between initial HBV infection and acute onset or reactivation of chronic HBV infection. Thus, serum HBsAg levels noted in previous medical records, blood donation screening, labor and delivery screening, or employment health screening, were obtained or were followed until negative of HBsAg and/or positive of hepatitis B surface antibody (HBsAb). No patients were using chemotherapeutic and immune modulating agents involved in HBV reactivation. Informed consent was obtained from all patients, and the study was carried out in accordance with the 1975 Helsinki Declaration. Serum was stored at −80°C for virological examinations.

Assay methodology

Hepatitis B virus DNA was isolated from peripheral blood with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Nested polymerase chain reaction (PCR) analysis and direct sequencing of the preS, polymerase and precore/core regions were performed as reported previously.[7] In brief, each 50-uL PCR reaction contained 100 nM each primer, 1 ng template DNA, 5 uL GeneAmp 10 × PCR buffer, 2 uL deoxyribonucleotide triphosphate and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers were: preS region sense 5′-TCACCTATTCT TGGGAACAAGA-3′ and antisense 5′-GGCACTAGTAAACTG AGCCA-3′; polymerase region, sense 5′-CCTGCTGGTGGCT CCAGTTC-3′ and antisense 5′-GGTTGAGTCAGCAAACAC ACTTG-3′; and precore/core region, sense 5′-ATGTCGACAA CCGACCTTGA-3′ and antisense 5′-GTATGGTGAGGTGAAC AATG-3′. Amplification conditions consisted of 5 min at 94°C followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was done in the same reaction buffer with the first-round PCR product as template and the following sets of primers: preS region, sense 5′-TCACCTATTCTT GGGAACAAGA-3′ and antisense 5′-AGAAGATGAGGCATAG CAGC-3′; and polymerase region, sense 5′-GGATGTGTCTGC GGCGTTT-3′ and antisense 5′-ACCCCATCTTTTTGTTTTG TTAGG-3′. PCR products were detected by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator ver. 1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). The neighbor-joining method[8] was used for phylogenetic analysis of the preS region to identify HBV subgenotypes. The bootstrap test with 1000 replicates was performed to confirm the reliability of the phylogenetic tree.[9]

Results

The results of the phylogenetic analyses of HBV subgenotypes of the 41 patients are shown in Figure 1. The HBV subgenotypes A2 ($n = 18$), B1 ($n = 1$), B2 ($n = 3$), B3 ($n = 2$), C1 ($n = 1$), C2 ($n = 19$) and C6 ($n = 1$) were detected. The prevalence of subgenotype A2 was increased, as previously reported. LMV resistance-associated mutations were detected within the HBV polymerase region (positions 116–214) by direct sequencing. Alignment of the amino acid sequence of the HBV polymerase region with LMV resistance-associated mutations was analyzed, and LMV-associated mutations could be detected in two patients at acute hepatitis onset. LMV-resistance mutations (L180M, M204I) were detected in a patient with subgenotype C2. The other patient with subgenotype A2 had LMV-resistance mutations (L180M, M204V). There were no resistant HBV mutants for other nucleoside/nucleotide analogs such as V173L, L180M or M204V/I. The clinical and virological characteristics of patients with LMV-resistant HBV strains are summarized in Table 1.

Discussion

Hepatitis B virus reverse transcriptase is an error-prone enzyme without proofreading capacity, and it is easy for frequent mutations to occur during viral replication. As a result, there are many well-known mutations that are associated with the pathogenesis of HBV infection.[10] LMV-resistant strains that have mutations in the polymerase region are induced by long-term administration of LMV.[11,12] LMV had been used widely for treatment for chronic hepatitis B and was available from 2000 in Japan. LMV-resistant strains have emerged in patients with chronic hepatitis. However, the prevalence and clinical impact of LMV-resistant strains in patients with acute hepatitis B are unknown. Thus, surveillance of LMV-resistant strains associated with acute hepatitis B had been conducted, but LMV-resistant strains could not be detected in 2006.[7] The possibility of acute hepatitis B caused by LMV-resistant strains exists, and the surveillance has continued. Of 45 patients with acute hepatitis, two were found to have LMV-associated mutations. We previously hypothesized that LMV-resistant strains may not have enough power to cause acute hepatitis. However, the present study demonstrated that LMV-resistant strains would have infectivity and would be capable of causing acute hepatitis. Less opportunity for infection may explain why previous studies failed to find acute hepatitis caused by LMV-resistant strains.

The infectious source of the LMV-resistant strains could not be confirmed. The subgenotypes of the patients infected with LMV-resistant strains were subgenotype A1 and C2, respectively. The patient infected with subgenotype C2 plus LMV-resistant strain had a history of sex with a prostitute 1 month before admission. Subgenotype C2 was the predominant subgenotype found in Japanese patients with chronic hepatitis B.[7,13–15] The infectious source would be a chronic hepatitis patient who developed resistant HBV mutants during long-term LMV treatment. The route of infection for the other patient with subgenotype A2 was unknown. HBV subgenotype A2 has been rarely reported in Japanese patients with chronic hepatitis B. However, subgenotype A2 has been increasing and has become responsible for the majority of patients with acute hepatitis B.[4,7,16] This study also confirmed that HBV subgenotype A2 has become widespread among Japanese patients with acute hepatitis. However, the origin of subgenotype A2 with an LMV-resistant mutation is not clear. The possibility of it coming from a patient with chronic hepatitis B is low, because subgenotype A2 is rarely found in Japanese patients with chronic hepatitis B who receive long-term LMV treatment. The other possible infectious source is a patient co-infected with HIV. Nucleoside/nucleotide analogs (NA) such as LMV were effective for both HBV and HIV. NA were used not only for treatment of HBV but also for treatment of HIV, and LMV-resistant strains have been reported.[17] HBV genotype A and HIV co-infection have been found among male

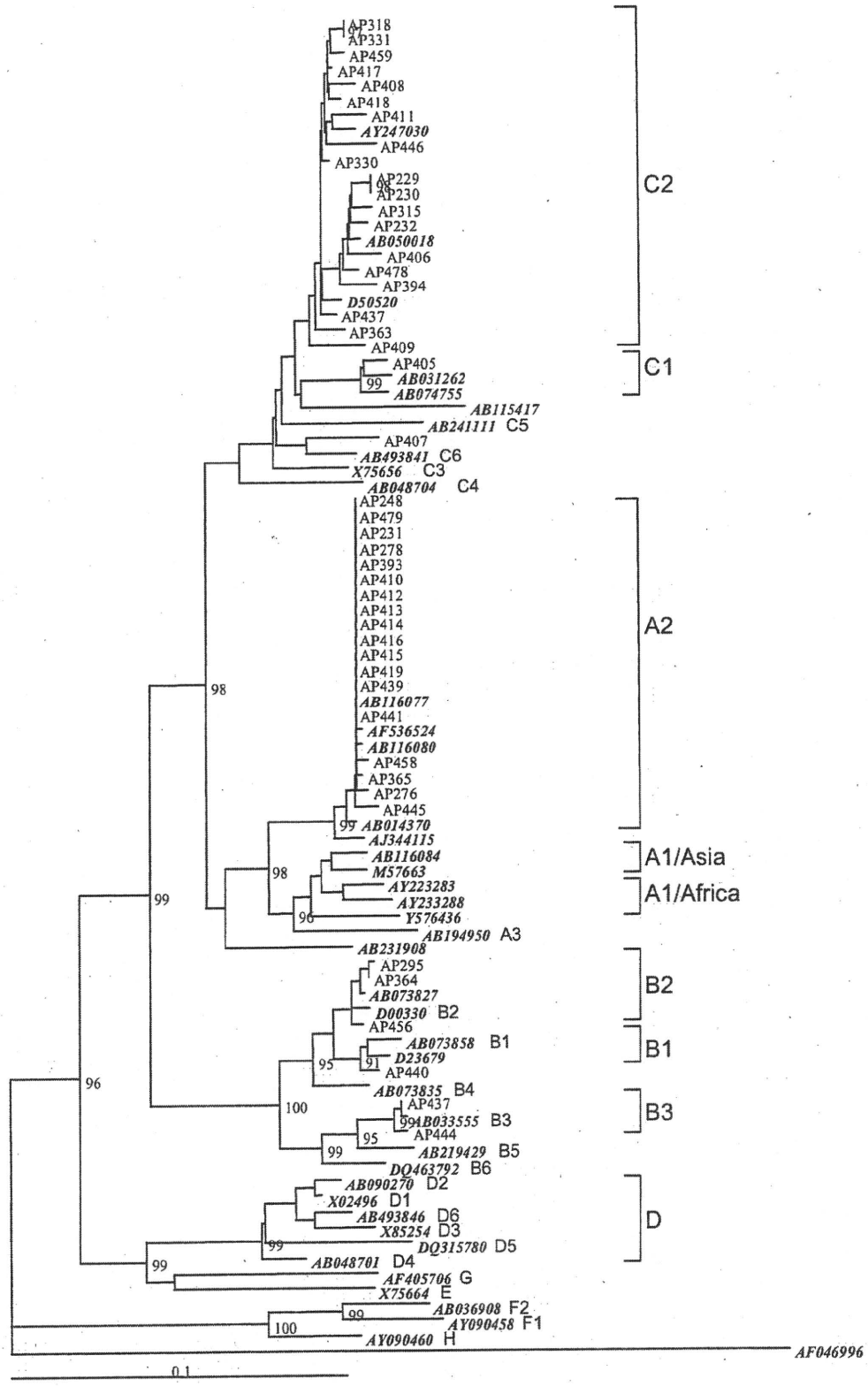

Figure 1 Results of phylogenetic analysis of 45 sequences from the preS region of hepatitis B virus (HBV) of acute hepatitis patients and 42 reference strains from a database and shown by accession number. Strains isolated from patients with acute hepatitis are indicated as AP. Phylogenetic analysis was performed by the neighbor-joining method with Woolly monkey HBV (AF046996) as out-group. Percentages of bootstrap values greater than 90% are shown on the nodes. The scale bar indicates genetic distance.

Table 1 Clinical characteristics

	Case 1	Case 2
Age (years)	32	32
Sex	Male	Male
ALT (IU/L)	4429	2820
AST (IU/L)	2709	1620
T Bil (mg/dL)	3.0	4.1
HBeAg	Positive	Positive
HBV (log copies/mL)	5.2	7.4
BCP1762/1764	T/A	A/G
PC1896	G	G
Route	STD	Unknown
Subgenotype	C2	A2

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; STD, sexually transmitted disease; T Bil, total bilirubin.

patients who have sex with men in Japan.[18] Because the patient infected with subgenotype A2 that was LMV-resistant was not co-infected with HIV, this was also inconclusive. The other possibility was that the infectious source could have been a foreign patient with subgenotype A2 in whom an LMV-resistant strain emerged. This study has the following limitations: a small number of patients, patients without symptom were not recruited, the identification of the infectious source. Thus, further studies such as a nationwide survey including blood banks to investigate asymptomatic patients, the need to make conclusion of the prevalence of patients with acute hepatitis B induced by LMV-resistant strains in Japan.

The patient with LMV-resistant mutations with subgenotype C2 developed self-limited hepatitis, while the other patient with LMV-resistant mutations with subgenotype A1 developed severe acute hepatitis. Basal core promoter (BCP) and precore (PC) variants have been shown to be associated with the severity of the clinical course of acute hepatitis. In particular, mutations at BCP/PC of HBV subgenotype C2 and B1 can increase the risk of progression to fulminant hepatic failure. The clinical impacts of basal core promoter and precore variants in other genotypes are unclear.[7,16] In the present study, both patients with acute hepatitis caused by LMV-resistant strains had wild-type BCP/PC variants. The wild-type BCP/PC variants were linked with mild self-limited hepatitis in the patient with subgenotype C2. The clinical impact of LMV-resistant strains on acute hepatitis appears to be not serious for subgenotype C2. Meanwhile, the mutations in the BCP/PC regions were not associated with the severity of acute hepatitis in the patient with subgenotype A2. Therefore, LMV-resistant mutations in subgenotype A2 might be associated with the severity of the clinical course. However, the present sample size was too small to allow evaluation of the clinical course in acute hepatitis B with LMV-resistant strains and to determine whether LMV-resistant strains have different effects on each subgenotype. Further studies are needed to clarify the influence of LMV-resistant strains on the clinical course of acute hepatitis B.

Lamivudine has begun to be used to treat patients with acute hepatitis to prevent progression to fulminant hepatic failure or chronic hepatitis. Some reports have shown the safety and effectiveness of LMV for the treatment of acute hepatitis B.[19,20] However, one clinical study that has been published did not confirm its efficacy.[21] Thus, the administration of LMV in acute hepatitis B is controversial. The use of LMV for all acute hepatitis was not of benefit and was not recommended for use in all patients. However, selected patients who have a high risk for progression to fulminant hepatic failure and chronic infection may benefit from LMV to prevent disease progression. There is a small possibility that acute hepatitis B can be caused by LMV-resistant strains, but previous studies did not consider LMV-resistant strains before they started to use LMV. Caution must be exercised when determining whether LMV should be used to treat acute hepatitis B because of the possibility of the development of LMV-resistant strains. In the present study, the patient with LMV-resistant mutations who progressed to severe hepatitis was treated with LMV and steroid. Despite the limited efficacy of LMV in suppressing viral replication of LMV-resistant strains, this patient recovered from severe acute hepatitis. Patients with severe acute hepatitis have a high risk for progression to fatal liver failure. However, patients not treated with LMV may have a full recovery and not progress to fulminant liver failure, either because of the efficacy of other treatment, such as steroid, or because the patients' immune reaction could clear the HBV infection. It is difficult to judge the clinical role of LMV-resistant strains in acute hepatitis based on this case. The present study included insufficient information about the magnitude of screening for LMV-resistant strains in acute hepatitis.

Lamivudine is associated with a high incidence of resistance.[22] Thus, the first-line agent for HBV infection has been changed from LMV to adefovir or entecavir because of their powerful antiviral effect and the lower likelihood of drug resistance mutations emerging. The emergence of drug resistance during long-term adefovir or entecavir therapy in chronic hepatitis B was not frequent compared to that with LMV.[23,24] With adefovir or entecavir, the incidence of LMV-resistant strains would be remarkably decreased, but the risk for other HBV drug-resistant strains still remains. Clinical use of anti-HBV agents such as adefovir, entecavir, telbivudine, clevudine and tenofovir has started, and multiple anti-HBV drug-resistant strains could occur in patients undergoing long-term treatment in the near future. Therefore, maintaining surveillance to detect drug-resistant strains of HBV may have a small impact, but it is important for clinical practice.

In conclusion, LMV-resistant mutations were previously rare but now appear to be prevalent among patients in Japan with acute hepatitis B. LMV-resistant strains must be considered in patients with acute hepatitis B.

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Regulation of hepatic branched-chain α-keto acid dehydrogenase kinase in a rat model for type 2 diabetes mellitus at different stages of the disease

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ABSTRACT

Branched-chain α-keto acid dehydrogenase (BCKDH) kinase (BDK) is responsible for the regulation of BCKDH complex, which is the rate-limiting enzyme in the catabolism of branched-chain amino acids (BCAAs). In the present study, we investigated the expression and activity of hepatic BDK in spontaneous type 2 diabetes using hyperinsulinemic Zucker diabetic fatty rats aged 9 weeks and hyperglycemic, but not hyperinsulinemic rats aged 18 weeks. The abundance of hepatic BDK mRNA and total BDK protein did not correlate with changes in serum insulin concentrations. On the other hand, the amount of BDK bound to the complex and its kinase activity were correlated with alterations in serum insulin levels, suggesting that hyperinsulinemia upregulates hepatic BDK. The activity of BDK inversely corresponded with the BCKDH complex activity, which was suppressed in hyperinsulinemic rats. These results suggest that insulin regulates BCAA catabolism in type 2 diabetic rats by modulating the hepatic BDK activity.

Introduction

Branched-chain α-keto acid dehydrogenase (BCKDH) kinase (BDK) is responsible for phosphorylation and inactivation of the BCKDH complex, which catalyses the committed step in branched-chain amino acid (BCAA) catabolism. The BDK is considered a primary regulator of the activity of the BCKDH complex [1]. Because the kinase activity corresponds to the amount of BDK bound to the complex under many physiological conditions [2–6], BDK activity towards its substrate appears to be dependent on the tight binding of this enzyme to the BCKDH complex. In addition, it has been reported that the activity of the hepatic BCKDH complex is inversely correlated with the amount of bound BDK [2]. On the other hand, BDK expression is not necessarily associated with changes in its activity [7].

Insulin is known to increase BDK expression in cultured rat hepatocytes [8] and to decrease BCKDH E1α expression in rat hepatoma cells [9], resulting in suppression of the BCAA catabolism. During type 1 diabetes, which is characterized by elevated plasma glucose concentration and defective insulin secretion, the circulating levels of BCAAs are markedly elevated because of increased proteolysis in body tissues, especially in skeletal muscle [10]. Furthermore, it has been reported that the BCKDH complex activity was increased and the BDK activity was decreased in the livers of streptozotocin-induced diabetic rats [11]. In contrast to animal models of type 1 diabetes, BCAA catabolism has been shown to be downregulated in animal models of type 2 diabetes, Otsuka Long-Evans Tokushima Fatty (OLETF) rats and Zucker diabetic fatty (ZDF) rats, in which the serum insulin levels were extremely high [5,12]. Development of insulin resistance causes compensatory hyperinsulinemia to maintain a normoglycemic state; however, when insulin secretion decreases because of dysfunctional beta cells in the pancreas, hyperglycemia characterizes the onset of diabetes. Although these specific states may affect BCAA catabolism, the influence of the shift in diabetic stages, from hyperinsulinemia (pre-diabetes) to hyperglycemia, on the regulation of BDK remains unclear. Thus, in the present study, we examined the activity and expression of hepatic BDK in hyperinsulinemic and hyperglycemic ZDF rats.

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Materials and methods

Materials. Lambda protein phosphatase was obtained from New England BioLabs (Beverly, MA). Antiserum against the E2 subunit of the BCKDH complex and monoclonal antibody against BDK were prepared as described previously [13]. Goat anti-rabbit and anti-mouse secondary antibodies used in the Western blotting analyses were purchased from Bio-Rad Laboratories (Hercules, CA). Protein A-agarose was purchased from Upstate Biotechnology (Lake Placid, NY). TaqMan® Gene Expression Assays for rat BDK (Rn00709396_g1) and rat β-actin (Rn00667869_m1) were purchased from Applied Biosystems (Foster City, CA). All other reagents were of analytical grade and were purchased from Wako (Osaka, Japan), Oriental Yeast (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma–Aldrich Japan (Tokyo, Japan).

Animals and experimental design. All procedures were approved by the Animal Care Committee of Nagoya University Graduate School of Bioagricultural Sciences. Male rats aged 6 weeks were obtained from Japan Charles River Laboratories (Yokohama, Japan). Zucker diabetic fatty rats (ZDF/CrlCrlj-fa/fa; Fatty rats) were used as rat models for spontaneous type 2 diabetes and age-matched non-diabetic rats (ZDF/CrlCrlj-?/+; Lean rats) were used as normal controls. All animals were housed in a conventional animal room with controlled temperature (22 ± 2 °C) and a 12-h light/dark cycle. Rats had free access to tap water and pellet type AIN-93G diet (Research Diet; New Brunswick, NJ) during the experimental period, unless otherwise stated. Serum insulin and glucose concentrations were measured once a week using blood samples obtained from the tail vein in order to monitor the diabetic stage of the Fatty rats. Based on the results of serum insulin and glucose determinations, we decided to sacrifice a part of the rats at 9 weeks of age (when Fatty rats were hyperinsulinemic) and the remaining rats at 18 weeks of age (when Fatty rats were hyperglycemic) (see Table 1). Body weights (mean ± SE) were 343 ± 4 g for Fatty rats and 234 ± 3 g for Lean rats at 9 weeks of age (n = 5 each group), and 426 ± 9 g for Fatty rats and 364 ± 2 g for Lean rats at 18 weeks of age (n = 7 each group).

On the day of sacrifice, rats were deprived of food for around 6 h (from 08:00 to 14:00) and then injected intraperitoneally with sodium pentobarbital (60 mg/kg body weight). They were sacrificed by blood sampling from the inferior vena cava, to prepare serum and plasma. Subsequently, livers were rapidly removed, freeze-clamped at liquid nitrogen temperature, and stored at −80 °C until analyses.

Blood component analyses. Concentrations of plasma insulin were measured using a rat insulin ELISA kit (Shibayagi Co.; Gunma, Japan). Plasma concentrations of the three BCAAs were determined by the HPLC method. Other blood components were determined by routine laboratory methods.

Enzyme assays. The activity of the BCKDH complex was measured by a spectrophotometric assay [14]. One unit of enzyme activity refers to the formation of 1 μmol of NADH/min. The actual activity (the activity in vivo of the partially phosphorylated enzyme) and the total activity (the activity of the fully dephosphorylated enzyme) of the BCKDH complex were measured separately. Dephosphorylation of the complex was accomplished by incubating the enzyme extract with lambda protein phosphatase, as described previously [14]. The activity state of the BCKDH complex is defined as the percentage of actual activity relative to total activity. Assay of BDK was performed by measuring the ATP-dependent inactivation of the BCKDH complex [3]. Kinase activity is expressed as the first-order rate constant of BCKDH complex inactivation over time.

Immunoprecipitation and immunoblotting. Rat liver extracts were prepared as reported previously [3] and used for immunoprecipitation with polyclonal antibody against the E2 subunit of the BCKDH complex [3]. Liver extracts and precipitated proteins were used in immunoblotting analyses for the determination of the amounts of total and bound BDK, respectively [3,13]. Target immunoreactive proteins on the membranes were visualized using ECL Western blotting detection reagents (GE Healthcare UK Limited; Buckinghamshire, UK) and quantified using the AE6962 Light Capture system (ATTO; Tokyo, Japan). The intensities of the bands are expressed relative to the mean values of the 9-week Lean group of rats.

Real-time PCR quantification of BDK mRNA. Total liver RNA (1 μg) was reversely transcribed in the presence of random hexamers and oligo(dT)$_{20}$ primers using the ReverTra Ace qPCR-RT kit (Toyobo; Osaka, Japan). Quantitative real-time PCR was performed using an ABI step-one plus real-time PCR system with a TaqMan detection Gene Expression Assay (Japan Applied Biosystems; Tokyo, Japan) for the amplification of 1 μl of cDNA. At least three independent assays were performed for each experiment, with duplicate amplification reactions for each target gene product. The cycle threshold number (C_T), at which amplification entered the exponential phase, was used as an indicator of the relative amount of initial target RNA in each sample. Results are presented as ratios of BDK mRNA normalized to the internal control (β-actin) compared with the control group according to the $\Delta\Delta C_T$ method, as previously reported [15].

Statistical analysis. All values are expressed as means ± standard error (SE). Data for Fatty and Lean rats were analyzed by the Tukey–Kramer multiple-comparison test. Statistical analyses were performed using the StatView software (Version 5.0) from SAS Institute (Cary, NC). Differences with $P < 0.05$ were considered significant.

Table 1
Concentrations of blood components in Lean and Fatty ZDF rats.

Blood components	9 weeks of age		18 weeks of age	
	Lean	Fatty	Lean	Fatty
Glucose (mg/dL)	154 ± 4	165 ± 12	176 ± 3	430 ± 24*,**
Insulin (ng/mL)	1.9 ± 0.2	19.8 ± 0.6*	3.7 ± 0.3	5.1 ± 0.8**
FFA (μM)	233 ± 11	718 ± 49*	554 ± 40**	966 ± 66*,**
Corticosterone (ng/mL)	311 ± 28	655 ± 55*	493 ± 20	691 ± 74*
BCAA (μM)	373 ± 20	496 ± 19*	382 ± 6	464 ± 21*
Leucine (μM)	131 ± 10	167 ± 8*	138 ± 3	147 ± 7*
Isoleucine (μM)	55 ± 3	90 ± 5*	60 ± 3	89 ± 6*
Valine (μM)	186 ± 13	239 ± 11*	184 ± 5	228 ± 12*

Values are means ± SE (n = 5 for both groups of rats at 9 weeks of age and n = 7 for both groups of rats at 18 weeks of age).
* Significantly different from the group of age-matched Lean rats ($P < 0.05$).
** Significantly different from the 9-week-old in the same group ($P < 0.05$).

Results

Concentrations of blood components in ZDF rats

At 9 weeks of age, plasma insulin concentrations were markedly higher in Fatty rats than in Lean rats, while plasma glucose concentrations were not significantly different between both groups of rats (Table 1). At 18 weeks of age, the plasma insulin concentration in Fatty rats was declined to similar level as that in Lean rats, whereas plasma glucose concentration was markedly higher in Fatty rats than in Lean rats (Table 1). In Lean rats, plasma glucose and insulin concentrations were similar at the two ages, although these concentrations tended to increase with age. Serum free fatty acid (FFA) and corticosterone concentrations were significantly higher in Fatty rats than in Lean rats at both 9 and 18 weeks of age (Table 1). In addition, serum FFA concentrations in Fatty rats were significantly higher at 18 weeks of age than at 9 weeks of age. These data for Fatty rats show typical phenotypes of type 2 diabetes. The concentrations of plasma BCAAs in Fatty rats were significantly higher than those in Lean rats at both ages; however, BCAA levels did not differ between Fatty rats aged 9 and 18 weeks (Table 1).

Abundance of hepatic BDK mRNA and total BDK protein in ZDF rats

The relative abundance of BDK mRNA was significantly greater in Fatty rats at 18 weeks of age than in Lean rats at the same age and Fatty rats at 9 weeks of age, and did not differ between the two ages in Lean rats (Fig. 1A). On the other hand, the protein amount of total BDK was significantly higher in Fatty rats than in Lean rats at 9 weeks of age, whereas it showed the reverse trend in rats at 18 weeks of age (Fig. 1B). In Lean rats, the protein amount of total BDK was greater at 18 weeks of age, whereas it was greater at 9 weeks of age in Fatty rats.

Amount of bound BDK and BDK activity in the liver

Among all groups of rats, both bound BDK and kinase activity had the highest levels in Fatty rats at 9 weeks of age and these high levels declined significantly with age. These values in Lean rats, however, were not significantly different between the two ages, suggesting that the amount of bound BDK corresponded to the activity of BDK in the liver (Fig. 2). Changes in the amount of bound form and the activity of BDK practically corresponded with plasma insulin concentrations, and the only inconsistent observation was that the kinase activity was significantly lower in Fatty rats at 18 weeks of age than in Lean rats at the same age, but the plasma insulin levels were not significantly different between these two groups of rats.

Activity of hepatic BCKDH complex

Total BCKDH complex activities were significantly lower in Fatty rats than in Lean rats at both ages (Table 2). Although the total enzyme activity in Lean rats was not different between the two ages, that in Fatty rats was significantly higher at the age of 18 weeks than at the young age. Total BCKDH complex activities were inversely correlated with BDK activity levels. The activity state of the hepatic BCKDH complex tended to be lower in Fatty rats than in Lean rats at both ages and to be inversely correlated with the activity of BDK (Table 2).

Discussion

In the present study, we showed that hepatic BDK activity and the amount of the bound form of BDK, which may be the active form, practically corresponded with serum insulin levels in ZDF rats, suggesting that insulin may regulate the association of BDK with the BCKDH complex, being responsible for the control of the kinase activity. In contrast to the BDK activity, the abundance of hepatic BDK mRNA did not correspond with the plasma insulin levels, and the protein amount of total BDK was only partly associated with plasma insulin in the rats. Although it has been reported that insulin upregulates the expression of BDK mRNA and protein in cultured rat cells [8], our results suggest that

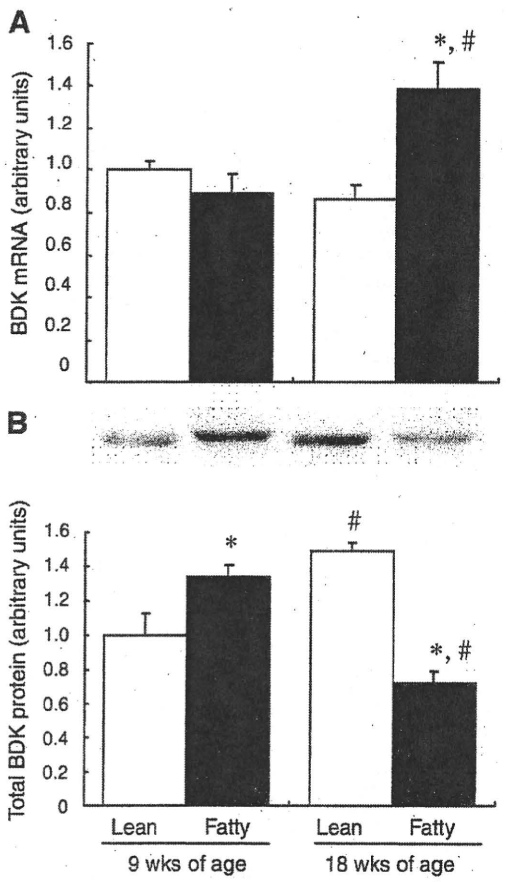

Fig. 1. Abundance of hepatic BDK mRNA (A) and protein (B) in ZDF rats. The abundance of BDK mRNA was normalized to that of ß-actin mRNA. mRNA abundance and band intensities for BDK protein are expressed relative to the mean values of Lean rats at 9 weeks of age. White bars represent Lean rats and black bars Fatty rats. Values are means ± SE. *Significantly different from the group of age-matched Lean rats ($P < 0.05$). #Significantly different from the 9-week-old in the same group ($P < 0.05$).

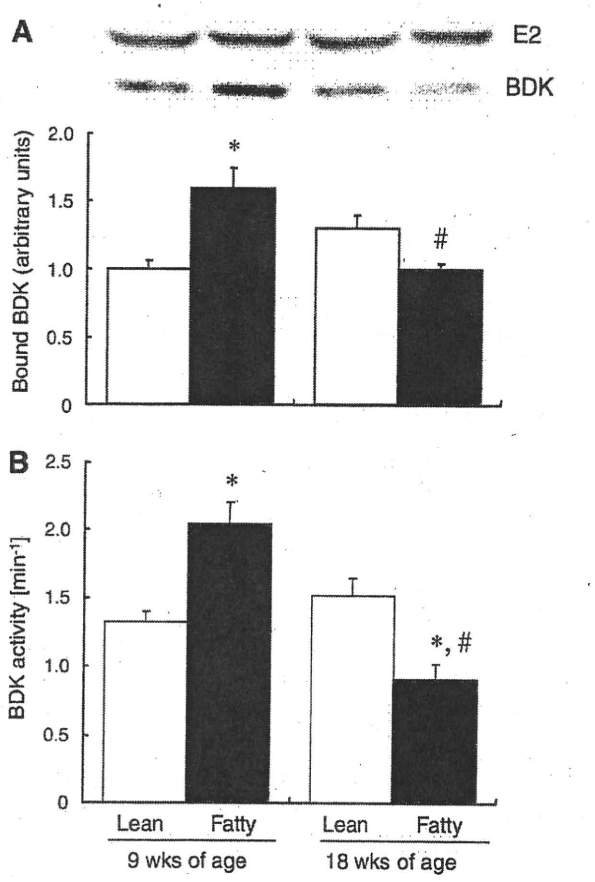

Fig. 2. Amount of the bound form of BDK (A) and activity of hepatic BDK (B) in ZDF rats. For the amount of the bound form of BDK, equivalent protein loading for immunoprecipitated proteins was verified by reprobing membranes with E2 antiserum [3]. Band intensities are presented relative to the mean values of Lean rats at 9 weeks of age. Typical images of Western blots are shown above each bar. White bars represent Lean rats and black bars Fatty rats. Values are means ± SE. *Significantly different from the group of age-matched Lean rats ($P < 0.05$). #Significantly different from the 9-week-old in the same group ($P < 0.05$).

Table 2
Activities of hepatic BCKDH complex in Lean and Fatty ZDF rats.

Enzyme	9 weeks of age		18 weeks of age	
	Lean	Fatty	Lean	Fatty
BCKDH complex				
Total activity (U/g tissue)	1.74 ± 0.05	0.82 ± 0.10*	1.85 ± 0.05	1.31 ± 0.16*,**
Actual activity (U/g tissue)	0.63 ± 0.22	0.01 ± 0.01*	0.23 ± 0.09**	0.08 ± 0.03
Activity state (%)	34.8 ± 11.4	0.5 ± 0.5*	11.8 ± 4.7**	5.5 ± 2.2

Values are means ± SE ($n = 5$ for both groups of rats at 9 weeks of age and $n = 7$ for both groups of rats at 18 weeks of age).
* Significantly different from the group of age-matched Lean rats ($P < 0.05$).
** Significantly different from the 9-week-old in the same group ($P < 0.05$).

insulin is not the primary factor to regulate hepatic BDK expression in vivo.

It has been reported that hyperinsulinemia in type 2 diabetic animals downregulates hepatic BCKDH complex in association with upregulation of the BDK activity [5,16]. In this study, we obtained consistent results in the Fatty rats at 9 weeks of age, which had markedly high plasma insulin concentration. However, although the insulin level was ~10-fold higher in Fatty rats than in Lean rats at 9 weeks of age, the activity and amount of bound form of BDK were only ~1.6-fold higher in the former than in the latter. Since Fatty rats have severe insulin resistance, we hypothesize that the effects of hyperinsulinemia on the hepatic BDK may be attenuated in these rats. Nevertheless, the hyperinsulinemia to maintain normoglycemia in the Fatty rats at 9 weeks of age still showed a significant effect on the hepatic BDK. In this context, despite the fact that plasma insulin concentrations were similar in Fatty and Lean rats at 18 weeks of age, the low BDK activity in Fatty rats may be attributed to insulin resistance.

It has been demonstrated that dexamethasone treatment results in the downregulation of BDK gene expression in rat hepatoma cell lines as well as in vivo [17]. In this study, although the serum corticosterone concentration was significantly higher in Fatty rats than in Lean rats at both 9 and 18 weeks of age (approximately 2-fold and 1.4-fold, respectively), the abundance of hepatic BDK mRNA did not correlate with concentrations of this hormone, suggesting that glucocorticoids may not directly regulate hepatic BDK gene expression in type 2 diabetes.

In the present study, the plasma BCAA concentrations were significantly higher in Fatty rats than in Lean rats, in accordance with previous reports [5,16]. As suggested previously, the low activity of hepatic BCKDH complex may contribute to the rise in plasma BCAA concentrations [5,16]. However, the BCAA concentrations were not different between the two ages in Fatty rats, although the BDK activity was markedly decreased with age in the same rats. This is likely related to the fact that the actual activity of the BCKDH complex at 18 weeks of age in Fatty rats was still very low as that at 9 weeks of age. We show here that declined serum insulin levels in Fatty rats aged 18 weeks may be reflected in the relatively low kinase activity, but not in the activity of the complex, suggesting that BCKDH phosphatase, which dephosphorylates and activates the complex, might be suppressed in the hyperglycemic stage in ZDF rats. Because this phosphatase has been characterized very recently [18,19], information on its function is quite limited; this enzyme is a PP2C-type mitochondrial phosphatase that shows Mn^{2+}-dependent phosphoserine phosphatase activity [18]. A commercially available Mn^{2+}-dependent lambda protein phosphatase is used for activation of BCKDH complex in the assay of the total complex activity in tissue extracts, and we have previously noted that incubation of the BCKDH complex present in liver extracts with the lambda protein phosphatase plus Mn^{2+} causes partial inactivation of BDK [14]. In a recent preliminary experiment, we observed that incubation of partially purified BCKDH-kinase complex with the lambda protein phosphatase plus Mn^{2+} also causes a decrease in the kinase activity towards the complex (S. Kazama, Y. Shimomura, unpublished results). These findings imply that BCKDH phosphatase might be involved in the regulation of BDK activity through modification of its binding to the BCKDH complex.

In conclusion, we showed that the BDK activity was correlated with the amount of enzyme tightly bound to the BCKDH complex in the livers of type 2 diabetic rats. Hyperinsulinemia elevated the hepatic BDK activity by increasing the bound form of BDK, resulting in decreased hepatic BCKDH complex activity. Although declined serum insulin levels and hyperglycemia at the following stage of type 2 diabetes decreased the activity of BDK, the activity state of hepatic BCKDH complex was not significantly altered. These findings suggest that the robust regulatory mechanism of BCAA catabolism may be absent in the type 2 diabetic conditions. It is known that type 2 diabetes and obesity patients have increased plasma BCAA concentrations [16,20,21]. These findings suggest that suppression of BCAA catabolism caused by insulin resistance may contribute the rise of plasma BCAA levels in the patients. Full understanding of the influence of insulin on BCAA catabolism requires further studies to clarify (1) the molecular mechanism responsible for the interconversion between bound and free forms of BDK, (2) insulin action on the interconversion system of BDK, and (3) regulation of the BCKDH phosphatase activity.

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CLINICAL STUDIES

Impact of amino acid substitutions in the hepatitis C virus genotype 1b core region on liver steatosis and hepatic oxidative stress in patients with chronic hepatitis C

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Keywords
8-hydroxy-2′-deoxyguanosine – amino acid substitutions – hepatitis C virus – glutamine – liver steatosis – methionine – oxidative stress

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Abstract

Background: Liver steatosis and hepatic oxidative stress are the histopathological features of chronic hepatitis C. Hepatitis C virus (HCV) genotype 1 core protein induces hepatic steatosis and reactive oxygen species production in transgenic mice. The amino acid substitutions in the HCV core region appear to be related to hepatocarcinogenesis. *Aims:* The aim of this study was to clarify the impact of mutations in the HCV core region on oxidative stress and lipid metabolism in patients with chronic hepatitis C. *Methods:* Sixty-seven patients (35 men, 32 women; mean age, 58.4 ± 10.2 years) with chronic hepatitis C with high titres (> 5 log IU/ml) were enrolled. Substitutions in amino acids 70, 75 and 91 of the HCV genotype 1b core region, the percentage of hepatic steatosis, and hepatic 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels were investigated in all patients. Urinary 8-OHdG levels were measured in 35 patients. *Results:* Body mass index, alanine aminotransferase, γ-glutamyl transferase, and triglyceride levels and substitutions of amino acid 70/Q (glutamine) were significantly associated with the presence of steatosis on univariate analysis. Multivariate analysis showed that substitution of amino acid 70 of glutamine and triglyceride levels were the independent factors related to liver steatosis. Hepatic and urinary 8-OHdG levels were significantly higher in patients with methionine at amino acid 91 of the HCV core region than in those with leucine. *Conclusion:* Substitutions in the amino acids of the HCV genotype 1b core region are associated with hepatic steatosis and oxidative stress in patients with chronic hepatitis C.

Hepatitis C virus (HCV) infection is widespread, with 170 million carriers worldwide (1). HCV infection leads to chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC), and it is a major global health issue. HCV, a hepatotropic Flavivirus, is formed by a positive, single-stranded RNA genome of about 9600 nucleotides that contains an open reading frame. HCV has two structural proteins, core protein and envelope protein. HCV core protein induces oxidative stress, which contributes to the pathogenesis of chronic hepatitis C. Transgenic mice that express HCV core protein develop hepatic steatosis and increased reactive oxygen species (ROS) production without inflammation (2, 3). Oxidative stress plays an important role in patients with chronic hepatitis C. It has been reported that 8-hydroxy-2′-deoxyguanosine (8-OHdG) reflects oxidative DNA damage by hydroxyl radicals (4, 5) and increases in the liver tissue and circulating leucocytes of patients with chronic hepatitis C (6–8). Hepatic oxidative stress is associated with the progression of fibrosis and increases the risk for HCC (9, 10). Therefore, the evaluation of oxidative stress in patients with chronic hepatitis C would be useful for understanding the pathogenesis of hepatitis. Not only hepatic oxidative stress but also lipid metabolism abnormalities, such as liver steatosis, are the histopathological features of human chronic hepatitis C. Several factors, such as metabolic syndrome, obesity and a high body mass index (BMI) have an influence on liver steatosis in chronic hepatitis C patients (11). Viral factors such as HCV genotype 3 and core protein, as well as host factors, contribute to the development of hepatic steatosis (12, 13). Therefore, several studies demonstrated that HCV core protein has close relationships both with induction of ROS and lipid metabolism, such as liver steatosis (14). However, these associations are not fully understood. Recently, some studies have indicated that amino acid substitutions in the HCV core region of genotype 1b were related to response to interferon

(IFN)–ribavirin combination therapy (15, 16) and insulin resistance (17). We speculated that these mutations in the HCV core region may be related to hepatic steatosis and oxidative stress in chronic hepatitis C patients. To investigate this hypothesis, we analysed the impact of amino acid substitutions in the HCV core region on histological hepatic steatosis and hepatic and urinary 8-OHdG levels.

Methods

Patients

Sixty-seven patients with chronic hepatitis C who were treated at Kakegawa City General Hospital between December 2004 and December 2008 were enrolled. The patients were 35 men and 32 women, with a mean age of 58.4 years. The exclusion criteria were: co-infection with hepatitis B virus and human immunodeficiency virus, drug addiction, alcoholism, autoimmune hepatitis, coexisting serious psychiatric or mental illness and pregnancy. All patients were genotype 1b with high titres (> 5 log IU/ml), and they were treated using a combination therapy with pegylated IFN and ribavirin. Peginterferon-α-2b (PegIntron; Schering Plough, Osaka, Japan) was given once a week for 48–72 weeks. Oral ribavirin (Rebetol; Schering Plough) was given at a dose of 600 mg/day to patients who weighed ≤ 60 kg and at a dose of 800 mg/day to those who weighed > 60 kg during the treatment period. Before therapy, blood and urine were sampled in the morning of the day of the liver biopsy after overnight fasting.

Liver histology

Ultrasound-guided percutaneous needle liver biopsies were performed in all 67 patients. All liver biopsies were reviewed by a pathologist who was blinded to the clinical and laboratory findings. Liver biopsy specimens were classified in terms of fibrosis and necroinflammatory activity according to the classification by Desmet *et al.* (18). Findings of steatosis were assessed according to the percentage of hepatocytes containing fat droplets and graded as follows: 0 (no steatosis), 1 (< 33% of hepatocytes affected), 2 (33–66% of hepatocytes affected) and 3 (> 66% of hepatocytes affected).

Histological examination for 8-hydroxy-2′-deoxyguanosine in the liver

Histological 8-OHdG in the liver was quantified using liver biopsy samples taken before IFN therapy, as reported previously (19). The sections were stained using mouse monoclonal antibody against 8-OHdG (5 μg/ml; Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan). Nuclei positive for 8-OHdG were counted in two non-overlapping randomly selected fields. The degree of oxidative stress of the liver was expressed by the average percentage of hepatocytes with 8-OHdG-positive nuclei in the two fields.

Urinary 8-hydroxy-2′-deoxyguanosine

Urine samples were collected before treatment in 35 of 67 patients. Urinary 8-OHdG levels were measured in 35 patients. The 8-OHdG concentration was determined using an enzyme-linked immunosorbent assay kit (Japan Institute for the Control of Aging; Nikken SEIL Corporation; Fukuroi, Shizuoka, Japan), and the urinary creatinine concentration was determined by a standard automated colorimetric assay. Then, urinary 8-OHdG was normalized for the urinary creatinine level, and it was presented as the urinary [8-OHdG (ng/ml)/creatinine (mg/ml)] ratio.

Amino acid substitutions in the hepatitis C virus core region of genotype 1b

Direct sequencing of the core region was carried out as reported previously but with modifications (20). In brief, RNA was extracted from 140 μl sera with a commercial kit (QIAamp viral RNA kit; Qiagen, Valencia, CA, USA) and dissolved in 50 μl diethylpyrocarbonate-treated water. Ten nanograms of RNA were used for reverse transcription with oligo and random hexamer primers with a commercial kit (iScript cDNA synthesis kit; Bio-Rad, Hercules, CA, USA). The core region was amplified by nested PCR. In brief, each 50 μl PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μl GeneAmp × 10 PCR buffer, 2 μl dNTPs and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primer sequences were as follows: sense, 5′-GGG AGGTCTCGTAGACCGTGCACCATG-3′ and antisense, 5′-GAGMGGKATRTACCCCATGAGRTCGGC-3′. Amplification conditions consisted of 10 min at 94 °C followed by 40 cycles at 94 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was performed in the same reaction buffer with the first-round PCR product as template, the sense primer 5′-AGACC GTGCACCATGAGCAC-3′ and antisense primer 5′-TAC GCCGGGGGTCAKTRGGGCCCCA-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle sequencing kit; Applied Biosystems) and an ABI 310 DNA sequencer (Applied Biosystems).

Statistical analysis

Quantitative values were expressed as means ± SD or medians. Each data set was first evaluated for normality of distribution by the Shapiro–Wilk test to decide whether a non-parametric rank-based analysis or a parametric analysis should be used. Between-group

differences in mean quantitative values were analysed using the Kruskal–Wallis *H*-test, and differences between two groups in non-parametric data were analysed by the Mann–Whitney *U*-test, χ^2-test and Fisher's exact probability test. Differences in parametric data were analysed using the paired *t*-test. Correlations between variables were tested using Spearman's rank-correlation analysis. The parameters that were found to be significant on univariate analysis were further processed through multivariate logistic regression analysis. All *P* values were two tailed, and $P < 0.05$ was considered statistically significant. Statistical analysis was carried out using SPSS version 16.0 for WINDOWS (SPSS Inc., Chicago, IL, USA).

Results

The patients' clinical characteristics are shown in Table 1. Comparisons of clinical characteristics according to the presence of steatosis are shown in Table 2. There were significant positive associations between the presence of steatosis and BMI, alanine aminotransferase levels, γ-glutamyltransferase levels, triglyceride levels and substitutions of amino acid 70. On multivariate logistic regression analysis, factors associated with steatosis were substitutions of amino acid 70/Q (glutamine) and triglyceride levels (Table 3).

Table 1. Clinical characteristics ($n = 67$)

Characteristics	
Age (years)	58.4 ± 10.2
Gender (male/female)	35/32
Body mass index	21.9 ± 2.8
Laboratory data	
AST (IU/L)	61.0 ± 36.2
ALT (IU/L)	62.4 ± 37.4
γ-GTP (IU/L)	52.9 ± 45.4
Cholesterol (mg/dl)	181.14 ± 39.38
Triglycerides (mg/dl)	92.69 ± 41.33
Platelet count ($\times 10^4$/ml)	16.4 ± 4.3
Serum ferritin (ng/ml)	156.8 ± 144.9
HOMA-IR	3.55 ± 4.58
Liver histology	
Inflammatory activity (A0/A1/A2/A3)	16/44/6/1
Fibrosis staging (F0/F1/F2/F3/F4)	10/28/15/14/0
Liver steatosis (Grade 0/1/2/3)	22/40/5/0
Liver steatosis (%)	8.43 ± 12.1
Hepatic 8-OHdG (absent/present)	19/48
Hepatic 8-OHdG (%)	42.67 ± 31.56
Urinary 8-OHdG (ng/mg Cr)*	10.99 ± 5.27
Amino acid substitutions of HCV core region	
aa 70 (arginine/glutamine/histidine)	42/23/2
aa 75 (threonine/alanine/serine/valine)	26/38/2/1
aa 91 (leucine/methionine)	43/24

Data are expressed as mean ± SD, and the numbers represent number of patients.

*35 patients were available.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; core aa, core amino acid; γ-GTP, γ-glutamyltransferase; HCV, hepatitis C virus; HOMA-IR, the index of homeostasis model assessment-insulin resistance; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

Table 4 shows correlations between hepatic/urinary 8-OHdG levels and amino acid substitutions at positions 70/75/91 in the HCV core region. The hepatic 8-OHdG levels were significantly lower ($P = 0.0453$) in the patient group without methionine (non-M) than in the patient group with methionine (M) at amino acid 91 of the HCV core region. The urinary 8-OHdG levels were significantly lower ($P = 0.0112$) in the patient group without M than in the patient group with M at amino acid 91 of the HCV core region. There were significant correlations between expression of hepatic and urinary 8-OHdG levels. We defined absence of oxidative stress as 8-OHdG ≤ 10%, while 8-OHdG > 10% indicated the presence of oxidative stress. Substitutions at amino acid 91/M were detectable in 22 of 48 (45.8%) patients with hepatic oxidative stress, but in only two of the 19 (10.5%) patients without hepatic oxidative stress. There were significant positive associations between the presence of hepatic oxidative stress and substitutions of amino acid 91/M ($P = 0.00977$). A sustained virological response (SVR) was achieved by 28 of 59 (47.5%) patients. In the patient group with non-Q (arginine or histidine) at amino acid 70, SVR was achieved by 24 of 40 (60%) patients. In the patient group with Q (glutamine) at amino acid 70, SVR was achieved by four of 19 (21.1%) patients. There was a significantly higher rate of SVR ($P = 0.0117$) in the patient group with non-Q (arginine or histidine) than in the patient group with Q (glutamine) at amino acid 70. There were no significant differences in substitutions of other amino acids (75, 91) on the efficacy of treatment.

We retrospectively assessed changes in urinary 8-OHdG levels before and after treatment in 18 patients. Twelve patients had achieved SVR, another six patients were non-virological response (NVR). The urinary 8-OHdG levels were significantly decreased in SVR patients after treatment ($P = 0.0164$). On the other hand, no significant change was observed in NVR patients.

Discussion

A high prevalence (67%) of steatosis was found among patients with chronic hepatitis C. Liver steatosis is one of the most important histopathological features in patients with chronic hepatitis C. However, it remains uncertain whether liver steatosis is related directly to the virus or is secondary to host factors. It has been suggested that HCV genotype 3 is closely associated with liver steatosis (21). However, in patients who did not have genotype 3, it has been reported that liver steatosis occurred through oxidative stress and insulin resistance with HCV infection (22–24). A recent report has shown that only HCV core protein is necessary for liver steatosis, and the interaction of HCV core protein leads to the activation of the sterol regulatory element-binding protein − 1c promoter and development of liver steatosis (25–27).

Table 2. Clinical characteristic of the patients according to the prevalence of steatosis ($n = 67$)

Characteristics	Steatosis absent ($n = 22$)	Steatosis present ($n = 45$)	*P* values
Age (years)	57.14 ± 12.87	59.06 ± 8.69	NS
Gender (male/female)	12/10	23/22	NS
Body mass index	20.57 ± 2.46	22.57 ± 2.79	0.0080
AST	48.45 ± 20.77	66.96 ± 40.62	NS
ALT	48.09 ± 27.95	69.40 ± 39.59	0.023
γ-GTP	35.36 ± 23.0	61.49 ± 51.03	0.0067
Cholesterol	181.59 ± 40.72	180.41 ± 39.15	NS
Triglycerides	80.91 ± 39.21	98.59 ± 41.53	0.043
Platelet count	16.35 ± 4.93	15.09 ± 3.93	NS
HOMA-IR	1.79 ± 0.87	4.29 ± 5.43	0.064
Serum ferritin	134.21 ± 164.57	167.87 ± 134.84	0.093
Hepatic 8-OhdG (absent/present)	7/15	12/33	NS
Substitutions of aa			
aa 70 (non-Q/Q)	20/2	24/21	0.00243
aa 75 (non-A/A)	12/10	14/31	NS
aa 91 (non-M/M)	14/8	29/16	NS
Inflammatory activity (A0/A1/A2/A3)	(8/13/0/1)	(8/31/6/0)	NS
Fibrosis staging (F0/F1/F2/F3/F4)	(4/11/3/4/0)	(6/17/12/10/0)	NS

Data analysis were performed using Mann–Whitney *U*-test, χ^2-test and Fisher's exact probability test and Kruskal–Wallis *H*-test.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltransferase, HOMA-IR, the index of homeostasis model assessment-insulin resistance; NS, not significant; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

Table 3. Associations with steatosis found in multivariable logistic regression ($n = 67$)

Characteristics	Category	Odds ratio	95% CI	*P* values
BMI	1: < 22.5 2: ≥22.5	2.069	0.540–7.928	0.289
Core amino acid 70 substitution	1: 70 non-Q 2: 70 Q	7.336	1.178–45.685	0.033
ALT	1: < 44 2: ≥44	3.012	0.661–13.729	0.154
γ-GTP	1: < 26 2: ≥26	1.923	0.406–9.097	0.410
Triglycerides	1: < 86 2: ≥86	4.025	1.038–15.612	0.044
Serum ferritin	1: < 96 2: ≥96	1.306	0.311–5.489	0.715

ALT, alanine aminotransferase; BMI, body mass index; 95% CI, 95% confidence interval; γ-GTP, γ-glutamyltransferase.

Thus, HCV core protein interacts with several pathways, including lipid metabolism, and could be a factor causing liver steatosis. HCV genotype 3 induced much more steatosis than genotype 1. Sequence analysis to identify the mutations that are associated with steatosis in genotype 3 has been performed, and it has been reported that amino acids 164, 182 and 186 of the HCV genotype 3 core region affect lipid metabolism (28, 29).

Hepatic steatosis in HCV genotype 1 and the expression of HCV core protein were evaluated, and the sequence variations related to steatosis were unclear. We found that there was a significant association between the prevalence of steatosis and substitutions of amino acid 70 on multivariate logistic regression analysis. Substitutions at amino acid 70/Q were detectable in 21 of 45 (46.7%) patients with steatosis, but only in two of the 22 (9.1%) patients without steatosis ($P = 0.00243$). The major amino acid 70 of genotype 3a was arginine, which defined the non-Q group. Thus, HCV genotype 1b with the amino acid 70/Q, which frequently caused steatosis, was not common in genotype 3a. HCV genotype 1b with 70/Q would enhance fatty acid synthase promoter or accelerate intracellular lipid accumulation as well as genotype 3, but further investigations are needed to confirm these speculations. Although the effect of substitutions of the core amino acid 70 is unclear, liver steatosis was increased by substitutions of the amino acid 70/Q of HCV genotype 1b core region.

The other factor that was related to liver steatosis was triglyceride levels. Lipid metabolism should be associated with liver steatosis, and triglyceride levels would be expected to be an index of the degree of liver steatosis. Several investigators have reported that host factors such as high BMI, obesity and insulin resistance were related

Table 4. Hepatic 8-OHdG ($n = 67$) and urinary 8-OHdG ($n = 35$) according to amino acid substitutions in core region

	Amino acid 70		Amino acid 75		Amino acid 91	
	Non-Q	Q	Non-A	A	Non-M	M
Hepatic 8-OhdG (%)	42.71 ± 31.0	42.60 ± 33.2	47.54 ± 29.5	39.95 ± 32.7	37.14 ± 31.1	52.58 ± 30.6
P values	NS		NS		$P = 0.045$	
Urinary 8-OhdG (ng/ml Cr)	10.26 ± 4.9	12.56 ± 5.9	12.14 ± 7.3	10.39 ± 3.9	9.31 ± 3.7	15.87 ± 6.3
P values	NS		NS		$P = 0.011$	

Data are expressed as mean ± SD. Data analysis were performed using Mann–Whitney *U*-test.
Core amino acids: Q, glutamine; A, alanine; M, methionine.
NS, not significant; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

to steatosis in patients with chronic hepatitis C. The prevalence of steatosis was significantly associated with increased BMI in the present study ($P = 0.0080$). There was a slight association with an increased index of homeostasis model assessment-insulin resistance, though this was not statistically significant ($P = 0.064$). The prevalence of steatosis (67%) observed was compatible with previous reports (30). However, the BMI (mean = 21.9) in the present study was less than in previous reports, and there were no patients with a BMI over 30; thus, the influence of obesity on liver steatosis was avoided. Although the BMI was low, the fact that the same level of liver steatosis was observed in the present study as in the past shows that HCV has an influence on liver steatosis that is independent of the BMI.

The substitutions of amino acid 91 of the HCV genotype 1b core region were associated with oxidative stress. Previous studies have shown that expression of the HCV core protein causes oxidative injury as a direct effect on mitochondria and increased ROS production in transgenic mice (14). One clinical study revealed that the rates of hepatocarcinogenesis in patients infected with the wild type of HCV core region were lower than in those with the mutant type (31). These results demonstrated that oxidative stress was related to substitutions of the HCV core region and the expression of HCV core protein. However, the effects of substitutions in the core region on core proteins are unknown. This might be due to differences in function through changes in the secondary structure or in the regulation of the expression level of HCV core protein and metabolic pathways. Further experiments are needed to clarify the mechanisms of these mutations.

Anti-oxidative stress treatment would be an option, especially for patients with substitution of amino acid 91/M of the HCV core region. Urinary 8-OHdG is a useful indicator that reflects systemic oxidative stress, as does hepatic 8-OHdG (32). Urinary 8-OHdG was superior to hepatic 8-OHdG because it is easy to measure. Therefore, serial urinary 8-OHdG levels in patients who received IFN therapy were evaluated, and it was confirmed that patients who achieved SVR had reduced oxidative stress. While the patient is on anti-oxidative stress treatment, the urinary 8-OHdG level would be a useful biomarker of oxidative stress for routine clinical use.

Oxidative stress as measured by 8-OHdG levels and hepatic steatosis were the common characteristics of chronic hepatitis C. Oxidative stress and hepatic steatosis were independent factors, but they are related; oxidative stress and hepatic steatosis have similar relationships with the HCV core region but different mutations are involved. The HCV core would be a key to the solution of the mechanism of HCV infection. Large clinical studies and formal basic research are needed to clarify these issues.

In conclusion, our results indicate that substitutions in amino acid 70 of the HCV core region were associated with hepatic steatosis, and similar relationships between substitutions in amino acid 91 and hepatic oxidative stress exist. Therefore, HCV core protein would be one of the most important factors for the development of liver steatosis and hepatic oxidative stress in chronic hepatitis C patients.

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CLINICAL STUDIES

Efficacy of peginterferon-α-2b plus ribavirin in patients aged 65 years and older with chronic hepatitis C

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Abstract

Objectives: The aim of this study was to evaluate the efficacy and indication of combination therapy with ribavirin plus peginterferon-α-2b in chronic hepatitis C virus (HCV) patients aged 65 years and older. *Methods:* Five hundred and ninety-one consecutive HCV patients were treated with combination therapy. These patients were divided into elder patients (≥65 years) ($n = 115$) and younger patients (< 65 years) ($n = 476$). The clinical characteristics, sustained virological response (SVR) rates and discontinuation rates were compared between the two groups. *Results:* Compared with younger patients, baseline haemoglobin levels and baseline platelet counts were significantly lower ($P < 0.0001$, $P = 0.013$ respectively) and fibrosis was more advanced in elderly patients ($P = 0.0310$). Moreover, the SVR rate was significantly lower (37.4 vs. 51.5%; $P = 0.0067$) while the combination therapy discontinuation rate was significantly higher (32.2 vs. 17.0%; $P = 0.0003$) in elderly patients. A multivariate analysis revealed that HCV load and genotype were significantly associated with an SVR in elderly patients. An SVR was achieved in over 50% of elderly male patients with genotype 1 and HCV RNA concentrations under 2 000 000 IU/ml. In contrast, the SVR rate was under 30% in elderly male patients with genotype 1 and with HCV RNA concentrations over 2 000 000 IU/ml and in all elderly female patients with genotype 1. *Conclusions:* The SVR rate was lower in elderly patients than in younger patients. However, in elderly patients combination therapy was most beneficial for genotype 1 patients, male patients with HCV RNA concentrations < 2 000 000 IU/ml and patients with genotype 2.

Hepatitis C virus (HCV) infection is a widespread viral infection that often leads to chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). The need for chronic HCV therapies for elderly patients is increasing in Japan and is expected to rise in the US and other Western countries (1). Moreover, HCC has become a recent and growing problem in elderly patients with chronic hepatitis C.

Sustained virological responders who are negative for serum HCV RNA 6 months after interferon (IFN) treatment are reported to be likely to remain in virological and biochemical remission with histological improvement (2, 3). Moreover, IFN therapy reduces the risk of HCC among virological or biochemical responders (4–6). Ribavirin is now generally used in combination with IFN to treat chronic hepatitis C, and this combination therapy is reportedly more effective than IFN monotherapy, with a higher rate of HCV eradication (7–10).

It is important to determine whether elderly hepatitis C patients should be treated with IFN. Arase *et al.* (11) reported that HCV clearance after IFN therapy significantly reduced the risk of HCC and death in older hepatitis C patients. In addition, Veldt *et al.* (12) reported that a sustained virological response (SVR) to treatment is associated with improved clinical outcomes in the general population with chronic hepatitis C and advanced fibrosis.

Several studies have shown that IFN monotherapy has comparable efficacy in elderly and younger patients with chronic hepatitis C (13, 14). IFN and ribavirin combination therapy has greater efficacy than IFN monotherapy

(7, 9). However, elderly patients with genotype 1 and high HCV loads have a lower SVR rate than younger patients because of higher dose reduction rates and discontinuation rates because of ribavirin-related anaemia (15, 16). In a previous study, we examined patients with a similar background, except for age, and found that treating chronic hepatitis C with combination therapy was comparably effective between patients ≥60 years old and those < 60 years old, although the ribavirin discontinuation rate was higher among older patients (17). Similar results were obtained from chronic hepatitis patients treated with peginterferon and ribavirin; although the probability of a positive response to peginterferon-α plus ribavirin combination therapy was decreased for genotype 1- or 4-infected patients older than 40 years, patients older than 65 years had a response rate similar to those aged 40–64 years (18). There are few reports on the efficacy of ribavirin and peginterferon in the elderly patients with chronic hepatitis C. Moreover, no study has determined which patients will benefit from combination therapy among elderly patients with chronic hepatitis C. This study was designed to examine the background and treatment efficacy of peginterferon and ribavirin combination therapy according to gender in older patients with chronic hepatitis C and to identify which patients will achieve an SVR in this patient population.

Methods

Patients

This nonrandomized, prospective study was originally discussed in December 2004 by a committee composed of members from Nagoya University Hospital and 63 affiliated hospitals in Japan. Diagnostic criteria for chronic hepatitis C patients, peginterferon and ribavirin regimens and follow-up protocols were determined. Patients were divided by age into two groups: those aged ≥65 years and those aged < 65 years. Patients were compared with respect to background and treatment efficacy according to gender and tolerability of combination therapy with peginterferon and ribavirin. The study protocol was approved by the ethics committee of each hospital, and written informed consent was obtained from each patient before therapy.

Five hundred and ninety-one consecutive patients with chronic hepatitis C were treated with peginterferon and ribavirin combination therapy between December 2004 and February 2007 at 64 institutions: Nagoya University Hospital and affiliated hospitals. The indications for treatment were under 75 years old, positive for antibody to HCV and a serum HCV RNA level > 100 000 IU/ml by a quantitative PCR assay (Amplicor GT-HCV Monitor version 2.0; Roche Molecular Systems, Pleasanton, CA, USA) within 12 weeks preceding the treatment. In Japan, combination with peginterferon and ribavirin therapy for patients with an HCV RNA level > 100 000 IU/ml (high viral load in Japan) was approved for medical insurance coverage. Exclusion criteria included pretreatment haemoglobin (Hb) levels < 10 g/dl, positive for serum hepatitis B surface antigen, drug addiction, alcohol abuse, autoimmune hepatitis, primary biliary cirrhosis, human immunodeficiency virus, coexisting serious psychiatric or medical illness and pregnancy. To exclude any patient bias, only complete cohorts from each hospital were enrolled. HCV genotypes were determined by PCR with genotype-specific primers that were described previously by Ohno *et al.* (19). All genotyping was performed at one institution.

All patients were treated with 1.5 μg peginterferon-α-2b (Pegintron®; Schering-Plough K. K., Osaka, Japan) per kilogram of body weight subcutaneously once weekly for 24 weeks for genotype 2 patients and for 48 weeks for genotype 1 patients. When the virus was eradicated between 16 and 24 weeks from the beginning of treatment, the treatment duration was prolonged up to 72 weeks for genotype 1 patients. Treatment was discontinued when a patient's Hb concentration declined below 8.5 g/dl because of drug-induced haemolytic anaemia or when a patient's white blood cell count declined below 1000/mm^3, the neutrophil count declined below 500/mm^3 or the platelet count declined below 50 000/mm^3. Some patients discontinued treatment because the virus could not be eradicated after 24 weeks, as determined by the physician. We considered these cases to be discontinued. Oral ribavirin (Rebetol; Schering-Plough K. K.) was administered for the same duration as peginterferon at 600 mg/day for patients who weighed < 60 kg, 800 mg/day for those who weighed > 60 kg but < 80 kg and 1000 mg/day for those who weighed > 80 kg during the treatment period. The dose of ribavirin was reduced by 200 mg/day when the patient's Hb concentration declined below 10 g/dl because of drug-induced haemolytic anaemia. Ribavirin was discontinued when peginterferon therapy was discontinued. In Japan, peginterferon and ribavirin combination therapy was not approved for medical insurance coverage until November 2004.

Liver histology

Pretreatment liver biopsy specimens were analysed for fibrosis on a scale of F0–F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis) and for necroinflammatory activity on a scale of A0–A3 (A0, no histological activity; A1, mild activity; A2, moderate activity; and A3, severe activity) (20).

Assessment of efficacy

A virological response was assessed using a qualitative HCV RNA assay with a lower detection limit of 100 IU/ml (Amplicor HCV version 2.0; Roche Molecular Systems). According to the qualitative HCV RNA results, the responses were defined as follows: SVR (no HCV RNA

detected at the end of the 24-week follow-up period after completion of treatment), relapse (no HCV RNA at the end of treatment and reappearance of serum HCV RNA during the 24-week follow-up period) or nonresponse (persistent positive serum HCV RNA throughout treatment).

Comparison of characteristics and efficacy of treatment according to age

Patients were divided by age into two age groups: (1) ≥65 years old ($n=115$) and (2) < 65 years old ($n=476$). The following baseline parameters were compared between the two groups: gender ratio, age, body weight, body mass index (BMI), alanine aminotransferase (ALT) levels, γ-glutamyl transpeptidase (GGT), Hb levels, platelet counts, HCV genotype and viral load, histological activity and fibrosis. The SVR rate, rapid virological response (RVR) (HCV RNA negative by a qualitative assay at week 4) rate, early virological response (HCV RNA negative by a qualitative assay at week 12) rate and end of treatment virological response (ETR) rate were obtained by an intention-to-treat (ITT) analysis and per-protocol (PP) analysis, and the ribavirin or peginterferon reduction rate and combination therapy discontinuation rate were compared between the two age groups.

Comparison of treatment efficacy between patients who did and did not achieve a sustained virological response

To identify factors that predict an SVR among patients treated with combination therapy, we first determined the factors associated with an SVR in combination therapy with respect to the same factors above baseline parameters by a univariate analysis. Next, we identified the factors associated with an SVR in combination therapy, including gender, age, BMI, baseline serum ALT, GGT, Hb, platelet counts, genotype and HCV RNA, using a multivariate stepwise analysis with forward inclusion methods.

Comparison of treatment efficacy between older patients who did and did not achieved a sustained virological response

To identify elderly patients who may particularly benefit from combination therapy, we determined factors associated with an SVR using a univariate analysis of the same background factors as above. Then we determined factors associated with an SVR in elderly patients treated with combination therapy by a multivariate stepwise analysis with forward inclusion methods. In addition, we analysed the virological responses to combination therapy according to the age and gender of patients infected with each genotype because the age distribution of the treated patients differed according to gender.

Statistical analysis

Values are expressed as means ± standard deviation (SD). Between-group differences in the mean quantitative values were analysed using Student's *t*-test, and differences in nonparametrical data were analysed using the Mann–Whitney *U*-test. Differences in proportions were tested by a χ^2-test. The SVR rate between age generations in females was assessed using Fisher's exact test. Multiple logistic regression analysis was used to identify factors related to SVR. Statistical analyses were performed using SPSS software version 16.0 (SPSS Japan Inc., Tokyo, Japan) for multiple logistic regression analysis and SAS software (SAS Institute Inc., Cary, NC, USA) for another analysis. All *P* values were two-tailed, and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

A total of 658 patients were screened, and 591 patients were enrolled in this study (Fig. 1). The patients included 327 men and 264 women aged 20–74 years (mean ± SD, 54.7 ± 11.6). Patients ≥65 years old comprised 19.5% of the patient population (115/591). The clinical characteristics of the two study groups are shown in Table 1. Body weight was significantly lower in patients aged ≥65 years than that in patients aged < 65 years ($P=0.0006$). Hb levels and platelet counts were significantly lower in patients aged ≥65 years than those in patients aged < 65 years ($P < 0.0001$ and $P=0.0013$ respectively). The fibrosis stage was more advanced in patients aged ≥65 years than that in patients aged < 65 years ($P=0.0310$).

Response to therapy

The ribavirin dose reduction rate was significantly higher in patients aged ≥65 years than that in patients aged < 65 years ($P=0.00013$) (Table 2), while the peginterferon dose reduction rate did not differ significantly between the two groups. The treatment discontinuation rate in patients aged ≥65 years was significantly higher than that in patients < 65 years ($P=0.0003$). As a result, the SVR rate by ITT analysis in patients aged ≥65 years was significantly lower than that in patients aged < 65 years ($P=0.0067$). However, the SVR and ETR rate by PP analysis were not significantly different between the two groups.

The factors associated with an SVR were determined by univariate analysis. The SVR rate was significantly higher in male patients than that in female patients ($P=0.0153$) (Table 3). Age was significantly lower in patients who achieved an SVR than in patients who did not achieve an SVR ($P < 0.0001$). Hb levels and platelet counts were significantly higher in patients who achieved an SVR than those in patients who did not achieved an SVR ($P=0.0202$ and $P=0.0002$ respectively). The HCV load in patients who achieved an SVR was significantly lower than that in patients who did not achieved an SVR

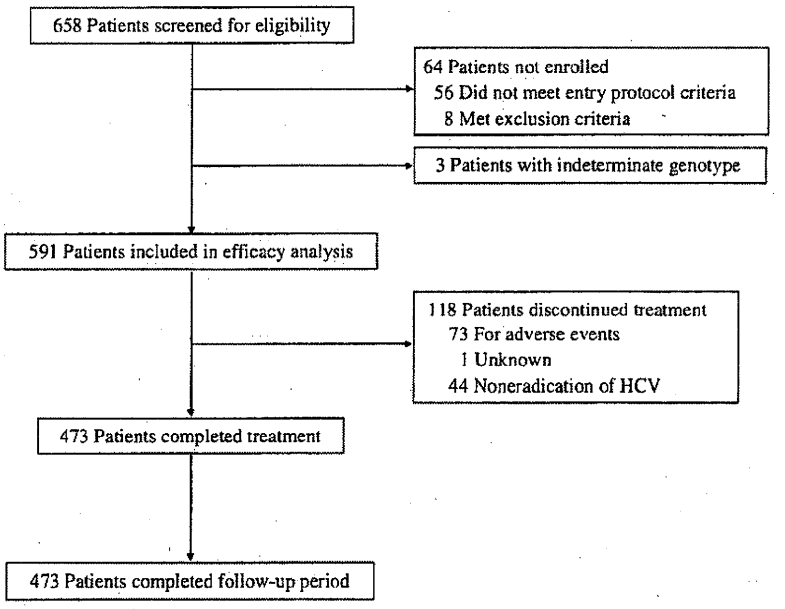

Fig. 1. Flow chart for patient selection.

Table 1. Baseline clinical characteristics of patients treated with combination therapy

	Total patients ($n = 591$)	Patients aged < 65 years ($n = 476$)	Patients aged ≥65 years ($n = 115$)	*P* value
Sex ratio (male/female)	327/264	270/206	57/58	0.1659
Age (years)	54.7 ± 11.6	51.5 ± 10.6	67.9 ± 2.2	< 0.0001
Body weight (kg)	60.1 ± 11.3	60.9 ± 11.4	56.7 ± 10.1	0.0006
Body mass index	22.9 ± 3.2	22.9 ± 3.2	22.9 ± 3.2	0.9221
Baseline serum ALT (IU/L)	64.8 ± 57.3	66.5 ± 60.6	57.7 ± 40.4	0.1425
GGT (IU/L)	57.8 ± 76.7	58.9 ± 78.9	53.3 ± 67.3	0.4880
Haemoglobin (g/dl)	14.1 ± 1.3	14.2 ± 1.4	13.7 ± 1.2	< 0.0001
Platelets (× 10^4/μl)	17.7 ± 5.7	18.0 ± 5.9	16.1 ± 4.3	0.0013
Genotype (1/2)	467/124	374/102	93/22	0.5870
HCV RNA (kIU/ml)	1863.3 ± 1456.3	1896.4 ± 1454.9	1726.2 ± 1460.5	0.2611
Activity (A0/A1/A2/A3)	16/255/141/19	13/202/115/13	3/53/26/6	0.6053
Fibrosis (F0/F1/F2/F3/F4)	37/228/107/56/5	31/191/83/37/3	6/37/24/19/2	0.0310

ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; HCV RNA, hepatitis C virus RNA; kIU, kilo international units.

($P = 0.0132$). The rate of genotype 2 patients who achieved an SVR was significantly higher than that in patients who did not achieve an SVR ($P < 0.0001$). The fibrosis stage was more advanced in patients who did not achieve an SVR than that in those who did achieve an SVR ($P = 0.0186$).

The factors associated with an SVR in combination therapy were determined by multivariate analysis (Table 4). Age [$P < 0.0001$, odds ratio 0.959 (0.942–0.975)] and genotype [$P < 0.0001$, odds ratio 0.415 (0.255–0.676)] were significantly associated with an SVR. Including the RVR (ITT) factor after starting treatment, the factors associated with an SVR in combination therapy were determined using a multivariate analysis (Table 5). Age [$P < 0.0001$, odds ratio 0.961 (0.944–0.978)] and RVR [$P < 0.0001$, odds ratio 8.168 (4.511–14.789)] were significantly associated with an SVR.

Then, we separately analysed male and female patients in different age groups. The virological responses to combination therapy according to the gender of genotype 1 patients are shown by age groups in Figure 2. In both males and females, the SVR rate decreased with age, and the SVR rates of patients < 40 years old were over 50%. In patients aged ≥65 years, the SVR rate of female patients was lower than that in patients aged < 65 years and was lower than that of male patients aged ≥65 years [20.8% (10/48) vs. 40.5% (64/158) and 20.8% (10/48) vs. 42.2% (19/45); $P = 0.0261$ respectively].

Table 2. Efficacy of combination therapy

	Total patients ($n=591$)	Patients aged < 65 years ($n=476$)	Patients aged ≥65 years ($n=115$)	*P* value
SVR rate (intention-to-treat)	48.7 (288/591)	51.5 (245/476)	37.4 (43/115)	0.0067
SVR rate (per-protocol)	59.2 (280/473)	60.3 (238/395)	53.8 (42/78)	0.2927
RVR rate (intention-to-treat)	20.0 (118/591)	21.2 (101/476)	14.8 (17/115)	0.1213
RVR rate (per-protocol)	22.0 (104/473)	22.8 (90/395)	17.9 (14/78)	0.3460
EVR rate (intention-to-treat)	62.6 (370/591)	64.5 (307/476)	54.8 (63/115)	0.0534
EVR rate (per-protocol)	71.0 (336/473)	71.1 (281/395)	70.5 (55/78)	0.9113
ETR rate (intention-to-treat)	81.0 (479/591)	83.2 (396/476)	72.2 (83/115)	0.0068
ETR rate (per-protocol)	92.8 (439/473)	92.9 (367/395)	92.3 (72/78)	0.8504
Ribavirin dose reduction rate	43.1 (255/591)	39.9 (190/476)	56.5 (65/115)	0.0013
PEGIFN dose reduction rate	34.3 (203/591)	33.2 (158/476)	39.1 (45/115)	0.2289
Combination therapy discontinuation rate	20.0 (118/591)	17.0 (81/476)	32.2 (37/115)	0.0003
Combination therapy discontinuation rate*	12.5 (74/591)	9.9 (47/476)	23.5 (27/115)	< 0.0001

*Except genotype 1 patients which therapy was discontinued because the virus could not be eradicated after 24 weeks.
ETR, end of treatment virological response; EVR, early virological response; PEGIFN, peginterferon; RVR, rapid virological response; SVR, sustained virological response.

Table 3. Factors associated with a sustained virological response in combination therapy by a univariate analysis

	Total patients ($n=591$)	Patients who achieved an SVR ($n=288$)	Patients who did not achieved an SVR ($n=303$)	*P* value
Sex ratio (male/female)	327/264	171/114	153/150	0.0153
Age (years)	54.7 ± 11.6	51.9 ± 12.6	57.3 ± 9.8	< 0.0001
Body weight (kg)	60.1 ± 11.3	60.7 ± 10.8	59.6 ± 11.8	0.2661
Body mass index	22.9 ± 3.2	23.0 ± 2.9	22.9 ± 3.5	0.8785
Baseline serum ALT (IU/L)	64.8 ± 57.3	65.8 ± 63.8	63.8 ± 50.5	0.6758
GGT (IU/L)	57.8 ± 76.7	54.7 ± 91.0	60.7 ± 60.2	0.3425
Haemoglobin (g/dl)	14.1 ± 1.3	14.2 ± 1.4	14.0 ± 1.3	0.0202
Platelets (× 10^4/μl)	17.7 ± 5.7	18.6 ± 6.1	16.8 ± 5.1	0.0002
Genotype (1/2)	467/124	204/84	263/40	< 0.0001
HCV RNA (kIU/ml)	1863.3 ± 1456.3	1711.2 ± 1415.4	2007.8 ± 1482.0	0.0132
Activity (A0/A1/A2/A3)	16/255/141/19	7/121/70/9	9/134/71/10	0.9596
Fibrosis (F0/F1/F2/F3/F4)	37/228/107/56/5	18/122/46/20/0	19/106/61/36/5	0.0186

ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; HCV RNA, hepatitis C virus RNA; kIU, kilo international units.

Table 4. Multivariate analysis of factors associated with a sustained virological response in combination therapy

Variable		Odds ratio (95% CI)	*P* value
Age		0.959 (0.942–0.975)	< 0.0001
Genotype	1 vs. 2	0.415 (0.255–0.676)	< 0.0001

CI, confidence interval.

Table 5. Multivariate analysis of factors (including treatment response) associated with a sustained virological response in combination therapy

Variable		Odds ratio (95% CI)	*P* value
Age		0.961 (0.944–0.978)	< 0.0001
RVR	RVR vs. nonRVR	8.168 (4.511–14.789)	< 0.0001

CI, confidence interval; RVR, rapid virological response.

Virological responses to combination therapy according to the gender of genotype 2 patients are shown by age groups in Figure 3.

The SVR rate was similar for all age groups among male patients. In both male and female patients < 40 years old, the SVR rate was over 75%. In both male and female patients over 40 years old, the SVR rate was approximately 60%.

Response to therapy in older patients

In patients aged ≥65 years, the factors associated with an SVR were determined by univariate analysis (Table 6). The SVR rate of male patients was significantly higher than that of female patients ($P=0.0284$). The ratio of genotype 1 in patients who achieved an SVR was significantly lower than that in patients who did not