J Gastroenterol (2011) 46:545–555
DOI 10.1007/s00535-010-0358-6

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Factors predictive of sustained virological response following 72 weeks of combination therapy for genotype 1b hepatitis C

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Received: 1 November 2010 / Accepted: 25 November 2010 / Published online: 19 January 2011

Abstract
Background Treatment of genotype 1b chronic hepatitis C virus (HCV) infection has been improved by extending peg-interferon plus ribavirin combination therapy to 72 weeks, but predictive factors are needed to identify those patients who are likely to respond to long-term therapy.
Methods We analyzed amino acid (aa) substitutions in the core protein and the interferon sensitivity determining region (ISDR) of nonstructural protein (NS) 5A in 840 genotype 1b chronic hepatitis C patients with high viral load. We used logistic regression and classification and regression tree (CART) analysis to identify predictive factors for sustained virological response (SVR) for patients undergoing 72 weeks of treatment.
Results When patients were separately analyzed by treatment duration using multivariate logistic regression, several factors, including sex, age, viral load, and core aa70 and ISDR substitutions ($P = 0.0003$, $P = 0.02$, $P = 0.01$, $P = 0.0001$, and $P = 0.0004$, respectively) were significant predictive factors for SVR with 48 weeks of treatment, whereas age, previous interferon treatment history, and ISDR substitutions ($P = 0.03$, $P = 0.01$, and $P = 0.02$,

Electronic supplementary material The online version of this article (doi:10.1007/s00535-010-0358-6) contains supplementary material, which is available to authorized users.

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respectively) were the only significant predictive factors with 72 weeks of treatment. Using CART analysis, a decision tree was generated that identified age, cholesterol, sex, treatment length, and aa70 and ISDR substitutions as the most important predictive factors. The CART model had a sensitivity of 69.2% and specificity of 60%, with a positive predictive value of 68.4%.

Conclusions Complementary statistical and data mining approaches were used to identify a subgroup of patients likely to benefit from 72 weeks of therapy.

Keywords CART analysis · Core protein · Decision tree · ISDR · LDL cholesterol

Abbreviations

HCV	Hepatitis C virus
ISDR	Interferon sensitivity determining region
CART	Classification and regression tree analysis
SVR	Sustained virological response
NR	Non-viral response

Introduction

Chronic hepatitis C virus (HCV) infection is a major global cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1–3]. The treatment of chronic hepatitis C has improved with the advent of peg-interferon (IFN) plus ribavirin combination therapy [4–7], but fewer than half of the patients with high viral loads of genotype 1b show a sustained virological response (SVR), defined as testing negative for HCV RNA 24 weeks after cessation of the therapy. To overcome this limitation, recent therapeutic regimens have extended the treatment period to 72 weeks [8–11]. This extension is especially effective in patients whose HCV RNA declines relatively slowly [9–11]. Accordingly, recent treatment protocols have recommended extending the treatment period to 72 weeks in patients who become negative for HCV RNA after 12 weeks of treatment but before 24 weeks [10, 11]. This response-guided decision-making approach to therapy has resulted in improvements of the SVR rate [10, 11]. Following this approach, patients with a non-viral response (NR), i.e., patients who show very poor response to the therapy (defined as less than 2-log decline of HCV RNA during 12 weeks of treatment), should be advised to discontinue therapy because SVR is rare in such patients. While response-guided therapy is useful in determining the appropriate duration of treatment for patients who are likely to respond eventually, predictors that can be assessed before the start of therapy will aid in differentiating which difficult-to-treat patients are likely to achieve an SVR with extended therapy and which may be better served by considering alternative therapy options.

To predict NR, recent studies recommend analysis of amino acid (aa) substitutions in the HCV core protein at positions 70 and 91 [12, 13]. The substitution of arginine with glutamine or other amino acids at core protein aa 70 has been reported to be associated with NR, and this finding was confirmed by several other groups [14–16]. Analysis of core aa 70 has also been shown to be useful to predict the outcome of 72 weeks of combination therapy [17]. While many factors have been reported to be useful predictors of the effect of combination therapy [18–26], many of these factors are mutually interdependent. Furthermore, because almost all of these factors have been reported under conditions in which a majority of patients were receiving 48 weeks of treatment, it is necessary to consider the effect of the treatment period.

In this study, we compiled a database of clinical data from 840 patients from 16 national centers in Japan. We used logistic regression and classification and regression tree analysis (CART) to identify factors predictive of SVR for 48- and 72-week therapy and to assess which patients are most likely to benefit by long-term 72-week therapy.

Methods

Study subjects

In this retrospective study, data from 840 patients with chronic hepatitis C treated at 16 different hospitals in Japan were analyzed for predictive factors for SVR based on

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Table 1 Patient characteristics for 48- and 72-week treatments

	All patients (n = 840)	48-Week therapy (n = 619) 73.69%	72-Week therapy (n = 221) 25.12%
Age (years)	54.4 ± 10.73	53.8 ± 11.21	56.2 ± 9.03
Gender (male/female)	449/391	357/262	92/129
Body weight (kg)	60.9 ± 10.8	61.3 ± 10.6	59.8 ± 11.4
Height (cm)	162.2 ± 9.1	162.7 ± 9.1	160.7 ± 9.0
BMI	23.0 ± 3.05	23.0 ± 2.92	23.0 ± 3.4
HCV core protein aa 70 (wild/mutant)	539/301	396/223	143/78
HCV core protein aa 91 (wild/mutant)	504/336	369/250	135/86
ISDR (0–1/≥2)	714/126	513/106	201/20
Hypertension (present/absent/ND)	538/113/189	395/78/146	143/35/43
Diabetes (present/absent/ND)	634/47/159	457/38/124	177/9/35
Transfusion (present/absent/ND)	505/227/108	379/162/78	126/65/30
Fibrosis stage (0–2/3–4/ND)	604/128/108	448/90/81	156/38/27
Activity stage (0–1/2–3/ND)	382/343/115	287/245/87	95/98/28
Steatosis (present/absent/ND)	158/344/338	119/250/250	39/94/88
AST (IU/l)	65 ± 49	66 ± 47	63 ± 53
ALT (IU/l)	68 ± 56	68 ± 56	66 ± 55
White blood cell count (/mm^3)	4832 ± 1455	4882 ± 1488	4693 ± 1352
Hemoglobin (g/dl)	14.2 ± 1.36	14.3 ± 1.39	14.1 ± 1.29
Platelets ($\times 10^4$/mm^3)	16.9 ± 5.18	17.0 ± 5.11	16.8 ± 5.35
γGTP (IU/l)	56 ± 59	59 ± 64	49 ± 42
Albumin (g/dl)	4.02 ± 0.348	4.01 ± 0.350	4.03 ± 0.343
Uric acid (mg/dl)	5.41 ± 1.29	5.46 ± 1.27	5.25 ± 1.35
Iron (μg/dl)	147.0 ± 69.65	151.0 ± 75.71	136.1 ± 47.45
Ferritin (μg/l)	173.9 ± 167.9	181.7 ± 175.7	153.0 ± 143.7
Fasting blood sugar (mg/dl)	99.8 ± 19.8	99.3 ± 19.1	101.2 ± 21.5
Alpha-fetoprotein (μg/l)	16.3 ± 50.4	14.2 ± 44.8	22.0 ± 62.7
Total cholesterol (mg/dl)	175 ± 32.3	173 ± 31.8	179 ± 33.4
LDL cholesterol (mg/dl)	100.8 ± 29.8	100.2 ± 30.3	102.5 ± 28.4
HDL cholesterol (mg/dl)	52.1 ± 15.5	51.4 ± 15.0	53.9 ± 16.6
Triglycerides (mg/dl)	103.2 ± 48.8	103.8 ± 46.1	101.7 ± 55.1
HCV-RNA (KIU/ml)	3239 ± 4669	3170 ± 4828	3427 ± 4205
Response to treatment (SVR/TR/NR)	465/246/129	341/164/114	124/82/15

BMI body mass index, *HCV* hepatitis C virus, *aa* amino acid, *ISDR* interferon sensitivity determining region, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γGTP* γ-glutamyl transpeptidase, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *SVR* sustained virological response, *TR* transient response/relapsers, *NR* non-viral response, *ND* not determined

treatment duration. Inclusion criteria included testing positive for HCV RNA for longer than 6 months and testing negative for both hepatitis B virus surface antigen and anti-HIV antibody. Patients with confounding conditions such as hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease were excluded. We excluded patients who were lost for follow up and those who did not show a high level of viremia for genotype 1b, as well as patients for whom we failed to determine both core and IFN sensitivity determining region (ISDR) of nonstructural protein (NS) 5A sequences; 385 patients were treatment-naïve. All subjects gave their written informed consent to participate in the study according to the process approved by the ethics committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki. Patient profiles are listed in Table 1.

All patients initially received weekly injections of peg-IFN-alpha-2b for 48 weeks (60 μg for body weight (BW) 35–45 kg, 80 μg for BW 46–60 kg, 100 μg for BW 61–75 kg, 120 μg for BW 76–90 kg, and 150 μg for BW 91–120 kg). Ribavirin was administered orally, and the dosage was determined based on the patient's BW (600 mg for <60 kg, 800 mg for 60–80 kg, and 1,000 mg

for >80 kg). Ribavirin dosage was reduced when hemoglobin levels were reduced to 10.0 g/dl and stopped if hemoglobin levels reached 8.5 g/dl. Successful treatment was ascertained based on SVR, defined as HCV RNA-negative 6 months after cessation of therapy. Using response-guided therapy, slow viral responders, i.e., patients for whom HCV RNA levels became negative after 12 weeks of therapy but before 24 weeks, and some non-responders were recommended for extension of therapy to 72 weeks.

Biochemical tests were performed at the individual hospitals, and pathological diagnosis was made by pathologists in each hospital according to the criteria of Desmet et al. [27]. Fibrosis and activity data were compared among hospitals to ensure that there were no systematic differences.

Analysis of viral titer and amino acid sequences in the core and ISDR region

The HCV RNA level was analyzed using reverse transcription polymerase chain reaction (RT-PCR)-based methods (Amplicor^{TM} high-range test; Roche Diagnostics, Basel, Switzerland, or TaqMan RT-PCR test; Applied Biosystems, CA). The measurement ranges of these assays were 5–5000 KIU/ml and 1.2–7.8 log IU/ml, respectively. For values exceeding the measurable range, the limit value was used as an approximation. The values obtained by the Amplicor test were converted to logarithmic values [28].

Nucleotide and amino acid sequences of the core and the ISDR region were determined by direct sequencing of cDNA fragments amplified by PCR. Arginine and leucine were considered wild-type for core protein aa 70 and aa 91, respectively [12, 13]. The number of aa substitutions in the ISDR was determined by comparison with the reference sequence reported by Kato et al. [29] using the method of Enomoto et al. [30, 31].

Statistical analysis

Statistical analysis was performed using the R software package (http://www.r-project.org). The χ^2 or Fisher's exact and Mann–Whitney U-tests were used to detect significant associations. All statistical analyses were two-sided, and $P < 0.05$ was considered significant. Simple and multiple logistic regression analyses were used to examine the association between viral substitutions and clinical factors, using $P < 0.05$ as the criterion for inclusion in the initial multivariate model. Multivariate logistic regression analysis was performed using forward/backward stepwise selection based on the akaike information criterion (AIC) score and validated by bootstrapping, using the rms package in R. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for each factor.

CART analysis

CART analysis was used to generate a decision tree by classifying patients by SVR, based on a recursive partitioning algorithm with minimal cost-complexity pruning to identify optimal classification factors. The SimpleCart classifier in the WEKA data mining package [32] was used with a minimal terminal node size of 4 and trained with the variables listed in Table 1. Performance was assessed using tenfold cross-validation, and the sensitivity, specificity, and precision of the model were calculated. Receiver operating characteristic (ROC) curves were generated and results were compared with the logistic regression model.

Results

Patient characteristics

Patients were partitioned into two groups based on whether they received 48 or 72 weeks of therapy (Table 1). In this study 465 patients achieved an SVR, whereas 375 patients were either non-responders or relapsers, yielding an overall SVR rate of 55.4%. The rate of SVR did not differ significantly between the 48- and 72-week treatment groups (55.3 vs. 56.4%, respectively; $P = 0.81$), but the NR rate was significantly lower in patients who were treated for 72 weeks (18.3 vs. 6.4%; $P = 9.3 \times 10^{-6}$).

Predictive factors for SVR

The association between SVR and individual clinical factors was assessed using logistic regression. A number of factors were significant at the $P < 0.05$ level, including age, sex, viral load, aa70/ISDR substitutions, hypertension, fibrosis, steatosis, prior IFN treatment, low-density lipoprotein (LDL) cholesterol, total cholesterol, white blood cell count, platelet count, hemoglobin, γ-glutamyl transpeptidase (γGTP), and albumin (Table 2). On multivariate logistic regression, only age, sex, core aa70, ISDR, LDL, and γGTP were identified as significant independent predictors of SVR. Although length of treatment was not identified as a significant predictor in this analysis, exploratory analysis suggests the presence of potential interactions between treatment length and age and/or sex that are not captured by the first-order terms in the model. When second-order terms were selected a posteriori, however, a significant interaction was found between sex and treatment length ($P = 0.0034$). When analyzed separately, independent predictive factors for SVR for 48 weeks

Table 2 Factors associated with sustained virological response to combination therapy

Variable	Simple			Multiple			
	n	OR	*P*	*n*	OR	(95% CI)	*P*
Age	840	0.393	3.16×10^{-11}***	517	0.386	(0.27–0.56)	5.08×10^{-7}***
Sex (male vs. female)	840	0.521	3.61×10^{-6}***	517	0.52	(0.35–0.78)	0.001459**
BMI (kg/m^2)	834	0.8	0.1094				
Viral load (Log IU/ml)	840	0.761	0.001828**				
Core aa70 substitution	840	0.537	1.98×10^{-5}***	517	0.507	(0.35–0.74)	0.000521***
Core aa91 substitution	840	0.818	0.1568				
ISDR (0–1 vs. ≥2)	840	2.36	5.19×10^{-5}***	517	2.12	(1.19–3.77)	0.01037*
Hypertension	651	0.625	0.02389*				
Diabetes	681	0.794	0.4464				
Blood transfusion	732	1	0.9788				
Fibrosis (F0–1 vs. F2–4)	732	0.674	0.008287**				
Activity (A0–1 vs. A2–4)	725	0.779	0.09567				
Steatosis	502	0.645	0.03413*				
Prior IFN treatment	830	1.37	0.02648*				
HDL cholesterol (mg/dl)	493	0.761	0.1333				
LDL cholesterol (mg/dl)	529	1.46	0.03223*	517	1.61	(1.10–2.38)	0.01521*
Triglyceride (mg/dl)	726	0.913	0.5412				
Total cholesterol (mg/dl)	814	1.25	0.11				
AST (IU/l)	783	0.933	0.6316				
ALT (IU/l)	840	0.972	0.837				
WBC (/mm^3)	836	1.55	0.001831**				
Hemoglobin (g/dl)	838	1.34	0.00276**				
Platelets ($\times 10^4$/mm^3)	838	1.74	7.92×10^{-5}***				
Gamma-GTP (IU/l)	823	0.735	0.0288*	517	0.656	(0.43–0.99)	0.04588*
Albumin (g/dl)	809	1.41	0.01699*				
Ferritin (μg/l)	532	0.898	0.5404				
Treatment period (weeks)	840	1.02	0.6095				

Simple and multiple logistic regression was used to examine the association between SVR and patient and viral factors. Factors with $P < 0.05$ were considered for inclusion in the multiple regression model and the best model selected by backwards stepwise selection using AIC

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

IFN interferon, *OR* odds ratio, *CI* confidence interval, *AIC* akaike information criterion

of treatment included age, sex, viral load, core aa70, LDL, platelets, and white blood cell counts, whereas for 72 weeks of treatment only age, ISDR, and prior IFN treatment were significant, although LDL cholesterol was marginally significant (Table 3).

Among patients who underwent 48 weeks of therapy, 61% of patients with core aa 70 wild-type achieved an SVR compared to only 44% of patients with mutant core aa 70 ($P = 1.8 \times 10^{-5}$, Fig. 1a), whereas for 72-week patients, the ratio was 1:1 (Fig. 3a). Conversely, in the 48-week group, 71% of patients with two or more mutations in the ISDR were able to achieve an SVR compared to 52% with the wild-type ISDR, and in the 72-week group (Fig. 1b), 80% of patients with two or more ISDR mutations achieved an SVR compared to 54% with zero or one ISDR mutations (Fig. 3b). Median baseline viral load was significantly lower in 48-week SVR patients compared to that in non-SVR patients ($P = 0.001$, Fig. 1c), whereas there was no significant difference between viral load and SVR in 72-week therapy patients ($P = 0.625$, Fig. 4c). There was a significant effect of age and treatment outcome among 48-week patients ($P = 9.3 \times 10^{-6}$, Fig. 2), but the difference was not significant among 72-week therapy patients. However, the proportion of patients achieving an SVR tended to decrease with age in both groups, particularly in females over age 70 years in the 72-week group (Figs. 2, 4).

Table 3 Independent factors associated with sustained virological response to 48- and 72-week peg-interferon plus ribavirin combination therapy

Variable	48 Weeks			72 Weeks			
	n	OR	*P*	*n*	OR	(95% CI)	*P*
Age	535	0.642	0.0165*	133	0.4	(0.176–0.91)	0.02877*
Sex (male vs. female)	535	0.481	0.000284**				
Viral load (Log IU/ml)	535	0.738	0.01033*				
Core aa70 substitution	535	0.454	9.95×10^{-5}**				
ISDR (0–1 vs. ≥2)	535	2.75	0.000358**	133	7	(1.35–36.2)	0.02047*
Fibrosis (F0–1 vs. F2–4)	535	0.66	0.03954*				
Prior IFN treatment				133	2.67	(1.22–5.85)	0.01431*
LDL cholesterol (mg/dl)				133	2.04	(0.952–4.35)	0.06673
WBC (/mm^3)	535	1.53	0.03342*				
Platelets ($\times 10^4$/mm^3)	535	1.54	0.03707*				

Simple and multiple logistic regression analysis was used to examine the association between SVR and patient/viral factors separately for patients receiving 48 and 72 weeks of treatment

** $P < 0.001$, * $P < 0.05$

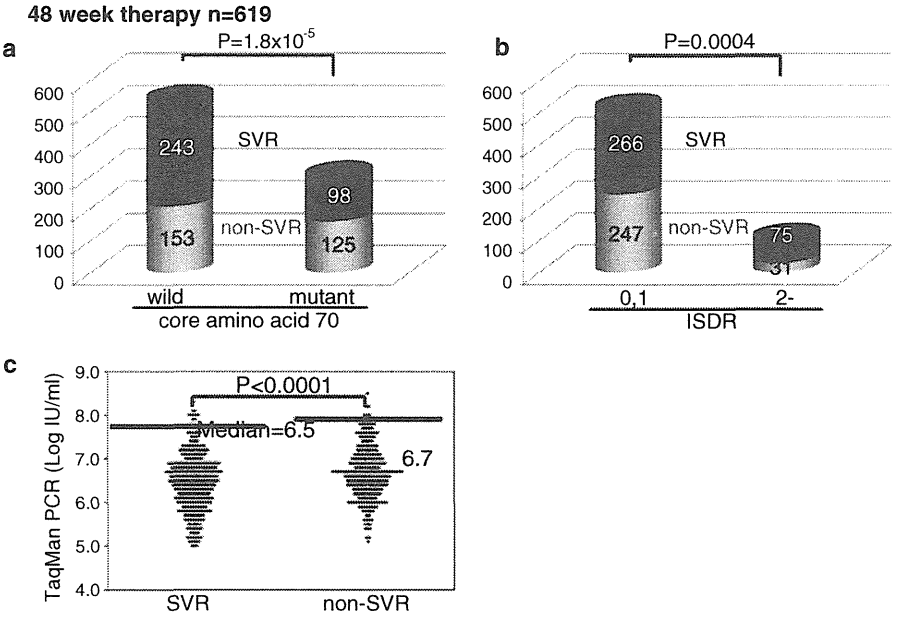
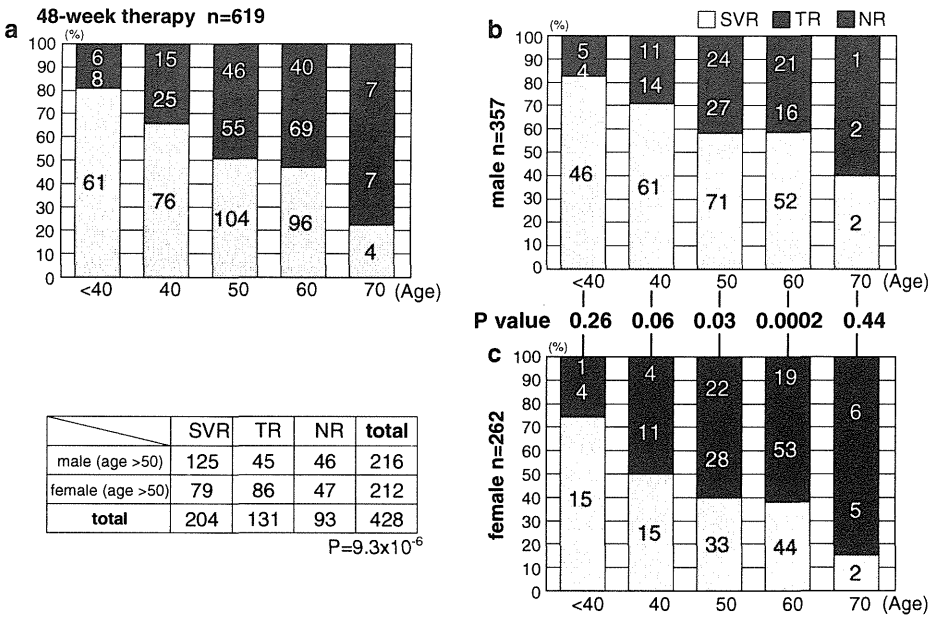


Fig. 1 Viral factors for 48-week treatment. Relationships between sustained virological response (*SVR*) and **a** core amino acid 70 substitutions, **b** amino acid substitutions in the interferon sensitivity determining region, and **c** baseline viral titers grouped by SVR and non-SVR for patients treated for 48 weeks. *PCR* Polymerase chain reaction

CART analysis

Figure 5 shows the decision tree generated by CART analysis. All variables were included during model construction, and the SimpleCart algorithm generated a tree based on the following fields: age, cholesterol, sex, γGTP, 48 versus 72 weeks of treatment, and aa substitutions in the ISDR and at core aa70. Age was used as the first cutoff, and patients younger than 46.5 years were classified as having a high probability for SVR (78%). Total cholesterol was identified as the next decision point, and patients with cholesterol higher than 211.5 mg/dl were classified as SVR if they were younger than 62.5 years (84%) and NR (65%) otherwise. Patients with cholesterol lower than 211.5 mg/dl were subdivided next by sex. Females who received 48 weeks of treatment were classified as NR (71%), whereas females receiving 72 weeks of treatment were classified as SVR if they were younger than 58.5 years (71%) or NR otherwise (64%). Males who were infected with aa70 wild-type were classified as SVR (62%), whereas males with aa70 substitutions were classified as NR if total cholesterol was less than 130 mg/dl (97%). Males with ISDR substitutions were classified as SVR (75%), and those with wild-type ISDR were classified



	SVR	TR	NR	total
male (age >50)	125	45	46	216
female (age >50)	79	86	47	212
total	204	131	93	428

P=9.3x10^{-6}

Fig. 2 Relationship between age and response to treatment for 48-week therapy. Treatment outcomes by age in 10-year intervals are shown for **a** all patients, **b** males only, and **c** females only. *NR* non-viral response

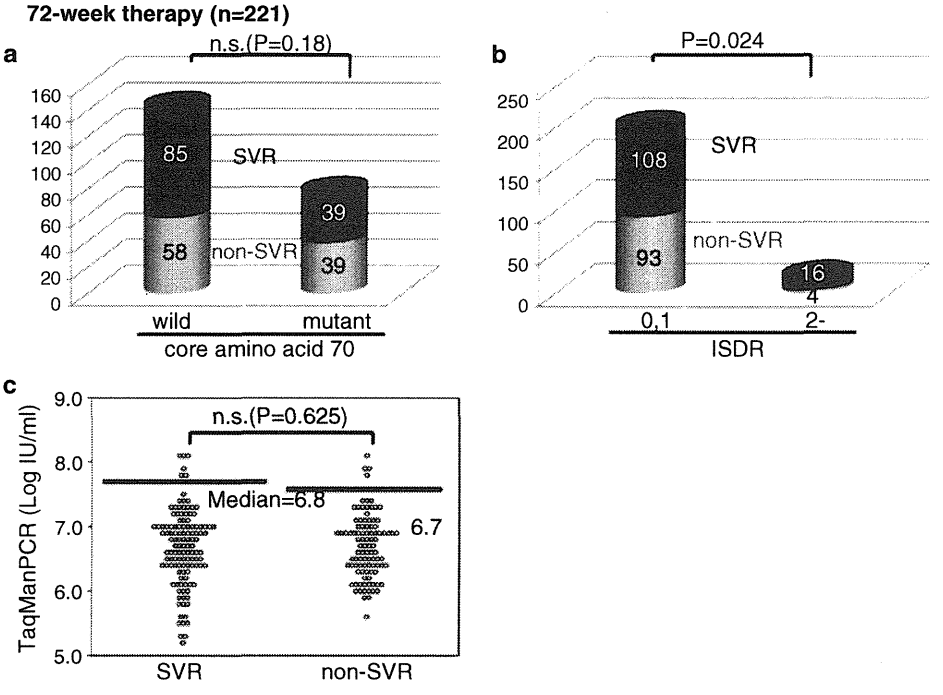
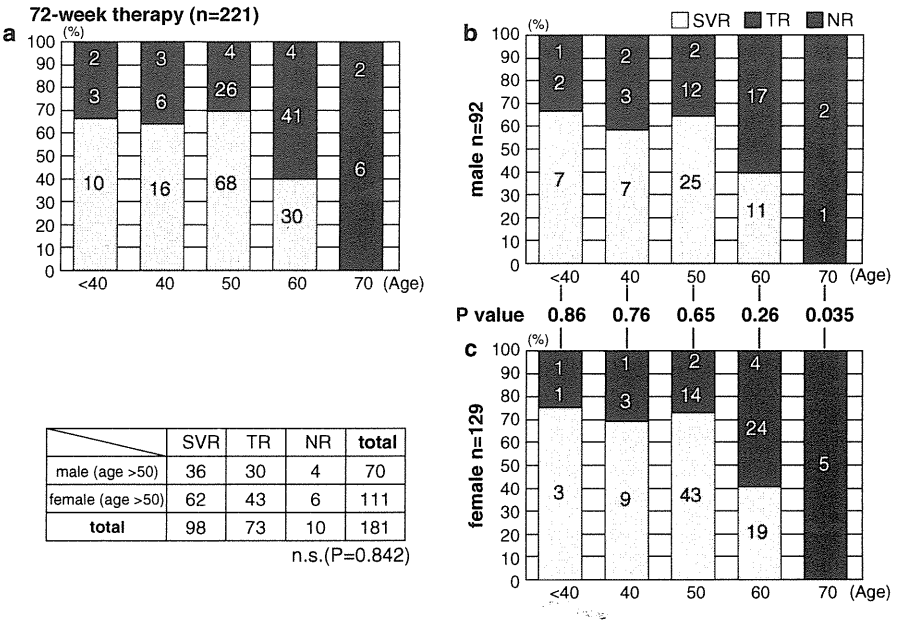


Fig. 3 Viral factors for 72-week treatment. Relationships between sustained virological response and **a** core amino acid 70 substitutions, **b** amino acid substitutions in the interferon sensitivity determining region, and **c** baseline viral titers grouped by SVR and non-SVR for patients treated for 72 weeks. *n.s.*, Not significant

as SVR if γGTP was less than 48.5 IU/l (57%) and NR otherwise (77%).

All factors selected during tree construction were found to be significant in univariate analysis, except for treatment length and cholesterol, and each remained significant in multivariate logistic regression. Although LDL was included in the multivariate logistic model, it was not selected during tree construction. Tenfold cross-validation resulted in 65.2% correctly classified instances with a kappa statistic of 0.29. The true positive rate was 69.2%, the false positive rate was 39.7%, and precision was 68.4%.

To compare the performance of SVR prediction between the logistic and CART models, the WEKA Logistic classifier was used to perform tenfold validation based on the



	SVR	TR	NR	total
male (age >50)	36	30	4	70
female (age >50)	62	43	6	111
total	98	73	10	181

n.s.(P=0.842)

Fig. 4 Relationship between age and response to treatment for 72-week therapy. Treatment outcomes by age in 10-year intervals are shown for **a** all patients, **b** males only, and **c** females only

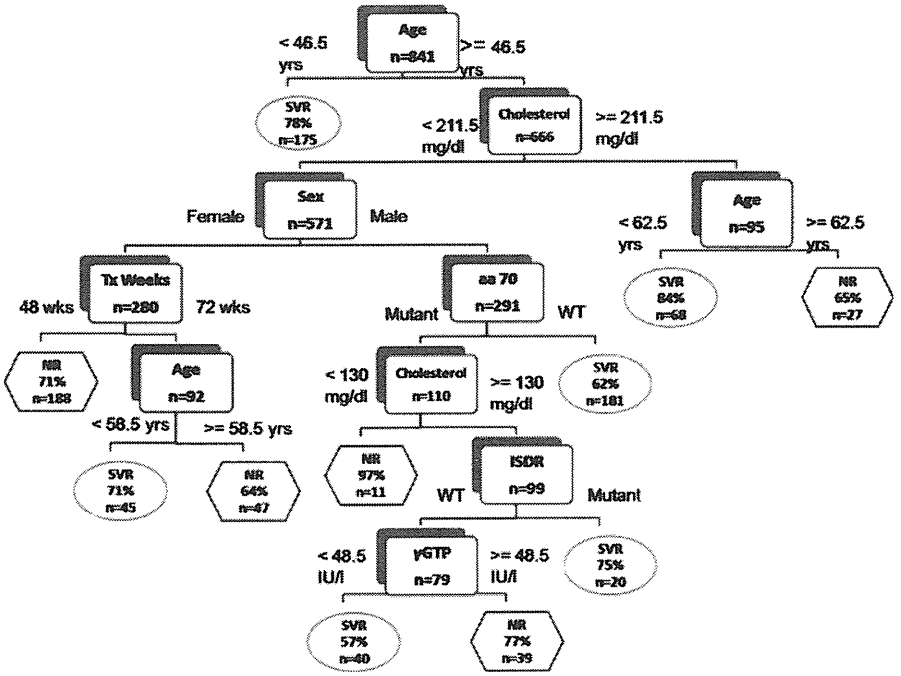


Fig. 5 Decision tree for SVR prediction. *Boxes* represent branch points based on cutoff values for factors determined by the tree generation algorithm. Each branch contains two choices, and each path ends in a prediction for either SVR or NR with an associated probability. *yrs* Years, *Tx* treatment, *ISDR* interferon sensitivity determining region, *aa* amino acid, *WT* wild-type, *γGTP* γ-glutamyl transpeptidase

multivariate logistic regression model above. The true positive rate for the logistic classifier was somewhat higher, at 73.1%, but with a slightly worse false-positive rate of 48%, and 63.7% correctly classified instances with a kappa statistic of 0.25 and precision 0.65. Receiver operating characteristic (ROC) curves were very similar, and the area under the curve was 0.677 for the CART model and 0.696 for the logistic model.

Discussion

Using two complementary approaches we identified several pretreatment factors predictive for SVR in patients treated for 48 and 72 weeks. Logistic regression and CART analysis both suggest that sex, age, cholesterol, and substitutions at core aa70 and ISDR are associated with SVR in patients with a high viral load of genotype 1b. Based on

the decision tree topology and a significant interaction between sex and treatment duration, it appears that 72 weeks of treatment may be most beneficial in women between the ages of 46 and 58 years who have low cholesterol. In general, patients who are younger, male, have cholesterol over 130 mg/dl, or who have wild-type core aa70 or mutant ISDR are the most likely to achieve an SVR.

Because each of the above values can be determined prior to treatment and are interpretable by clinicians, they may be useful as a guide when establishing a treatment regimen in the case of potentially difficult-to-treat patients. Once IFN treatment has been started, early and/or rapid viral response is likely to be the strongest predictor of SVR [33], and slow responders have been shown to be the most likely to benefit from extended treatment [34, 35]. However, because of the expense, low success rate, and potential side effects of IFN-based therapy, predictors available prior to treatment are also needed. Factors predictive of NR may help guide the decision to avoid or discontinue IFN therapy in patients with a low probability of SVR, and factors predictive of SVR may help identify subsets of patients who are likely to achieve an SVR if treated longer than the standard 48-week regimen.

Several other recent studies have examined predictors for SVR for 72 weeks of treatment, although nearly all focus on on-treatment predictors and conclude that 72-week therapy significantly improves SVR rates in slow responders [9, 10, 35]. Ferenci et al. [11] also showed that extension to 72 weeks decreased the relapse rate among early viral responders. In a large retrospective cohort study, Watanabe et al. [36] dissected a complex relationship between SVR and age, sex, and viral load similar to that reported here, although results are difficult to compare because they did not measure cholesterol or viral substitutions. While they recommend 72-week therapy for all slow-responding patients regardless of sex or age, they note that the SVR rate was surprisingly high among elderly female patients following 72-week treatment, noting that the SVR for 48-week treatment was typically low among older female patients in Japan, which they suggest could be related to the development of insulin resistance associated with menopause [36]. Other studies discourage the use of 72-week therapy for all patients except in the specific case of slow responders [8]. Moreover, in a large prospective study, Buti et al. [34] conclude that 48-week combination therapy should remain the standard of care even for slow responders, due to the increased cost and incidence of adverse events relative to a modest increase in the SVR rate. They clarify, however, that patients with a less than 2 log decline at week 8 and undetectable HCV RNA at week 24 are the most likely to benefit from 72-week treatment. Unfortunately they did not examine other predictors in a multivariate analysis. Because each of these studies hinges on rapid versus slow viral response and an on-treatment predictor requiring up to 24 weeks of treatment to establish, pretreatment predictors of early viral kinetics, including those presented here (e.g., viral substitutions and baseline cholesterol levels [12]), may be useful for predicting the outcome of extended therapy prior to treatment [17].

The combination of multiple approaches to identify predictive factors should help improve confidence in the results and partially protect against the bias inherent in any single approach. Comparing the results of a standard analysis with an alternative technique may reveal which variables are robust and which are sensitive to methodological differences. There are many different classification tools, including neural networks, Bayesian networks, and support vector machines, but models based on these may be more difficult to interpret or apply in clinical practice. On the other hand, decision tree approaches such as C4.5 and CART are widely used in biomedical studies [37–39] and provide a simple and intuitive hierarchical format that in many cases can be used without a computer.

The lack of randomized assignment of patients to duration of treatment limits the conclusions that can be drawn from the present study, and additional predictive factors, particularly interleukin (IL) 28B single-nucleotide polymorphism (SNP) genotype and viral kinetics, should be included in future prospective studies. Comparison of ROC curves suggests that the performance of the two models in the present study is similar, although neither is sufficiently sensitive or specific for accurate clinical prediction based on the number of patients analyzed. Nonetheless the strong overlap between the variables selected by each method suggests that several patient factors, including age, sex, and cholesterol level, as well as several viral factors, including core aa70 and ISDR substitutions, are robust predictors for SVR. Differences in the variables selected between the two approaches suggest that several models with similar predictive ability are also possible. In the regression model, LDL cholesterol but not total cholesterol was an independent factor associated with SVR, whereas in the CART analysis total cholesterol was selected instead. This may be due to the hierarchical nature of decision tree models, which may yield better results in the face of missing data, higher-order interactions, or nonlinear relationships. Comparison of separate models for 48 and 72 weeks also suggests that age and ISDR substitutions are important predictors of SVR for patients undergoing 72 weeks of treatment, whereas the decision tree suggests that the 72-week treatment length is important mainly for a subgroup of female patients. Without greater understanding of the role of HCV core and ISDR substitutions, it is difficult to interpret the role of these predictors, as well as

potential interactions with cholesterol level and other clinical factors. Further studies should be performed to investigate these interactions and to better characterize the subgroup of patients who are most likely to respond to long-term IFN therapy.

Acknowledgments This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Health, Labor and Welfare and Ministry of Education, Culture, Sports, Science and Technology, Government of Japan. We thank Sakura Akamatsu and Mika Tsuzuno for their assistance.

Conflict of interest None of the authors have conflicts of interest to declare.

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Research Article

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Comparison of resection and ablation for hepatocellular carcinoma: A cohort study based on a Japanese nationwide survey

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Background & Aims: The treatment of choice for early or moderately advanced hepatocellular carcinoma (HCC) with good liver function remains controversial. We evaluated the therapeutic impacts of surgical resection (SR), percutaneous ethanol injection (PEI), and radiofrequency ablation (RFA) on long-term outcomes in patients with HCC.

Methods: A database constructed on the basis of a Japanese nationwide survey of 28,510 patients with HCC treated by SR, PEI, or RFA between 2000 and 2005 was used to identify 12,968 patients who had no more than 3 tumors (≤3 cm) and liver damage of class A or B. The patients were divided into SR (n = 5361), RFA (n = 5548), and PEI groups (n = 2059). Overall survival and time to recurrence were compared among them.

Results: Median follow-up was 2.16 years. Overall survival at 3 and 5 years was respectively 85.3%/71.1% in the SR group, 81.0%/61.1% in the RFA, and 78.9%/56.3% in the PEI. Time to recurrence at 3 and 5 years was 43.3%/63.8%, 57.2%/71.7%, and 64.3%/76.9%, respectively. On multivariate analysis, the hazard ratio for death was significantly lower in the SR group than in the RFA (SR *vs.* RFA:0.84, 95% confidence interval, 0.74–0.95; $p = 0.006$) and PEI groups (SR *vs.* PEI:0.75, 0.64–0.86; $p = 0.0001$). The hazard ratios for recurrence were also lower in the SR group than in the RFA (SR *vs.* RFA:0.74, 0.68–0.79; $p = 0.0001$) and PEI groups (SR *vs.* PEI:0.59, 0.54–0.65; $p = 0.0001$).

Conclusions: Our findings suggest that surgical resection results in longer overall survival and shorter time to recurrence than either RFA or PEI in patients with HCC.

Keywords: Hepatectomy; Surgical resection; Radiofrequency ablation; Percutaneous ethanol injection.

Received 3 October 2012; received in revised form 2 November 2012; accepted 7 November 2012

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Abbreviations: HCC, hepatocellular carcinoma; SR, surgical resection; RFA, radiofrequency ablation; PEI, percutaneous ethanol injection; TACE, transcatheter hepatic arterial chemoembolization.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women, worldwide [1]. Outcomes remain disappointing, despite recent progress in the techniques of diagnosis and therapy. Japanese [2], European [3] and American [4] clinical practice guidelines strongly recommend surgical resection (SR) and percutaneous ablation, including radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI), for the management of early or moderately advanced HCC (i.e., up to 3 tumors 3 cm or less in diameter) in patients with adequately maintained liver function. Although comparative studies of these treatments have been conducted previously [5–7], the most suitable treatment strategy still remains controversial.

By nationwide surveys initiated in 1965, the Liver Cancer Study Group of Japan has prospectively collected data on patients with HCC in Japan. The Group conducted two retrospective analyses to define the treatment with the best outcomes [8,9]. However, each of the analyses was flawed, and had several problems: data on RFA were not included in the first report [8], and the follow-up period was short in the second one [9]. Although the second analysis demonstrated that surgical resection was superior to RFA and PEI for preventing recurrence [9], no apparent difference in the overall survival could be discerned between surgery and percutaneous ablation therapies (RFA and PEI). Thus, the treatment of choice for less advanced HCC still remains under debate.

Before starting this study, the results of 2 randomized controlled trials (RCT) were available [10,11]. As we pointed out in a previous report [12], however, the study designs of these 2

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trials were critically flawed by factors such as insufficient sample size, excessively optimistic hypotheses, and high conversion ratios. Because of these problems, the results of the two RCTs do not allow firm conclusions to be drawn concerning the important clinical question: is surgery or percutaneous ablation the treatment of choice for early or moderately advanced HCC? To answer this question, we conducted this cohort study based on the latest data available from a Japanese nationwide survey.

Patients and methods

Patients and settings

The Liver Cancer Study Group of Japan has performed nationwide surveys of patients with primary liver cancer since 1965. Patients are registered and followed up, as reported previously [9]. Although this study protocol was not submitted to the Institutional Review Board of each institution participating in the nationwide survey, the collection and registration of data of patients with HCC were performed with the approval of each institution. Because RFA has been available for clinical use since 1999 in Japan, we set the study period from 2000 to 2005, to exclude preliminary experiences with RFA. During this period, a total of 28,510 patients with HCC were registered and received surgical resection, RFA or PEI as the primary treatment with curative intent for HCC. We identified 12,968 patients who met the following criteria: (1) liver function classified as liver damage A or B defined by the Liver cancer Study Group of Japan [13]; (2) number of tumors 3 or less; (3) maximum tumor diameter $\leqslant$3 cm. The 12,968 patients were divided into 3 groups according to the treatment received: SR group (n = 5361, 41.3%), RFA group (n = 5548, 42.8%), and PEI group (n = 2059, 15.9%). The diagnostic criteria and details of follow-up were described previously [8]. Because it has been unusual for biopsies to be performed in cases treated by percutaneous ablation in Japan, histological findings such as microscopic vascular invasion, tumor grading, and microscopic intrahepatic metastasis were not evaluated in this study. Relevant clinical data were collected and analyzed.

Statistical analyses

The baseline characteristics of the three groups (Table 1) were compared by analysis of variance for continuous variables and by Chi-square or Mantel-trend tests for categorical variables. Consistent with our preliminary report [9], the SR group had a higher proportion of younger patients and male patients than the RFA and PEI groups. Hepatitis C virus infection was less prevalent in the SR group than in the RFA and PEI groups. Based on the liver damage class, serum albumin and total bilirubin levels, platelet counts, and the indocyanine green retention rate at 15 min, liver function was better in the SR group than in the RFA and PEI groups, consistent with our previous report [9]. As for tumor-related factors, the number of tumors was smaller, and the maximum tumor diameter was larger in the SR group than in the RFA or PEI group. The SR group had the lowest proportion of patients with abnormally elevated alpha-fetoprotein levels ($\geqslant$15 ng/ml) and the highest proportion of patients with abnormally elevated des-γ-carboxy prothrombin levels ($\geqslant$40 AU/ml).

Overall survival and time to recurrence curves were plotted using the Kaplan–Meier method and compared with the use of the log-rank test. Recurrence was diagnosed on the basis of imaging studies, clinical data, and/or histopathological studies at each institution [9].

The therapeutic impacts of surgical resection, RFA and PEI were estimated using a Cox proportional hazards model including the following 10 covariates: age, gender, liver damage class, hepatitis C virus antibody, hepatitis B surface antigen, platelet count, number of tumors, tumor size, and serum alpha-fetoprotein and des-γ-carboxy prothrombin levels. The results of multivariate analysis were expressed as hazard ratios with 95% confidence intervals. p values of <0.05 were considered to indicate statistical significance.

For the subgroup analyses, the study populations were classified into 8 subgroups according to the tumor size (< or $\geqslant$2 cm), tumor number (single or multiple), and liver damage class (A or B). Macroscopic vascular invasion was excluded from the subgroup analyses because its presence is a contraindication to percutaneous ablation therapies. The therapeutic impacts of the three treatments were evaluated in each of these subgroups, and hazard ratios with 95% confidence intervals and p values were calculated according to the above three factors (tumor size, number of tumors, and liver damage class).

Results

The median follow-up after treatment was 2.16 years, and the 5th and 95th percentiles were 0.14 and 5.19 years, respectively. The overall survival rates at 3/5 years were 85.3%/71.1% in the SR group, 81.0%/61.1% in the RFA group, and 78.9%/56.3% in the PEI group (Fig. 1). The median survival times were 8.4, 5.9, and 5.6 years in the three groups, respectively. The time to recurrence rates at 3/5 years in the 3 groups were 43.3%/63.8%, 57.2%/71.7%, and 64.3%/76.9%, respectively (Fig. 2).

According to the results of the multivariate analysis, the hazard ratio for death in the SR group was 0.84 (0.74–0.95, p = 0.006) relative to that in the RFA group, and 0.75 (0.64–0.86, p = 0.0001) relative to that in the PEI group (Table 2A). The hazard ratios for recurrence in the SR group were 0.74 (0.68–0.79, p = 0.0001) and 0.59 (0.54–0.65, p = 0.0001) relative to those in the RFA and PEI groups, respectively (Table 2B). These results indicated that the overall survival and time to recurrence rates were both significantly better in the SR group than in the RFA and PEI groups.

The overall survival rates following surgical resection, RFA and PEI in the 4 subgroups with a single tumor are shown in Fig. 3A–D. The results of the subgroup analyses (summarized in Fig. 4A) showed that the overall survival was significantly longer in the SR group than in the RFA group in 2 subgroups of patients, namely, those who had a single tumor smaller than 2 cm in diameter with liver damage class A, and those who had a single tumor 2 cm or larger in diameter with liver damage class B.

As shown in Fig. 4B, the time to recurrence was shorter in the SR group than that in the RFA group in the 4 following subgroups: patients with a single tumor with liver damage class A (regardless of the tumor size), those with multiple tumors 2 cm or larger in diameter with liver damage class A, and those with a single tumor 2 cm or larger in diameter with liver damage class B.

Discussion

Our study showed that surgical resection was associated with significantly lower risk of both death and recurrence as compared to RFA and PEI in patients with early or moderately advanced HCC. Our previous preliminary report [9] suggested that surgery reduces the risk of recurrence, but failed to demonstrate any difference in the overall survival between surgery and percutaneous ablation therapies in patients with early or moderately advanced HCC. The present study reconfirms that surgery is associated with a reduced recurrence rate and newly shows that surgery yields a longer overall survival than percutaneous ablation therapies.

Differences in the results between the present study and previous investigations are most likely related to the sample size and length of follow-up. The total number of subjects increased markedly from 7185 in our previous study to 12,968 in this study, and the median follow-up period increased from 10.4 months to 2.16 years (25.9 months). These factors are considered not only to have enhanced the reliability of our findings, but also to have strengthened our conclusions. We believe that our results, which are, of course, subject to the inherent drawbacks of the study design, are meaningful, given the current lack of credible data derived from well-designed RCTs.

The large sample size and prolonged follow-up period also allowed us to perform several subgroup analyses, which were not feasible in our previous study [9]. We classified the patients

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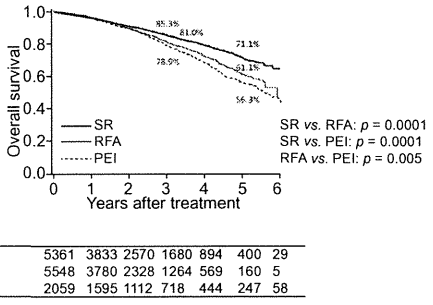
Table 1. Baseline characteristics.

Variables	SR n = 5361	RFA n = 5548	PEI n = 2059	*p* value
Age, median (5, 95 percentile), yr	66 (48, 77)	69 (52, 80)	69 (52, 80)	<0.0001[a]
Sex				<0.0001[b]
Male, No. (%)	3967 (74.0)	3569 (64.3)	1303 (63.3)	
Female, No. (%)	1394 (26.0)	1979 (35.7)	756 (36.7)	
Hepatitis virus infection				<0.0001[b]
HBs Ag(+)/HCV-Ab(-), No. (%)	908 (16.9)	462 (8.3)	141 (6.8)	
HBs Ag(-)/HCV-Ab(+), No. (%)	3393 (63.3)	4263 (76.8)	1632 (79.3)	
HBs Ag(+)/HCV-Ab(+), No. (%)	106 (2.0)	87 (1.6)	32 (1.6)	
HBs Ag(-)/HCV-Ab(-), No. (%)	760 (14.2)	512 (9.2)	160 (7.8)	
Unknown	194 (3.6)	224 (4.0)	94 (4.6)	
Liver damage				<0.0001[b]
A, No. (%)	4000 (74.6)	3349 (60.4)	1204 (58.5)	
B, No. (%)	1361 (25.4)	2199 (39.6)	855 (41.5)	
Serum albumin, median (5, 95 percentile), g/dl	3.9 (3.1, 4.6)	3.7 (2.9, 4.4)	3.7 (2.8, 4.4)	<0.0001[a]
Serum total bilirubin, median (5, 95 percentile), mg/dl	0.8 (0.4, 1.5)	0.9 (0.4, 1.9)	0.9 (0.4, 2.2)	<0.0001[a]
Platelet count, median (5, 95 percentile), x 10^4/μl	12.6 (5.8, 24.0)	9.9 (4.5, 20.4)	9.5 (4.4, 19.6)	<0.0001[a]
ICG R15, median (5, 95 percentile), %	15 (5, 35)	22 (7, 51)	24 (8, 51)	<0.0001[a]
Tumor number				<0.0001[c]
Single, No. (%)	4458 (83.2)	4068 (73.3)	1449 (70.4)	
Two, No. (%)	706 (13.2)	1096 (19.8)	443 (21.5)	
Three, No. (%)	197 (3.7)	384 (6.9)	167 (8.1)	
Tumor size, median (5, 95 percentile), mm	23 (12, 30)	20 (10, 30)	17 (10, 30)	<0.0001[a]
Alpha-fetoprotein				<0.0001[b]
≥15 ng/ml, No. (%)	2726 (50.9)	3028 (54.6)	1125 (54.6)	
<15 ng/ml, No. (%)	2457 (45.8)	2301 (41.5)	828 (40.2)	
Unknown, No. (%)	178 (3.3)	219 (3.9)	106 (5.2)	
Des-γ-carboxy prothrombin				<0.0001[b]
≥40 AU/ml, No. (%)	2182 (40.7)	1593 (28.7)	541 (26.3)	
<40 AU/ml, No. (%)	2651 (49.5)	3322 (59.9)	1169 (56.8)	
Unknown, No. (%)	528 (9.9)	633 (11.4)	349 (17.0)	

HBsAg, hepatitis B virus antigen; HCV-Ab, hepatitis C virus antibody; ICG R15, indocyanine green retention rate at 15 min.
[a]ANOVA.
[b]Chi-square.
[c]Mante-trend test.



Patients at risk

SR	5361	3833	2570	1680	894	400	29
RFA	5548	3780	2328	1264	569	160	5
PEI	2059	1595	1112	718	444	247	58

Fig. 1. Overall survival curves after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI).

into 8 subgroups according to 3 factors (liver damage class, tumor size, and number of tumors), which have repeatedly been shown to be clinically relevant prognostic factors. The results of the subgroup analyses indicated that surgical resection would effectively prevent recurrence in patients with relatively advanced HCC (2–3 cm in diameter) among the study populations, irrespective of liver damage class or number of tumors. This finding suggests that surgery might be superior to percutaneous ablation therapies in patients with a more advanced tumor stage. As for the subgroups with a single tumor, surgical resection yielded better overall survival and time to recurrence rates than RFA or PEI. Especially in the subgroup with a single tumor smaller than 2 cm in diameter, both the overall and time to recurrence rates were statistically significantly better after surgery than after RFA, whereas no such statistically significant differences in these two parameters between the two treatment groups were detected in a few subgroups with a single tumor, maybe due to the insufficient sample size of the subgroups. Thus, surgical resection would be considered as the treatment modality of first choice for a single HCC, as recommended by the Japanese clinical practice guideline [2]. Overall, there was a trend toward superior

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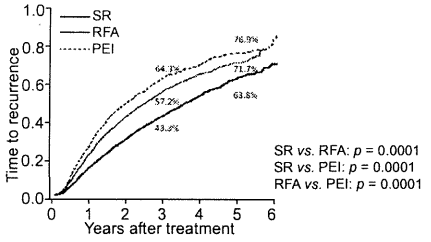
Research Article



Patients at risk							
SR	5361	3265	1844	1039	451	189	15
RFA	5548	2954	1396	591	225	62	4
PEI	2059	1154	583	304	172	90	15

Fig. 2. Time to recurrence curves after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI).

overall and time to recurrence rates after surgery than after RFA and PEI.

The reason why the long-term outcomes of the SR group were better than those of the PEI and RFA groups cannot be definitely clarified from the results of this study, however, in theory, surgical resection has the advantage of offering better local control of HCC over PEI and RFA, both of which have some potential risks of local recurrence associated with insufficient ablation. In addition, anatomic resection to remove minute tumor satellites [14] might have decreased the recurrence rate in the SR group, although this remains a speculation.

Recently, the latest trial from China [15], which had an adequate sample size (total 230 patients), reported that surgical resection yielded significantly better long-term outcomes than RFA. Although the study design was better than that of the two previously reported RCTs [10,11], it appeared to have limitations with respect to the results, such as drop in the overall survival in the RFA group as compared with that in the surgery group during the early period after treatment. The early deaths in the RFA group could have been treatment-related rather than cancer-related. Thus, no conclusion can be drawn from the three currently available RCTs.

One of the limitations of our study is the diversity of demographic factors in the study population, which would have been

Table 2. Hazard ratios for death and recurrence adjusted by multivariate analysis.

A For death

Variables		Hazard ratio	95% CI	*p* value
Treatments	SR *vs.* RFA	0.84	0.74, 0.95	0.006
	SR *vs.* PEI	0.75	0.64, 0.86	0.0001
	RFA *vs.* PEI	0.88	0.77, 1.01	0.08
Age	<65 *vs.* ≥65	0.71	0.63, 0.79	0.0001
Sex	Female *vs.* male	0.87	0.78, 0.98	0.03
HBsAg	Positive *vs.* negative	0.91	0.74, 1.11	0.34
HCV Ab	Positive *vs.* negative	0.93	0.79, 1.10	0.40
Liver damage	A *vs.* B	0.62	0.56, 0.69	0.0001
Platelet count	$\geq 10^4$ *vs.* $<10^4/\mu l$	0.76	0.68, 0.85	0.0001
Tumor size	<2 *vs.* ≥2 cm	0.82	0.73, 0.92	0.0007
Tumor number	Single *vs.* multiple	0.72	0.64, 0.80	0.0001
AFP	<15 *vs.* ≥15 ng/ml	0.66	0.59, 0.74	0.0001
DCP	<40 *vs.* ≥40 AU/ml	0.59	0.53, 0.66	0.0001

B For recurrence

Variables		Hazard ratio	95% CI	*p* value
Treatments	SR *vs.* RFA	0.74	0.68, 0.79	0.0001
	SR *vs.* PEI	0.59	0.54, 0.65	0.0001
	RFA *vs.* PEI	0.81	0.74, 0.88	0.0001
Age	<65 *vs.* ≥65	0.83	0.78, 0.89	0.0001
Sex	Female *vs.* male	0.88	0.82, 0.95	0.0001
HBsAg	Positive *vs.* negative	1.04	0.92, 1.17	0.53
HCV Ab	Positive *vs.* negative	1.15	1.04, 1.27	0.007
Liver damage	A *vs.* B	0.87	0.81, 0.93	0.0001
Platelet count	$\geq 10^4$ *vs.* $<10^4/\mu l$	0.92	0.86, 0.98	0.02
Tumor size	<2 *vs.* ≥2 cm	0.84	0.79, 0.90	0.0001
Tumor number	Single *vs.* multiple	0.69	0.64, 0.74	0.0001
AFP	<15 *vs.* ≥15 ng/ml	0.71	0.67, 0.76	0.0001
DCP	<40 *vs.* ≥40 AU/ml	0.72	0.67, 0.77	0.0001

HBsAg, hepatitis B virus surface antigen; HCV, hepatitis C virus; Ab, antibody; AFP, alpha-fetoprotein; DCP, des-γ-carboxy prothrombin; SR, surgical resection; RFA, radiofrequency ablation; PEI, percutaneous ethanol injection; CI, confidence interval.

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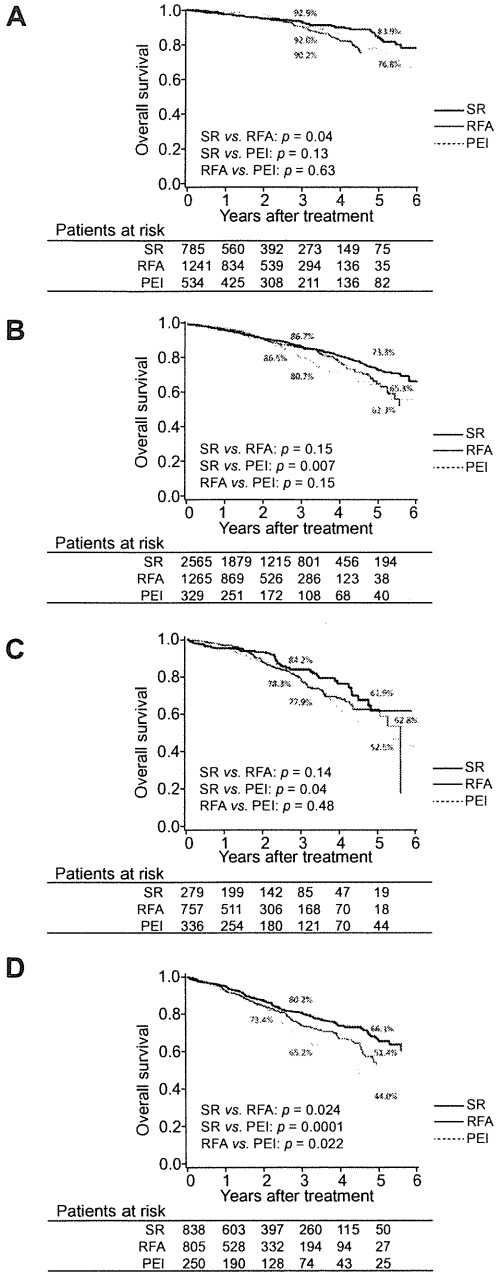

Fig. 3. Overall survival rates after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI) in the subgroup of cases with single tumor and liver damage class A and B. (A) Liver damage class A, a single tumor (<2 cm); (B) liver damage class A, a single tumor (2–3 cm); (C) liver damage class B, a single tumor (<2 cm); (D) liver damage class B, a single tumor (2–3 cm).

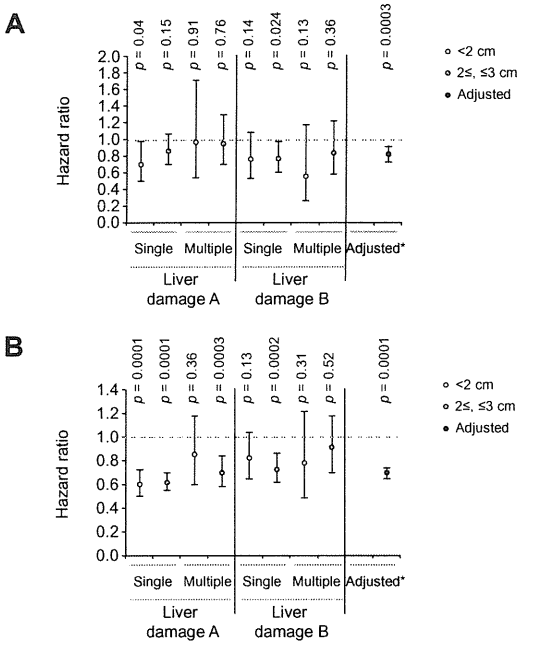

Fig. 4. Hazard ratios for death and recurrence with 95% confidence intervals and *p* values after surgical resection relative to those after radiofrequency ablation in the 8 subgroups. *The adjusted values for death and recurrence were calculated according to the three factors (tumor size, number of tumors, and liver damage class), as done in each subgroup. (A) Hazard ratios for death; (B) hazard ratios for recurrence.

caused by the selection process of treatment modalities. As similar to the previous retrospective studies [5–9], the patients amenable to surgery had had younger age, less prevalence of hepatitis C virus infection, better liver function, less association with portal hypertension, fewer number of tumors and lower alpha-fetoprotein level, whereas their tumor size was larger and their des-γ-carboxy prothrombin level was higher. To minimize potential effects of confounding factors, we studied patients who had similar tumor-related and liver function-related factors and performed multivariate analysis using 10 clinically important factors, similar to our previous study [9]. Although it is impossible to completely eliminate potential negative impacts of demographic diversity, we believe that our results are clinically meaningful, because of the large sample size of our study. In Japan, a nationwide RCT in patients with HCC is now ongoing, and the results are expected to lead to more definitive conclusions [16].

Another potential limitation of our study is the lack of data on liver function during the follow-up, which precluded assessment of the relationship between the liver function status and the choice of treatment at recurrence. In HCC, the influence of the first treatment is considered to be smaller than that in other primary malignant diseases, because the liver function remarkably affects the recurrence rate. Further investigations, particularly prospective clinical trials, are needed to address these issues.

In conclusion, this large cohort study based on data obtained by a nationwide survey in Japan, suggests that surgical resection may offer some advantage over RFA and PEI in terms of both overall survival and time to recurrence in patients with less advanced HCC. Although our results are considered as being more reliable than those of previous studies comparing the treatment

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outcomes in HCC, our conclusions need to be confirmed by future RCTs.

Financial support

This study was supported by the Liver Cancer Study Group of Japan. There were no other sources of funding for any authors.

Conflicts of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgements

We all express our sincere thanks to Mr. Tomohiro Shinozaki, Ph.D. for his great contribution to the preparation of the data for statistical analyses.

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Human Molecular Genetics, 2011, Vol. 20, No. 17 **3507–3516**
doi:10.1093/hmg/ddr249
Advance Access published on June 9, 2011

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

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Received February 9, 2011; Revised May 16, 2011; Accepted May 31, 2011

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