INTERNAL MEDICINE

ORIGINAL ARTICLE

The Development of Chronic Kidney Disease in Japanese Patients with Non-alcoholic Fatty Liver Disease

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Abstract

Objective Chronic kidney disease (CKD) is present in patients with nonalcoholic fatty liver disease (NAFLD). The aim of this retrospective study was to assess the cumulative development incidence and predictive factors for new onset of CKD in Japanese patients with NAFLD.

Methods A total of 5,561 NAFLD patients without CKD were enrolled. CKD was defined as either an estimated glomerular filtration rate of <60 mL/min/1.73 m^2 or dipstick proteinuria ($\geq +1$). A blood sample and a urine sample were taken for routine analyses during follow-up. The mean observation period was 5.5 years. The primary goal is the new development of CKD. Independent factors associated with new development of CKD were analyzed by using the Kaplan-Meyer method and the Cox proportional hazards model.

Results Of 5.561 NAFLD patients, 263 patients developed CKD. The cumulative development rate of CKD was 3.1% at the 5th year and 12.2% at the 10th year. Multivariate Cox proportional hazards analysis showed that CKD development in patients with NAFLD occurred when patient had low level of GFR of 60-75 mL/min/1.73 m^2 [hazard ratio:2.75; 95% confidence interval (CI) =1.93-3.94; p<0.001], age of ≥50 years (hazard ratio: 2.67; 95% CI=2.06-3.46; p<0.001), diabetes (hazard ratio: 1.92; 95% CI=1.45-2.54; p<0.001), hypertension (hazard ratio: 1.69; 95% CI=1.25-2.29; p<0.001), and elevated serum gamma-glutamyltransferase of ≥109 IU/L(hazard ratio: 1.35; 95% CI=1.02-1.78; p=0.038).

Conclusion Our retrospective study indicates that the annual incidence of CKD in Japanese patients with NAFLD is about 1.2%. Five factors of low eGFR level, aging, type 2 diabetes, hypertension, and elevated gamma-glutamyltransferase, increases the risk of the development of CKD.

Key words: nonalcoholic fatty liver disease, chronic kidney disease, gamma-glutamyltransferase

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the more common causes of chronic liver disease in Western world (1-4) and in many Asian nations (5, 6). NAFLD is considered to be the liver component of metabolic syndrome (7-9). It is associated with obesity, dyslipidemia, pituitary dysfunction, hypertension, sleep apnea, and type 2 diabetes mellitus (T2DM) (10-16). Moreover, NAFLD often causes cardiovascular disease and stroke (17, 18). Thus, NAFLD is emerging as a new significant health problem in many countries.

On the other hand, there has been a recent dramatic in-

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crease in the prevalence of end-stage renal disease (ESRD) in USA and Asia (19-22). Chronic kidney disease (CKD) often progresses to ESRD with its attendant complications. CKD, a disease entity including mild to ESRD due to any etiology, was defined as an estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2 and/or the presence of proteinuria (21). Recently, metabolic syndrome and NAFLD have been reported to enhance the new onset of CKD (23, 24). Although there is growing evidence to support the concept that metabolic syndrome is a risk factor for developing CKD, little research has been done to evaluate whether NAFLD is associated with the long-term development of CKD.

The present cohort study was initiated to investigate the cumulative incidence and risk factors of CKD after long-term follow-up in patients with NAFLD. The strengths of the current study are the large numbers of patients included and the long-term follow-up of patients.

Methods

Patients

The number of Japanese patients who were diagnosed with fatty liver by ultrasonography (25) between January 1997 and December 2007 in the Department of Hepatology and/or Health Management Center, Toranomon Hospital, Tokyo, Japan was 9,120. Of these, 5,561 Japanese patients satisfied with the following enrolled criteria; 1) no evidence of CKD based on eGFR calculated with serum creatinine level (eGFR≥60 [mL/min/1.73 m^2]); 2) the absence of proteinuria (≥ +1); 3) current and past daily alcohol intake of <20 g/day; 4) negativity for hepatitis B surface antigens (HBsAg), hepatitis C virus antibodies, antinuclear antibodies, or antimitochondrial antibodies in serum, as determined by radioimmunoassay, enzyme-linked immunosorbent assay or spot hybridization; 5) no underlying neoplasm or systemic disease, such as systemic lupus erythmatosus, rheumatic arthritis; 6) no evidence of nodules of hepatocellular carcinoma as shown by ultrasonography and/or computed tomography. Patients with the above criteria were enrolled regardless of whether the serum level of aminotransferase was normal or abnormal. Patients with any of the following criteria were excluded from the study: 1) illness that could seriously reduce their life expectancy, 2) findings suggestive of other chronic liver disease, and 3) refusal to be followed up after the diagnosis of NAFLD. A total of 3,559 out of 9,120 patients were excluded based on the following findings; 169 had a dipstick-positive proteinuria; 1,685 had an eGFR of <60 mL/min/1.73 m^2; 2,098 had alcohol intake of ≥20 g/day; 133 had positive serologic findings for either hepatitis B or C virus, a reported history of known liver disease, or decompensated liver cirrhosis; 36 had a history of malignancy; 26 had a history of cardiovascular disease; 11 refused the participation of prospective follow-up. Because some individuals were excluded for multiple reasons, the total number of eligible patients for the study was 5,561.

Patients were classified into three groups according to fasting plasma glucose (FPG): 1) those with FPG level of < 110 mg/dL (normal glucose group), 2) those with FPG level of 110-125 mg/dL (pre-diabetes group), and 3) those with FPG level of ≥126 mg/dL (diabetes group) (25). Patients were regarded as hypertension by the confirmation of blood pressure ≥140 mmHg systolic and/or ≥90 mmHg diastolic.

The primary goal was the new onset of CKD in patients with NAFLD. The end-point was defined as the first eGFR <60 mL/min/1.73 m^2 or dipstick proteinuria (≥ +1) for more than three months. Serum creatinine level was also measured using an enzymatic method, and the GFR was estimated from the Japanese Society of Nephrology CKD Practice Guide; eGFR (mL/min/1.73 m^2) =194× (serum creatinine level [mg/dL]) -1.094× (age [y]) -0.287. The product of this equation was multiplied by a correction factor of 0.739 for women. CKD and its stages were defined from estimated eGFR of <60 mL/min/1.73 m^2 or dipstick proteinuria (≥ +1) as follows: stage I, eGFR≥90 and proteinuria (≥ +1); stage II, 90>eGFR≥60 and proteinuria (≥ +1); stage III, 60>eGFR≥30; stage IV, 30>eGFR≥15; and stage V, 15>eGFR. Patients with stage III-V were regarded as having CKD regardless of the absence of other markers of kidney damage (21, 22).

All of the studies were performed retrospectively by collecting and analyzing data from the patient records. This study was approved by Institutional Review Board of our hospital.

Medical evaluation

Fatty liver was diagnosed by the presence of an ultrasonographic pattern consistent with bright liver with stronger echoes in the hepatic parenchyma than in the renal or spleen parenchyma (26). Ultrasonography test was performed with a high-resolution, real-time scanner (model SSD-2000; Aloka Co., Ltd, Tokyo Japan. Mode Logic-700 MR; GE-Yokokawa Medical Systems, Tokyo, Japan). Body weight was measured in light clothing and without shoes to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm. Height and weight were recorded at baseline and the body mass index (BMI) was calculated as weight (in kg) / height (in m^2). All of the patients were interviewed in the Toranomon Hospital using a questionnaire that gathered information on demographic characteristics, medical history, and heath-related habits including questions on alcohol intake at the time of diagnosis of fatty liver.

Laboratory investigation

At the first consultation anti-HCV and HBsAg were examined. Anti-HCV was detected using a third-generation enzyme-linked immunosorbent assay (Abbott Laboratories, North Chicago, IL). HBsAg was tested by radioimmunoassay (Abbott Laboratories, Detroit, MI). Anti-HBs was not evaluated in the present study. Serum creatinine concentration was measured by a modified Jaffe method (creatinine

Table 1. Characteristics of Subjects Enrolled

	Total
Number of cases	5561
Age (years)	48.0±8.4
Sex(male/female)	4916/645
Systolic blood pressure(mmHg)	134±18
Diastolic blood pressure(mmHg)	76±10
Hypertension(+)	725(13.0%)
Height(cm)	167.8±7.3
Body Weight (kg)	70.7±9.9
BMI (kg/m^2)	25.1±2.8
Smoking (+)	1028 (18.5%)
FPG(mg/dL)	104.7±24.8
Glucose status (Normal/ preDM/DM)	4436(79.8%)/667(12.0%)/458(8.2%)
eGFR (mL/min/1.73m^2)	74.6±11.9
WBC(×10^3/mm^3)	5.8±1.5
Hemoglobin (g/dL)	15.1±1.2
Platelet (×10^4/mm^3)	23.1±5.0
Triglyceride (mg/dL)	164±117
Total cholesterol (mg/dL)	210±34
HDL cholesterol (mg/dL)	48.1±11.9
Total Protein(g/dL)	7.5±0.4
Albumin (g/dL)	4.2±0.3
Uric Acid (mg/dL)	6.2±1.3
AST (IU/L)	29.2±16.4
ALT (IU/L)	37.5±27.0
GGT(IU/L)	78.2±81.0
Follow-up period (years)	5.5±4.8

Data are number of patients (percent) or mean ± standard deviation
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DM, diabetes mellitus, eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; GGT, gamma-glutamyltransferase; HDL, high density lipoprotein; WBC, white blood cell;

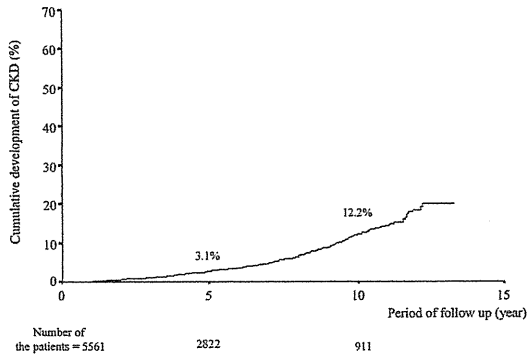

Figure 1. Cumulative development rate of CKD in 5,561 patients with NAFLD.

HR, Wako Pure Chemicals Industries, Ltd, Osaka, Japan) using an autoanalyzer (Hitachi 7350, Hitachi Ltd, Tokyo, Japan or RX-20, JEOL Ltd., Tokyo, Japan). Proteinuria was tested using dipsticks (Ames Hemacombistics; Bayer-Sankyo Ltd, Tokyo, Japan). A test result of $\geq +1$ was defined as positive.

Follow-up

Starting time of follow-up was the day that the fatty liver was confirmed by ultrasonography. After that, patients were followed up monthly to six-monthly in the Toranomon hospital. A blood sample and a urine sample were taken for routine analyses. Four hundred and ninety-two patients were lost to follow-up. Because the appearance of CKD was not identified in these 492 patients, they were considered as censored data in statistical analysis (27).

Statistical Analysis

The cumulative appearance rate of CKD was calculated from the starting time of follow-up to the development of CKD by using the Kaplan-Meier method. Differences in the development of CKD were tested using the log rank test. The Cox proportional hazard model analyzed independent factors associated with the development rate of CKD. The following variables were analyzed for potential covariates for incidence of CKD: age, BMI, T2DM, hypertension, and levels of eGFR, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), total protein, triglyceride (TG), total cholesterol level, high density lipoprotein (HDL) cholesterol uric acid, hemoglobin, white blood cell, platelet at the time of diagnosis of NAFLD. A p value of less than 0.05 was considered significant. Data analysis was performed using the computer program SPSS package (SPSS 11.5 for Windows, SPSS, Chicago, IL).

Results

Patients' characteristics

Table 1 shows the characteristics in the 5,561 patients diagnosed as NAFLD in the present study. The mean age was 48 years. The mean BMI was 25.1. Patients with hypertension accounted for 13.0% and patients with T2DM accounted for 8.2%. The eGFR level was 74.6±11.9 mL/min/1.73 m^2. The mean follow-up period was 5.5 years.

Incidence of CKD in Patients with NAFLD

Of 5.561 NAFLD patients, 263 developed CKD. Figure 1 shows that the cumulative development rate of CKD was 3.1% at the 5th year and 12.2% at the 10th year in all patients with NAFLD. Cox proportional hazards analysis showed that CKD development in NAFLD patients occurred when patient had eGFR of 60-75 mL/min/1.73 m^2 [hazard ratio:2.75; 95% confidence interval (CI) =1.93-3.94; p< 0.001], age of $\geq$50 years (hazard ratio:2.67; 95% CI =2.06-3.46; p<0.001), T2DM (hazard ratio:1.92; 95% CI=1.45-2.54; p<0.001), hypertension (hazard ratio:1.69; 95% CI= 1.25-2.29; p<0.001), and elevated serum GGT (hazard ratio: 1.35; 95% CI=1.02-1.78; p=0.038) at the initiation of follow up (Table 2).

Figure 2 shows the cumulative development rate of CKD based on the difference of age and eGFR level at the starting time of follow-up. Figure 3 shows the cumulative development rate of CKD based on the difference of FPG, blood pressure, and serum GGT at the starting time of follow-up. On the difference of serum GGT level, the cumulative rate of CKD at 10th year in NAFLD was 11.3% in patients with

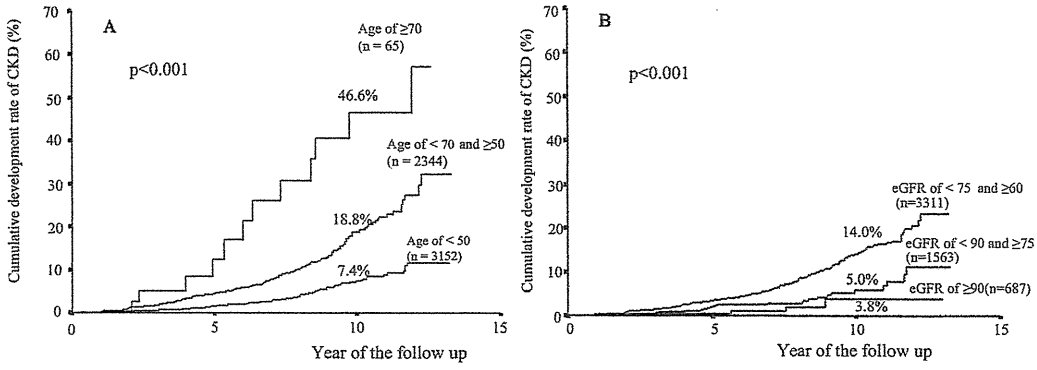

Figure 2. Cumulative development rate of CKD in NAFLD patients. Panel A: Cumulative development rate of CKD based on the difference of age at the starting time of follow-up, Panel B: Cumulative development rate of CKD based on the difference of eGFR level at the starting time of follow-up

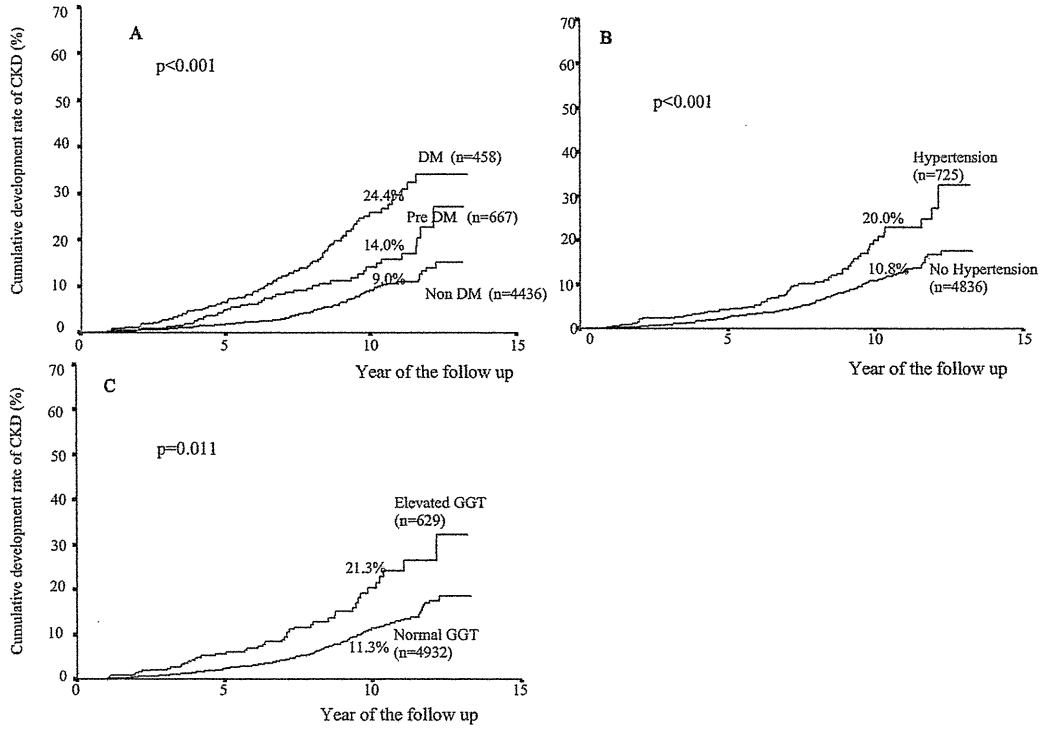

Figure 3. Cumulative development rate of CKD in NAFLD patients. Panel A: Cumulative development rate of CKD based on the difference of glucose level at the starting time of follow-up, Panel B: Cumulative development rate of CKD in patients with hypertension or without hypertension at the starting time of follow-up, Panel C: Cumulative development rate of CKD based on difference of serum GGT level at the starting time of follow-up

normal GGT level and 21.3% in those with elevated GGT level.

Impact of GGT on the incidence of CKD

In addition to elevated level of serum GGT, the four factors of ≥50 years, eGFR of 60-75 mL/min/1.73 m^2, and T2DM, hypertension were high risk factors of developing CKD with statistical significance. Figure 4 shows the cumulative development of CKD based on the difference of serum GGT in NAFLD patients with each risk factor of age of ≥50 years, eGFR of 60-75 mL/min/1.73 m^2, T2DM, or hypertension. Elevated serum GGT enhances the new development of CKD with statistically significant differences in NAFLD patients with each risk factor of ≥50 years, eGFR of 60-75 mL/min/1.73 m^2, or hypertension. In NAFLD patients with T2DM, elevated serum GGT tended to facilitate the new development of CKD (p=0.068).

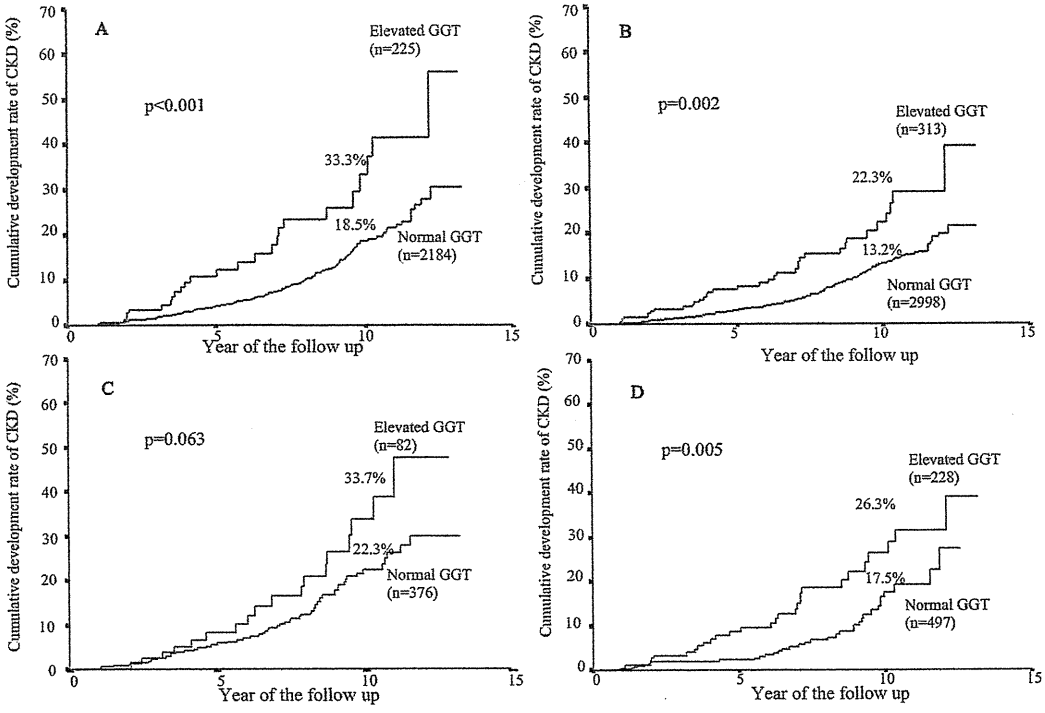

Figure 4. Cumulative development rate of CKD in NAFLD patients. Panel A: Cumulative development rate of CKD based on the difference of serum GGT level at the starting time of follow-up in NAFLD patients aged ≥50 years, Panel B: Cumulative development rate of CKD based on the difference of serum GGT level at the starting time of follow-up in NAFLD patients with eGFR of 60-75 mL/min/1.73 m^2 and absence of dipstick proteinuria (≥+1), Panel C: Cumulative development rate of CKD based on the difference of serum GGT level at the starting time of follow-up in NAFLD patients with T2DM, Panel D: Cumulative development rate of CKD based on the difference of GGT levels at the starting time of follow-up in NAFLD patients with hypertension

Discussion

We have described the incidence of development of CKD in NAFLD patients. The present study indicates that the annual incidence of CKD for a prolonged follow-up among NAFLD patients is about 1.2% based on a follow-up of 10 years. The present study was limited by a retrospective cohort trial. A blood sample and a urine sample were taken for routine analyses during follow-up. Next limitation of the study was that patients were treated with different types of exercise and diet for the NAFLD during follow-up. Moreover, although the NAFLD can be categorized into simple steatosis and steatohepatitis, the present study was undertaken without histological differentiation of simple steatosis and steatohepatitis. Next, prescribed agents during the follow-up were not considered in the present study. Finally, the interval of follow-up was different for each patient. This heterogeneity makes it slight difficult to precisely interpret the results of the study. On the other hand, the strengths of the present study are a long-term follow-up with a large numbers of patients included.

The present study shows several findings with regard to development of CKD in NAFLD patients. First, the CKD development rate in NAFLD patients with an elevated level of GGT was higher than that in those with a normal level of GGT. The fact that elevated GGT enhanced the onset of CKD is in accordance with the data reported by Chang et al (28), Ryu et al (29), and Fraser et al (30). Though the role of elevated GGT in the pathogenesis of CKD remains speculative, the following possible mechanism have been reported, 1) GGT is related to T2DM and/or insulin resistance by meta-analysis; insulin resistance may be associated with an increased risk for CKD (31-33). 2) GGT is linked with systemic low-grade inflammation; low grade inflammation may cause a change in kidney function (34). 3) GGT has been proposed as a sensitive marker of oxidative stress; oxidative stress plays an important role in renal damage (35).

Second, in addition to the elevation of GGT, the present study suggests that aging, eGFR of 60-75 mL/min/1.73 m^2, T2DM, and hypertension enhanced the development of CKD in NAFLD patients. The present findings of factors of metabolic syndrome such as T2DM and hypertension, which enhanced the new development of CKD is in accordance with the data reported by Chen et al (36), and Luk et al (37). Moreover, when GGT was elevated in NAFLD patients with each factor of ≥50 years, eGFR of 60-75 mL/min/1.73 m^2, or hypertension, the cumulative development rate of CKD increased with significant difference compared to those with a normal GGT level. In NAFLD patients with T2DM, an

Table 2. Predictive Factors for CKD Development Based on the Clinical Data at the Starting Time of Follow-up

Variables	Univariate analysis		Cox-regression	
	HR (95%CI)	p	HR (95%CI)	p
Age (years, ≥50/<50)	2.92(2.27-3.75)	< .001	2.67(2.06-3.46)	< .001
Gender (female/male)	1.08(0.73-1.60)	.706		
BMI (≥25/<25)	1.15(0.90-1.46)	.270		
Hypertension (+/-)	2.04(1.55-2.69)	< .001	1.69(1.25-2.29)	<.001
Smoking (+/-)	1.19(0.63-2.24)	.588		
AST(IU/L, ≥34/<34)	1.25(0.95-1.65)	.113		
ALT(IU/L, ≥43/<43)	1.06(0.82-1.38)	.640		
GGT (IU/L, ≥109/<109)	1.43(1.09-1.88)	.011	1.35(1.02-1.78)	.038
Diabetes (+/-)	2.42(1.85-3.17)	< .001	1.92(1.45-2.54)	< .001
WBC ($\times 10^3/mm^3$, <5.0/≥5.0)	1.04(0.80-1.35)	.770		
Hemoglobin (g/dL, <15/≥15)	1.08(0.84-1.39)	.552		
Platelet ($\times 10^4/mm^3$, <25/≥25)	1.04(0.80-1.34)	.770		
Total protein(g/dL, ≥7.5/<7.5)	0.84(0.45-1.50)	.588		
Triglyceride(mg/dL, ≥150/<150)	1.58(1.24-2.00)	< .001	1.32(0.99-1.76)	.059
Total Cholesterol (mg/dL, ≥220/<220)	1.17(0.87-1.57)	.314		
HDL Cholesterol (mg/dL, <40/≥40)	0.94(0.73-1.23)	.693		
Uric acid (mg/dL, ≥7/<7)	1.15(0.86-1.53)	.330		
eGFR (≥60 and <75 /≥75)	2.73(1.92-3.88)	< .001	2.75(1.93-3.94)	< .001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; GGT, gamma-glutamyltransferase; HDL, high density lipoprotein; HR, hazards ratio

elevated GGT indicated tendency to increase the cumulative development rate of CKD compared to those with normal GGT level.

Thus, the present results indicate that T2DM, hypertension, and elevated GGT enhance the new development of CKD in NAFLD patients. This means that in addition to the improvement of glucose level and hypertension, normalization of serum GGT could reduce the aggravation of kidney function.

NAFLD that is considered to be a risk factor for developing CKD is emerging into a new significant health problem in many countries. In addition, the life span in Japan has recently become long. In the near future, a large number of patients with NAFLD will be >60 years of age. CKD occurs more frequently in elderly patients than in young patients. Thus, it is reasonable to conclude that CKD will be increasing in NAFLD patients. CKD often progresses to ESRD with its accompanying complications. Medical physicians regarding the daily management of patients with NAFLD should check on the development of CKD in addition to the aggravation of liver function.

In conclusion, our retrospective study indicates that the annual incidence of CKD in Japanese patients with NAFLD is about 1.2%. The following five factors enhance the risk of development of CKD: low eGFR level, aging, type 2 diabetes, hypertension, and elevated GGT.

The authors state that they have no Conflict of Interest (COI).

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Antiviral effects of peginterferon alpha-2b and ribavirin following 24-week monotherapy of telaprevir in Japanese hepatitis C patients

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Abstract
Background/aims Anemia is commonly observed as a side effect in a treatment with protease inhibitors combined with peginterferon alpha and ribavirin for hepatitis C virus infection. This study assessed the safety, tolerability, viral kinetics, and selection of variants in telaprevir monotherapy for 24 weeks, and outcomes of the off-study treatment with peginterferon alpha-2b and ribavirin among Japanese female patients at a median age of 54 years who were difficult to treat with the standard therapy (peginterferon alpha-2b and ribavirin) alone in Japan.
Methods Four treatment-naïve patients with chronic hepatitis C virus subtype 1b infection received telaprevir (750 mg every 8 h) alone for 24 weeks. All patients then started the off-study treatment with peginterferon alpha-2b and ribavirin. Safety, tolerability, hepatitis C virus RNA levels, and emergence of telaprevir-resistant variants were monitored.
Results During the 24 weeks of telaprevir monotherapy, there was no discontinuation due to adverse events, but 2 patients stopped the intake at weeks 6 and 15 because of viral breakthrough. Emergence of telaprevir-resistant variants was observed in 3 patients who showed viral breakthrough. These variants were eliminated by the off-study treatment, and sustained virological response was achieved in all patients.
Conclusions Anemia was manageable by carefully adjusting the ribavirin dosage in the standard therapy that followed telaprevir monotherapy. This sequential regimen seems to be safer and more tolerable than the triple combination of telaprevir, peginterferon alpha, and ribavirin, especially among elderly females with low baseline hemoglobin.

Keywords Hepatitis C therapy · Telaprevir · Ribavirin · Anemia

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Introduction

Hepatitis C virus (HCV) is a major cause for concern worldwide. More than 3% of the world's population is chronically infected with HCV, and 3–4 million people are newly infected each year [1]. Chronic HCV infection is relatively mild and progresses slowly; however, about 20% of chronic hepatitis C (CHC) carriers progress to potentially serious end-stage liver disease [2–4]. The current standard treatment for HCV infection is administration of pegylated alpha interferon (PEG-IFN) in combination with ribavirin (RBV) for 48 weeks. The overall sustained virological response (SVR) rates with this intervention are

40–50% for patients with genotype 1 [5, 6]. Several direct-acting antiviral agents (DAAs) for HCV infection have been clinically evaluated [7]. Telaprevir (VX-950/MP-424) is a novel peptidomimetic slow- and tight-binding inhibitor of HCV NS3-4A protease, which was discovered using a structure-based drug design approach [8]. As one of the most advanced DAAs against HCV, phase 3 clinical trials of telaprevir are on-going in the US, EU, and Japan. Recent clinical trials of telaprevir in combination with the standard treatment have indicated a promising advancement in therapy for treatment-naïve CHC patients as well as patients who did not respond previously to the standard treatment alone [9–11]. However, compared with the standard treatment alone, telaprevir is associated with an increased incidence of several side effects, such as anemia and skin rash.

The epidemiology of HCV in Japan is different from that in the US and EU; the majority of Japanese HCV carriers are of age >55 years, and three-fourths of Japanese HCV carriers are infected with genotype 1, which consists almost entirely of subtype 1b [12–14]. The dose reduction rate and the frequency of discontinuation of this treatment are high in elderly patients [15]. The SVR rate of the standard therapy is lower in females than males, especially in older patients in Japan [16]. In addition to the need for a therapy yielding higher SVR rates than the current standard therapy, there is also the need for a treatment regimen with a lower incidence of severe side effects because of the characteristics of HCV carriers in Japan.

Since our institution is a site of the phase 2a trial of telaprevir monotherapy among Japanese patients infected with HCV subtype 1b, our primary objective was to evaluate the safety, tolerability, and efficacy of telaprevir alone for up to 24 weeks. We also assessed the selection of HCV subtype 1b variants under prolonged telaprevir monotherapy and the susceptibility of these selected variants to the standard PEG-IFN and RBV therapy.

Patients and methods

Study design and organization

This single-arm, open-label study was conducted between January 2008 and September 2008 at Sapporo Kosei General Hospital, Sapporo, Japan, as a site of the telaprevir phase 2a monotherapy trial in Japan. The study was conducted in compliance with the Good Clinical Practice guidelines and the Declaration of Helsinki. Before the study, the protocol and informed consent form were approved by an institutional review board. Written informed consent was obtained from each patient after sufficient explanation before participation in the study.

All patients received telaprevir at a dose of 750 mg every 8 h orally for a maximum of 24 weeks, which was determined by the stopping rule of viral kinetics [2 $\log_{10}$ increase from the nadir or 3 $\log_{10}$ IU/ml if the nadir was below the lower limit of quantification (LOQ)]. Telaprevir was administered in the fed state. After the patients met the stopping rule of viral kinetics, the investigators recommended the patients to begin the standard treatment for HCV infection (weight-based PEG-IFN alpha-2b and RBV) in order to prevent them from the earlier treatment failure. This standard treatment was off-study. The dose of PEG-IFN alpha-2b was specified in the package insert. The doses of RBV were based on total body clearance (CL/*F*) calculated by the following equation:

$$\mathrm{CL}/F\ (\mathrm{L/h}) = 3.23 \times \text{body weight (kg)} \times (1 - 0.0094 \times \text{age}) \times (1 - 0.42 \times \text{gender}) / \text{serum creatinine}\ (\mu\mathrm{mol/L}),$$

where gender = 0 for male and 1 for female. The RBV dose was set for a targeted blood concentration of 2250 ng/ml.

Telaprevir was supplied as 250-mg tablets for oral administration provided by Mitsubishi Tanabe Pharma Corp., Osaka, Japan. PEG-IFN alpha-2b and ribavirin (Pegintron® and Rebetol®) were obtained from Schering-Plough, KK, Osaka, Japan.

Participants

Patients were enrolled in this study according to the following inclusion criteria: diagnosis of CHC; infection with HCV genotype 1b as determined by phylogenetic analysis on the NS5B region; no prior antiviral therapy for HCV; Japanese (Mongoloid) lineage; age 20–70 years at enrollment. Patients were excluded from the study if they met any of the following criteria: diagnosis of decompensated liver cirrhosis and/or hepatitis B surface antigen in serum; diagnosis or history of hepatocellular carcinoma; previous treatment for malignant neoplasm; diagnosis of autoimmune hepatitis, alcoholic liver disease, hemochromatosis, or chronic liver disease other than CHC; history of allergy to medication or anaphylactoid symptoms; women who were pregnant, breast feeding, or who planned to become pregnant.

Safety assessments

The safety and tolerability of the study treatments were assessed by clinical laboratory results, vital signs, physical examination results, and occurrence of adverse events. These safety parameters were recorded at regular intervals from day −28 through the follow-up visits. Adverse events

were classified according to the Medical Dictionary for Regulatory Activities (MedDRA), version 12.0.

HCV RNA measurement

The HCV subtype was determined by direct sequencing followed by phylogenetic analysis on the NS5B region [17]. The serum HCV RNA levels were determined using the COBAS TaqMan® HCV test (Roche Diagnostics, Tokyo, Japan). The linear dynamic range of the assay was from 1.2 to 7.8 $\log_{10}$ IU/ml. The LOQ of the assay was 1.2 $\log_{10}$ IU/ml, and the qualitative result below LOQ was also determined as positive (+) and negative (−). Blood samples in this study were collected on days −28, 1 (before the first dose), 3, 8, 15, 29, 43, 57, 71, 85, 99, 113, 127, 141, 155, and 169 of the study drug dosing period, at the 2-week follow-up, and on the days when the patients met the stopping rules. During the off-study treatment, blood samples were collected before the first injection, 1 and 2 weeks after the off-study treatment was initiated, and every 4 weeks thereafter.

Viral sequencing analysis

The HCV interferon sensitivity determining region (ISDR) on NS5A [18] and the core region [19] were analyzed by the direct sequencing method. The DNA fragment containing the 534-bp (181 amino acids) NS3 protease domain was amplified by the nested reverse transcription-polymerase chain reaction and cloned. At least 39 clones per specimen were sequenced and determined bidirectionally. The sequences of the NS3 protease domain registered in the public databases of the National Center for Biotechnology Information (NCBI), except the protease-resistant variants reported previously [20–23], were considered to be a naturally occurring variant and treated as a wild type in the analysis. The limit of detection for the sequencing analysis was approximately 3 $\log_{10}$ IU/ml.

Viral dynamics model analysis

The basic mathematical model for the analysis of HCV infection in vivo, which is a system of three ordinary differential equations for uninfected cells (T), productively infected cells (I), and free virus (V), has been reviewed elsewhere [24]. The solved Eq. 1 was fitted to the HCV RNA levels ($\log_{10}$ IU/ml) obtained in this study via nonlinear regression using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). The positive and negative qualitative values below LOQ were treated as 1.0 and 0.5, respectively.

$$V(t) = V_0 + \log_{10}\left[e^{-ct} + \frac{(1-\varepsilon)c}{c-\delta}(e^{-\delta t} - e^{-ct})\right] \quad (1)$$

where c is the virion clearance rate from serum, δ is the clearance rate of infected cells, and ε is the effectiveness in blocking virion production.

Genetic variation near the IL28B gene

Analysis of genetic variation near IL28B gene was performed by use of Invader assay, TaqMan assay, or direct sequencing as described previously [25, 26]. In this study, a single nucleotide polymorphism (SNP) near IL28B gene (rs8099917), reported as one of the predictors of non-response to PEG-IFN and RBV therapy [27], was retrospectively checked.

Results

Patient characteristics

Four females at a median age of 54 years (range 48–58) were enrolled in the study. Patient baseline characteristics are summarized in Table 1. The mean baseline HCV RNA level was 6.1 $\log_{10}$ IU/ml (range 5.2–6.9). The amino acid (aa) sequences of the HCV core region at positions 70 and 91 and the ISDR were also analyzed. The substitution of arginine at core aa 70 was observed in 2 of the 4 patients, whereas the substitution of leucine at core aa 91 was not observed. The number of amino acid substitutions at ISDR aa 2209–2248 was 1 in 2 of the patients and 2 or more in 2 of the patients. We retrospectively checked rs8099917, which is the typical SNP near the IL28B locus associated with non-response to the standard therapy, and confirmed that all 4 study subjects possessed the major allele (T/T).

HCV RNA kinetics

Two of the 4 study patients completed the scheduled telaprevir dosing period of 24 weeks. Patient 2 showed an HCV RNA level below 1.2 $\log_{10}$ IU/ml at the end of treatment, whereas patient 1 showed a negative HCV RNA level at week 8 and had viral breakthrough at week 20 while receiving the study drug. The other 2 patients also showed a rapid decline in viral load to below 2 $\log_{10}$ IU/ml, but they met the stopping rule of viral breakthrough and ceased the study drug at weeks 15 and 6 (patients 3 and 4, respectively).

After the telaprevir monotherapy was stopped, each study patient agreed to enroll in the off-study treatment with PEG-IFN and RBV. By mutual agreement between the patients and investigators, the duration of the standard

Table 1 Baseline characteristics of enrolled patients

Factor	Patient			
	1	2	3	4
Age (years)	48	51	58	57
Sex	Female	Female	Female	Female
Height/body weight (cm/kg)	160.0/51.2	161.4/48.9	165.0/51.6	153.0/49.0
Body mass index (kg/m^2)	20.0	18.8	19.0	20.9
Subtype	1b	1b	1b	1b
Core aa 70/aa 91	R70H/wild	R70H/wild	Wild/wild	Wild/wild
ISDR substituted aa sites	1	2	1	3
IL28B SNP (rs8099917)[a]	T/T	T/T	T/T	T/T

[a] T/T is homozygote of the major allele

Table 2 Summary of the off-study treatment

	Patient			
	1	2	3	4
Baseline (TVR mono/off-study)				
Neutrophils (/μg)	3762/2142	2258/2995	2284/2503	1677/2013
Hemoglobin (g/dl)	14.6/10.8	13.4/10.9	12.9/10.7	12.3/11.7
Platelets (×10^4/μl)	22.4/16.8	28.1/25.9	12.4/14.3	15.5/17.6
ALT (IU/l)	20/11	28/11	40/18	66/91
HCV RNA ($\log_{10}$ IU/l)	6.2/3.7	5.9/3.3	6.9/5.1	5.2/5.0
Dosage				
PEG-IFN α-2b (μg)	80	80	80	80
RBV, max/min (mg)	400/–	600/400	600/200	600/200
Mean RBV (mg/kg/day)	7.8	8.8	8.3	7.2
Accumulated RBV, entire period (g/kg)	2.6	3.9	4.3	2.5
Outcome				
Time after the last TVR (days)	20	26	13	0
HCV RNA negativity (weeks)	2	13	8	8
Duration of treatment (weeks)	48	60	72	48
Treatment response	SVR	SVR	SVR	SVR

TVR telaprevir, *PEG-IFN* peginterferon, *RBV* ribavirin, *SVR* sustained virological response

treatment was decided according to gender, age, substitutions at core aa 70 and 91, the number of substitutions at the NS5A ISDR domain [28], and the time to HCV RNA becoming undetectable (Table 2). Patients 1 and 4 received the off-study treatment for 48 weeks. Patients 2 and 3 were treated beyond 48 weeks, and patient 3 completed 72 weeks of treatment. In patient 2, the off-study treatment was discontinued at week 60 because of the aggravation of subjective symptoms including malaise and insomnia. The HCV RNA levels became negative at 2, 13, 8, and 8 weeks in patients 1, 2, 3, and 4, respectively. SVR was attained in all patients after completion of the off-study treatment (Table 2). Viral kinetics during the 2 courses of treatment are shown in Fig. 1a.

Safety

During the telaprevir monotherapy, all subjects had at least one adverse event with mild to moderate severity. No serious adverse reactions occurred that caused the discontinuation of telaprevir. All patients exhibited a decrease in their hemoglobin levels. Other biochemical blood changes were found in one of each study patient (changes such as increased uric acid, decreased white blood cell count,

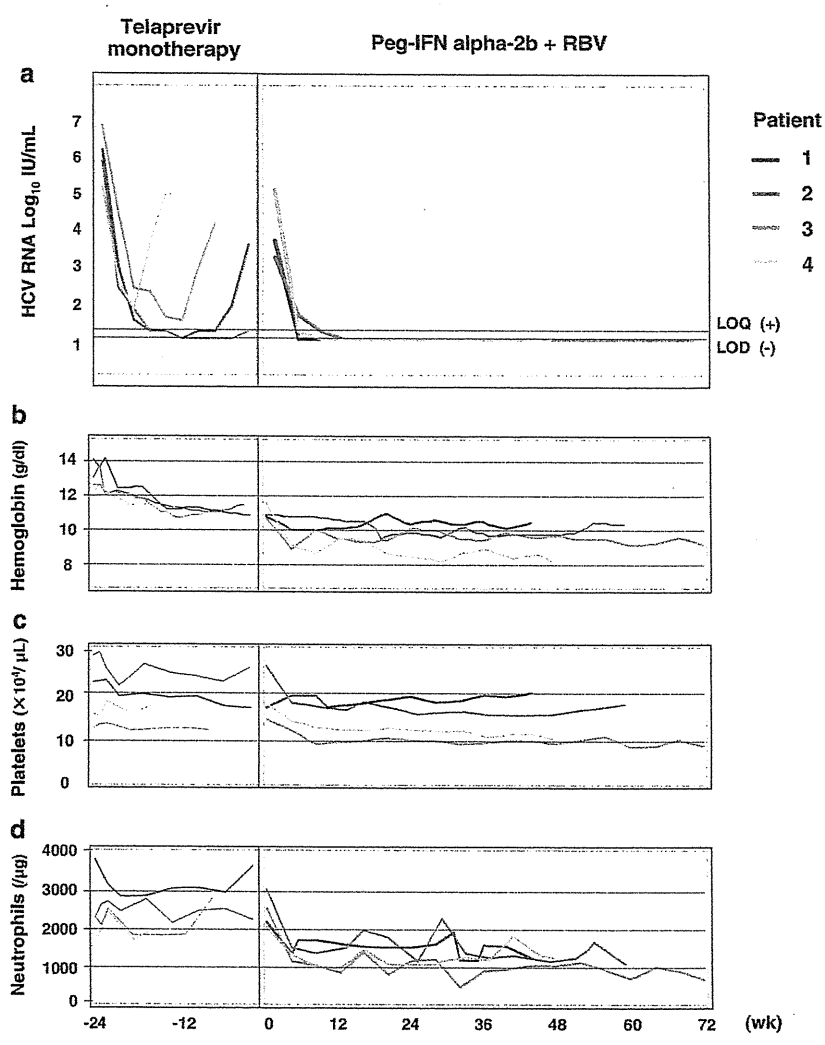

Fig. 1 Changes in plasma HCV RNA, hemoglobin, platelets, and neutrophils for individual patients during administration of telapreivir alone, or peginterferon alpha-2b and ribavirin. Panels on left are telaprevir alone, and those on right are peginterfron alpha-2b and ribavirin therapy. The HCV RNA levels were monitored by the COBAS TaqMan HCV test; limit of quantification (LOQ) is 1.2 $\log_{10}$ IU/ml, with qualitative values below LOQ a positive (+) and limit of detection (LOD) negative (−)

decreased platelet count, and increased serum creatinine level). The observed clinical symptoms were rash, headache, and gastrointestinal symptoms including nausea, stomach discomfort, and gastroesophageal reflux disease, peripheral edema, and pyrexia. No notable adverse event occurred during the off-study treatment with PEG-IFN and RBV except what is usually observed with the standard therapy (Table 3).

The median hemoglobin concentration at the beginning of this study was 13.2 g/dl (range 12.3–14.6) and decreased to 10.9 g/dl (range 10.7–11.7) at the beginning of the off-study treatment (Fig. 1b). No fixed tendency was observed for platelet count and neutrophil count during the course of telaprevir monotherapy, whereas these counts mildly decreased during the course of off-study treatment (Fig. 1c, d).

NS3 protease genotypic analysis

Clonal sequencing analysis on the NS3 protease domain was investigated (Fig. 2). Before the administration of telaprevir, only the wild-type variants were observed in all patients at two time points. Before viral breakthrough, a telaprevir-resistant variant (A156V) could be detected in only 1 patient (patient 3) on day 8 because of rapid viral decline below 3 $\log_{10}$ IU/ml. After emergence of A156V in this patient, the HCV RNA load was still suppressed under the telaprevir monotherapy until week 8; however, another double-substituted variant (T54A+I132L) was detected as the major variant after viral breakthrough. Although patient 4 showed a decrease in the HCV RNA level to 1.8 $\log_{10}$ IU/ml at week 1, viral breakthrough was observed at week 2, and there were two types of resistant variants (A156T and T54A). As the telaprevir treatment was prolonged, the major variant shifted to the double-substituted variant (T54S+A156T). Patient 1 completed the dosing schedule for 24 weeks, but experienced viral breakthrough at week 20. At the end of treatment, the novel substitution of A156F was observed as the major variant. After the withdrawal of telaprevir, other variants including A156Y and T54S+A156T emerged. However, the HCV

Table 3 Adverse events

	Telaprevir monotherapy *n* (%)	Peg-IFN α-2b+RBV *n* (%)
Anemia	4 (100)	4 (100)
Headache	2 (50)	
Rash	2 (50)	
Blood uric acid increased	1 (25)	
Pruritic rash	1 (25)	
Pruritus	1 (25)	
Nausea	1 (25)	
Stomach discomfort	1 (25)	
Gastroesophageal reflux disease	1 (25)	
Peripheral edema	1 (25)	
Pyrexia	1 (25)	
Musculoskeletal stiffness	1 (25)	
White blood cell count decreased	1 (25)	4 (100)
Platelet count decreased	1 (25)	2 (50)
Blood creatinine increased	1 (25)	1 (25)
General fatigue		2 (50)
Loss of appetite		2 (50)
Insomnia		1 (25)
Lack of concentration		1 (25)
Palpitations		1 (25)
Dyspnea		1 (25)

RNA levels remained lower than the baseline (around 4 $\log_{10}$ IU/ml) for 3 weeks, and the major variant further shifted to A156V+V158I just before initiation of the off-study treatment. Patient 2 completed the dosing schedule, with the HCV RNA level below 1.2 $\log_{10}$ IU/ml at the end of treatment. After completion, HCV RNA levels increased, and only the wild-type variant was observed at the 4-week follow-up.

Viral dynamics model analysis

In order to compare the viral dynamics in the initial phase of both treatments, the solved equation from the conventional mathematical model [24] was fitted to the observed values (Fig. 3). The best fit values are summarized in Table 4. At treatment initiation, the HCV RNA levels were equivalent or lower in the off-study treatment than in the telaprevir treatment. The first-phase clearing of telaprevir-resistant variants by the PEG-IFN+RBV treatment was comparable to that of the wild-type variants by telaprevir alone in 3 of the patients. However, in patient 2, the wild-type variants were less susceptible to PEG-IFN+RBV than telaprevir.

Discussion

In this study, 4 treatment-naïve patients with CHC participated in the phase 2a telaprevir monotherapy study in Japan. The subjects were all middle-aged to elderly females infected with HCV subtype 1b, the predominant subtype in Japan. The study patients possessed the baseline viral factors that suggest "difficult to treat" by the standard therapy [28]: the substitution at core aa 70 was observed in patients 1 and 2, and the number of aa substitutions at the NS5A ISDR domain was <1 in patients 1 and 3.

After the completion or discontinuation of the telaprevir monotherapy, PEG-IFN and RBV therapy was initiated as soon as possible to preserve the telaprevir-resistant variants as the majority of the viral population. The standard therapy was initiated soon because the in vivo viral fitness of the telaprevir-resistant variants was estimated to be lower than that of the wild type [20], and some select variants under the telaprevir treatment were susceptible to the PEG-IFN and RBV therapy [21]. Three patients who met viral breakthrough criteria during telaprevir monotherapy definitely had only the telaprevir-resistant variants, including novel substitutions of A156F and A156Y. In addition, the T54S and V158I substitutions, which were reported from the clinical trial of boceprevir [22, 23], were all observed to be a secondary mutation associated with A156 substitutions in this telaprevir trial. Moreover, the other patient (patient 2) had only the wild-type variants at 26 days after the completion of 24 weeks of telaprevir monotherapy. Although it is unclear whether the wild type arose as a reverse mutation, Suzuki et al. [29] recently reported a patient who achieved SVR in the same telaprevir monotherapy trial. These observations suggest a higher genetic barrier for telaprevir among Japanese patients with HCV subtype 1b than in patients observed previously in the EU and US [20, 21]. At least, the telaprevir-resistant variants observed in this study showed some susceptibility to the off-study treatment (Fig. 3).

Anemia has been described as a major adverse event caused by the triple combination therapy including telaprevir [9–11], but the onset mechanism of anemia has not been elucidated. In the phase 1b clinical trial of the triple combination regimen for 12 weeks in Japan, the discontinuation rate due to adverse events was 35% (7 of 20 patients); in 5 of these 7 patients, the triple therapy was discontinued because the hemoglobin decreased to <8.5 g/dl [30]. In the present study, all the patients developed mild anemia after the administration of telaprevir alone for up to 24 weeks (Fig. 1); the median baseline hemoglobin concentration of 13.2 g/dl had decreased to 10.9 g/dl at the initiation of the off-study treatment (Table 2). In 3 of the 4 study patients, the hemoglobin concentration further decreased with the

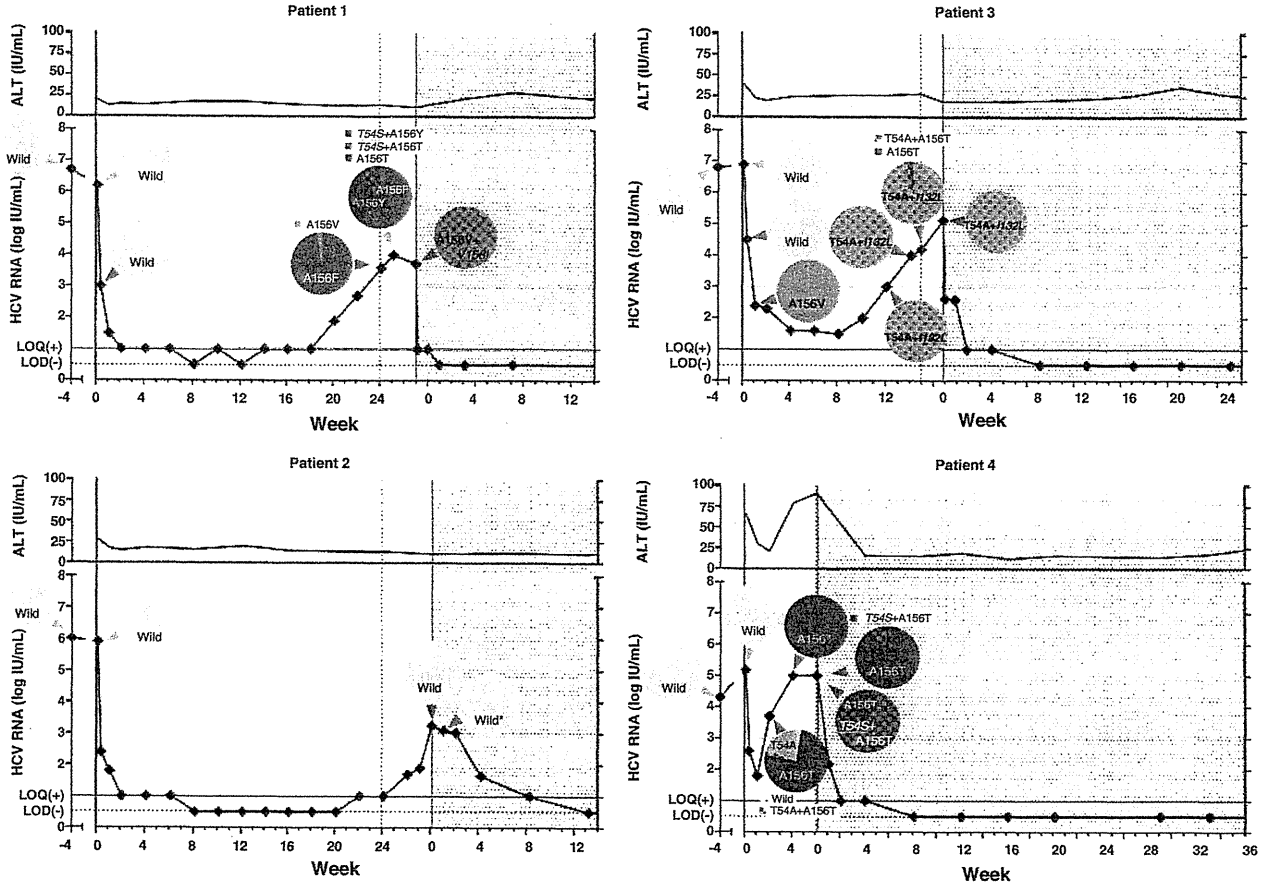

Fig. 2 Viral sequencing results and alanine aminotransferase (ALT) elevation after viral breakthrough for individual patients. *Shaded backgrounds* indicate the off-study treatment. *Circular charts* indicate the population of NS3 protease variants in >39 clones, except the chart indicated by an *asterisk* in 16 clones. *Arrowheads*: *aqua* pretreatment, *red* during treatment, *green* at initiation of off-study treatment

standard treatment; therefore, a dose reduction of RBV was required. Especially in patient 4, the dose of RBV was reduced to 200 mg from the initial dose of 600 mg to maintain the hemoglobin concentration above 8 g/dl. Thus, we managed the decrease in hemoglobin without using erythropoietin. No discontinuation due to anemia occurred during the off-study treatment. Hiramatsu et al. [31] reported that maintaining the RBV dose at >12 mg/kg/day was important even after complete early virological response to avoid relapse after the standard therapy. Although the RBV doses among our 4 study patients ranged from 7.2 to 8.8 mg/kg/day, and the accumulative RBV doses for 48 weeks in patients 1 and 4 were <3 g/kg, SVR was achieved in all cases. Besides relatively lower exposure to RBV, our patient demography of females at a median age of 54 years (range 48–58) is noteworthy. In their study on the standard therapy among Japanese patients infected with HCV subtype 1b, Sezaki et al. [16] reported SVR stratified rates as 53% in males and 22% in females in patients older than 50 years, and no significant gender difference was observed in patients younger than 50 years. However, a study performed in the US suggested higher SVR rates among females than males in patients infected with HCV subtypes 1a and 1b [32]. Although this controversy on gender difference may be attributed to different ethnic groups, the HCV subtypes 1a and 1b were considered to spread in a different epoch [33]. Therefore, we speculate that age distribution of HCV carriers in a certain geographic region exerts an impact on the response rates and severity of anemia with the standard therapy. The recent study on SNPs near the IL28B gene also confirmed that female gender and elderly age remain as factors related to non-virological response [27]. In conclusion, we can avoid treatment failure caused by anemia by carefully adjusting the RBV dosage in the standard therapy that

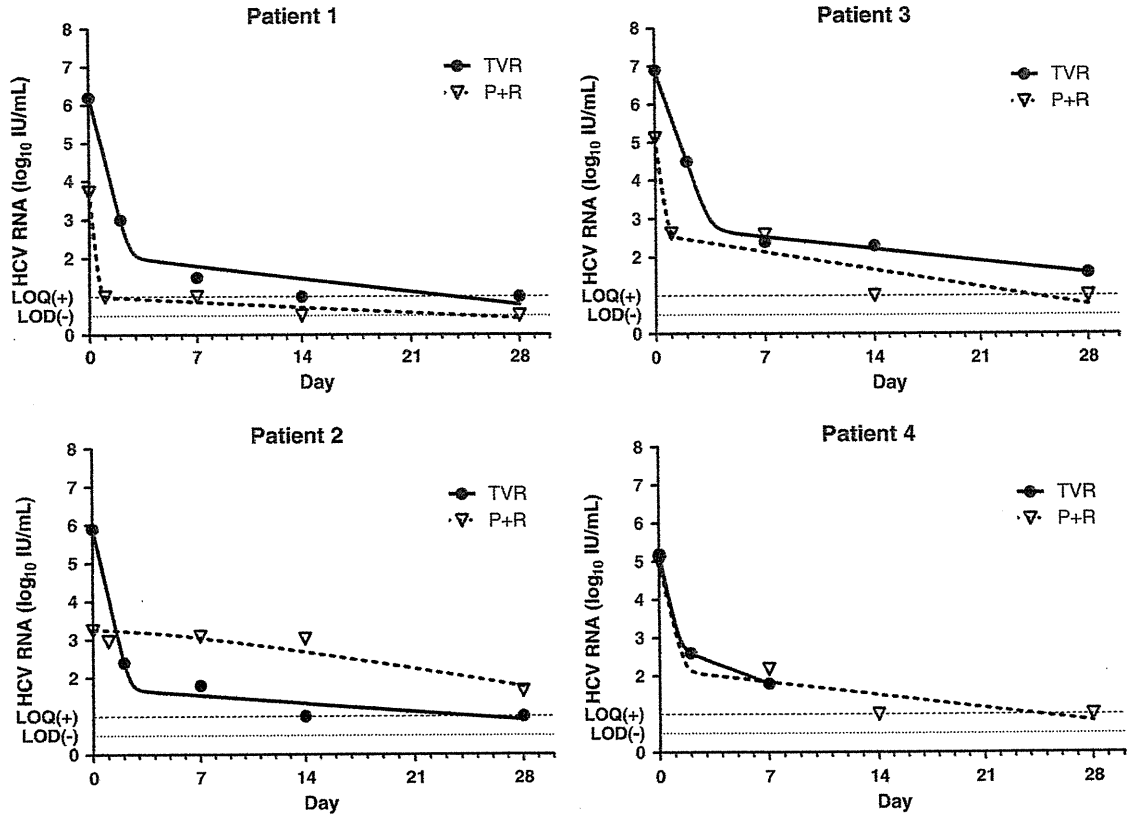

Fig. 3 Viral kinetics modeling on initial 4 weeks of telaprevir alone and peginterferon alpha-2b and ribavirin. *Solid lines* are telaprevir (TVR) alone and *dotted lines* are peginterferon alpha-2b and ribavirin (P+R)

Table 4 Estimates from the viral dynamics modeling analysis

Patient	Treatment	Baseline		Estimated parameters[a]		
		Viral load ($\log_{10}$ IU/ml)	NS3 aa substitution	ε	c (day^{-1})	δ (day^{-1})
1	Telaprevir mono	6.2	Wild	(0.9999)	(3.745)	0.1117
	PEG-IFN+RBV	3.7	A156V+V158I	0.9981	9.342	0.04699
2	Telaprevir mono	5.9	Wild	(0.9999)	(4.139)	0.07018
	PEG-IFN+RBV	3.3	Wild	$<10^{-11}$	0.1913	0.1828
3	Telaprevir mono	6.9	Wild	(0.9999)	(2.772)	0.1018
	PEG-IFN+RBV	5.1	T54A+I132L	0.9971	7.382	0.1494
4	Telaprevir mono[b]	5.2	Wild	(0.9954)	(4.572)	(0.3598)
	PEG-IFN+RBV	5.0	T54S+A156T	(0.9985)	(4.278)	0.1109

[a] ε is the effectiveness in blocking virion production, c is the virion clearance rate from serum, and δ is the clearance rate of infected cells. Software reported parenthetic values as ambiguous

[b] Estimated from days 0–7 because of viral breakthrough

follows telaprevir monotherapy. SVR was initially achieved in all cases. However, relapses occurred in patients who received telaprevir alone, suggesting that the current standard therapy remains important in this sequential regimen.

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Common Genetic Polymorphism of ITPA Gene Affects Ribavirin-Induced Anemia and Effect of Peg-Interferon Plus Ribavirin Therapy

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An association between a single nucleotide polymorphism (SNP) in the inosine triphosphate pyrophosphatase (ITPA) gene and reduction of hemoglobin during peg-interferon plus ribavirin combination therapy for patients with chronic hepatitis C virus (HCV) infection has been reported. However, the effect of the SNP on outcome of therapy has not been fully elucidated. Factors associated with anemia during combination therapy, including rs1127354 genotype, were analyzed in 1,002 treated patients. The effect of the SNP on outcome of therapy was analyzed in a subset of 830 patients with genotype 1. A rapid initial decrease in hemoglobin levels was observed in patients with rs1127354 genotype CC compared with a slow decrease in non-CC patients. Cumulative reduction of ribavirin was significantly more frequent in genotype CC patients than non-CC patients (odds ratio 1.928, $P = 8.6 \times 10^{-8}$). The frequency of patients who received at least the recommended 80% of scheduled ribavirin was significantly lower among genotype CC patients, especially among those who had pretreatment hemoglobin levels between 13.5 and 15 g/dl ($P < 0.03$), and the sustained viral response rate was significantly lower in this group of patients. Independent predictive factors for sustained virological response included a SNP in the IL28B locus (rs809991), age, fibrosis, ITPA SNP rs1127354 as well as pretreatment hemoglobin levels. Our data suggests that measures to prevent anemia should be considered for patients who have pretreatment hemoglobin levels less than 13.5 g/dl or who have rs1127354 genotype CC and pretreatment hemoglobin levels between 13.5 and 15 g/dl. ***J. Med. Virol. 83:1048–1057, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: inosine triphosphate pyrophosphatase; single nucleotide polymorphism; peg-interferon; anemia; dose reduction

INTRODUCTION

Hepatitis C virus (HCV), a positive-strand RNA flavivirus, chronically infects 170 million people worldwide and is responsible for up to 300,000 deaths due

Abbreviations: HCV, hepatitis C virus; ITPA, inosine triphosphate pyrophosphatase; SNP, single nucleotide polymorphism.

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to progression to liver cirrhosis and hepatocellular carcinoma [Alter, 1995; Chevaliez and Pawlotsky, 2007]. Currently, peg-interferon plus ribavirin combination therapy (PEG-RBV) is the most effective treatment, but it is only effective in 50% of patients with genotype 1b, and the therapy has severe side effects often requiring dose modification or discontinuation [Hadziyannis et al., 2004]. However, there are several factors that may help predict outcome of therapy, including HCV genotype [Zeuzem et al., 1996], virus titer [Zeuzem et al., 1996; Dienstag and McHutchison, 2006], age, fibrosis of the liver, obesity, race, hepatic steatosis [Dienstag and McHutchison, 2006], LDL cholesterol, gamma-GTP [Akuta et al., 2007], insulin resistance [Romero-Gómez et al., 2005], amino acid substitutions at positions 70 and 91 of the HCV core protein and accumulation of substitutions in the interferon sensitivity determining region (ISDR) of the NS5A protein [Enomoto et al., 1995a; Akuta et al., 2005]. A series of recent studies have also identified common genetic variants in the IL28B locus on chromosome 19 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] that are strongly associated with outcome of combination therapy.

Ribavirin-induced anemia is a serious side effect of therapy which results in dose reduction of ribavirin and possibly of interferon as well. The precise mechanism of induction of anemia remains to be determined. Ribavirin-induced hemolytic anemia accompanied by an increase in reticulocyte counts has been reported to be associated with membrane oxidative damage as well as impairment of erythrocyte Na–K pump activity and increase in dithiotreitol-sensitive fraction, malondialdehyde, and methemoglobin levels [De Franceschi et al., 2000]. Treating patients with erythropoietin, which induces erythropoiesis and helps alleviate anemia, has been reported to be effective in preventing ribavirin dose reduction and leads to better therapy outcome [Dieterich et al., 2003].

Recently, single nucleotide polymorphisms (SNPs) in the inosine triphosphate pyrophosphatase (ITPA) locus have been found to be associated with anemia in patients treated with combination therapy [Fellay et al., 2010; Ochi et al., 2010; Thompson et al., 2010]. In Caucasian patients there are two SNPs that are associated with ITPA enzyme activity [Fellay et al., 2010; Thompson et al., 2010], although one of these SNPs appears to be absent in Japanese patients [Ochi et al., 2010]. Although the effect of the ITPA polymorphism on ribavirin-induced anemia has been clearly demonstrated by these studies, the effect of the SNP on outcome of therapy has not been fully explored. Our previous report suggested an association of the polymorphism with sustained virological response (SVR) [Ochi et al., 2010], whereas other reports found no association [Fellay et al., 2010; Thompson et al., 2010].

In the current study, 1,002 patients who were treated with peg-interferon 2b plus ribavirin combination therapy were analyzed to elucidate the precise effect of the ITPA SNP on hemoglobin reduction. A subset of 830 of the patients with genotype 1 were further examined to assess the effect of the SNP on therapy outcome. The results show that reduction of ribavirin was frequent among patients with low pretreatment hemoglobin levels (<13 g/dl) as well as those with the ribavirin-sensitive ITPA genotype (rs1127354 CC) and intermediate pretreatment hemoglobin levels (13.5–15 g/dl). Our results suggest that anemia-preventing measures, such as administration of erythropoietin, should be considered for patients likely to develop anemia.

MATERIALS AND METHODS

Patients

Data from 1,002 patients who were treated with peg-interferon alpha 2b and ribavirin combination therapy for chronic hepatitis C infection between December 2004 and January 2010 were collected from Toranomon Hospital (Tokyo) and hospitals belonging to the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html) in Hiroshima, Japan. Patient profiles are shown in Table I. All patients tested positive for HCV RNA for more than 6 months and were negative for hepatitis B and HIV and showed no evidence for other liver diseases including alcoholic hepatitis, hemochromatosis, Wilson's disease, and autoimmune hepatitis. Patients received weekly injections of peg-interferon-alpha-2b at 1.5 g/kg body weight for 48 weeks, and ribavirin was administered orally. The amount of ribavirin was adjusted based on body weight (600 mg for <60 kg, 800 mg for 60–80 kg, and 1,000 mg for >80 kg). Ribavirin dose was reduced when hemoglobin levels fell to 10 g/dl, and both peg-interferon and ribavirin were discontinued when hemoglobin levels dropped to <8.5 g/dl. Patients who remained positive for HCV RNA during the first 12 weeks of treatment but became negative by week 32 received extended administration of both drugs until 72 weeks. The successful endpoint of treatment was considered SVR, defined as undetectable HCV RNA levels 24 weeks after cessation of treatment. A subset of patients showed transient response (TR), in which HCV RNA dropped to undetectable levels but then later rebounded. The remaining patients in which HCV RNA never became undetectable were considered non-responders (NVR). Histopathological diagnosis was made by pathologists at each hospital according to the criteria of Desmet et al. [1994]. All subjects gave written informed consent to participate in the study according to the process approved by the ethical committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

HCV RNA Levels

HCV RNA levels were measured throughout the course of therapy via RT-PCR using the original

TABLE I. Characteristics of Patients by ITPA rs1127354 SNP Genotype

	All patients	Patients with HCV genotype 1		
	Total (n = 1,002)	Total (n = 830)	CC (n = 628)	CA/AA (n = 202)
Age (years)	58 (51–64)	58 (51–64)	58 (52–64)	58 (51–64)
Sex (M/F)	539/463	448/382	328/300	120/82
Height (cm)	161 (154–168)	161 (154–168)	161 (154–168)	161 (155–168)
Weight (kg)	58.5 (52–67)	58.2 (52–66.2)	58.05 (51.8–66.45)	59 (52–65)
rs8099917 (TT/GT/GG)	720/253/25	585/222/20	437/174/15	148/48/5
rs12979860 (CC/CT/TT)	543/198/52	541/197/52	403/151/44	138/46/8
rs1127354 (CC/CA/AA)	753/227/22	628/183/19	628/0/0	0/183/19
Core70 (W/M/ND)	240/143/619	239/143/448	175/114/339	64/29/109
Core91 (W/M/ND)	217/168/617	216/168/446	168/123/337	48/45/109
ISDR (0–1/≥2/ND)	287/80/635	287/80/463	216/61/351	71/19/112
Fibrosis (1/2/3/4/ND)	252/191/124/29/401	252/190/124/29/230	194/138/90/23/179	58/52/34/6/51
Activity (0/1/2/3/ND)	9/252/280/42/419	9/251/280/42/248	6/187/213/31/191	3/64/67/11/57
WBC (/mm^3)	4,700 (3,900–5,600)	4,700 (3,900–5,600)	4,700 (3,900–5,530)	4,900 (4,000–5,942)
Plt ($\times 10^4$/mm^3)	15.6 (12.2–19.7)	15.4 (12.2–19.35)	15.3 (12.1–19.33)	15.9 (12.45–19.4)
Hb (g/dl)	14 (13.2–14.9)	14 (13.2–14.9)	14.1 (13.2–14.9)	14 (13.4–15)
AST (IU/L)	45 (34–66)	45 (34–66)	45 (34–67)	45.5 (34–64.5)
ALT (IU/L)	53 (36–85)	53 (36–85)	52 (36–84.5)	55 (34.5–85)
γGTP (IU/L)	40 (25–73)	40 (25–73)	39.5 (25–72)	43.5 (25.25–77.25)
Total cholesterol (mg/dl)	172 (151–193)	172 (151–193)	172 (150–194)	171 (154–190)
HDL cholesterol (mg/dl)	51 (40–64)	51 (40–64)	52 (40.25–64)	50 (38–63.75)
Fasting blood sugar (mg/dl)	98 (89–112.8)	98 (89–113)	99 (89–113)	95 (88–108)
Virus titer (log IU/ml)	6.5 (6–7)	6.5 (6–7)	6.5 (6–7)	6.5 (6.1–6.9)
Viral genotype (1b/1a/others)	814/9/179	814/9/7	618/6/4	196/3/3
RBV treatment period (weeks)	48 (37–59)	48 (37–59)	48 (34.75–57)	48 (47–64.75)
RBV reduction (no/yes/ND)	316/450/236	315/448/67	212/366/50	103/82/17
Weeks to first RBV reduction	16 (5–48)	16.5 (5–48)	12 (4–47)	44 (12–51.75)
Outcome of therapy (NR/TR/SVR)	154/157/283	154/156/281	125/120/202	29/36/79

ND, not determined or data unavailable.
Categorical variables are reported as counts, and continuous variables are reported as median and interquartile range.

Amplicor method, the high range method, or the TaqMan RT-PCR test. The measurement ranges of these assays were 0.5–850 KIU/ml, 5–5,000 KIU/ml, and 1.2–7.8 log IU, respectively. Samples exceeding the measurement range were diluted with PBS and reanalyzed. All values are reported as log IU/ml.

ISDR and Core aa Substitutions

Amino acid substitutions in the HVC core and ISDR regions were determined by direct sequencing of PCR products following extraction and reverse transcription of HCV RNA using serum samples kept frozen at −80°C. Core amino acid substitutions at positions 70 and 91 (core70 and core91, respectively) were determined according to Akuta et al. [2007, 2006], and the number of ISDR substitutions was established as in Enomoto et al. [1995b, 1996].

SNP Genotyping

Each patient was genotyped for two IL28B SNPs previously reported to be associated with therapy outcome: rs12979860 and rs8099917, and a SNP reported to be associated with ribavirin-induced anemia: rs1127354. Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip, the Invader assay, or the TaqMan assay, as described previously [Ohnishi et al., 2001; Suzuki et al., 2003]. The two SNPs in the IL28B locus are in strong linkage disequilibrium, with a correlation coefficient of 0.99.

Statistical Analysis

The χ^2 and Mann–Whitney U-tests were applied to detect significant associations. Simple and multiple regression analyses were used to examine the association between treatment outcome and the values of other markers, using $P < 0.1$ as the criterion for inclusion in the multivariate model. All of the statistical analyses were two sided, and $P < 0.05$ was considered significant. All statistical analysis was performed using the PASW Statistics 18 program (SPSS, Inc., Chicago, IL).

RESULTS

Reduction of Hemoglobin Levels During Therapy by ITPA Genotype

Decrease in hemoglobin levels during therapy was analyzed by rs1127354 genotype (CC vs. non-CC). As shown in Figure 1, a rapid decrease in hemoglobin levels during the initial 4 weeks was observed in genotype CC patients. Hemoglobin levels in genotype

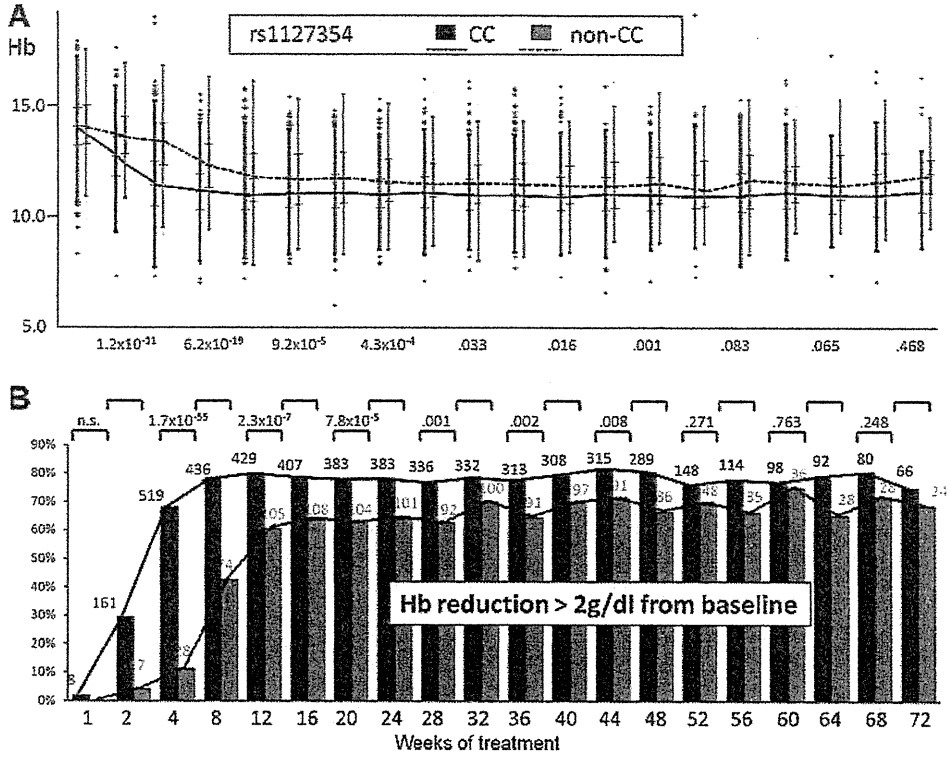

Fig. 1. Reduction of hemoglobin levels by ITPA polymorphism during peg-interferon plus ribavirin combination therapy. **A**: Hemoglobin levels in patients who were treated during the course of therapy. Patients were grouped by ITPA SNP rs1127354 genotype (CC or non-CC). Follow-up hemoglobin levels following cessation of therapy are not shown. **B**: Number of patients who showed >2 g/dl of hemoglobin. Statistical significance was assessed using the χ^2 and Mann–Whitney *U*-tests.

CC patients stabilized by week 8 and did not decrease further. In contrast, a slow but continuous decrease in hemoglobin level was observed in non-CC patients until week 48 (Fig. 1A). Reduction of hemoglobin by more than 2 g/dl was observed significantly more frequently in CC genotype patients than in non-CC patients (Fig. 1B). Differences between the two groups of patients were most pronounced between weeks 2 and 8 (Fig. 1B).

Ribavirin Dose Reduction by ITPA Genotype and Pretreatment Hemoglobin Levels

Decrease in hemoglobin levels resulted in ribavirin dose reduction. The frequency of hemoglobin decrease was higher in genotype CC patients compared with non-CC patients (Fig. 2A). Based on the assumption that initial hemoglobin levels influence incidence of ribavirin dose reduction, reduction frequency was analyzed by initial hemoglobin levels. As shown in Figure 2B–D, reduction of ribavirin was more frequent in genotype CC patients than non-CC patients in all three subsets of patients but was more prominent in patients with intermediate pretreatment hemoglobin levels between 13.5 and 15 g/dl (Fig. 2B–D).

Effect of ITPA Genotype and Pretreatment Hemoglobin Levels on Patients Receiving at Least 80% of Planned Ribavirin Administration

The reduction of ribavirin dosage during therapy resulted in reduction of the total amount of ribavirin given to each patient. As 80% of planned ribavirin administration appears to be a threshold associated with treatment outcome in patients with genotype 1b [McHutchison et al., 2002], the proportion of patients who received more than 80% of the initially planned dosage of ribavirin in 830 patients with genotype 1 and treated with the combination therapy (Table I) were analyzed. As shown in Figure 3, patients with non-CC genotypes tended to tolerate more than 80% of the predetermined dose of ribavirin compared with patients with CC. The difference was statistically significant only in patients whose pretreatment hemoglobin level was 13.5–15 g/dl, however (Fig. 3).

Factors Associated With Successful Administration of at Least 80% of Planned Ribavirin Dose

As it is possible that several factors including ITPA genotype and pretreatment hemoglobin levels are associated with dose reduction of ribavirin, the