [13]) who developed entecavir-resistant mutations. None of the five patients had mutation(s) for entecavir at baseline. VBT was defined as any increase in serum HBV DNA by >1 $\log_{10}$ copies/ml from nadir or redetection of serum HBV DNA at levels 10-fold the lower limit of detection of the HBV DNA assay after having an undetectable result. Table 4 shows the patient baseline demographics, HBV DNA levels, and viral resistance profiles. All patients were positive for HBeAg and had serum levels of HBV DNA >6 $\log_{10}$ copies/ml at baseline. The median period until the appearance of the mutation was 120 (68–224) weeks. Two of the 49 (4%) patients who had detectable HBV DNA at the end of the first year subsequently developed resistance to entecavir. Furthermore, 3 of 49 (6%) patients who had detectable HBV DNA at the end of the first year developed VBT. Fig. 4A and B show the cumulative percentages of VBT and entecavir-resistance cases analyzed by the Kaplan–Meier test.

Discussion

Long-term data are rare for nucleoside-naïve patients treated continuously for more than 4 years with entecavir at the recommended dose of 0.5 mg daily. The only available data [6,8] were generated from follow-up studies of two phase III registration trials [3,4] in which patients showing complete response and non-responders were taken off entecavir. In the rollover studies, entecavir was administered to these patients at 1-mg dose at varying periods after cessation of the initial treatment. This double dose of entecavir was also given to patients showing a partial virological response after 48–96 weeks of entecavir at 0.5 mg daily. The present study has several unique features addressing specific and unanswered questions about entecavir treatment. It provided long-term results with respect to antiviral potency, viral resistance, and clinical safety for treatment-naïve patients who were treated continuously with entecavir at 0.5 mg daily for 4 years. Specifically, we found excellent viral suppression with 96% of patients achieving undetectable HBV DNA levels, only 1.1% (5/475) chance of viral breakthrough, and no clinically serious side effects after 4 years of treatment.

Genotype B was a significant factor associated with undetectable HBV DNA after the first year, although there were no significant differences after subsequent years. Previous studies showed conflicting results on the effect of HBV genotype on the response to lamivudine, with genotypes A, B, and C not affecting the antiviral response to lamivudine [14–16]. However, we have previously found that 47%, 84%, and 76% of patients had undetectable HBV DNA after the third year among patients of genotype

Table 4. Characteristics of patients with virologic breakthrough.

Patient No.	1	2	3	4	5
Age (yr)/gender	40/M	28/M	39/M	51/F	64/M
At start of entecavir therapy					
HBeAg status	+	+	+	+	+
HBV DNA ($\log_{10}$ copies/ml)	>7.6	>7.6	7.2	7.2	6.2
HBV genotype	H	A	C	C	C
Viral load at maximum suppression ($\log_{10}$ copies/ml)	<2.6	<2.6	<2.6	3.1	<2.6
Time of detection of mutation (wk)	83	224	120	68	145
HBV DNA ($\log_{10}$ copies/ml), maximum	6.8	7.2	7.1	7.6	7.8
Mutational pattern	L180M+/S202G+/M204V	L180M+/T184I+/S202G+/M204V	L180M+/M204V, L180M+/M204I	A181T	A181S+/T184A+/M204I

A, B, and C, respectively [17,18]. The difference among these groups was probably due to the younger age of patients of genotype A and that they were often positive for HBeAg compared to those of genotype B or C. However, the genotype was not a significant predictor of HBV DNA loss after >2 years of entecavir therapy in the present study. There was also no difference in HBeAg seronegativity with entecavir among patients infected with genotype A, B, or C virus. These results were consistent with studies on lamivudine therapy [14,18].

In this study, HBeAg positivity was a significant factor associated with detectable HBV DNA at years 1 through 3, and these results were consistent with those reported by Zoutendijk *et al.* [10]. In addition, lower HBV DNA and HBeAg negativity at baseline were associated with enhanced response to lamivudine therapy [18–20]. We have also previously reported that lamivudine induced a better response in HBeAg-negative patients with higher levels of serum ALT [17]. The most important factor of long-term entecavir therapy therefore was low HBV DNA level.

Low HBV DNA level at baseline correlated significantly with HBeAg seroclearance, but not with seroconversion. One of the reasons was that patients who showed HBeAg seroclearance but no seroconversion had lower HBV DNA (median; 6.7 $\log_{10}$ copies/ml) at baseline compared to patients with seroconversion (median; 7.5 $\log_{10}$ copies/ml, $p = 0.005$).

Univariate analysis showed that age (>40 years), serum albumin level (<3.5 g/dl), and platelet count ($<20 \times 10^4/mm^3$) correlated with HBeAg seroconversion rate. We also investigated the correlation between serum albumin and other factors. Serum albumin level correlated significantly with age ($r = -0.378$, $n = 216$, $p < 0.001$), platelet count ($r = 0.262$, $n = 215$, $p < 0.001$), AFP ($r = -0.372$, $n = 161$, $p < 0.001$), cirrhosis ($P < 0.001$) and male sex ($p = 0.004$). Multivariate analysis identified low serum albumin level (<3.5) as the only significant determinant of HBeAg seroconversion. In this regard, Chien *et al.* [21] reported that pre-treatment ALT was the only significant determinant of HBeAg seroconversion during lamivudine therapy. The reasons for the different findings are probably related to the study design. In our study, the age of patients at baseline was higher (47 *vs.* 32 years) and the duration of treatment was longer (2.4 [median] *vs.* 1 year) than in the study of Chien *et al.* [21]. Furthermore, differences in the pharmacodynamics of lamivudine and entecavir could also contribute to the observed differences between the two studies.

On the other hand, resistant mutants and breakthrough hepatitis seemed to be less frequent during long-term therapy with entecavir than with lamivudine [16–19], indicating that entecavir is better than lamivudine for long-term treatment of CHB and cirrhosis patients. Tenney *et al.* [6] reported that 9 out of 663 (1.4%) patients had baseline lamivudine-resistant mutations, and other studies also found only small numbers of preexisting lamivudine-resistant mutations in treatment-naïve patients [22–24]. It is known that the HBV rtM204V (usually with concomitant rt180M) mutation often acquires one of the entecavir signature mutations at rt184, rt202, or rt250 over long-term treatments and patients develop clinical HBV DNA breakthroughs. Although *in vitro* studies showed that rt204I mutations with or without rt180M conferred 3- to 21-fold decrease in entecavir susceptibility [25], in clinical practice, patients with rt204I mutations, even with the entecavir signature mutations, have lower levels of phenotypic resistance to entecavir and can often achieve undetectable HBV DNA levels [6,9,26]. Interestingly, there were three patients in the present study with VBT who had no HBV DNA mutations at rt184, rt202, or rt250 with rt180M and rt204V (entecavir-resistance). The rtM204V/I mutation, lamivudine's signature mutation, is necessary but not sufficient for entecavir-resistance, causing an 8- to 10-fold decrease in susceptibility to entecavir compared with wild-type HBV. Other mutations at positions rtT184, rtS202, and rtM250 confer additional decreases in entecavir susceptibility [25,27,28]. In the present study, two patients (Patients #3 and 5) with mutations at position rtM204V/I, without rtT184, rtS202, or rtM250 mutations, showed emergence of VBT, as did one patient (Patient #4) with an rtA181T mutation, which was first reported in a LAM-treated patient [29]. Although the rtA181T mutation is related to resistance to adefovir dipivoxil, this mutation has not been linked to additional decreases in entecavir susceptibility. Future *in vitro* analyses using replication-competent HBV clones in patients with rtA181T mutations are therefore necessary.

In conclusion, long-term treatment of treatment-naïve CHB patients with 0.5 mg/day entecavir for 4 years suppressed HBV DNA to undetectable levels in more than 90% of patients, regardless of HBeAg status and genotype. Moreover, the drug was very safe and rarely induced resistance mutations. Further studies exploring the therapeutic efficacy over longer durations may be necessary to confirm these findings.

Conflict of interest

Hiromitsu Kumada has received speaker's honoraria from Bristol-Myers Squibb. All other authors declare no conflict of interest.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Determinants of the clinical outcome of patients with severe acute exacerbation of chronic hepatitis B virus infection

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Abstract
Background Severe acute exacerbation of chronic hepatitis B can sometimes occur and lead to hepatic failure and death. The objective of this study was to elucidate the predictors of progression to hepatic decompensation during severe acute exacerbation.
Methods We prospectively analyzed 37 consecutive patients with acute exacerbation of chronic hepatitis B (accompanied by jaundice and coagulopathy) for clinical outcome and factors that influenced the development of severe acute exacerbation, including viral kinetics.
Results Fourteen (37.8%) patients progressed to severe acute exacerbation (accompanied by encephalopathy). Multivariate analysis identified serum bilirubin (>5 mg/dl, $P = 0.002$) as a significant determinant of progression to hepatic failure and prothrombin activity (<45%, $P = 0.028$) and as a determinant of liver-related death. The hepatitis B virus (HBV) DNA level before therapy was measured in 25 patients. HBV DNA levels increased or did not change from before commencement of treatment in all 11 patients who progressed to severe acute exacerbation. On the other hand, HBV DNA levels did not change or increased in 8 of 14 patients (57%) with acute exacerbation ($P = 0.02$).
Conclusions Serum bilirubin and prothrombin activities were significant predictors of clinical outcome in patients with severe acute exacerbation of chronic hepatitis B. Viral kinetics until commencement of therapy can predict the severity of acute exacerbation of chronic hepatitis B.

Keywords Hepatitis B · Acute exacerbation · HBV DNA · Genotype · Encephalopathy

Abbreviations

AE	Acute exacerbation
ALT	Alanine aminotransferase
BCP	Basal core promoter
CS	Corticosteroid
HBV	Hepatitis B virus
IFN	Interferon
LMV	Lamivudine
NA	Nucleos(t)ide analogue
PC	Pre-core
PT	Prothrombin activity
SAE	Severe acute exacerbation

Introduction

More than 3 billion people worldwide and approximately 1.5 million people in Japan are chronically infected with hepatitis B virus (HBV), and chronic HBV infection is one of the most common causes of chronic hepatic failure and hepatocellular carcinoma (HCC) [1, 2]. Other complications of HBV infection include fulminant hepatitis and acute liver failure [3, 4]. Acute exacerbation (AE) in HBV carriers occurs either through a natural course [5, 6] or following intensive chemotherapy or immunosuppressive

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therapy [7, 8]. Some abrupt flares may be so severe that decompensation or even fulminant hepatic failure may occur [9–11]. Previous studies have identified pre-existing cirrhosis, high serum bilirubin levels, prolonged prothrombin time, pre-core/core promoter mutants, and high HBV DNA levels as factors associated with hepatic decompensation during AE in HBV carriers, though little is known about the predictive factors [9, 12, 13].

Liver transplantation is suitable therapy for acute hepatic failure, but the rate of liver transplantation has remained about 20% in Japan, where living donor liver transplantation is dominant [14, 15]. Thus, it is necessary to establish other effective therapies for patients with AE apart from liver transplantation. Steroids can rapidly inhibit excessive immune response and inflammatory reactions, and have been reported to be effective in cases of severe and potentially life-threatening exacerbation of chronic HBV (CHB) infection [16]. With the advent of oral nucleos(t)ide analogues (NAs), most guidelines recommend NAs for patients with AE of CHB infection [17–19], and several observational studies reported the use of NAs [9–11, 20, 21]. Timely use of potent anti-HBV agents, such as NAs, interferon (IFN), and steroids [22], during and/or after the development of hepatic decompensation could be potentially effective against various host- and virus-related factors.

The aim of the present study was to investigate the factor(s) that influence the rapid development of hepatic decompensation during AE of CHB.

Materials and Methods

Patients

The study subjects were patients with AE admitted to the Department of Hepatology, Toranomon Hospital, Tokyo, between 1984 and 2010. All patients were either followed up at our hospital with clinicopathologically proven CHB infection or were new patients with sudden-onset hepatic flares who visited our hospital outpatient clinic or were referred to our hospital from other clinics/hospitals. The diagnosis of CHB carrier state was established based on either positivity for hepatitis B surface antigen (HBsAg) for at least 6 months prior to the development of AE, or the presence of a high titer of anti-hepatitis B core antibodies (anti-HBcAb), together with negativity or a low titer of IgM anti-HBcAb. Chronic hepatitis and cirrhosis were confirmed by laparoscopy, needle biopsy, or ultrasonography, or treatment for these conditions for 1 year before the development of AE. AE of CHB infection was diagnosed by the following criteria: (1) an abrupt increase in serum alanine aminotransferase (ALT) levels to >300 IU/l in patients with original ALT levels of less than 5× the upper limit of normal or an abrupt two-fold increase in the serum ALT level to greater than 5× the upper limit of normal, (2) hyperbilirubinemia [serum bilirubin (Bil) >3.0 mg/dl], (3) evidence of coagulopathy with plasma prothrombin activity (PT) of <60% during the clinical course, and (4) lack of encephalopathy at admission. We also applied the following exclusion criteria: (1) the presence of viral markers other than HBV (hepatitis A, C, D, E, Epstein-Barr virus, cytomegalovirus, herpes simplex virus), (2) HBV reactivation induced by immunomodulators or chemo-/immunosuppressive therapy, (3) asymptomatic HBV carriers, (4) recent exposure to drugs and chemical agents as well as recent heavy alcohol intake, (5) breakthrough hepatitis caused by NAs, (6) evidence of decompensated liver disease before the onset of exacerbation as characterized previously, (7) HCC diagnosed by ultrasonography or computed tomography, and (8) coexistence of other serious medical conditions and other liver diseases, or metabolic diseases. Progression to severe acute exacerbation (SAE) was diagnosed by the development of hepatic encephalopathy of more than grade 2 within 8 weeks of onset associated with coagulopathy (PT <40%).

HBV DNA levels were measured serially to investigate the effects of HBV kinetics on the prognosis of patients with severe AE. HBV DNA levels were measured before treatment in 25 patients. "Before treatment" represented 1–8 weeks before commencement of treatment. HBV DNA levels were also measured after treatment in 27 patients. "After treatment" was defined as 2 weeks after commencement of therapy. Viral kinetics was assessed using the same assay in all individuals. The Local Ethics Committee of Toranomon Hospital approved the study, and informed consent was obtained from all patients.

Virological markers

Serial blood samples were obtained during the clinical course of AE and stored at −80°C until used for HBV molecular analysis. Serological tests for HBsAg, HBsAb, hepatitis e antigen (HBeAg), IgM anti-HBcAb, total anti-HBcAb, and anti-HBeAb were conducted using radioimmunoassay kits (Abbot Diagnostics, Chicago, IL, USA) according to the instructions provided by the manufacturer. Precore (PC) mutations were analyzed by PCR enzyme-linked mini-sequence assay (Roche Diagnostics, Tokyo, Japan), and basal core promoter (BCP) mutations were analyzed by PCR specific probe assay (Roche Diagnostics, Tokyo, Japan). HBV DNA was measured by Amplicor monitor assay (dynamic range 2.6–7.6 log copies/ml, Roche Diagnostics, Tokyo, Japan), COBAS TaqMan v.2.0 (dynamic range 2.1–9.0 log copies/ml, Roche Diagnostics), transcription-mediated amplification and hybridization

protect assay (TMA-HPA) (dynamic range 3.7–8.7 LGE/ml, Chugai Diagnostics Science Co., Tokyo) or sandwich hybridization assay with signal amplification using branched DNA (bDNA, dynamic range 0.7–3800 Meq/ml). The major genotype of HBV was determined using enzyme-linked immunosorbent assay (ELISA, Institute of Immunology, Tokyo, Japan) or PCR-invader assay (BML, Inc, Tokyo, Japan) based on the methods described previously [23, 24]. HBVDNA levels assessed by bDNA were re-measured by TaqMan PCR assay using stored serum samples.

Statistical analysis

Continuous variables were expressed as median (range), and compared by Mann–Whitney *U* test. Categorical variables were compared by χ^2 test or Fisher's exact test, as appropriate. Univariate analysis was applied to determine the relationship between SAE and each of the following factors: sex, age, presence of compensated cirrhosis, and various biological and virological markers as measured at baseline (bilirubin, PT, ALT, albumin, HBeAg, HBV DNA, and HBV genotype, PC and BCP mutations). Each continuous variable was transformed into two categories based on the value with the largest capacity to discriminate between patients for univariate and multivariate analyses. Factors that correlated significantly with SAE were entered into multiple logistic regression analysis, and the odds ratio (OR) with 95% confidence intervals (95% CI) were determined. All analyses were performed using The Statistical Package for Social Sciences (SPSS II v. 11.0, Chicago, IL, USA), and statistical significance was taken as a two-sided P value <0.05.

Results

Clinical features of severe acute exacerbation

A total of 37 patients (30 men and 7 women) fulfilled the criteria of AE and were included in this study. The baseline characteristics at the commencement of therapy of these 37 patients are shown in Table 1. Twenty-two patients were observed at our hospital, and 15 patients were referred from another hospital after the onset of hepatic flares. The majority of patients had genotype C, and 27 patients (72.9%) were HBeAg positive. The PC and BCP mutations were determined in 27 patients; 22 patients had mutations in the PC region, 16 patients had mutations in the BCP region, and 12 patients had mutations in both the PC and BCP regions. During the clinical course, the peak median values were: ALT 713 IU/l (range 307–2857), bilirubin 8.4 mg/dl (3.0–51.4), and PT 47.6% (12.0–60.0).

Table 1 Baseline characteristics of the 37 patients infected with HBV who developed severe acute exacerbation at the commencement of therapy

Number	37
Sex (male/female)	30/7
Age (years)	45 (23–63)
Family history (yes/no)	21/16
Cirrhosis (present/absent)	7/30
Albumin (g/dl)	3.4 (2.5–4.6)
Bilirubin (mg/dl)	4.7 (1.0–30.7)
AST (IU/l)	601 (64–2593)
ALT (IU/l)	657 (124–2142)
LDH (IU/l)	297 (106–594)
Platelets ($\times 10^4$/mm^3)	12.3 (6.2–32.0)
α-Fetoprotein (μg/ml)	62.0 (3.0–1600)
Prothrombin activity (%)	53 (26–80)
Genotype (A/B/C)	0/5/32
HBeAg (positive/negative)	27/10
HBV-DNA ($\log_{10}$ copies/ml)	8.5 (6.8–8.9)
PC (wild/mutant/ND)	5/22/10
BCP (wild/mutant/ND)	11/16/10

Data are median values (range) or number of patients

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactate dehydrogenase, *HBeAg* hepatitis B envelope antigen, *PC* pre core, *BCP* basal core promoter, *ND* not done

Treatment

NAs were used in 19 patients, IFN in 8, and corticosteroids (CS) in 20 patients. In addition, 7 patients were treated with a combination of NAs and CS; 2 patients were treated with three drugs (NAs, IFN, and CS). At the time of the study, lamivudine (LMV) was not yet available for the treatment of chronic hepatitis B, and thus IFN was used; 6 patients were treated with both IFN and CS. None of the patients underwent liver transplantation.

Prognosis of severe acute exacerbation and factors associated with progression to hepatic failure

Of the 37 patients admitted with CHB infection and AE, 23 (62.2%) did not develop SAE. The remaining 14 (37.8%) patients developed SAE; 9 (24.3%) patients died of liver-related death, but 5 (13.5%) survived. Further analysis showed that 8 (36.4%) of 22 patients who were observed in our hospital developed AE, and 6 (27.3%) of these patients died, whereas 6 (40.0%) of 15 patients who were referred from other hospitals after the onset of exacerbation developed AE, and 3 (20.0%) of these patients died. There was no significant difference in prognosis by treatment facility before AE. Ten of 37 patients experienced AE before 2000 when LMV was available in Japan, and 19

Table 2 Biochemical, virological and histological features of patients with severe acute exacerbation at the commencement of therapy

Case	Age (years)/ sex	Genotype	HBeAg	HBV-DNA (log copies/ ml)	Preexisting cirrhosis	Serum bilirubin (mg/dl)	ALT (IU/l)	PT (%)	Platelets ($\times 10^4$/ mm^3)	Therapy	Outcome (time from treatment to death, weeks)
1	63/M	B	–	8.4	No	5.8	1680	43	6.2	LMV + CS	Death (11)
2	32/M	B	–	>8.7	No	6.9	1340	41	13.4	CS	Death (1)
3	58/M	B	–	8.6	No	7.4	1446	36	7.7	CS	Death (2)
4	29/M	B	–	>8.7	No	15.6	307	26	10.0	LMV	Recovery (alive)
5	54/F	C	+	>8.7	No	2.4	2077	79	21.0	LMV + CS	Recovery (alive)
6	37/M	C	+	>8.7	No	4.1	552	53	8.9	CS	Recovery (alive)
7	62/M	C	+	7.0	No	12.0	220	53	7.1	LMV + CS + IFN	Recovery (alive)
8	33/F	C	+	>8.7	No	14.0	632	39	13.1	CS	Recovery (alive)
9	55/M	C	+	>8.7	Yes	4.0	1089	55	10.3	LMV + CS	Death (1)
10	37/F	C	+	7.1	Yes	5.8	1444	34	22.0	LMV + CS + IFN	Death (10)
11	49/M	C	+	8.0	Yes	8.8	834	58	9.9	CS	Death (10)
12	33/M	C	+	8.5	No	9.6	657	26	7.4	LMV + CS	Death (2)
13	54/M	C	+	7.8	Yes	12.1	364	36	15.8	LMV + CS	Death (2)
14	55/M	C	+	>8.7	No	24.2	520	44	8.3	CS	Death (5)

Abbreviations as in Table 1, *PT* prothrombin activity, *LMV* lamivudine, *CS* corticosteroids, *IFN* interferon-α

patients experienced AE after 2000. The other 8 patients experienced AE before 2000, but received LMV through participation in clinical trials or paid for the drug privately. The clinical features at the commencement of therapy of 14 patients who developed SAE are shown in Table 2 (median age 52 years, range 29–63). The mean time period between admission and death of 9 patients who developed SAE was 2 (range 1–11) weeks. Six patients who were admitted before the availability of LMV were treated with CS alone, 5 patients were treated with the combination of LMV and CS, 1 patient was treated with LMV alone, and 2 other patients were treated with LMV, CS, and IFN. Among 8 patients treated with LMV, of those who developed SAE, 5 died, and 2 patients developed complications caused by bacterial infection. Four patients had genotype B, while 10 patients had genotype C. HBeAg status was positive in 10 patients. The mean HBV DNA level was 8.7 (range 7.0–>8.7) log copies/ml, ALT 746 (220–2077) IU/l, serum bilirubin 8.1 (2.4–24.2) mg/dl, PT 42 (26–79)%, and platelet count was 10.0 (62–220) $\times$ $10^4/mm^3$.

Of the 5 patients who were treated successfully after progression to SAE, one later died of severe breakthrough hepatitis caused by emergence of LMV-resistant virus 3 years after SAE (case 7, Table 2). The other four survived (cases 4–6 and 8, Table 2).

Table 3 shows the results of univariate analysis. The following factors showed significant relationship with the development of SAE at the commencement of treatment: serum bilirubin (>5 mg/dl) and PT (<60%). Multivariate analysis identified serum bilirubin as a significant and independent determinant of the development of SAE (Table 3). On the other hand, two parameters showed significant relationships with liver-related death: serum bilirubin (>7 mg/dl, $P = 0.049$) and PT (<45%, $P = 0.003$). Multivariate analysis identified PT (OR 9.50, 95% CI 1.3–71.0, $P = 0.028$) as a significant determinant of death.

Viral kinetics associated with fulminant hepatic failure

To investigate the relationship between viral kinetics and SAE, HBV DNA levels were measured in 25 patients both before and commencement of treatment and also after treatment in 27 patients. Figure 1 shows the viral load of patients who developed and did not develop SAE at commencement of treatment compared with before treatment. Falls in the HBV DNA level occurred naturally. However, in 11 patients who developed SAE, HBV DNA levels increased in 6 patients and did not change in 5 patients. Among the latter 5, HBV DNA levels of 4 patients were >8.7 log copies/ml. In 14 patients who did not develop SAE, HBV DNA levels increased in 4 patients, were unchanged in 4 patients, and decreased in 6 patients. Hence, the HBV DNA level increased/was unchanged in 8 of 14 (57%) patients who did not develop SAE, compared with 11 of 11 (100%) patients who developed SAE. A significantly higher proportion of patients with SAE showed an increase/was unchanged in viral load compared to those who without SAE ($P = 0.02$). We also examined the viral kinetics in 27 patients by comparing HBV DNA levels at the commencement of treatment to after treatment.

Table 3 Univariate and multivariate analyses of host and viral factors associated with progression of severe acute exacerbation at commencement of treatment

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Sex (female)	1.30 (0.15–4.11)	0.76		
Age (>55 years)	2.64 (0.57–12.3)	0.22		
Cirrhosis (present)	1.90 (0.39–9.26)	0.43		
Albumin (<3.5 g/dl)	1.75 (0.44–6.97)	0.85		
Bilirubin (>5 g/dl)	17.0 (2.92–99.1)	0.002	11.2 (1.71–73.8)	0.01
ALT (>800 IU/l)	1.88 (0.48–7.26)	0.36		
AST/ALT ratio (>1)	1.27 (0.31–5.19)	0.74		
Prothrombin activity (<60%)	11.9 (1.33–106.7)	0.03	8.22 (0.73–92.6)	0.09
Platelets (<15 × 10^4/mm^3)	0.81 (0.19–3.58)	0.89		
Genotype (B)	8.82 (0.87–89.1)	0.06		
HBeAg (positive)	0.89 (0.20–3.90)	0.89		
HBV-DNA (>8.7 log copies/ml)	2.34 (0.60–9.20)	0.70		
PC mutation	2.29 (0.22–24.1)	0.49		
BCP mutation	0.19 (0.034–1.08)	0.06		

Abbreviations as in Tables 1 and 2, *OR* odds ratio, *CI* confidence level

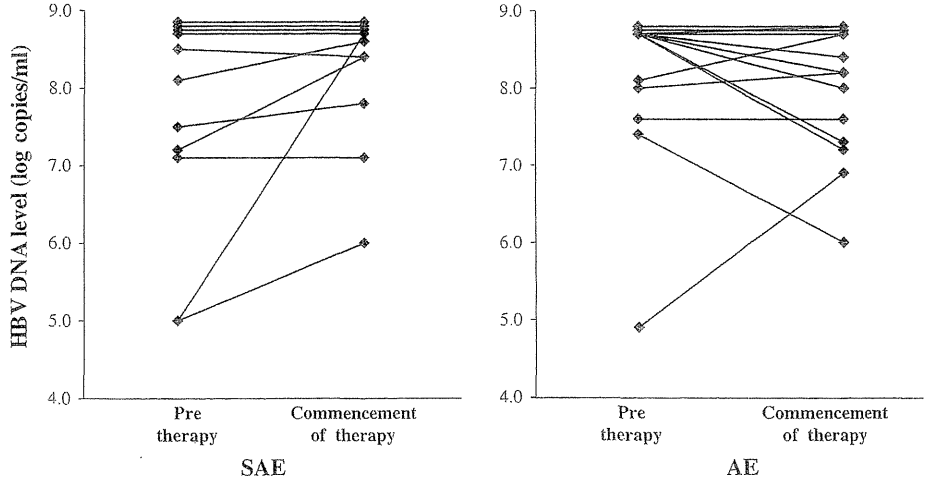

Fig. 1 Viral kinetics from pre-treatment to commencement of treatment in patients with acute exacerbation. Viral kinetics tended to increase or remained unchanged until treatment in 8 patients with acute exacerbation course ($n = 14$), while the viral load in all patients with severe acute exacerbation ($n = 11$) increased or remained unchanged ($P = 0.02$)

The HBV DNA level decreased more than 1 log copies/ml in 9 of 17 (52.9%) patients who did not develop SAE, compared with 3 of 10 (30.0%) patients who developed SAE, but the difference between the two groups was not significant.

Discussion

The results of the present study examined the predicting factors of progression to SAE accompanied by coagulopathy and encephalopathy in patients with AE of chronic hepatitis B, as well as the pattern of viral kinetics before and after commencement of therapy. Up to 30% of patients with CHB infection experience reactivation of hepatitis every year [5, 6], while some patients develop acute exacerbation with jaundice and coagulopathy, a severe life-threatening condition with high mortality [9, 12]. It is important to determine the predicting factors of progression to liver decompensation in patients with acute exacerbation. Multivariate analyses in previous studies indicated that pre-existing cirrhosis, a high Child–Pugh score, low albumin level, high serum bilirubin level, prolonged PT, and high HBV DNA levels were associated with the severity or mortality during acute exacerbation [9, 12, 13]. Our results are almost comparable to those of the above studies. Multivariate analysis in the present study identified the serum bilirubin level as a predictor of progression to liver decompensation. Moreover, there were no significant differences in viral load or therapeutic regimen. Genotype B was the predominant HBV strain in patients with SAE compared to patients with variable severity of liver diseases [25]. The frequencies of HBV genotype in patients with chronic hepatitis B admitted to our hospital were 3.0, 12.3, and 84.5%, for genotypes A, B, and C, respectively [26]. In the present study, although patients

with genotype B were only 5 of the total 37 (13.5%), 4 of 14 (28.6%) patients with SAE and 3 of 9 (33.3%) patients who died of liver failure were infected with genotype B. The different HBV genotypes also cause different clinical and epidemiological features. In a study from Japan, a high prevalence of genotype B HBV was found among patients with acute fulminant hepatitis [27]. In two case control studies conducted in Hong Kong, genotype B was the predominant HBV strain among patients with SAE compared to control patients with various severities of liver diseases [25, 28]. In this regard, another study indicated that genotype Bj was associated with high extracellular expression of HBV DNA in vitro [29]. The tendency of genotype Bj to produce high extracellular virion levels would be associated with a more vigorous immune response, leading to a higher risk of hepatic decompensation during the hepatitis flare. Several studies examined the association between specific mutations in the HBV genome and fulminant hepatitis or acute-on-chronic liver failure, especially in the PC (nt 1896) and BCP (nt 1762 and 1764) regions [30–32]. The PC and BCP regions are crucial replications of HBV [33], so alteration of the phenotype by the emergence of mutations in the PC and BCP regions might causes changes in the relationship between the virus and hepatocytes [30], and lead to fulminant hepatitis and acute exacerbation of chronic hepatitis. In the present study, genotype B and PC/BCP mutations were not significant predictors associated with the development of SAE or liver-related death, which is probably related to the small number of cases.

Jeng et al. [13] reported that HBV DNA levels greater than 1.55×10^9 copies/ml in patients with AE may predict subsequent occurrence of hepatic decompensation. While the overall viral load in our subjects was high (8.5 log copies/ml, Table 1), there was no relationship between viral load and the severity of AE or mortality. In addition, the HBV DNA level could not be estimated correctly when it was above the upper limit. Interestingly, the level of HBV DNA re-measured by TaqMan PCR in stored blood samples was higher than the upper limit (>9.1 log copies/ml) in one-third of the patients. The extremely high HBV DNA levels in patients with AE suggest that the vigorous immune attack on HBV and resultant liver injury will continue and may progress into hepatic decompensation. The present results showed that the decrease of viral load was significantly lower in patients with fulminant hepatic failure than in those with AE. These findings suggest that viral kinetics before the commencement of therapy are an important predictor of hepatic decompensation in patients with CHB infection complicated with AE. Interestingly, there was no significant difference in viral kinetics after the commencement of therapy between the two groups. To our knowledge, this is the first report that identifies viral kinetics before the commencement of therapy as a predictor of prognosis of patients with AE of chronic hepatitis B.

LMV monotherapy does not seem to improve short-term mortality in patients with AE [9], although other studies showed a possible decrease in the mortality rate with earlier administration [21]. In a recent randomized trial designed for the treatment of acute-on-chronic liver failure due to severe reactivation of hepatitis B, the use of tenofovir significantly reduced the mortality rate compared with placebo [11], and the results suggested that rapid suppression of HBV DNA replication with potent antiviral therapy could inhibit the ongoing necroinflammation and permitted hepatic regeneration. Although 8 of 14 patients were treated with LMV in the present study, two patients had to start LMV after the development of SAE because of the rapid exacerbation soon after admission. Five patients developed SAE within a median period of 8 days (range 1–17 days) after the commencement of LMV. The other one patient developed complications caused by bacterial infection and gradually progressed to liver failure over 2 months. Thus, it is thought that most of these patients developed SAE earlier than the available effect of LMV.

The prevailing idea is that AE is the result of a robust quantitative recovery of HBV specific T cells, which directly cause liver injury [34]. Other mechanisms of the effects of CS in AE may be related to the prevention of endotoxin-induced secondary liver injury [35], prevention of cytolysis of ballooned hepatocytes by stabilization of the lysosomal membrane [36], and improvement of the functional activity of the remaining hepatocytes [37]. Other studies showed that the preferential increase in the number of HBV-specific CD8 T and CD4 T cells is associated with viral control rather than liver damage [38, 39]. Whatever the mechanism of AE, a few weeks are needed for sufficient suppression of the production of HBV-related proteins by preventing HBV replication even when NAs are used [40]. Thus, earlier introduction of CS in combination with potent antiviral therapy is a reasonable approach for the initial treatment of AE to prevent excessive immunological reactions and progression of liver cell injury [22, 41]. NA or CS used on its own has limits in the resolution of the serious conditions. Considered together, it is necessary to establish effective standardized strategies, such as the combination of NA and CS. Moreover, to provide cover for NA, especially for the time until NA starts to exert its potent antiviral effect, IFN could be added with NA and CS.

In conclusion, the results of this study suggest that viral kinetics before therapy may influence the clinical course and fate of patients with SAE complicating chronic hepatitis B. Antiviral therapies, including NA and/or IFN with CS, should be started as soon as possible in cases with high serum bilirubin and/or low PT levels, genotype B, and viral

load to prevent progression into hepatic decompensation. Although ethical issues could be an obstacle to randomized trials in such severe cases, more effective strategies are necessary for the treatment of AE associated with chronic hepatitis B.

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Conflict of interest The authors declare no conflict of interest.

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Original Article

Efficacy of reduction therapy of natural human β-interferon and ribavirin in elderly patients with chronic hepatitis C, genotype 1b and high viral load

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Aim: To evaluate the efficacy of reduction therapy of natural human interferon (IFN)-β and ribavirin in elderly patients with hepatitis C virus (HCV) genotype 1b and high viral load who had complications of anemia, low bodyweight (<50 kg), diabetes mellitus and/or hypertension.

Methods: Inclusion criteria were age of 65 years or older, HCV genotype 1b, and serum HCV RNA level of 5.0 logIU/mL or higher. A total of 23 subjects with hemoglobin level of less than 13 g/dL, low bodyweight, diabetes mellitus and/or hypertension were enrolled in this study (reduction-dose group). IFN-β was administrated i.v. at a dose of 6 million units daily for 4 weeks initially, followed by three times a week for 44 weeks. Ribavirin was given daily for 48 weeks at a decreased dose of one tablet per day compared to the ordinary dose described based on bodyweight. As a control, another 22 patients without anemia, low bodyweight and/or complications treated with the standard dose of ribavirin (standard-dose group) were enrolled.

Results: Patients' rates with further dose reduction or discontinuation of treatment was 26.1% (6/23) in the reduction-dose group and 77.3% (17/22) in the standard-dose group. The sustained virological response (SVR) was 39.1% (9/23) in the reduction-dose group and 27.3% (6/22) in the standard-dose group ($P = 0.404$). Based on genetic variations near the IL28B gene (rs8099917), SVR was 44.1% (15/34) in patients with TT and 0% (0/11) in patients with TG ($P = 0.008$).

Conclusion: The reduction therapy of IFN-β and ribavirin in elderly HCV patients with genotype 1b, high viral load, IL28B gene (rs8099917) of TT who had complications of anemia, low bodyweight, diabetes mellitus and/or hypertension is one possible selection of treatment.

Key words: β-interferon, chronic hepatitis C, hepatitis C virus genotype 1b, natural ribavirin

INTRODUCTION

COMBINATION THERAPY OF peginterferon and ribavirin has been widely recommended as a first choice for chronic hepatitis C patients with high viral load.[1–7] In addition, recent study suggests that combination therapy of peginterferon, ribavirin and protease inhibitor is more effective compared to combination therapy of peginterferon and ribavirin against hepatitis C virus (HCV) of genotype 1 and high viral load.[8,9] The sustained virological response (SVR) rate was approximately 75% in naïve cases with genotype 1 and high viral load treated with three-drug combination therapy of peginterferon, ribavirin and protease inhibitor for 24 weeks. Thus, combination therapy of peginterferon, ribavirin and protease inhibitor might be recommended as a first choice for chronic hepatitis C patients with genotype 1 and high viral load in future.

However, the big problem in combination therapy of peginterferon and ribavirin or combination therapy based on three drugs of peginterferon, ribavirin, and protease inhibitor is the side-effects due to treatment.[9–11] Combination therapy of peginterferon, ribavirin and protease inhibitor might cause severe dermatitis and anemia compared to conventional treatments. The adverse events due to combination therapy of

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peginterferon and ribavirin or combination therapy of peginterferon, ribavirin and protease inhibitor have a tendency to occur in elderly patients compared to young patients. Several authors have reported that interferon (IFN)-β plus ribavirin therapy might seem to have a strong effect and mild side-effects from reports of treatment to date.[12–14] This indicates the possibility that IFN-β plus ribavirin therapy could be given to elderly patients for eradication of HCV. In particular, dose reduction might enhance the tolerability of IFN-β plus ribavirin therapy.

However, there is little information regarding efficacy of dose reduction in IFN-β plus ribavirin for elderly patients with chronic hepatitis C. Thus, in the present study, we performed a retrospective study to examine the efficacy of reduction therapy of IFN-β and ribavirin in elderly patients of 65 years or older with HCV genotype 1b and high viral load who had complications of anemia, low bodyweight (<50 kg), diabetes mellitus and/or hypertension.

METHODS

Patients

ELIGIBILITY CRITERIA FOR entry into the study included the following: (i) age of 65 years or older; (ii) HCV genotype 1b; (iii) serum level of HCV RNA of 5.0 logIU/mL or higher before treatment; (iv) no corticosteroid, immunosuppressive agents or antiviral agents used within 6 months; (v) no hepatitis B surface antigens, antinuclear antibodies or anti-mitochondrial antibodies detectable in serum, as determined by radioimmunoassay, enzyme-linked immunosorbent assay or indirect immunofluorescence assay; (vi) leukocytes of more than 2000/mm^3, platelet count of more than 80 000/mm^3 and bilirubin of less than 2.0 mg/dL; (vii) follow up for more than 6 months before treatment; (viii) complication of anemia (hemoglobin <13 g/dL), low bodyweight (<50 kg), diabetes mellitus and/or hypertension. We excluded from the study all of the patients with the following: (i) a history of alcohol abuse; (ii) complication of malignancy; (iii) advanced liver cirrhosis of encephalopathy, bleeding esophageal varices or ascites. From December 2007 to October 2010, a total of 23 HCV patients were enrolled in this retrospective cohort study at the study hospital. In these 23 patients, combination therapy was started with dose reduction of ribavirin. As control, another 22 patients without complications anemia, low bodyweight, and/or diabetes mellitus and/or hypertension treated with the standard dose of IFN-β and ribavirin were enrolled (standard-dose group). All collection and analysis of patient data for the dose-reduction group and standard-dose group was performed retrospectively from the patient records. This study had been approved by Institutional Review Board of our hospital.

Combination therapy of IFN-β and ribavirin

Treatment was provided for 48 weeks. IFN-β (Feron; Toray Industries, Tokyo, Japan) was administrated i.v. at a dose of 6 million units (MU) daily for 4 weeks, followed by three times a week for 44 weeks. Ribavirin (Rebetol; MSD, Whitehouse Station, NJ, USA) were given at the dose described based on bodyweight. In the standard-dose group, the ribavirin dose was adjusted according to bodyweight (600 mg for ≤60 kg, 800 mg for >60 kg and ≤80 kg, and 1000 mg for >80 kg). Twenty-two patients were given the standard dose of ribavirin as described above at the initiation of combination therapy (standard-dose group). On the other hand, 23 patients were given a reduced dose of ribavirin that decreased by one tablet per day compared to the standard group due to complications of having a hemoglobin level of less than 13 g/dL, bodyweight of less than 50 kg, diabetes and/or hypertension (reduction-dose group).

Aspartate aminotransferase to platelet ratio index (APRI) calculation method and prevalence of significant fibrosis

The hepatic fibrosis was evaluated by the APRI, which was calculated according to the following formula: APRI = (AST level / ULN) × 100 / platelet count (10^9/L), where ULN was the aspartate aminotransferase (AST) upper limit of normal (33 IU/L).

As previously reported, an APRI of more than 1.50 is predictive of significant fibrosis (positive predictive value, 88%; negative predictive value, 64%).[15]

Laboratory investigation

In this study, HCV RNA levels were evaluated at least once every month before, during and after therapy. HCV RNA concentrations were determined using the COBAS TaqMan HCV test (Roche Diagnostics, Basel, Switzerland). The linear dynamic range of the assay was 1.2–7.8 logIU/mL, and the undetectable samples were defined as negative. An SVR was defined as clearance of HCV RNA by COBAS TaqMan HCV test (Roche Diagnostics) at 6 months after the cessation of combination therapy.