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Association of IL28B Variants With Response to Pegylated-Interferon Alpha Plus Ribavirin Combination Therapy Reveals Intersubgenotypic Differences Between Genotypes 2a and 2b

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Genetic polymorphisms of the interleukin 28B (IL28B) locus are associated closely with outcomes of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. The aim of this study was to investigate the relationship between IL28B polymorphism and responses to therapy in patients infected with genotype 2. One hundred twenty-nine chronic hepatitis C patients infected with genotype 2, 77 patients with genotype 2a and 52 patients with genotype 2b, were analyzed. Clinical and laboratory parameters, including genetic variation near the IL28B gene (rs8099917), were assessed. Drug adherence was monitored in each patient. Univariate and multivariate statistical analyses of these parameters and clinical responses were carried out. Univariate analyses showed that a sustained virological response was correlated significantly with IL28B polymorphism, as well as age, white blood cell and neutrophil counts, adherence to RBV, and rapid virological response. Subgroup analysis revealed that patients infected with genotype 2b achieved significantly lower rapid virological response rates than those with genotype 2a. Patients with the IL28B-major allele showed higher virus clearance rates at each time point than those with the IL28B-minor allele, and the differences were more profound in patients infected with genotype 2b than those with genotype 2a. Furthermore, both rapid and sustained virological responses were associated significantly with IL28B alleles in patients with genotype

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; PEG-IFN, pegylated-interferon; RBV, ribavirin; IL28B, interleukin 28B; SNPs, single nucleotide polymorphisms; BMI, body mass index; ALT, alanine transaminase; ISDR, the interferon sensitivity determining region; ITPA, inosine triphosphatase

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2b. IL28B polymorphism was predictive of PEG-IFN plus RBV combination treatment outcomes in patients infected with genotype 2 and, especially, with genotype 2b. In conclusion, IL-28B polymorphism affects responses to PEG-IFN-based treatment in difficult-to-treat HCV patients. ***J. Med. Virol. 83:871–878, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus (HCV); chronic hepatitis C; genotype 2; PEG-IFN plus RBV therapy; combination therapy; IL28B; interferon-λ3

INTRODUCTION

Hepatitis C virus (HCV) infects around 170 million people worldwide and is characterized by a high probability of developing chronic inflammation and fibrosis of the liver, leading to end-stage liver failure and hepatocellular carcinoma (HCC) [Alter, 1997; Sakamoto and Watanabe, 2009]. Since the first report in 1986, type I interferons have been the mainstay of HCV therapy [Hoofnagle, 1994]. Current standards of care consist of a combination of ribavirin (RBV) plus pegylated interferon (PEG-IFN)-alpha for 48 weeks for infection with genotypes 1 and 4, and for 24 weeks for the other genotypes [Zeuzem et al., 2000; Fried et al., 2002]. Although this treatment improved substantially sustained virological response rates, it may result also in serious adverse effects and a considerable proportion of patients require early discontinuation of treatment. Patients of African origin have even poorer treatment outcomes [Rosen and Gretch, 1999]. Given this situation, a precise assessment of the likely treatment outcomes before the initiation of treatment may improve substantially the quality of antiviral treatment.

Recently, several studies have reported that genetic polymorphisms of the IL28B locus, which encodes interferon-λ3 (interleukin 28B), are associated with response to interferon-based treatment of chronic HCV infections with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and also spontaneous clearance of HCV [Thomas et al., 2009].

While chronic HCV infections with genotype 2 are associated with good treatment outcome, there are some refractory cases among patients infected with genotype 2, similar to genotype 1. The aims of this study were to analyze retrospectively clinical and virological factors associated with treatment response in patients with chronic HCV infection with genotype 2 who were treated with PEG-IFN plus RBV combination therapy and to clarify the relationship between IL28B polymorphism and the response to combination therapy.

PATIENTS AND METHODS

The authors analyzed retrospectively 129 patients with chronic HCV infection with genotype 2 who received combination therapy with PEG-IFN plus RBV between December 2004 and December 2009 at 10 multicenter hospitals (liver units with hepatologists) throughout Japan. All patients had chronic active hepatitis confirmed histologically or clinically and were positive for anti-HCV antibodies and serum HCV RNA by quantitative or qualitative assays. Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

Study Design

Each patient was treated with combination therapy with PEG-IFN-α2b (Peg-Intron, Schering-Plough Nordic Biotech, Stockholm, Sweden, at a dose of 1.2–1.5 μg/kg subcutaneously once a week) or PEG-IFN-α2a (Pegasys; Roche, Basel, Switzerland, at a dose of 180 μg subcutaneously once a week) plus RBV (Rebetol, Schering-Plough Nordic Biotech or Copegus; Roche) 600–1,000 mg daily depending on the body weight (b.w.) (b.w. <60 kg: 600 mg po daily; b.w: 60–80 kg: 800 mg po daily; b.w. >80 kg: 1,000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 24 weeks, but treatment reduction or discontinuation was permitted by doctor's decision. The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of treatment. Biochemical and hematological testing was carried out in a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4 weekly intervals, and after therapy at 4 weekly intervals for 24 weeks, by quantitative or qualitative assays.

Patient Evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, gender, body mass index (BMI), previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pretreatment biochemical parameters, such as white blood cells, neutrophils, hemoglobin, platelet count, alanine transaminase (ALT) level, serum HCV RNA level (log IU/ml), and single nucleotide polymorphism (SNPs) in the *IL28B* locus (rs8099917). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity and A3 shows severe activity. Fibrosis was staged on a scale of 0–4:

F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis and F4 shows cirrhosis.

Informed written consent was obtained from each patient who participated in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

SNP Genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphism of IL28B was determined by DigiTag2 assay by typing one tag SNP located within the IL28B locus, rs8099917 (22). Heterozygotes (T/G) or homozygotes (G/G) of the minor allele (G) were defined as having the IL28B minor allele, whereas homozygotes for the major allele (T/T) were defined as having the IL28B major allele.

TABLE I. Baseline Characteristics of Participating Patients Infected With HCV Genotype 2

Total number	129
Genotype (2a/2b)	77/52
IL28B SNPs (rs8099917)	
TT/TG/GG	100/28/1
Age (years)[a]	64 (20–73)
Gender (male/female)	64/65
Body mass index (kg/m^2)[a] (N = 80)	23.7 (16.9–33.5)
Previous interferon therapy (no/yes)	102/21 (unknown 6)
Histology at biopsy (N = 96)	
Grade of inflammation	
A0/1/2/3	10/53/29/4
Stage of fibrosis	
F0/1/2/3	7/59/19/11
White blood cells (/μl)[b] (N = 94)	5,115 ± 1,630
Neutrophils (/μl)[b] (N = 94)	2,765 ± 1,131
Hemoglobin (g/dl)[b] (N = 95)	14.2 ± 1.3
Platelet count (×10^{-3}/μl)[b] (N = 98)	187 ± 95
ALT (IU/L)[b] (N = 95)	82 ± 78
Serum HCV-RNA level (log(IU/ml))[a,c]	6.2 (3.6–7.4)
Treatment duration (>16, ≦24)	19/110

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase.
[a]Data are shown as median (range) values.
[b]Data are expressed as mean ± SD.
[c]Data are shown as log(IU/ml)).

Outcomes

The primary end point was a sustained biochemical and virological response. A sustained virological response was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were a rapid virological response (HCV RNA undetectable in serum at week 4) and end-of-treatment virological response. In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response explored.

Statistical Analysis

SPSS software package (SPSS 18J, SPSS, Chicago, IL) was used for statistical analysis. Discrete variables were evaluated by Fisher's exact probability test and distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All *P*-values were calculated by two-tailed tests, and those of less than 0.05 were considered statistically significant.

RESULTS

Clinical Characteristics and Response to Therapy

The clinical characteristics and response rates to therapy of 129 patients are summarized in Tables I and II. Sixty-eight patients achieved a rapid virological response, whereas 44 patients remained HCV-RNA positive at week 4. Treatment reduction or cessation was permitted also to avoid side effects, and one patient stopped treatment at week 12 because he was anticipated to be a non-responder. On an intention-to-treat analysis, serum HCV-RNA levels were negative at the end of treatment in 125 of the 129 patients (97%) treated and, among them, 98 (76%) achieved a sustained virological response. The rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a ($P = 0.036$) (Table II). The sustained virological response rate decreased with RBV drug discontinuation and dose reduction (84% and 66% with ≧80% and <80% of RBV dose, $P = 0.021$, Table III). Adherences to PEG-IFN did not influence a sustained virological response or end of treatment response significantly, while RBV adherence was associated significantly with a sustained virological response (Table III).

Factors Associated With a Sustained Virological Response

Next the host clinical and viral factors associated with a sustained virological response were analyzed. Univariate statistical analysis showed that six parameters were associated significantly with the sustained virological response rates, including age, white blood cells, neutrophils, adherence to RBV, rapid virological response and an IL28B SNP (rs8099917) (Table IV). There was no significant association of sustained virological response with gender, previous interferon therapy, stage of fibrosis, pretreatment HCV titer or adherence to PEG-IFN. Further multivariate analyses were conducted using significant factors identified by the univariate analysis (Table V). The multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response (OR = 0.170, $P = 0.019$).

TABLE II. Response Rates to Therapy

Character			Number/total number (%)
Overall			
RVR			68/112 (61)
ETR			125/129 (97)
SVR			98/129 (76)
Genotype	2a	2b	*P*-value
RVR	46/67 (69)	22/45 (49)	**0.036**
ETR	74/77 (96)	51/52 (98)	NS
SVR	56/77 (73)	42/52 (81)	NS

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.
Bold indicated *P*-value of less than 0.05.

TABLE III. Response Rates to Treatment According to Drug Adherence

	≧80%	<80%	*P*-value
PEG-IFN adherence			
ETR	94/96 (98)	31/33 (94)	NS
SVR	75/96 (78)	23/33 (70)	NS
RBV adherence			
ETR	72/73 (99)	53/56 (95)	NS
SVR	61/73 (84)	37/56 (66)	**0.021**

ETR, end of treatment response; SVR, sustained virological response; PEG-IFN, pegylated interferon; RBV, ribavirin.
The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy.
Bold indicated *P*-value of less than 0.05.

Comparison of Sustained Virological Response Rates According to IL28B SNPs

The PEG-IFN plus RBV treatment efficacy was compared after dividing the study subjects into two groups based on IL28B alleles (Table VI). Patients homozygous for the IL28B major allele (TT allele) achieved significantly higher rapid and sustained virological response rates than those heterozygous or homozygous for the IL28B minor allele (TG/GG alleles) ($P < 0.05$). In addition, responses to PEG-IFN plus RBV treatment were analyzed after dividing the study subjects into those with genotype 2a and with genotype 2b. The rapid and sustained virological response rates tended to be higher in patients homozygous for the IL28B major allele than those heterozygous or homozygous for the

TABLE IV. Clinical and Virological Characteristics of Patients Based on Therapeutic Response

	SVR (n = 98)	Non-SVR (n = 31)	*P*-value
Genotype (2a/2b)		56/42	21/10
IL28B SNPs (rs8099917)			
TT/TG + GG	81/17	19/12	**0.024**
Age (years)[a]	56 (20–73)	61 (40–72)	**0.002**
Gender (male/female)	51/47	13/18	NS
Body mass index (kg/m^2)[a]	22.8 (16.9–33.5)	24.1 (20.3–27.6)	NS
Previous Interferon therapy (no/yes)	80/14	22/7	NS
Grade of inflammation (A0-1/2-3)	46/28	15/7	NS
Stage of fibrosis (F0-2/3-4)	64/10	21/1	NS
White blood cells (/μl)[b]	5,318 ± 1,617	4,489 ± 1,540	**0.032**
Neutrophils (/μl)[b]	2,913 ± 1,139	2,278 ± 983	**0.021**
Hemoglobin (g/dl)[b]	14.2 ± 1.4	14.1 ± 1.1	NS
Platelet count ($\times 10^{-3}$/μl)[b]	193 ± 105	171 ± 54	NS
ALT (IU/ml)[b]	79 ± 73	94 ± 92	NS
Pretreatment Serum HCV-RNA level (log(IU/ml))[a,c]	6.1 (3.6–7.4)	6.3 (4.0–6.7)	NS
PEG-IFN adherence (≧80%/<80%)	75/23	21/10	NS
RBV adherence (≧80%/<80%)	61/37	12/19	**0.024**
RVR/non-RVR	57/24	11/20	**0.001**

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase; RVR, rapid virological response.
[a]Data are show as median (range) values.
[b]Data are expressed as mean ± SD.
[c]Data are shown as log (IU/ml)).
Bold indicated *P*-value of less than 0.05.

TABLE V. Multivariate Analysis for the Clinical and Virological Factors Related to Sustained Response With Peg-IFN Plus RBV Therapy in 63 Patients

Factor	Category	Odds ratio (95% CI)	*P*-value
Regression analysis			
RVR	RVR	1	**0.019**
	Non-RVR	0.170 (0.039–0.744)	
RBV adherence	≧80%	1	0.061
	<80%	0.250 (0.059–1.064)	
IL28B SNPs (rs8099917)	TT	1	0.104
	TG + GG	0.252 (0.048–1.330)	
Age		1.087 (0.976–1.211)	0.128
Neutrophils		0.999 (0.997–1.001)	0.209
White blood cells		1.000 (0.999–1.002)	0.504

CI, confidence interval; SNPs, single nucleotide polymorphisms; RVR, rapid virological response, RBV, ribavirin.
Bold indicated *P*-value of less than 0.05.

IL28B minor allele infected with both genotype 2a and 2b, and these differences were more profound in patients infected with genotype 2b than with genotype 2a. The rapid and sustained virological response rates of patients with the major IL28B allele were higher significantly than those of patients with the minor IL28B allele infected only with genotype 2b (rapid virological response: 58% and 0% with IL28B major and hetero/minor, $P = 0.002$, sustained virological response: 88% and 44% with IL28B major and hetero/minor, $P = 0.009$).

Although the rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a, the sustained virological response rate was higher in patients infected with genotype 2b than with genotype 2a (Table II), In order to investigate that discrepancy, sustained virological response rates in patients with or without rapid virological response were analyzed according to IL28B SNPs. In patients infected with genotype 2b and a non-rapid virological response, the sustained virological response rates differed significantly between IL28B major and hetero/minor groups (sustained virological response with non-rapid virological response: 75% and 29% with IL28B major and hetero/minor, $P = 0.044$), and no one achieved a rapid virological response among the patients infected with genotype 2b and with the IL28B hetero/minor allele. In patients infected with genotype 2a, on the contrary, there was no significant correlation of rapid and sustained virological response rates between IL28B SNPs (sustained virological response with rapid virological response: 78% and 70% with IL28B major and hetero/minor, $P = 0.630$, sustained virological response with non-rapid virological response: 57% and 43% with IL28B major and hetero/minor, $P = 0.552$).

Next, changes in virological response rates over time were investigated in patients treated with PEG-IFN plus RBV and the time course was analyzed after separating the patients infected with genotype 2a and 2b (Fig. 1). Patients with IL28B-TG and -GG showed significantly lower rates of rapid and sustained virological response, compared to patients with IL28B-TT, and greater differences were observed according to IL28B SNPs among patients infected with genotype 2b than with 2a.

Side Effects

Side effects leading to Peg-IFN plus RBV discontinuation occurred in eight patients (6.2%) and discontinuation of RBV alone occurred in four patients (3.1%).

TABLE VI. Rapid and Sustained Virological Response Rates to Treatment According to IL28B SNPs

Character	IL28B major	IL28B hetero/minor	*P*-value
Number/total number (%)			
Overall			
RVR	58/88 (66)	10/24 (42)	0.031
SVR	81/100 (81)	17/29 (59)	0.013
Genotype 2a			
RVR	36/50 (72)	10/17 (59)	NS
SVR	43/57 (75)	13/20 (65)	NS
Genotype 2b			
RVR	22/38 (58)	0/7 (0)	0.002
SVR	38/43 (88)	4/9 (44)	0.009

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.

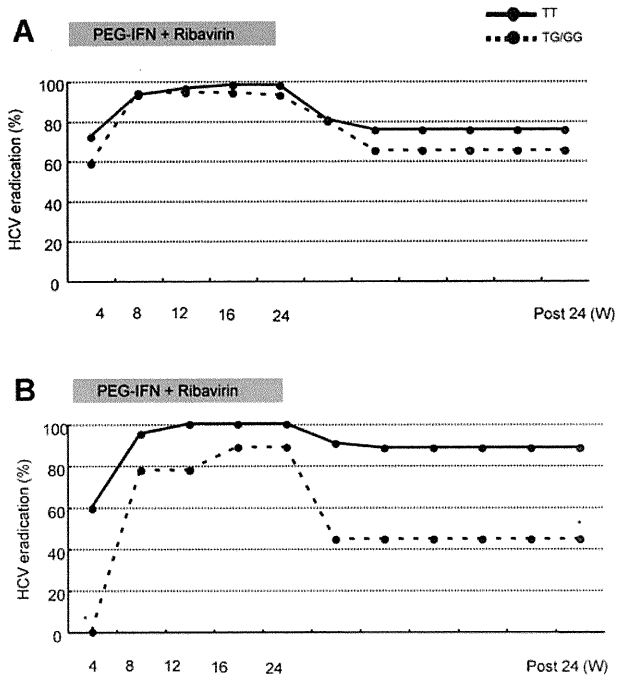

Fig. 1. Changes over time in virological response rates were confirmed in patients treated with PEG-IFN plus RBV, and the time courses were analyzed after separating the patients infected with genotypes 2a and 2b. Patients with the IL28B major (TT allele) are indicated in the figure by a continuous line and those with IL28B hetero or minor (TG or GG), by a dotted line. IL28B-TG and -GG patients showed significantly lower rates of rapid and sustained virological response, compared to IL28B-TT patients. *P*-values were two-tailed and those of less than 0.05 were considered to be statistically significant. $^{*}P < 0.01$.

Among the eight patients who withdrew from both drugs, four, including one who stopped at week 7, had achieved a sustained virological response. Among four patients who withdrew from RBV alone, three had achieved a sustained virological response. The events leading to drug withdrawal were HCC treatment (n = 2), general fatigue (n = 2), retinopathy, neuro-psychiatric event, severe dermatological symptoms suggestive of the drug-induced hypersensitivity syndrome, and arrhythmia.

DISCUSSION

Recent studies suggest that genetic variations in IL28B are strongly associated with response to therapy of chronic HCV infection with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and with spontaneous HCV clearance [Thomas et al., 2009]. In this study, univariate analyses showed that the sustained virological response was correlated significantly with IL28B polymorphism (rs8099917) as well as age, adherence to RBV and rapid virological response, and multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response in all patients infected with genotype 2 (Table V). Although the IL28B polymorphisms are not so useful for predicting the clinical outcomes of PEG-IFN plus RBV combination therapy among patients with genotype 2, compared to genotype 1, IL28B polymorphism was predictive of PEG-IFN plus RBV treatment outcomes among patients with genotype 2 and, more remarkably, among patients with genotype 2b in this study. Indeed, both rapid and sustained virological response rates according to the rs8099917 genotypes were different significantly in patients with genotype 2b but not in patients with genotype 2a. Furthermore, in the plot of virological response (Fig. 1), a stronger effect of the IL28B allele was observed in patients with genotype 2b than with genotype 2a.

It has been reported that there was no significant association between genetic variation in IL28B and response to therapy of HCV patients infected with genotype 2 or 3, indicating that the prognostic value of the risk allele for treatment response might be limited to individuals with difficult-to-treat HCV genotypes [Rauch et al., 2010]. This report lacks details of the distribution of the various genotypes. The present study agrees with a more recent report that the IL28B polymorphism was associated with a sustained virological response in patients with chronic HCV infection with genotype 2 or 3 who did not achieve a rapid virological response [Mangia et al., 2010]. In Japan, the percentage of HCV infection with genotype 1b is 70%, genotype 2a is 20% and genotype 2b is 10%, whilst other genotypes are observed only rarely. In this study, the association of IL28B polymorphism with response to therapy was analyzed in more detail, considering the subtypes 2a and 2b, and IL28B polymorphism (rs8099917) found to be linked more closely to the virological response of patients infected with genotype 2b than those with genotype 2a. A recent in vitro study, which constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), also supported subgenotypic differences between genotype 2a and 2b [Suda et al., 2010]. The authors speculated that the prognostic value of the risk allele for treatment response might be more pronounced in individuals with difficult-to-treat HCV subgenotypes, such as patients infected with genotype 2b, compared with 2a. In addition, the prevalence of the IL28B minor allele is much higher in Caucasians and African Americans than in eastern Asian populations [Thomas et al., 2009], which suggest that the effects of IL28B polymorphism could be more pronounced in non-Asian populations. In the present results, however, the sustained virological response rate of patients infected with genotype 2b was higher than that of patients with genotype 2a overall. We speculate that, among patients infected with genotype 2b, only those with the IL28B minor variant might be treatment-refractory. That possibility might be validated further by a larger cohort study with genotype 2b.

The sustained virological response rates decreased significantly with failure of adherence to RBV (Table III), which was extracted as a factor associated with sustained virological response by univariate

analysis (Table IV). Regardless of the drug adherence, end of treatment response rates of patients infected with genotype 2 were around 94–99%, but the sustained virological response rates of the patients who received a total cumulative treatment dose of RBV of <80% was reduced significantly. As reported previously, increased RBV exposure during the treatment phase was associated with an increased likelihood of a sustained virological response [McHutchison et al., 2009] and these results confirm the importance of RBV in order to prevent relapse. Furthermore, host genetic variation leading to inosine triphosphatase (ITPA) deficiency protects against hemolytic anemia in chronic hepatitis C patients receiving RBV as revealed recently [Fellay et al., 2010]. We have reported also that the *ITPA* SNP, rs1127354, is confirmed to be a useful predictor of RBV-induced anemia in Japanese patients and that the incidence of early dose reduction was significantly higher in patients with ITPA-major (CC) variant as expected and, more importantly, that a significant higher sustained virological response rate was achieved in patients with the *ITPA-hetero*/minor (CA/AA) variant with non-genotype 1 or low viral loads [Sakamoto et al., 2010].

A rapid virological response was extracted in this study as a factor associated with sustained virological response only by multivariate analysis. It has been reported recently that a rapid virological response is an important treatment predictor and that drug adherence, which is reported to affect the therapeutic efficacy in patients infected with genotype 1, had no impact on the both sustained and rapid virological responses in combination therapy for patients infected with genotype 2 [Inoue et al., 2010]. The reasons why several host factors useful for predicting the response to therapy in patients with genotype 1, such as gender, age, progression of liver fibrosis and IL28B polymorphism had no influence on the efficacy in patients with genotype 2, can be attributed to IFN-sensitive genotypes. Similarly, the other viral factors useful for predicting the response to therapy, such as viral load and amino acid substitutions in the Core and NS5A regions had no influence on treatment outcomes. In this study, patients who achieved a rapid virological response had a high sustained virological response rate, regardless of IL28B polymorphism in patients with genotype 2a but, interestingly, none of the IL28B-TG and -GG patients with genotype 2b achieved a sustained virological response (although there were nine IL28B-TG and -GG patients with genotype 2b, two could not be determined as rapid virological response because the times at which they became HCV-negative were not recorded clearly, being described as 4–8 weeks.) These results also suggest that patients with both genotype 2b and IL28B minor allele are refractory cases.

IL28B encodes a protein also known as IFN-λ3 [O'Brien, 2009]. *IL28A* (IFN-λ2) and *IL29* (IFN-λ1) are found adjacent to *IL28B* on chromosome 19. These three IFN-λ cytokines, discovered in 2003 by two independent groups [Kotenko et al., 2003; Sheppard et al., 2003] have been suggested to be involved in the suppression of replication of a number of viruses, including HCV [Robek et al., 2005; Marcello et al., 2006; Tanaka et al., 2010]. Humans have these three genes for IFN-λ, and this group of cytokines is now collectively referred to as type III IFN [Zhou et al., 2007]. IFN-λ functionally resembles type I IFN, inducing antiviral protection in vitro [Kotenko et al., 2003; Sheppard et al., 2003] as well as in vivo [Ank et al., 2006]. Type III IFN utilizes a receptor complex different from that of type I IFN, but both types of IFN induce STAT1, STAT2, and STAT3 activation by activation of a highly overlapping set of transcription factors, and the two types of IFN seem to have similar biological effects at a cellular level. Some in vitro studies have suggested that IFN-α induces expression of IFN-λ genes [Siren et al., 2005]. Other in vitro studies also suggest that IFN-λ inhibits hepatitis C virus replication through a pattern of signal transduction and regulation of interferon-stimulated genes that is distinct from IFN-α and that the anti-HCV activity of either IFN-α or IFN-λ is enhanced by a low dose of the other [Marcello et al., 2006]. A novel mechanism of the interaction between IFN-α and IFN-λ may play a key role in the suppression of HCV [O'Brien, 2009].

In conclusion, IL28B polymorphism is predictive of PEG-IFN plus RBV treatment outcomes in patients infected with genotype 2, and more remarkably with genotype 2b. These results suggest that IL-28B polymorphism affects responses to IFN-based treatment in more difficult-to-treat subpopulations of HCV patients, and that intersubgenotypic differences between genotype 2a and 2b are revealed by responses to PEG-IFN plus RBV treatment according to IL28B variants.

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Genome-wide association study identified *ITPA/DDRGK1* variants reflecting thrombocytopenia in pegylated interferon and ribavirin therapy for chronic hepatitis C

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Hematologic abnormalities during current therapy with pegylated interferon and ribavirin (PEG-IFN/RBV) for chronic hepatitis C (CHC) often necessitate dose reduction and premature withdrawal from therapy. The aim of this study was to identify host factors associated with IFN-induced thrombocytopenia by genome-wide association study (GWAS). In the GWAS stage using 900K single-nucleotide polymorphism (SNP) microarrays, 303 Japanese CHC patients treated with PEG-IFN/RBV therapy were genotyped. One SNP (rs11697186) located on *DDRGK1* gene on chromosome 20 showed strong associations in the minor-allele-dominant model with the decrease of platelet counts in response to PEG-IFN/RBV therapy [$P = 8.17 \times 10^{-9}$; odds ratio (OR) = 4.6]. These associations were replicated in another sample set ($n = 391$) and the combined *P*-values reached 5.29×10^{-17} (OR = 4.5). Fine mapping with 22 SNPs around *DDRGK1* and *ITPA* genes showed that rs11697186 at the GWAS stage had a strong linkage disequilibrium with rs1127354, known as a functional variant in the *ITPA* gene. The

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ITPA-AA/CA genotype was independently associated with a higher degree of reduction in platelet counts at week 4 ($P < 0.0001$), as well as protection against the reduction in hemoglobin, whereas the CC genotype had significantly less reduction in the mean platelet counts compared with the AA/CA genotype ($P < 0.0001$ for weeks 2, 4, 8, 12), due to a reactive increase of the platelet count through weeks 1–4. Our present results may provide a valuable pharmacogenetic diagnostic tool for tailoring PEG-IFN/RBV dosing to minimize drug-induced adverse events.

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) presents a significant health problem worldwide, with ~2.3% of the world population, i.e. more than 120–130 million people, being infected (1). Only 20–30% of HCV-infected individuals recover spontaneously. The remaining 70–80% go on to develop chronic infection, being at significant risk for progressive liver fibrosis and subsequent liver cirrhosis (LC) and hepatocellular carcinomas (HCC). Successful treatment of chronic hepatitis C (CHC) leads to a reduction of liver fibrosis stage of patients, and also prevents HCC development (2).

Antiviral treatment has been shown to improve liver histology and decrease incidence of hepatocellular carcinoma in CHC (3,4). Current therapy for CHC consists of treatment with pegylated interferon (IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral pro-drug that interferes with RNA metabolism (5,6). However, <50% of patients infected with HCV genotype 1 treated in this way achieve a sustained viral response (SVR) or cure of the infection (5,7). Older patients with liver fibrosis showed a significantly lower SVR rate due to poor adherence resulting from adverse events and laboratory abnormalities (8–10). In particular, hematologic abnormalities often necessitate dose reduction, and premature withdrawal from therapy in 10–14% of patients (5,11–14). New drugs and therapeutic approaches for CHC are actively developed and several candidates are in early trial phase (15,16). Given this background, effective pre-treatment screening for predictive biomarkers with the aim of evaluating possible risks over benefits of currently available treatment will avoid these side effects in patients who will not be helped by treatment, as well as reduce the substantial cost of treatment.

The completion of the Human Genome Project has led to the advent of a new era of scientific research, including a revolutionary approach: the genome-wide association study (GWAS). Several recent studies, including our study, have demonstrated marked associations between single-nucleotide polymorphisms (SNPs) within and around *IL28B* gene, which codes for IFN–λ3 (16–21). Another recent study indicated that genetic variants of *ITPA* gene leading to inosine triphosphatase (ITPA) deficiency could protect against hemolytic anemia (HA) in CHC patients receiving RBV (22).

In Japan, HCV-infected patients are relatively old and some of them have had severe fibrosis (9). Thrombocytopenia is one of the critical adverse events by IFN-based therapy among liver cirrhotic patients (23), because low platelet count (PLT), i.e. <30.0 (10^9/l), would be a risk factor for any bleeding, as well as it would lead to poor treatment efficiency due to the initial or early dose reduction of PEG-IFN. Based on its pathogenesis, drug-induced thrombocytopenia is usually due to bone marrow suppression, immune-mediated destruction and platelet aggregation (24). In this study, we firstly found that genetic variants in the *ITPA/DDRGK1* genes were associated with IFN-induced thrombocytopenia, and then examined the correlation between IFN-induced thrombocytopenia and RBV-induced HA in Japanese CHC patients under PEG-IFN/RBV treatment.

RESULTS

Genetic variants associated with IFN-induced thrombocytopenia

In this study, we conducted a GWAS to identify host genes associated with the decrease of platelets in response to PEG-IFN/RBV treatment in 303 Japanese HCV patients (107 patients with the decrease of PLT versus 196 patients without the decrease of PLT based on the criteria described in Materials and Methods), using a genome-wide SNP typing array (Affymetrix SNP 6.0 for 900K SNPs). The characteristics of patients for each GWAS stage and replication stage are summarized in Table 1. Figure 1 shows a genome-wide view of the single-point association data based on allele frequencies. One SNP (rs11697186) located on *DDRGK1* gene on chromosome 20 showed strong associations in the allele frequency model ($P = 8.17 \times 10^{-9}$) with the decrease of PLT in response to PEG-IFN plus RBV treatment. The association reached genome-wide level of significance [Bonferroni criterion $P < 8.40 \times 10^{-8}$ (0.05/595052)], and another SNP (rs6139030) near *ITPA* gene had a marginal significance ($P = 4.30 \times 10^{-7}$, in Table 2).

To validate the results of the GWAS stage, 22 SNPs were selected for the replication in a set of 391 Japanese HCV patients with and without platelet reduction (Supplementary Material, Table S1). The associations of the original significant SNP (rs11697186) and the marginal SNP (rs6139030) at the GWAS stage were replicated in the second set of 391 patients in the minor-allele-dominant model [$P = 5.88 \times 10^{-10}$, odds ratio (OR) = 4.6 for rs11697186; $P = 3.83 \times 10^{-10}$, OR = 4.3 for rs6139030, Table 2]. The combined *P*-values for both stages reached 5.29×10^{-17} (OR = 4.5; 95% CI = 3.1–6.5) and 1.33×10^{-15} (OR = 3.9; 95% CI = 2.8–5.5), respectively (Table 2).

Genetic variants associated with RBV-induced anemia

We also conducted a GWAS to identify host genes associated with a quantitative change in hemoglobin (Hb) levels from baseline to week 4 of PEG-IFN/RBV treatment in the above 303 Japanese HCV patients (94 patients with an Hb reduction of ≥3 g/dl at week 4 and 209 patients without Hb reduction), using a genome-wide SNP typing array (Affymetrix SNP 6.0 for 900K SNPs). Two SNPs (rs11697186 and rs6139030)

Table 1. Clinical characteristics of patients in this study

	GWAS ($n = 303$)	Replication ($n = 391$)
Age	57.4 (9.7)	56.8 (9.9)
Sex (M/F)	151/152	209/182
Weight (kg)	60.6 (10.4)	61.3 (10.7)
Body mass index	23.5 (3.1)	23.7 (4.1)
Baseline Hb (g/dl)	14.1 (1.4)	14.1 (1.4)
Baseline platelet count (10^9/l)	151.3 (54.3)	159.7 (55.0)
Baseline ALT (IU/l)	83.5 (79.4)	86.8 (71.9)
Baseline creatinine (mg/dl)	0.70 (0.15)	0.72 (0.16)
Baseline liver fibrosis (F0–2/F3–4/ND)	153/77/73	175/59/43
rs8099917: TT/non-TT	165/138	296/95
rs1127354: AA/CA/CC	4/79/220	6/101/284
Week 4 Hb (g/dl)	11.8 (1.7)	11.9 (1.5)
Week 4 platelet count (10^9/l)	127.6 (48.2)	132.4 (51.0)
Hb reduction at week 4	−2.3 (1.4)	−2.2 (1.4)
Platelet reduction at week 4	−22.2 (38.4)	−24.7 (30.4)

located on *DDRGK1* gene and *ITPA* gene on chromosome 20 showed strong associations in the allele frequency model ($P = 3.29 \times 10^{-10}$ and $P = 2.56 \times 10^{-9}$) with Hb reduction in response to PEG-IFN plus RBV treatment (Table 3).

The above 22 SNPs were selected for the replication study and fine mapping, including rs1127354, which was reported by the US group (22) to be strongly associated with Hb reduction (Supplementary Material, Table S2). All SNPs were genotyped using the DigiTag2 assay in an independent set of 391 Japanese HCV patients with quantitative change in Hb in response to PEG-IFN/RBV treatment [137 patients with Hb reduction versus 254 patients without Hb reduction (Table 3)]. The associations of the original SNPs were replicated in the second set of 391 patients in the minor-allele-dominant model ($P = 3.86 \times 10^{-16}$, OR = 0.02 for rs11697186; $P = 6.90 \times 10^{-18}$, OR = 0.03 for rs6139030, Table 3). The combined *P*-values for both stages reached 9.43×10^{-25} (OR = 0.03; 95% CI = 0.01–0.08) and 2.12×10^{-25} (OR = 0.04; 95% CI = 0.02–0.09), respectively (Table 3). The rs1127354 was also strongly associated with a quantitative change in Hb in response to PEG-IFN/RBV treatment in a set of 694 Japanese HCV patients (303 patients from the GWAS stage plus the second set of 391 patients) with and without Hb reduction ($P = 4.58 \times 10^{-26}$, OR = 0.03; 95% CI = 0.01–0.08).

Fine mapping with 22 SNPs around *DDRGK1* and *ITPA* genes showed that four significant SNPs (rs11697186, rs6139030, rs1127354 and rs13830) at the GWAS stage had a strong linkage disequilibrium (LD) ($r^2 > 0.86$) within the 22.7 kb region (Fig. 2). As the rs1127354 is known as a functional variant in the *ITPA* gene that caused ITPase deficiency and protected against RBV-induced HA (22,25), the representative SNP was applied for the following detailed studies.

ITPA/DDRGK1 variants reflect anemia and reactive increase of the platelet count

The mean quantitative reduction of blood cells from the baseline according to the *ITPA* rs1127354 genotypes is shown in Figure 3. Patients with the rs1127354 genotypes AA and CA showed lower degree of Hb reduction at weeks 2, 4, 8 and 12 during therapy compared with those with the CC genotype ($P < 0.0001$ for weeks 2, 4, 8 and 12 in Fig. 3A). The most difference of mean Hb reduction was found at week 4 (AA/CA −1.14 versus CC −2.72). These results show that the AA and CA genotypes are significantly associated with less absolute reduction in Hb levels, especially during the early weeks of therapy, and protect against the development of severe anemia. Interestingly, the CC genotype had significantly less reduction in the mean platelet count compared with the AA/CA genotype ($P < 0.0001$ for weeks 2, 4, 8; $P = 0.019$ for week 12 in Fig. 3B), due to a reactive increase of platelet count through weeks 1–4. The most difference of mean platelet reduction was found at week 4 [AA/CA −41.2 versus CC −18.0 (10^9/l)]. There was no difference in the neutrophil leukocyte count between genotypes (Fig. 3C). We then compared the percentage of patients with platelet count reduction in the *ITPA* rs1127354 genotypes at week 4 of PEG-IFN/RBV therapy (Fig. 4). The percentage of patients with a platelet count reduction of <30 (10^9/l) at week 4 was significantly higher in the rs1127354 genotypes CC ($P < 0.0001$), indicating that the degree of platelet count reduction was less in patients with the rs1127354 genotype CC. A multivariate analysis for factors associated with a platelet reduction >30 (10^9/l) at week 4 showed that lower platelet count at the baseline and the rs1127354 genotypes AA/CA were independently associated with platelet reduction (OR = 1.15; 95% CI = 1.11–1.20; $P < 0.0001$, OR = 5.92; 95% CI = 3.82–9.17; $P < 0.0001$, respectively).

Figure 5 showed reactive increase of the platelet count through weeks 1–4 of PEG-IFN/RBV therapy. Patients with anemia (Hb reduction ≥3.0 g/dl) at week 4 had a significantly higher degree of the reactive increase of the platelet count than those without anemia ($P < 0.0001$ in Fig. 5A). Within a subgroup of patients with the rs1127354 genotypes CC, patients with anemia still had a significantly higher degree of reactive increase of the platelet count than those without anemia ($P = 0.004$ in Fig. 5B). On the other hand, patients with the rs1127354 genotypes CC had a significantly higher degree of the reactive increase of the platelet count than those with genotypes AA/CA ($P < 0.0001$ in Fig. 5C), and a similar result was obtained in a subgroup of patients without anemia (Fig. 5D). To elucidate the significant factors associated with the rs1127354 genotypes by multivariate analysis, the rs1127354 genotypes AA/CA were independently associated with protection against the reduction in Hb and more reduction in platelet counts at week 4 due to a lower degree of the reactive increase of the platelet count (OR = 0.029; 95% CI = 0.009–0.092; $P < 0.0001$, OR = 4.73; 95% CI = 3.04–7.37; $P < 0.0001$, respectively). Indeed, the reactive increase of the platelet count through weeks 1–4 was positively correlated with a high platelet count at the baseline and anemia (Hb reduction ≥3.0 g/dl) at week 4, but was negatively correlated with rs1127354 genotypes AA/CA and a platelet count reduction of ≥30 (10^9/l) at week 4 (Table 4).

Relationship between *ITPA rs1127354* genotypes and treatment outcome due to dose reduction of PEG-IFN or RBV

In this population, a multivariate analysis showed that SVR was significantly associated with *IL28B* TT-genotype [OR

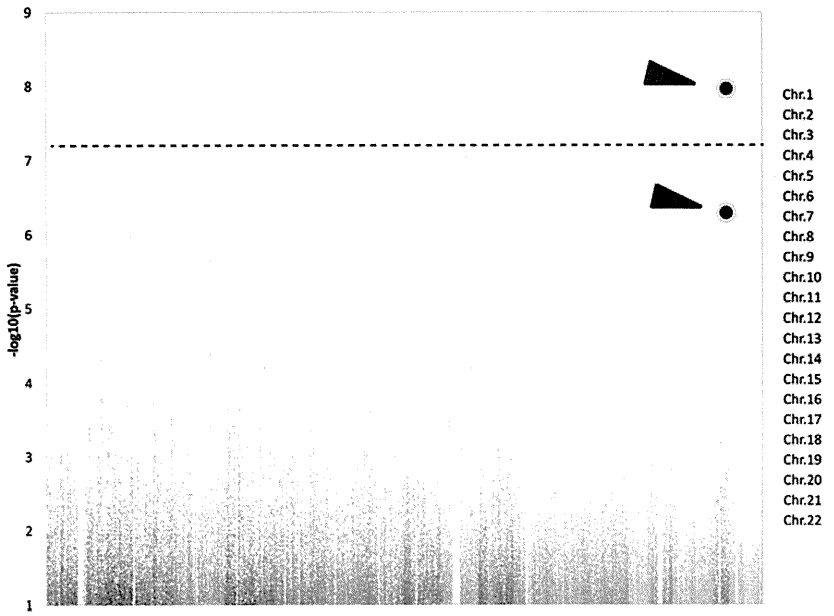

Figure 1. Genome-wide association results in 303 Japanese HCV patients with the decrease of platelets in response to PEG-IFN plus RBV treatment (107 patients with the decrease of PLT and 196 patients without the decrease of PLT). *P*-values were calculated using the χ^2 test for allele frequencies. Dots with arrow on chromosome 20 showed a significant SNP ($P = 8.17 \times 10^{-9}$ for rs11697186) and a candidate SNP with a marginal significance ($P = 4.30 \times 10^{-7}$ for rs6139030) associated with the decrease of PLT with response to PEG-IFN/RBV treatment. The dotted line indicates a genome-wide significance ($P < 8.40 \times 10^{-8}$).

Table 2. Two SNPs (rs11697186 and rs6139030) significantly associated with the decrease of PLT in response to PEG-IFN/RBV treatment

dbSNP rsID	Nearest gene	MAF[a] (allele)	Allele (1/2)	Stage	Patients with the decrease of PLT			Patients without the decrease of PLT			OR (95% CI)[b]	*P*-value[c]
					11	12	22	11	12	22		
rs11697186	*DDRGK1*	0.15 (T)	T/A	GWAS	3 (2.8)	48 (44.9)	56 (52.3)	0 (0.0)	32 (16.6)	161 (83.4)	4.6 (2.7–7.8)	8.17×10^{-9}
				Replication	3 (1.8)	65 (39.9)	95 (58.3)	3 (1.4)	25 (12.0)	181 (86.6)	4.6 (2.8–7.7)	5.88×10^{-10}
				Combined	6 (2.2)	113 (41.9)	151 (55.9)	3 (0.7)	57 (14.2)	342 (85.1)	4.5 (3.1–6.5)	5.29×10^{-17}
rs6139030	*ITPA*	0.17 (C)	T/C	GWAS	56 (52.3)	48 (44.9)	3 (2.8)	157 (80.1)	38 (19.4)	1 (0.5)	3.7 (2.2–6.1)	4.30×10^{-7}
				Replication	96 (54.9)	74 (42.3)	5 (2.9)	181 (83.8)	32 (14.8)	3 (1.4)	4.3 (2.7–6.8)	3.83×10^{-10}
				Combined	152 (53.9)	122 (43.3)	8 (2.8)	338 (82.0)	70 (17.0)	4 (1.0)	3.9 (2.8–5.5)	1.33×10^{-15}

[a]Minor allele frequency and minor allele in 184 healthy Japanese individuals.
[b]OR for the minor allele in a dominant model.
[c]*P*-value by χ^2 test for the minor allele dominant model.

6.12 (2.78–13.46), $P < 0.0001$] as well as platelet counts [OR 1.18 (1.11–1.26), $P < 0.00001$]. We analyzed whether the rs1127354 genotype could influence the treatment outcome by PEG-IFN/RBV therapy. When analyzed in the patients available for treatment outcome (172 with *ITPA*-AA/CA and 450 with *ITPA*-CC), the percentage of patients receiving >80% of the expected PEG-IFN and RBV dose at baseline and week 4 was not significantly different among the rs1127354 genotypes. However, the rate of SVR tended to be higher in patients with *ITPA*-AA/CA genotype than those with *ITPA*-CC (48.8 versus 37.3%), because the relapse rate was lower in patients with *ITPA*-AA/CA. To investigate the influence on treatment outcome by dose reduction of PEG-IFN, in a subgroup of patients with low platelet counts (<10) at baseline (19 with *ITPA*-AA/CA and 53 with *ITPA*-CC) we analyzed the treatment outcome according to rs1127354 genotypes. The SVR rate was very low in each group (21.1% in *ITPA*-AA/CA and 17.0% in *ITPA*-CC), because many patients had the initial dose reduction of PEG-IFN (<80% of standard dose)—36.8% of patients with *ITPA*-AA/CA and 44.6% of patients with *ITPA*-CC genotype. Further prospective studies are required among the pre-cirrhotic or cirrhotic patients with low platelet counts.

DISCUSSION

Recent genome-wide association studies, including our study on HCV infection, have identified two important host genetic variants: the SNP in *IL28B* gene, which is strongly associated with response to therapy for chronic genotype 1 HCV infection (16–21), and the SNP in *ITPA* gene, which precisely predicts RBV-induced anemia in

Table 3. Two SNPs (rs11697186 and rs6139030) significantly associated with quantitative change in Hb levels from baseline to week 4 of PEG-IFN/RBV treatment

dbSNP rsID	Nearest gene	MAF[a] (allele)	Allele (1/2)	Stage	Patients with quantitative change in Hb			Patients without quantitative change in Hb			OR (95% CI)[b]	*P*-value[c]
					11	12	22	11	12	22		
rs11697186	*DDRGK1*	0.15 (T)	T/A	GWAS	0 (0.0)	3 (3.3)	89 (96.7)	3 (1.5)	77 (37.0)	128 (61.5)	0.06 (0.02–0.16)	3.29×10^{-10}
				Replication	0 (0.0)	2 (1.5)	134 (98.5)	6 (2.5)	88 (37.3)	142 (60.2)	0.02 (0.01–0.09)	3.86×10^{-16}
				Combined	0 (0.0)	5 (2.2)	223 (97.8)	9 (2.0)	165 (37.2)	270 (60.8)	0.03 (0.01–0.08)	9.43×10^{-25}
rs6139030	ITPA	0.17 (C)	T/C	GWAS	88 (93.6)	6 (6.4)	0 (0.0)	125 (59.8)	80 (38.3)	4 (1.9)	0.08 (0.03–0.22)	2.56×10^{-9}
				Replication	134 (97.8)	3 (2.2)	0 (0.0)	143 (56.3)	103 (40.6)	8 (3.1)	0.03 (0.01–0.08)	6.90×10^{-18}
				Combined	222 (96.1)	9 (3.9)	0 (0.0)	268 (57.9)	183 (39.5)	12 (2.6)	0.04 (0.02–0.09)	2.12×10^{-25}

[a]Minor allele frequency and minor allele in 184 healthy Japanese individuals.
[b]OR for the minor allele in a dominant model.
[c]*P*-value by χ^2 square test for the minor allele dominant model.

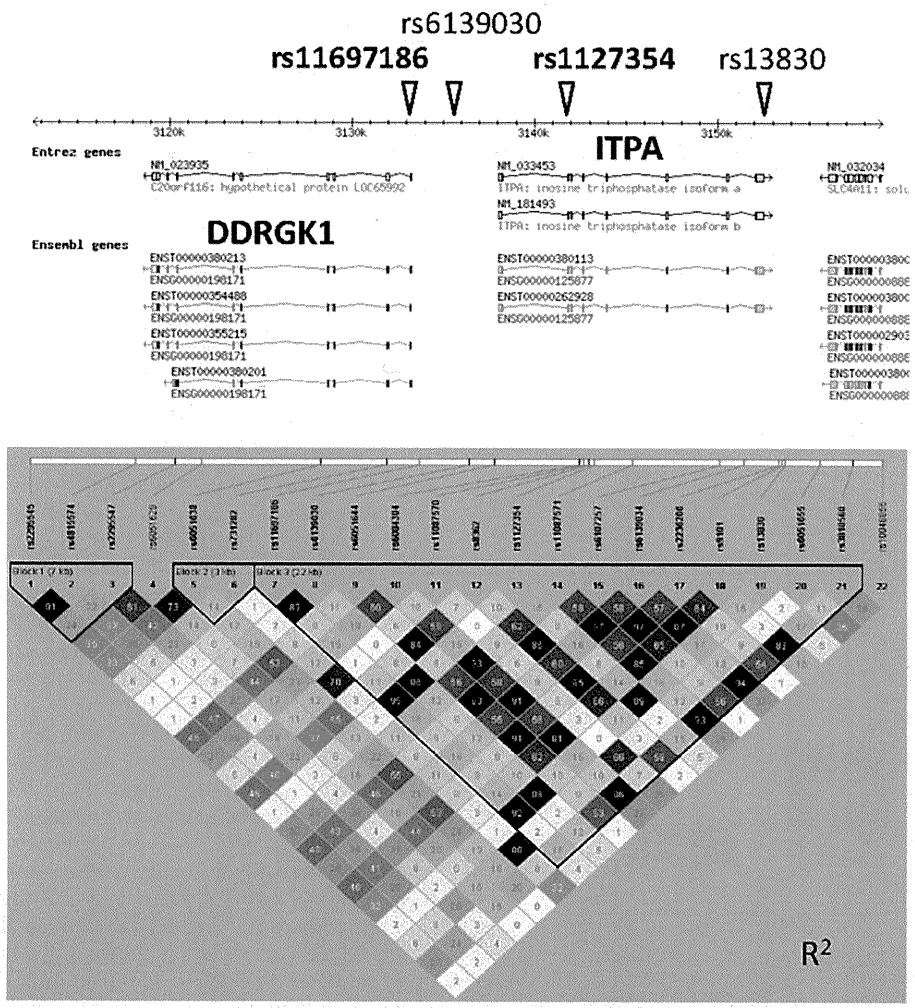

Figure 2. Pairwise LD (r^2) diagrams for *DDRGK1* and *ITPA*. Lower panel shows estimates of pairwise r^2 for 22 SNPs selected in the replication study using the second set of 391 Japanese HCV patients with and without quantitative change in PLT levels from baseline to week 4 of PEG-IFN/RBV treatment.

European-American population (22) and Japanese population (26). The genetic variation of *ITPA* causing an accumulation of inosine triphosphate (ITP) has been shown to protect patients against RBV-induced anemia during treatment for CHC infection. A recent report showed the biologic mechanism that ITP confers protection against RBV-induced ATP reduction by substituting for erythrocyte GTP, which is depleted by RBV, in the biosynthesis of ATP (25).

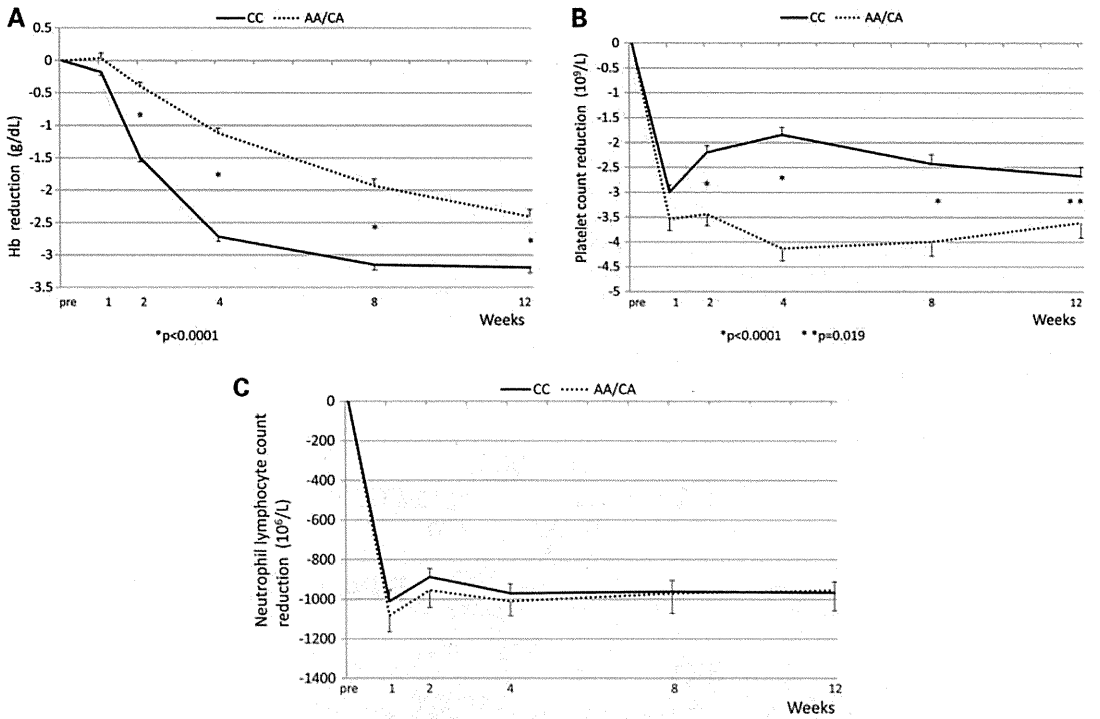

Figure 3. *ITPA* rs1127354 genotypes and the quantitative reduction of blood cells from baseline. Mean reduction of (**A**) Hb levels, (**B**) platelet counts and (**C**) neutrophil leukocyte counts during treatment according to rs1127354 genotype is shown. Solid and dotted lines indicate patients with CC and AA/CA genotypes, respectively. Error bars indicate standard error. CC genotype had more reduction in mean Hb levels during therapy compared with the AA/CA genotype ($^*P < 0.0001$ for weeks 2, 4, 8, 12). CC genotype had less of a reduction in mean platelet counts ($^*P < 0.0001$ for weeks 2, 4, 8, and $^{**}P = 0.019$ for week 12), and showed a reactive increase of platelet counts through weeks 1–4.

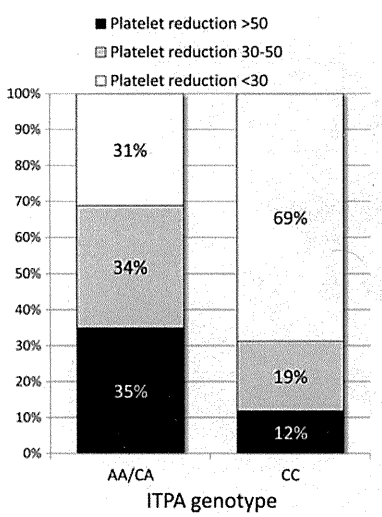

Figure 4. *ITPA* rs1127354 genotypes and reduction of platelet counts at week 4 of PEG-IFN/RBV therapy. The percentage of patients with platelet count reduction of >50 (10^9/l) (black bar), 30–50 (10^9/l) (gray bar) and <30 (10^9/l) (white bar) at week 4 is shown for rs1127354 genotypes. The incidence of platelet count reduction of >50 and <30 was significantly lower in patients with the rs1127354 genotypes CC compared with AA/CA genotypes: 12 versus 35%, $P < 0.0001$, and 69 versus 31%, $P < 0.0001$, respectively.

In this study, two SNPs, rs11697186 and rs6139030, which were within and around *DDRGK1* gene on chromosome 20, were strongly associated with thrombocytopenia as well as with Hb reduction at week 4. In clinical practice, the positive predictive value and negative predictive value by rs11697186 genotypes were 66.5 and 69.4% for thrombocytopenia, as well as 97.2 and 45% for RBV-induced anemia at week 4. As previously reported (22,26), a functional SNP (rs1127354) in the *ITPA* locus, which is in strong LD with rs11697186, was the most significant SNP associated with RBV-induced anemia and, in this study, IFN-induced thrombocytopenia in Japanese genetic populations. Note that severe Hb decline, which is mainly found in ITPA-CC patients, was inversely correlated with platelet reduction. This would contribute to an association between severe anemia and relative reactive increase of platelet count in this population, which attenuated the IFN effect on the platelet count. Our data supported a previous report which described that the current use of RBV, inducing severe anemia, might blunt the thrombocytopenic effect of IFNs as a result of reactive increase of platelet counts (27).

A previous paper showed hematological and bone marrow effects of RBV in rhesus monkeys (28). Hb values decreased significantly during RBV administration due to dose-related erythroid hypoplasia in bone marrow and returned to normal following withdrawal. On the other hand, increase of the platelet count occurred in both low- and high-dose treatment groups during RBV administration, with a fall of the platelet count to normal after drug withdrawal. The effect on platelet count was clearly dose related, with maximum counts rising to twice and three times above baseline levels in the low- and high-dose groups, respectively. This caused a significant increase of

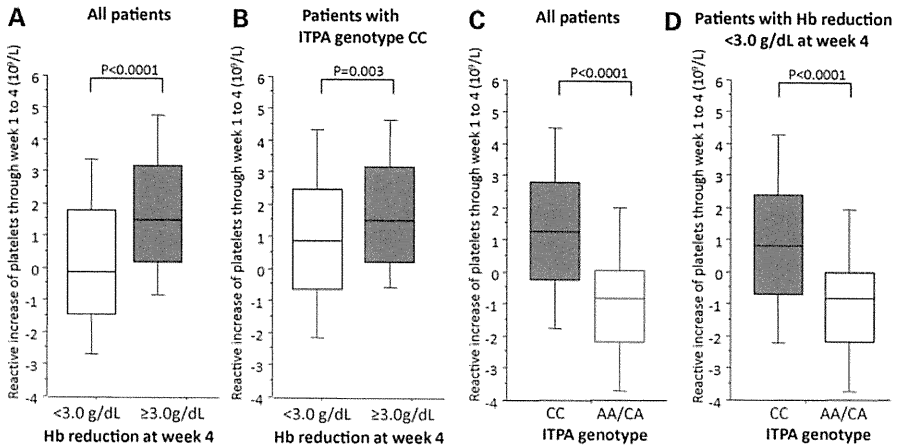

Figure 5. Reactive increase of platelet counts through weeks 1–4. Box plots of reactive increase of platelet count through weeks 1–4 according to the degree of anemia at week 4 are shown for all patients (**A**) and a subgroup of patients with the rs1127354 genotypes CC (**B**). Patients with anemia (Hb reduction ≥3.0 g/dl) at week 4 had a significantly higher degree of reactive increase of platelet count than those without anemia ($P < 0.0001$). Box plots of reactive increase of platelet counts according to the rs1127354 genotype CC are shown for all patients (**C**) and a subgroup of patients without anemia (**D**) (Hb reduction <3.0 g/dl) at week 4. Patients with the rs1127354 genotypes CC had a significantly high degree of reactive increase of platelet counts compared with those with genotypes AA/CA ($P < 0.0001$).

Table 4. Multivariate analysis of factors associated with reactive increase of platelets ≥20 (10^9/l) through weeks 1–4

	OR	95% CI	*P*-value
Baseline platelet counts	1.168	1.101–1.239	<0.0001
ITPA AA/CA	0.379	0.168–0.856	0.0196
Platelet reduction ≥30 (10^9/l) at week 4	0.051	0.021–0.120	<0.0001
Hb reduction ≥3.0 g/dl at week 4	1.602	0.914–2.809	0.0996

the platelet count associated with increased numbers of megakaryocytes. Additionally, the sequence homology of thrombopoietin (TPO) and erythropoietin (EPO) may explain the synergy of the physiologic role of TPO and EPO in platelet production. When EPO is elevated, as in iron deficiency anemia, an amino acid sequence similar to TPO may increase the platelet count (29).

Another possibility is a direct association between *ITPA* SNPs or the related SNPs with a strong LD and IFN-induced thrombocytopenia. *DDRGK1* (DDRGK domain-containing protein 1) is a novel C53/LZAP-interacting protein. C53/LZAP (also named as Cdk5rap3) is a putative tumor suppressor that plays important roles in multiple cell signaling pathways, including DNA damage response and NF-kappaB signaling (30); however, it remains largely unknown how the function of *DDRGK1* variants is regulated. Further studies are required to elucidate the possible association between *DDRGK1* variants and thrombocytopenia.

Multivariate analysis demonstrated that rs1127354 in the *ITPA* gene was independently associated with RBV-induced severe anemia and IFN-induced thrombocytopenia. This finding suggests that rs1127354 would be a useful marker to predict these hematological side effects by PEG-IFN/RBV therapy, indicating that genetic testing of *ITPA* variant might be applied to establish personalized dosages of PEG-IFN/RBV therapy. The rate of SVR tended to be higher in patients with *ITPA*-AA/CA genotype than those with *ITPA*-CC in this population. This might reflect decreased treatment efficacy (higher relapse rate) due to dose reduction of RBV in patients with *ITPA*-CC genotype. Our recent paper also demonstrated that the incidence of early dose reduction was significantly higher in *ITPA*-major (CC) patients as expected and, more importantly, that a significantly higher SVR rate was achieved in *ITPA*-hetero/minor (CA/AA) patients with HCV non-1b or low viral load strains (31) and in a subset of Japanese patients with the favorable TT genotype at rs8099917 of *IL28B* (32). Taken together, our results indicate that the *ITPA* minor variant A is not only a protective allele against PEG-IFN and RBV treatment-associated anemia in Japanese population, but also a significant predictor of SVR in certain HCV strains that show good response to IFN. The possible mechanism of protection against RBV-induced hemolysis is that ITP deficiency or low-activity variants (*ITPA* minor variant A) in turn lead to the accumulation of ITP in red blood cells (33,34), and the ITP confers protection against RBV-induced ATP reduction by substituting for erythrocyte GTP (25). On the other hand, half of the *ITPA*-major (CC) patients did not develop a significant Hb decline. This finding suggests other low-frequency *ITPA* variants or SNPs in other enzymes that are involved in erythrocyte purine nucleoside metabolism.

In Japan, the older HCV-infected patients developing liver fibrosis have been prevalent (mean age 62 years) (9). Thrombocytopenia by PEG-IFN/RBV therapy could lead to poor treatment efficiency among such Japanese patients with LC due to the initial or early dose reduction of PEG-IFN. In fact, ~40% of such population in this study had the initial dose reduction of PEG-IFN, resulting in a low SVR rate. Splenectomy or embolization of the splenic artery might be one of the options to increase the SVR rate, but a sufficient treatment outcome had not been obtained at present (35). Based on the recently accumulated SNP data, if patients had favorable *IL28B* genotype and *ITPA*-CC (lower reduction of platelet counts), a standard dose of PEG-IFN might be available for

the patients with lower platelet counts and the SVR rate might be increased due to sufficient dose of PEG-IFN.

Several STAT-C agents (specifically targeted antiviral therapies for hepatitis C) are being tested for clinical efficacy against hepatitis C (12,13,15,16). Most experts believe that when new drugs are approved to treat hepatitis C, they will be used in combination with PEG-IFN and RBV. Moreover, recent clinical trials, including NS3 protease inhibitors, have shown that PEG-IFN plus RBV would be necessary to achieve optimal treatment responses (12,13). Our present results may provide a valuable pharmacogenetic diagnostic tool for tailoring PEG-IFN and RBV dosing to minimize drug-induced adverse events and for further optimization of clinical anti-HCV chemotherapeutics.

MATERIALS AND METHODS

Patients

From April 2007 to April 2010, samples were obtained from 303 patients with chronic HCV (genotype 1) infection who were treated at 14 multi-center hospitals (liver units with hepatologists) throughout Japan. Each patient was treated with PEG-IFN-α2b (1.5 μg/kg body weight, subcutaneously once a week) or PEG-IFN-α2a (180 μg once a week) plus RBV (600–1000 mg daily according to body weight) for 48 weeks. Treatment duration was extended in some patients up to 72 weeks, according to the physicians' preferences. The dose of PEG-IFN or RBV was reduced according to the recommendations on the package inserts or the clinical conditions of the individual patients. EPO or other growth factors were not given. Written informed consent was obtained from each patient and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees. HBsAg-positive and/or anti-HIV-positive patients were excluded from this study.

In the following stage of replication study, SNP genotyping in an independent set of 391 Japanese HCV patients treated with PEG-IFN plus RBV treatment was completed using the DigiTag2 or TaqMan assay (ABI) following the manufacturer's protocol. The characteristics of patients for each GWAS stage and replication stage are summarized in Table 1.

SNP genotyping and data cleaning

In the GWAS stage, we genotyped 303 Japanese HCV patients with and without the decrease of platelet counts from baseline to week 4 of PEG-IFN/RBV treatment [107 patients with a decrease of >30 (10^9/l) in platelet counts and 196 patients without a decrease of >30 (10^9/l) in platelet counts], using the Affymetrix Genome-Wide Human SNP Array 6.0 according to the manufacturer's instructions. The cut-off value was calculated to maximize the difference, which was also close to the median change. The average overall call rate of patients with and without the decrease of PLT reached 98.69 and 98.72%, respectively. We then applied the following thresholds for SNP QC in data cleaning: SNP call rate $\geq$95% for all samples, MAF $\geq$1% for all samples. A total of 595 052 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots of SNPs showing $P < 0.0001$ in association analyses by comparing allele frequencies in both groups with and without the decrease of PLT were checked by visual inspection, and SNPs with ambiguous genotype calls were excluded.

In the following stage of the replication study and high-density association mapping, we selected 23 tag SNPs from the 44.7 kb region, including *DDRGK1* gene and *ITPA* gene by analyzing LD and haplotype structure based on the HapMap data of Japanese, using the Haploview software. Of these tag SNPs, rs1127354 within the *ITPA* gene, which was associated with RBV-induced anemia (22), was included; however, rs7270101 was excluded because recent papers studying Japanese patients showed no variants in rs7270101 (26,31,32). The SNP genotyping in an independent set of 391 Japanese HCV patients with and without quantitative change in PLT levels from baseline to week 4 of PEG-IFN/RBV treatment (175 patients with quantitative change in PLT and 216 patients without quantitative change in PLT) was completed using the DigiTag2 assay (36). Twenty-two of the 23 SNPs were successfully analyzed and were used for SNP genotyping and data cleaning. All 22 SNPs in the replication study cleared HWE P-value >0.001.

Based on the above SNPs data obtained from 303 Japanese HCV patients, using the Affymetrix Genome-Wide Human SNP Array 6.0, we also performed GWAS between 94 patients with a quantitative change of >3 g of reduction in Hb and 209 patients without quantitative change in Hb levels from baseline to week 4 of PEG-IFN/RBV treatment. SNP genotyping in an independent set of 391 Japanese HCV patients with and without quantitative change in Hb levels from baseline to week 4 of PEG-IFN/RBV treatment (137 patients with quantitative change in Hb and 254 patients without quantitative change in Hb) was also completed using the DigiTag2 assay (36). Twenty-two of the 23 SNPs were successfully analyzed and were used for SNP genotyping and data cleaning.

An application of the Cochrane–Armitage test on all the SNPs showed the genetic inflation factor $\lambda = 1.000$ for thrombocytopenia and $\lambda = 1.006$ for anemia in the GWAS stage (Supplementary Material, Figs S1 and S2). In addition, principal component analysis was performed in 303 samples for the GWAS stage together with the HapMap samples (CEU, YRI, CHB and JPT) (Supplementary Material, Fig. S3). These results implied that the effect of population stratification was negligible, except one sample, which was excluded from further analysis.

Laboratory and histological tests

Blood samples were obtained at baseline, 1, 2, 4, 8 and 12 weeks after the start of therapy and for hematologic tests after the start of therapy and for hematologic tests, blood chemistry and HCV-RNA. Genetic polymorphism in the *IL28B* gene (rs8099917) was determined using the ABI TaqMan assay (Applied Biosystems, Carlsbad, CA, USA). Fibrosis was evaluated on a scale of 0–4 according to the METAVIR scoring system. The SVR was defined as an undetectable HCV-RNA level by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems, CA, USA) or by Cobas Ampliprep/Cobas TaqMan assay (CAP/CTM) with a lower detection limit of

15 IU/ml (Roche Diagnostic Systems) 24 weeks after the completion of therapy.

Statistical analysis

The observed association between an SNP and the decrease of platelets/quantitative change in Hb levels with response to PEG-IFN plus RBV treatment was assessed by χ^2 test with a two-by-two contingency table in three genetic models: allele frequency model, dominant-effect model and recessive-effect model. SNPs on chromosome X were removed because gender was not matched between groups with and without the decrease of PLT and quantitative change in Hb levels. A total of 595 052 SNPs passed the quality control filters in the GWAS stage; therefore, significance levels after Bonferroni correction for multiple testing were $P = 8.40 \times 10^{-8}$ (0.05/595052) in the GWAS stage and $P = 2.27 \times 10^{-3}$ (0.05/22) in the replication stage.

The association between an SNP of the *ITPA* gene (rs1127354) and the incidence of platelet reduction at week 4 was analyzed by Fisher's exact test. The association between *ITPA* polymorphisms and the degree of reduction in platelet counts and Hb levels at each time point during therapy were analyzed by Mann–Whitney *U* test. Multivariable regression analysis was used to analyze the factors associated with *ITPA*, the rs1127354 genotype, factors associated with platelet count reductions and factors associated with the reactive increase in platelet counts. IBM-SPSS software v.15.0 (SPSS, Inc., Chicago, IL, USA) was used for these analyses.

Possible heterogeneity in allele frequencies at rs1127354 was assessed by Tarone's test. The association between the SNP and thrombocytopenia/anemia were analyzed by the Cochran–Mantel–Haenszel test. Both analyses were performed using the R (version 2.9.0) software (Supplementary Material, Table S3).

AUTHORS' CONTRIBUTIONS

Drafting of the paper, statistical analysis and approval of the final draft submitted: M.M.; drafting of the paper, statistical analysis, collecting samples and clinical data and approval of the final draft submitted: Y.T. and M.K.; statistical analysis and approval of the final draft submitted: N.N., M.S. and K.T.; collecting samples and clinical data and approval of the final draft submitted: K.M., N.S., N.E., H.Y., S.N., K.H., S.H., Y.I., E.T., S.M., M.H., Y.H., F.S., S.K. and N.I.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Highly Parallel and Short-Acting Amplification with Locus-Specific Primers to Detect Single Nucleotide Polymorphisms by the DigiTag2 Assay

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Abstract

The DigiTag2 assay enables analysis of a set of 96 SNPs using Kapa 2GFast HotStart DNA polymerase with a new protocol that has a total running time of about 7 hours, which is 6 hours shorter than the previous protocol. Quality parameters (conversion rate, call rate, reproducibility and concordance) were at the same levels as when genotype calls were acquired using the previous protocol. Multiplex PCR with 192 pairs of locus-specific primers was available for target preparation in the DigiTag2 assay without the optimization of reaction conditions, and quality parameters had the same levels as those acquired with 96-plex PCR. The locus-specific primers were able to achieve sufficient (concentration of target amplicon $\geq$5 nM) and specific (concentration of unexpected amplicons $<$2 nM) amplification within 2 hours, were also able to achieve detectable amplifications even when working in a 96-plex or 192-plex form. The improved DigiTag2 assay will be an efficient platform for screening an intermediate number of SNPs (tens to hundreds of sites) in the replication analysis after genome-wide association study. Moreover, highly parallel and short-acting amplification with locus-specific primers may thus facilitate widespread application to other PCR-based assays.

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Introduction

Polymerase chain reaction (PCR) is a commonly used technique in molecular biology. Several previously developed methods have employed multiplexed PCR in order to analyze genomic variations such as microsatellites or short tandem repeats (STRs), single nucleotide polymorphisms (SNPs) and insertions/deletions [1–3]. Multiplexed preparation of DNA templates in a single reaction is cost-effective, saving starting materials and run-time, while requiring careful optimization of assay conditions. The optimization process is highly empirical and time consuming, and depending on the combinations of markers, may or may not lead to successful assay development. For the conventional design of multiplex PCR, optimization of reaction conditions and careful pre-selection of targets are required in order to prevent excessive off-target priming by the numerous primers in the reaction. Moreover, the risk of generating errors in multiplex PCR, such as insufficient amplification, biased amplification and considerable primer-dimer formation within primers, tends to increase roughly as the square of the number of added primer pairs [4].

There are several approaches to resolving these drawbacks, including solid-phase assay formats (glass slide arrays, microbeads), oligonucleotides containing locked nucleic acid (LNA) residues and circularized amplification. Primers immobilized on the surface of the solid phase appear to markedly increase product yield on solid supports and may avoid the need for target pre-selection with a modification to enrich the input genomic DNA via a crude solution-phase multiplex PCR [5,6]. LNA pentamers showed high priming efficiency to achieve small biased priming in multiplex PCR [7]. Circularized amplification avoids generating artifacts associated with conventional multiplex PCR where two primers are used for each target [8]. This procedure was shown to perform a 96-plex amplification of an arbitrary set of specific DNA sequences. The arrayed primer extension-based genotyping method (APEX-2) allows efficient homogeneous 640-plex DNA amplification with locus-specific primers [9]. These approaches show effective consequences for multiplex amplification, however, a small number of approaches are practically used in the field of molecular genetics, presumably due to its cost and time consuming steps in preparation.

We developed the DigiTag2 assay for multiplex SNP typing as a simple and cost effective approach by combining multiplex PCR to enrich genetic regions including the target SNPs and an oligonucleotide ligation assay to encode all of the SNP genotypes into well-designed oligonucleotides designated DNA coded numbers (DCNs) [10]. For an effective primer design for multiplex PCR, there are several important physical properties for primer sequences, including melting temperature, Gibbs energy of duplex between primer and template, and interactions between primers and PCR amplicons. The DNA polymerase enzyme used in a multiplex PCR is one of the important factors for a successful unbiased amplification.

The DigiTag2 assay is a suitable approach to analyze an intermediate number of SNPs (tens to hundreds of locus) in the replication study after genome wide association study [11–12]. However, the most time consuming step for the DigiTag2 assay in a total running time of 13 hours is multiplex PCR for target preparation (5.5 hours). Here, we report an improved protocol for the DigiTag2 assay with a short-acting multiplex PCR through the use of Kapa 2GFast HotStart DNA polymerase, which reduces total running time and increases assay throughput. In this study, we also validate the applicability of the 192-plex PCR with locus specific primers to amplify the target regions from genomic DNA, which leads to save genomic DNA samples.

Methods

DNA samples

Genomic DNA samples from 96 unrelated healthy donors were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). All donors provided written informed consent and samples were anonymized. One microgram of purified genomic DNA was dissolved in 100 µl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

Primer design

A total of 192 pairs of primer were designed using the Visual OMP software version 7.1.0.0 (DNA software, Ann Arbor, MI, USA) with relatively long length (35–45-mer; average, 39.5-mer) to give amplicon sizes between 312 bp and 995 bp (average, 589 bp), each of which had an SNP site (Table S1). Prediction of DNA melting temperature was calculated using nearest-neighbor thermodynamic models. To avoid spurious amplification products, we employed a two-step protocol (denature and extension steps) using specifically designed primer pairs with an extension temperature at 68°C. The specificity of primer sequences was verified by Blat search in order to predict its location(s) on the human genome (GRCh37), and to confirm no unexpected SNP(s) within the primer sequence. The specificity of primer pairs was verified using MFE primer software, which can predict potential amplicon(s) generated from the human genome (GRCh37, up to 5 kb in amplicon size) [13]. All oligonucleotides (de-salted, 100 pmol/µl in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)) were purchased from Life Technologies (Carlsbad, CA, USA), and were stored at −20°C.

Multiplex PCR with Kapa 2GFast HotStart DNA polymerase

Multiplex PCR mix had a final volume of 10 µl, including 10 ng of genomic DNA, 25 nM each primer, 1.5× KAPA2G Buffer (including 2.25 mM Mg^{2+}), an additional 2.25 mM Mg^{2+} (final concentration of Mg^{2+}: 4.5 mM), 0.2 mM dNTPs and 0.4 U of Kapa 2GFast HotStart DNA polymerase (Kapa Biosystems, Woburn, MA, USA). PCR amplification was conducted using a TGradient (Biometra, Göttingen, Germany) or PTC-225 (MJ Research, Waltham, MA, USA) as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 68°C for 2 min. When necessary, the fragment length of PCR products was confirmed by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent, Santa Clara, CA, USA) in order to evaluate PCR efficiency. The total running times for multiplex PCR with Kapa 2GFast HotStart DNA polymerase using TGradient and PTC-225 were 1 h 48 min 55 s and 2 h 6 min 59 s, respectively.

Multiplex PCR with QIAGEN Multiplex PCR Kit

Multiplex PCR mix had a final volume of 10 µl, including 10 ng of genomic DNA, 25 nM each primer, 1× Multiplex PCR Buffer (including 3.0 mM Mg^{2+}), 0.2 mM dNTPs and HotStarTaq DNA polymerase (QIAGEN Multiplex PCR Kit; QIAGEN, Valencia, CA, USA). PCR amplification was conducted using a TGradient or PTC-225 as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 30 s and 68°C for 6 min. The total running times for multiplex PCR with QIAGEN Multiplex PCR Kit using TGradient and PTC-225 were 5 h 27 min 53 s and 5 h 46 min 39 s, respectively.

96-plex genotyping by the DigiTag2 assay

The DigiTag2 assay performs multiplex SNP typing by encoding all of the SNP genotypes into well-designed oligonucleotides, designated DNA coded numbers (Figure 1, DCNs: D1_i, ED-1 and ED-2) [10]. The DCNs are assigned to the target SNPs in an unconstrained manner; therefore, the DNA chips prepared to read out the types of DCNs are universally available for any type of SNP without optimization of assay conditions. The DigiTag2 assay proceeds in four steps; target preparation, encoding, labeling and detection.

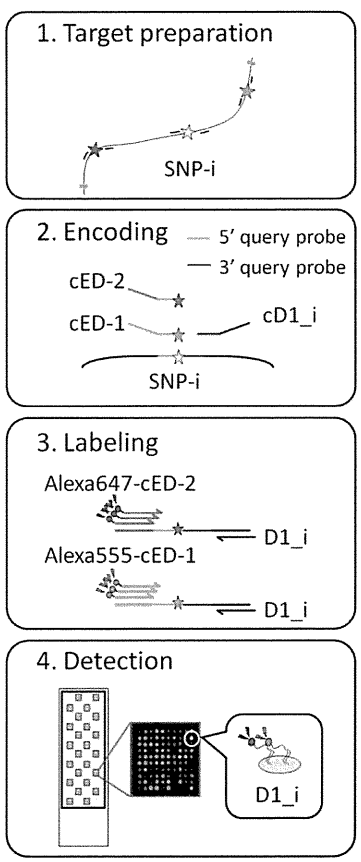

Figure 1. Schematic representation of the DigiTag2 assay. The assay has four steps: target preparation, encoding, labeling and detection. SNP genotypes are encoded into well-designed oligonucleotides, designated DNA coded numbers (DCNs: D1_i, ED-1 and ED-2). D1_i is a variable sequence assigned to each SNP. Reverse complement sequences are written by attaching the character 'c' before the sequence name.
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