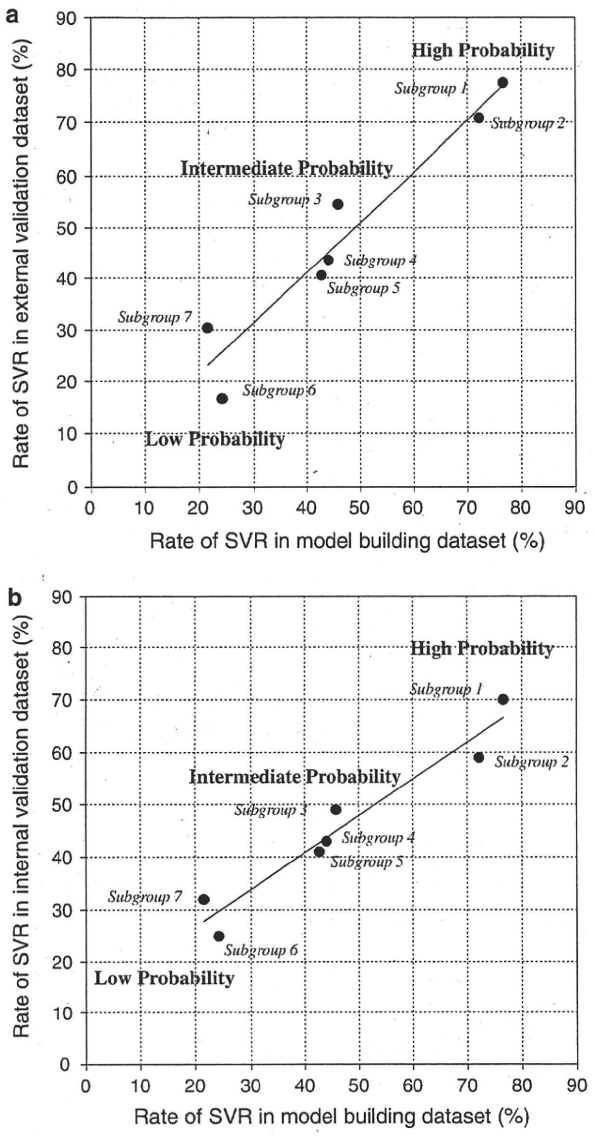

Fig. 2 Validation of the decision tree analysis by an internal and external validation dataset: subgroup-stratified comparison of the SVR rate. The rate of SVR in each subgroup was plotted. The *X* axis represents the model building, and the *Y* axis represents the validation datasets. **a** Internal validation and **b** external validation. There was a close correlation between the model building and the internal validation dataset (correlation coefficient $r^2 = 0.925$) and between the model building and the external validation dataset (correlation coefficient $r^2 = 0.936$)

original dataset used for model building. Each patient in the external validation set was allocated to subgroups 1–7 using the flow-chart form of the tree. The rates of SVR were 70% for subgroup 1, 59% for subgroup 2, 49% for subgroup 3, 43% for subgroup 4, 41% for subgroup 5, 25% for subgroup 6, and 32% for subgroup 7. The rates of SVR for each subgroup of patients were closely correlated

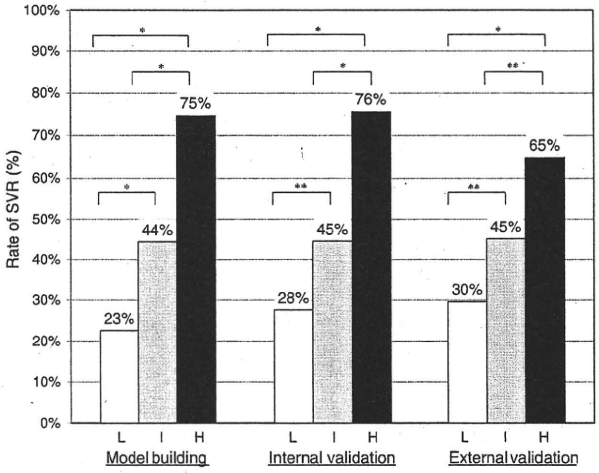

Fig. 3 Comparison of SVR rates between groups divided by the decision tree. The rate of SVR was compared among the 3 groups of patients divided by the decision tree analysis (*white*, *gray* and *black boxes*, indicating a low (*L*), intermediate (*I*) and high (*H*) probability group, respectively). The rate of SVR was significantly different among the 3 groups. $*p < 0.0001$, $**p < 0.001$

between the model-building dataset and the validation dataset ($r^2 = 0.936$) (Fig. 2b).

Construction of 3 groups according to the probability of SVR

Seven subgroups were reconstructed into 3 groups according to their predicted rates of SVR: the high probability group consisted of subgroups 1 and 2, the intermediate probability group consisted of subgroups 3, 4 and 5, and the low probability group consisted of subgroups 6 and 7. The rate of SVR was significantly different among the 3 groups (Fig. 3). The rate of SVR in the high probability group was consistently high: 75% for model building patients, 76% for internal validation patients and 65% for external validation patients. Conversely, the rate of SVR in the low probability group was consistently low: 23% for model building patients, 28% for internal validation patients and 30% for external validation patients. The rate of SVR in the intermediate probability group was 44% for model building patients, 45% for internal validation patients and 45% for external validation patients. Since 28–32% of patients were classified as high probability and 30–32% were classified as low probability, roughly 60% of patients were classified as having either a high or low probability of achieving SVR.

Effect of dose reductions of PEG-IFN and RBV on SVR

The cumulative dose of PEG-IFN and RBV was not included as a variable of analysis since the present study

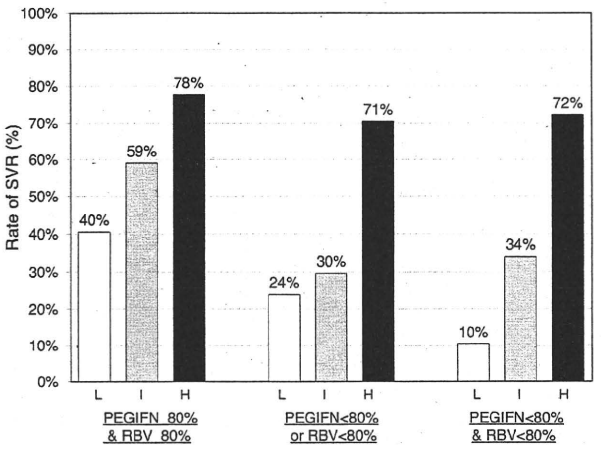

Fig. 4 Comparison of SVR rates among groups stratified by drug adherence. The 3 groups of patients divided by the decision tree analysis (*white*, *gray* and *black boxes* indicating a low (*L*), intermediate (*I*) and high (*H*) probability group, respectively) were further stratified according to the cumulative drug exposure of PEG-IFN and RBV. The good adherence group (≥80% planned dose of both PEG-IFN and RBV) had a higher rate of SVR compared with the poor adherence group (<80% planned dose of both PEG-IFN and RBV) in the low ($p = 0.0003$) and intermediate ($p = 0.007$) but not in the high probability group ($p = 0.53$)

aimed to develop a pre-treatment model for the prediction of response. To analyze the possible effect of drug reductions on the result of the decision tree analysis, 3 groups of patients divided by the decision tree analysis (low, intermediate and high probability group) were further stratified according to the cumulative drug exposure of PEG-IFN and RBV (Fig. 4). Even after adjustment for adherence, 3 groups of patients still had low, intermediate and high probability of achieving SVR, respectively. Of note, the good adherence group (≥80% planned dose of both PEG-IFN and RBV) had higher rates of SVR compared with the poor adherence group (<80% planned dose of both PEG-IFN and RBV) in the low ($p = 0.0003$) and intermediate ($p = 0.007$) probability group, but not in the high probability group ($p = 0.53$).

Factors associated with SVR by multivariate logistic regression analysis

We also explored the factors associated with SVR using a standard statistical analysis. By univariate analysis, age, gender, serum albumin, creatinine, alanine aminotransferase, GGT, red blood cell count, hemoglobin, hematocrit, platelet count and AFP were found to be associated with SVR (Table 2). HCVRNA load was not associated with SVR. By multivariate analysis, age, gender, GGT and platelet count were found to be independently associated with SVR (Table 3). Of note, AFP, which was selected as a significant predictor of response in the decision tree analysis, was not found to be an independent response predictor in the standard multivariate analysis. This indicates a unique feature of the decision tree analysis; i.e., it could identify significant predictors that specifically apply to selected patients, in this case patients younger than 50 years old.

Relationships between decision tree model and stage of fibrosis or HCV RNA load

Liver biopsy was performed in 664 patients. The distribution of fibrosis in three probability groups differed significantly. Advanced fibrosis (F3 or F4) was higher in the low probability group (39%) compared to the intermediate probability group (13%) ($p < 0.0001$) and to the high probability group (6%) ($p < 0.0001$). Advanced fibrosis was also higher in the intermediate group compared to the high probability group ($p = 0.01$). AFP was significantly associated with liver fibrosis stage: medians of AFP levels were 4.9, 5.9, 13.0 and 18.6 for F1, F2, F3 and F4, respectively ($p < 0.0001$, Spearman's rank correlations). Lower platelet counts correlated with advanced fibrosis stages (data not shown). The SVR rate was higher in the high probability group compared to the intermediate or low probability group after stratification by HCV RNA load. Among patients with low HCVRNA load (<400,000 IU/ml), the rate of SVR was 93, 59 and 50% for the high, intermediate and low probability group, respectively ($p = 0.002$ for high vs. intermediate and $p < 0.001$ for high vs. low probability groups). Among patients with a high HCVRNA load (≥400,000 IU/ml), the rate of SVR was 73, 42 and 21% for the high, intermediate and low probability group, respectively ($p < 0.001$ for high vs. low, high vs. intermediate and intermediate vs. low probability groups).

Discussion

Currently, the combination of PEG-IFN and RBV is the recommended therapy for chronic HCV infection. The rate of SVR with 48 weeks of therapy is around 50% in patients with HCV genotype 1b and a high HCV RNA titer [2, 3]. To date, the virological response during therapy is the most reliable means for predicting the likelihood of SVR [2, 24, 25]. More potent therapy, such as a triple combination of protease inhibitor, PEG-IFN and RBV, is being evaluated in clinical trials but is not readily available [26, 27]. Under the circumstances, pre-treatment prediction of the likelihood of SVR may be useful for both patients and physicians to support clinical decisions as to whether to start PEG-IFN/RBV therapy or delay treatment until a new more effective therapy becomes available.

Table 2 Comparison of pre-treatment factors between patients with and without sustained virological response (SVR) among the model building dataset ($n = 506$)

	SVR ($n = 240$)	Non-SVR ($n = 266$)	p
Age (years)	54 (25–75)	60 (36–73)	<0.0001
Male gender[a]	151/240 (63%)	171/266 (41%)	<0.0001
Body mass index (kg/m^2)	22.5 (16.8–32.0)	22.6 (15.5–33.3)	0.244
Albumin (g/dl)	4.1 (3.2–5.0)	4 (2.7–4.9)	0.004
Creatinine (mg/dl)	0.7 (0.44–1.14)	0.69 (0.39–1.47)	<0.0001
AST (IU/l)	59 (11–370)	61 (17–261)	0.457
ALT (IU/l)	58 (11–413)	53 (11–316)	0.031
GGT (IU/l)	31 (10–322)	43 (12–328)	0.005
Total cholesterol (mg/dl)	175 (87–297)	171 (73–274)	0.184
Triglyceride (mg/dl)	105 (36–474)	105 (33–294)	0.992
White blood cell count (/μl)	4,600 (2,200–10,900)	4,425 (1,800–10,810)	0.479
Neutrophils (/μl)	2,507 (667–7,870)	2,423 (900–7,281)	0.321
Red blood cell count (/μl)	455 (336–577)	441 (313–564)	0.001
Hemoglobin (g/dl)	14.3 (10.2–17.6)	13.9 (9.4–17.9)	0.004
Hematocrit (%)	42.1 (13.3–53.7)	41.2 (30.7–52.0)	0.031
Platelets (10^9/l)	178 (81–380)	142 (60–320)	<0.0001
AFP (ng/ml)	4.3 (0.9–680)	6.4 (1.9–468)	0.041
HCVRNA (10^3 IU/ml)	1,400 (100–5,100)	1,700 (100–5,100)	0.659
Fibrosis stage: F3–4[a]	21/198 (11%)	52/219 (24%)	<0.0001

Data expressed as median (range) unless otherwise indicated

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma-glutamyltransferase, *AFP* alpha-fetoprotein

[a] Data expressed as number/available data (percentage)

Table 3 Multivariate logistic regression analysis for factors associated with sustained virological response (SVR)

	Odds	95% CI	p value
Age (years)	0.96	0.94–0.98	0.001
Platelets (10^9/l)	1.09	1.04–1.14	<0.0001
ALT (IU/l)	1.01	1.00–1.01	0.001
GGT (IU/l)	0.99	0.98–0.99	<0.0001
Male gender	2.92	1.87–4.55	<0.0001

GGT gamma-glutamyltransferase

Using the data mining analysis, we constructed a simple decision tree model for the pre-treatment prediction of response to PEG-IFN/RBV. The analysis highlighted 5 variables relevant to response: age, gender, platelet count, AFP and GGT. Classification based on these variables identified subgroups of patients with high probabilities of achieving SVR among difficult to treat genotype 1b chronic hepatitis C patients. The reproducibility of the model was confirmed by the independent internal and external validation datasets. An advantage of the decision tree analysis over traditional regression models is that the decision tree model is user-intuitive and can be readily interpreted by medical professionals without any specific knowledge of statistics. Patients can be allocated to specific subgroups with a defined rate of response simply by following the flow-chart form. Using this model, an estimate of the response before treatment can be rapidly obtained, which may facilitate clinical decision making. Thus, this model could be readily applicable to clinical practice.

According to the results of the decision tree analysis, patients were categorized into 3 groups: the rate of SVR was 23–30% for the low probability group, 44–45% for the intermediate probability group and 65–76% for the high probability group. About 30% of patients were each categorized in the high and low probability group and the remaining 40% of patients in the intermediate probability group. These results support the evidence-based approach for selecting an optimum treatment strategy for individual patients. For example, patients in the high probability group may be the most suitable candidates for PEG-IFN/RBV therapy, while patients in the low probability group may be advised to wait for a future therapy, such as the combination of protease inhibitor, PEG-IFN and RBV. However, the estimation of low probability should not be used to preclude patients from therapy, and the final decision should be made on a case-by-case basis, taking into consideration the acceptance by the patient of a low likelihood of response and the potential risk of disease progression while waiting for a future therapy.

Another important finding was that poor adherence to drugs lowered the rate of SVR in the low and intermediate probability groups, which implies that effort should be made to maintain ≥80% of the planned dose of PEG-IFN and RBV in those patients. On the other hand, the rate of SVR was high irrespective of drug adherence in the high probability group. Whether shorter duration of therapy is sufficient in this group of patients should be confirmed in future study.

The variables used in the decision tree have been previously reported to associate with the efficacy of IFN therapy. Younger age and male gender are associated with a favorable response [28]. Lower platelet count is a hallmark of advanced fibrosis in chronic hepatitis C and is reported to be associated with poor response to IFN [29]. AFP is usually used for the screening or the diagnosis of hepatocellular carcinoma, but recent studies suggest an association between higher AFP levels and poor response to IFN therapy [30–33]. Previous report speculated that higher expression of AFP by hepatic progenitor cells may be associated with non-response to therapy [30]. Another report speculated that AFP levels predict poor response to therapy through the underlining link to advanced liver fibrosis [31]. Our data support the latter speculation since advanced fibrosis was associated with elevation of AFP levels. Fibrosis of the liver is an important predictor of response, but we did not include this factor in the decision tree analysis since liver biopsy may not always be available in general practice. As a result, two predictive factors that correlate with fibrosis stage (platelet counts and AFP) were selected in the model, and three probability groups reflected the different distribution of fibrosis stage. GGT is reported to be associated with insulin resistance and hepatic steatosis [34–37], a factor that confers resistance to IFN therapy [38–44]. What is unique to the present study is the visualization of response probability by combining these factors and its high reproducibility revealed by a high-quality validation of the model by internal and external validation datasets that were completely independent of the model building dataset. Since factors used in the model were clinical parameters that are readily available by the usual workup of patients, this model could be immediately applicable to clinical practice without imposing costs for additional examinations.

A potential limitation of this study is that data mining analysis has an intrinsic risk of showing relationships that fit to the original dataset but are not reproducible in different populations. Although internal and external validations showed that our model had high reproducibility, we recognize that further validation on a larger external validation cohort, especially in populations other than Japanese, may be necessary to further verify the reliability of our model.

In conclusion, we built a pre-treatment model for the prediction of virological response to PEG-IFN/RBV. Because this decision tree model was made up of simple variables, it can be easily applied to clinical practice. This model may have the potential to support decisions about patient selection for PEG-IFN/RBV based on a possibility of response weighed against the potential risk of adverse events or costs.

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Hepatic ISG Expression Is Associated With Genetic Variation in Interleukin 28B and the Outcome of IFN Therapy for Chronic Hepatitis C

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See related article, Younossi and Stepanova, on page 718 in *CGH*.

BACKGROUND & AIMS: Multiple viral and host factors are related to the treatment response to pegylated-interferon and ribavirin combination therapy; however, the clinical relevance and relationship of these factors have not yet been fully evaluated. **METHODS:** We studied 168 patients with chronic hepatitis C who received pegylated-interferon and ribavirin combination therapy. Gene expression profiles in the livers of 91 patients were analyzed using an Affymetrix genechip (Affymetrix, Santa Clara, CA). The expression of interferon-stimulated genes (ISGs) was evaluated in all samples by real-time polymerase chain reaction. Genetic variation in interleukin 28B (IL28B; rs8099917) was determined in 91 patients. **RESULTS:** Gene expression profiling of the liver differentiated patients into 2 groups: patients with up-regulated ISGs and patients with down-regulated ISGs. A high proportion of patients with no response to treatment was found in the up-regulated ISGs group (P = .002). Multivariate logistic regression analysis showed that ISGs (<3.5) (odds ratio [OR], 16.2; $P < .001$), fibrosis stage (F1-F2) (OR, 4.18; $P = .003$), and ISDR mutation (≥2) (OR, 5.09; $P = .003$) were strongly associated with the viral response. The IL28B polymorphism of 91 patients showed that 66% were major homozygotes (TT), 30% were heterozygotes (TG), and 4% were minor homozygotes (GG). Interestingly, hepatic ISGs were associated with the IL28B polymorphism (OR, 18.1; $P < .001$), and its expression was significantly higher in patients with the minor genotype (TG or GG) than in those with the major genotype (TT). **CONCLUSIONS: The expression of hepatic ISGs is strongly associated with treatment response and genetic variation of IL28B. The differential role of host and viral factors as predicting factors may also be present.**

Keywords: Pegylated Interferon, Ribavirin; Gene Expression; Single Nucleotide Polymorphism.

A human liver infected with hepatitis C virus (HCV) develops chronic hepatitis, cirrhosis, and, in some instances, hepatocellular carcinoma.[1] Interferon (IFN) and ribavirin (RBV) combination therapy is a popular modality for treating patients with chronic hepatitis C (CH-C); approximately 50% of patients usually relapse, particularly those with HCV genotype 1b and a high viral load.[2,3] Therefore, it is beneficial to predict the response of patients with the 1b genotype and a high viral load to pegylated-IFN (Peg-IFN) and RBV combination therapy before starting treatment because therapy can be long, costly, and have many adverse effects. Amino acid (aa) substitutions in the interferon sensitivity determining region (ISDR), located in the HCV nonstructural region 5A, are useful for predicting the response of patients with genotype 1b to IFN therapy.[4] However, viral factors alone do not sufficiently predict the outcome of treatment in every case.[5]

In addition to viral factors, hepatic gene expression before and during IFN treatment has been examined to determine host factors associated with the response to treatment.[6,7] Interferon-stimulated genes (ISGs) up-regulated in the liver prior to treatment might be related to the poor induction of ISGs and the impaired eradication of HCV during treatment.[6–9] This may be because the ISGs have already been maximally induced before treat-

Abbreviations used in this paper: **aa, amino acid; AST, aspartate aminotransferase; cDNA, complementary DNA; CH-C, chronic hepatitis C; Down-ISGs, down-regulated ISGs; EVR, early virologic response; GWAS, genome-wide association studies; HCV, hepatitis C virus; IFN, interferon; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IL, interleukin; IL28B, interleukin 28B; ISDR, interferon sensitivity determining region; ISGs, interferon stimulated genes; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); NR, no response; Peg, pegylated; RBV, ribavirin; ROC, receiver operating characteristic; RTD, real-time detection; PCR, polymerase chain reaction; RTD-PCR, real-time detection-polymerase chain reaction; SNP, single nucleotide polymorphism; SVR, sustained viral response; TR, transient response; Up-ISGs, up-regulated ISGs.**

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ment. However, the clinical relevance of the expression of ISGs as predictive factors for the outcome of treatment has not yet been fully evaluated.

In parallel to gene expression analysis, genome-wide association studies (GWAS) have been used to identify loci associated with the response to treatment; genetic variation in interleukin 28B (IL28B) was found to predict hepatitis C treatment-induced viral clearance.[10–12]

In this study, with a relatively large cohort of CH-C patients treated with Peg-IFN and RBV, we validated the clinical relevance of the expression of hepatic ISGs as predictive factors for the outcome of treatment. In addition, we demonstrated that the expression of hepatic ISGs was closely related to genetic variation in IL28B.

Materials and Methods

Patients

We enrolled 168 patients with CH-C at the Graduate School of Medicine, Kanazawa University Hospital and its related hospitals, Japan (Table 1, Supplementary Table 1). The cohort included 92 men and 76 women, ranging from 21 to 73 years of age, who were registered prospectively in 2005 and 2007. All patients had HCV

Table 1. Comparison of Clinical Factors Between Patients With and Without NR

Clinical category	SVR+TR		NR		Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
No. of patients	n = 125		n = 43			—	
Age and sex							
Age, *y*	57	(30–72)	56	(30–73)	.927	—	
Sex (M vs F)	68 vs 57		24 vs 19		.872	—	
Liver factors							
F stage (F1-2 vs F3-4)	95 vs 30		20 vs 23		.001	4.18 (1.61–11.5)	.003
A grade (A0-1 vs A2-3)	68 vs 57		19 vs 24		.248	—	
ISGs (Mx, IFI44, IFIT1)							
(<3.5 vs ≥3.5)	103 vs 22		12 vs 31		<.001	16.2 (6.21–47.8)	<.001
Laboratory parameters							
HCV-RNA (*KIU/mL*)	2300	(126–5000)	1930	(140−5000)	.725	—	
BMI (kg/m^2)	23.2	(16.3–34.7)	23.4	(19.5–40.6)	.439	—	.107
AST (*IU/L*)	46	(18–258)	64	(21–283)	.017	—	
ALT (*IU/L*)	60	(16–376)	82	(18–345)	.052	—	
γ-GTP (*IU/L*)	36	(4–367)	75	(26–392)	<.001	—	
WBC ($/mm^3$)	4800	(2100–11,100)	4800	(2500–8200)	.551	—	
Hb (*g/dL*)	14	(9.3–16.6)	14.4	(11.2–17.2)	.099	—	
PLT ($\times 10^4/mm^3$)	15.7	(7–39.4)	15.2	(7.6–27.8)	.378	—	
TG (*mg/dL*)	98	(30–323)	116	(45–276)	.058	—	
T-Chol (*mg/dL*)	167	(90–237)	160	(81–214)	.680	—	
LDL-Chol (*mg/dL*)	82	(36–134)	73	(29–123)	.019	—	
HDL-Chol (*mg/dL*)	42	(20–71)	47	(18–82)	.098	—	
FBS (*mg/dL*)	94	(60–291)	96	(67–196)	.139	—	
Insulin (*μU/mL*)	6.6	(0.7–23.7)	6.8	(2–23.7)	.039	—	
HOMA-IR	1.2	(0.3–11.7)	1.2	(0.4–7.2)	.697	—	
Viral factors							
ISDR mutations ≤1 vs ≥2	80 vs 44		34 vs 9		.070	5.09 (1.69–17.8)	.003
Treatment factors							
Total dose administered							
Peg-IFN (*μg*)	3840	(960–7200)	3840	(1920–2880)	.916	—	
RBV (*g*)	202	(134–336)	202	(36–336)	.531	—	
Achieved administration rate							
Peg-IFN (%)							
≥80%	84		28		.975	—	
<80%	42		14				
RBV (%)							
≥80%	76		24		.745	—	
<80%	50		18				
Achievement of EVR	101/125 (81%)		0/43 (0%)		<.001	—	

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; EVR, early virologic response; γ-GTP, γ-glutamyl transpeptidase; ISDR, interferon sensitivity determining region; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); WBC, leukocytes; HOMA-IR, homeostasis model assessment of insulin resistance; Hb, hemoglobin; RBV, ribavirin; PLT, platelets; TG, triglycerides; TR, transient response; T-chol, total cholesterol; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; FBS, fasting blood sugar; CI, confidence interval.

genotype 1b and high viral loads (≥100K IU/mL) measured by quantitative Cobas Amplicor assays (Roche Diagnostics Co Ltd, Tokyo, Japan). All patients had undergone liver biopsy before combination therapy. Exclusion criteria for patients not eligible for Peg-IFN and RBV combination therapy were as follows: (1) pregnant women or women of childbearing potential, nursing mothers, or male patients whose partner might become pregnant; (2) patients with hepatocellular carcinoma; (3) patients with serious complications in the heart, kidneys, or lungs; (4) patients with autoimmune diseases, such as autoimmune hepatitis, and primary biliary cirrhosis; and (5) patients infected with the hepatitis B virus. Informed consent was obtained from all patients, and ethics approval for the study was obtained from the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science.

All patients were administered Peg-IFN-α 2b (Schering-Plough KK, Tokyo, Japan) and RBV combination therapy for 48 weeks. Peg-IFN was given in weekly doses and adjusted to body weight according to the manufacturer's instructions (45 kg or less, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; and 91 kg or more, 150 μg/dose). Similarly, RBV (Schering-Plough KK) was administered in daily doses adjusted to body weight according to the manufacturer's instructions (60 kg or less, 600 mg/day; 61–80 kg, 800 mg/day; and 81 kg or more, 1000 mg/day).

The final outcome of treatment was assessed 24 weeks after the cessation of combination therapy. We defined treatment outcomes according to the decrease in viremia as follows: sustained viral response (SVR), clearance of HCV viremia 24 weeks after the cessation of therapy; transient response (TR), no detectable HCV viremia at the cessation of therapy but relapsed during the follow-up period; and no response (NR), HCV viremia detected at the cessation of therapy. An early virologic response (EVR) (complete EVR) was defined as undetectable HCV-RNA in the serum by 12 weeks. HCV genotypes were determined according to the method of Okamoto et al. Serum HCV RNA was determined using qualitative and quantitative COBAS Amplicor assays (Roche Diagnostics Co, Ltd, Tokyo, Japan). The grading and staging of chronic hepatitis were histologically assessed according to the method of Desmet et al (Table 1).[13]

Preparation of Liver Tissue Samples

Liver biopsy samples were taken from all patients before treatment. The biopsy samples were divided into 2 parts: the first part was immersed in formalin for histologic assessment, and the second was immediately immersed in RNAlater (QIAGEN, Valencia, CA) for RNA isolation. Liver tissue RNA was isolated using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. Isolated RNA was stored at −70°C until use.

Affymetrix Genechip Analysis

The quality of the isolated RNA was estimated after electrophoresis using an Agilent 2001 Bioanalyzer (Agilent, Santa Clara, CA). Aliquots of total RNA (50 ng) isolated from the liver biopsy specimens were subjected to amplification using the WT-Ovation Pico RNA Amplification System (NuGen, San Carlos, CA) according to the manufacturer's instructions. Approximately 10 μg of complementary DNA (cDNA) was amplified from 50 ng of total RNA, and 5 μg of cDNA was used for fragmentation and biotin labeling using the FL-Ovation cDNA Biotin Module V2 (NuGen) according to the manufacturer's instructions. Biotin-labeled cDNA was suspended in 220 μL of hybridization cocktail (NuGen), and 200 μL was used for hybridization to the Affymetrix Human 133U Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA) containing 54,675 probes. After stringent washing, the microarray chips were stained with streptavidin-phycoerythrin, and probe hybridization was determined using a GeneChip Scanner 3000 (Affymetrix). Data files (CEL) were obtained using the GeneChip Operating Software 1.4 (Affymetrix).

Hierarchical Clustering and Pathway Analysis of Genechip Data

Genechip data analysis was performed using BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.htm). The data were log transferred, normalized, centered, and applied to the average linkage hierarchical clustering with centered correlation.

For genechip analysis, we selected 37 representative ISGs. Hepatic gene expression profiling was obtained from 30 CH-C patients before and 1 week after the initiation of IFN and RBV combination therapy and the 100 most up-regulated genes were selected (submitted for publication). ISGs were suppressed in patients with a rapid viral response and up-regulated in patients with a slow viral response before treatment. Using the 100 treatment-induced genes, we evaluated hepatic gene expression in 30 patients before treatment. Hierarchical clustering analysis showed that a cluster of 37 ISGs was up-regulated in patients with a slow viral response.

Pathway analysis was performed using MetaCore (GeneGo, St. Joseph, MI). Functional ontology enrichment analysis was performed to compare the gene ontology process distribution of differentially expressed genes ($P < .01$).

Quantitative Real-time Detection-Polymerase Chain Reaction

We performed quantitative real-time detection (RTD)-polymerase chain reaction (PCR) (RTD-PCR) using TaqMan Universal Master Mix (PE Applied Biosystems, Carlsbad, CA). Primer pairs and probes for myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse) (Mx1), 2′-5′-oligoadenylate synthelase 3 (OAS3), interferon-induced protein 44 (IFI44),

interferon-induced protein 44-like (IFI44L), 2′-5′-oligoadenylate synthetase 2 (OAS2), ubiquitin specific peptidase 18 (USP18), radical S-adenosyl methionine domain containing 2 (RSAD2), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon induced with helicase C domain 1 (IFIH1), XIAP associated factor 1 (XAF1), cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial (CMPK2), epithelial stromal interaction 1 (EPSTI1), hect domain and RLD 6 (HERC6), poly (ADP-ribose) polymerase family, member 9 (PARP9), phospholipid scramblase 1 (PLSCR1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from the TaqMan assay reagents library. Primer pairs and probes for IL28B were designed as previously described.[12] The standard curve was obtained in every assay using the RNA obtained from a normal liver.[14,15] The expression values were normalized by GAPDH, and normalized values indicate the relative fold expression to a normal liver.

Amino Acid Substitutions of ISDR in the Nonstructural 5A Region

The nucleotide sequence of ISDR in the nonstructural 5A region was determined by direct sequencing of PCR amplified materials.[4] Mutant-type ISDR was defined as containing 2 or more aa substitutions.

Genetic Variation of IL28B Polymorphism

A single nucleotide polymorphism (SNP) of IL28B was evaluated in 91 patients whose hepatic gene expression profiling was obtained. We genotyped 32 patients using Affymetrix Genome-Wide Human SNP Array 6.0 as previously described.[12] The results for rs8105790, rs11881222, rs8099917, and rs7248668 were retrieved from a database to evaluate the association of these SNPs. rs12979860 was determined by direct sequencing, and rs8099917 was determined using TaqMan Pre-Designed SNP Genotyping Assays (PE Applied Biosystems) as recommended by the manufacturer.

Statistical Analysis

The Mann–Whitney U test was used to analyze continuous variables. Fisher exact test and χ^2 test were used for the analysis of categorical data. The overall plausibility of the treatment response groups was assessed using Fisher C statistic (Supplementary Table 2).[16,17] C is defined by $C = -2 \Sigma \ln(p_i)$, where p_i is the probability (P value) of each independent statement (clinical factors). C follows a χ^2 distribution with 2k degrees of freedom, k being the number of independent statements (clinical factors).[16] A nonsignificant C value means that the treatment response in the 2 groups was not statistically independent.

Multivariate analysis was performed using a stepwise logistic regression model. Each cut-off point for the continuous variables was decided by analysis of the receiver operating characteristic (ROC) curve. A P value of less than .05 was considered significant. Statistical analyses were performed using JMP7 for Windows (SAS Institute, Cary, NC).

Results

Response Rate and Clinical Characteristics

The clinical characteristics of the patients are shown in Table 1 and Supplementary Table 1. All of the patients were infected with HCV genotype 1b and had a high viral load (>100K IU/mL). No patients were coinfected with the hepatitis B virus (HBV). The intention-to-treat analysis showed that SVR, TR, and NR were observed in 70 (42%), 55 (33%), and 43 (25%) patients, respectively (Supplementary Table 1). Before comparing patients with 3 different responses, the overall plausibility of the treatment response groups was assessed using Fisher C statistic. Fisher C statistic utilizes the P values obtained by comparing pretreatment factors including age, gender, liver factors, laboratory parameters, and viral factors. Because the SVR and TR groups could not be defined as different, they were grouped together and compared with NR (Table 1, Supplementary Table 2).

Eleven patients with NR discontinued the therapy after 24 weeks because of an insufficient effect, namely, serum HCV-RNA was still detectable at this time. The remaining patients completed 48 weeks of Peg-IFN and RBV combination therapy. The administration rate of Peg-IFN with 80% or more was achieved in 67% of patients, and the administration rate of RBV with 80% or more was achieved in 60% of patients (Table 1).

Analysis of Hepatic Gene Expression

Prior to treatment, 91 of 168 patients (Supplementary Table 3) were randomly selected, and their hepatic gene expression was determined using Affymetrix genechip analysis.

Hierarchical clustering using 37 representative ISGs (see Materials and Methods) demonstrated 2 clear clusters of patients: one was a group composed of patients with up-regulated ISGs (Up-ISGs), and the other was a group consisting of patients with down-regulated ISGs (Down-ISGs) (Figure 1). In patients with Up-ISGs, 21 (49%) showed NR, whereas 8 (17%) patients with Down-ISGs showed NR ($P = .002$). In contrast, 14 (33%) patients with Up-ISGs showed SVR, whereas 27 (56%) patients with Down-ISGs showed SVR ($P = .03$). There were no significant differences in the frequency of advanced stages of liver fibrosis (F3-F4) between patients with Up-ISGs and patients with Down-ISGs (18 [42%] and 17 [35%], respectively, $P = .664$). These data indicated that the up-regulation of ISGs in the liver before treatment was strongly associated with resistance to IFN treatment.

Host and Viral Factors Associated With the Response to Combination Therapy

To evaluate the multiple host and viral factors associated with the response to Peg-IFN and RBV combination therapy in all patients, univariate and multivariate analyses were performed. To assess the expression of hepatic ISGs, 15 genes (Mx1, OAS3, IFI44, IFI44L, OAS2,

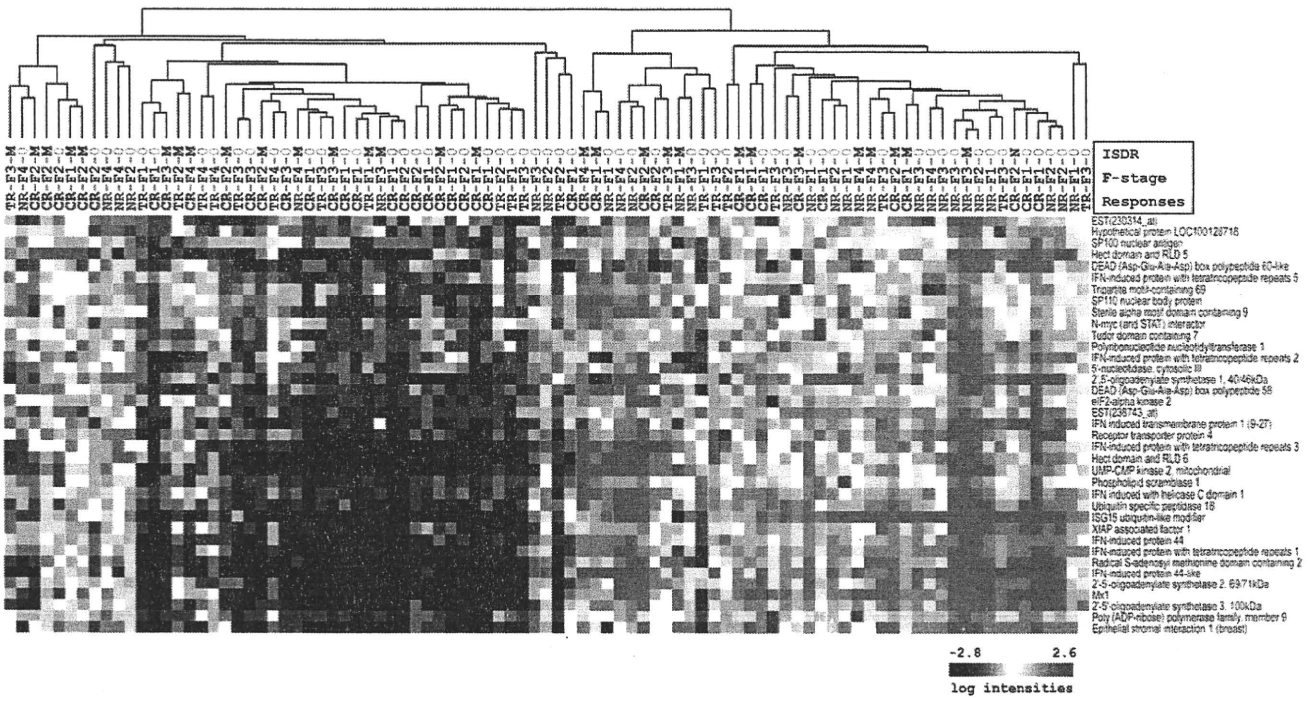

Figure 1. Hierarchical clustering analysis of 91 patients using 37 representative ISGs. Responses to therapy (SVR, TR, and NR), fibrosis stage (F1–F4), and status; ISDR mutations are also shown. ISDR mutation ≥2 = M, ≤1 = 0.

USP18, RSAD2, IFIT1, IFIH1, XAF1, CMPK2, EPSTI1, HERC6, PARP9, and PLSCR1) out of 37 representative ISGs were selected for their expression values of probe intensity, and their expression was confirmed in liver tissue obtained from 168 patients by RTD-PCR. Although there were significant correlations of their expression with each other, except RARP9 and PLSCR1 (Supplementary Table 4), the dynamic range of gene expression was high for 3 genes, namely, Mx1, IFI44, and IFIT1 (Supplementary Figure 1*A*). We averaged the expression values of Mx1, IFI44, and IFIT1 and used them for further study.

When we compared patients with SVR+TR and NR, the fibrosis stage of the liver ($P = .001$), expression of hepatic ISGs ($P < .001$), aspartate aminotransferase (AST) serum level ($P = .017$), γ-glutamyl transpeptidase (γ-GTP) ($P < .001$), low-density lipoprotein cholestrol (LDL-Chol) ($P = .019$), and insulin (μU/mL) ($P = .039$) were significantly different prior to treatment (Table 1). For treatment factors, the total dose and administration of IFN and RBV were not significantly different between these 2 groups. EVR was observed in 101 (81%) patients, and the proportion was significantly different ($P < .001$) between patients with SVR+TR and NR (Table 1).

Regression analysis of pretreatment factors showed a strong correlation among γ-GTP, alanine aminotransferase (ALT), and aspartate aminotransferase (AST); and homeostasis model assessment-insulin resistance (HOMA-IR), fasting blood sugar, and insulin; and total cholesterol (T-Chol), high-density lipoprotein cholestrol (HDL-Chol), and LDL-Chol (data not shown). We selected fibrosis stage, ISGs, HCV-RNA, ISDR mutation, and body mass index (BMI) as factors for multivariate analysis. Stepwise multivariate logistic regression analysis was performed using the selected factors. From the ROC curve, we set the cut-off value for the expression of ISGs as 3.5 (Supplementary Figure 1*B*). The results showed that expression of hepatic ISGs (<3.5), fibrosis stage (F1-F2), and ISDR mutation (≥2) were significant pretreatment factors contributing to SVR+TR (Table 1).

Clinical Parameters Associated With the Expression of Hepatic ISGs

Univariate and multivariate analyses revealed that the expression of hepatic ISGs was a strong predictor of the treatment outcome for SVR+TR patients. We next examined which clinical parameters were associated with the expression of hepatic ISGs (Table 2). Univariate analysis showed that the expression of ISGs was strongly correlated with the serum levels of γ-GTP ($P < .001$) and AST ($P < .001$) and weakly correlated with HCV-RNA, fasting blood sugar, insulin, HOMA-IR, triglyceride (TG), and LDL-Chol. Multivariate analysis showed that γ-GTP ($P < .001$), HCV-RNA ($P < .001$), and LDL-Chol ($P = .048$) were significantly associated with hepatic ISGs. Noticeably, the expression of ISGs was negatively correlated with HCV-RNA in SVR+TR patients ($P = .009$), whereas this correlation was not evident in NR patients ($P = .298$) (Table 2, Supplementary Figure 2). These results may indicate that endogenous ISGs suppress HCV in SVR+TR patients, whereas they are not active in NR patients.

Table 2. Clinical Factors Associated With Expression of Hepatic Interferon-Stimulated Genes

	Univariate analysis				Multivariate analysis			
Clinical factor	β	95% CI		*P* value	β	95% CI		*P* value
AST (*IU/L*)	0.274	0.13	0.42	<.001	—	—	—	
γ-GTP (*IU/L*)	0.326	0.18	0.47	<.001	0.288	0.14	0.43	<.001
HCV-RNA (*KIU/mL*)	−0.170	−3.19	−0.02	.025	−0.255	−0.40	−0.11	<.001
SVR+TR	−0.237	−0.32	−0.05	.009	—	—	—	
NR	−0.168	−0.57	0.18	.298	—	—	—	
FBS (*mg/dL*)	0.182	0.03	0.35	.021	—	—	—	
Insulin (*μU/mL*)	0.190	0.03	0.34	.016	—	—	—	
HOMA-IR	0.181	0.03	0.33	.017	—	—	—	.073
TG (*mg/dL*)	0.201	0.05	0.35	.011	—	—	—	.089
LDL-Chol (*mg/dL*)	−0.177	−0.33	−0.02	.025	−0.143	−0.28	0.00	.048

γ-GTP, γ-glutamyl transpeptidase; AST, aspartate aminotransferase; FBS, fasting blood sugar; TG, triglycerides; TR, transient response; NR, no response; SVR, sustained viral response; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-chol, low-density lipoprotein cholesterol; CI, confidence interval; β, β coefficient; CI, confidence interval.

Expression of Hepatic ISGs Before Treatment Is Associated With Genetic Variation of IL28B

Recently, a GWAS successfully identified the genomic locus associated with the treatment response to Peg-IFN and RVB combination therapy for CH-C. Genetic variation in IL28B predicts HCV treatment-induced viral clearance.[11,12] We determined the genetic variation in IL28B of 32 patients[12] (Table 3). The SNPs rs8105790, rs11881222, rs8099917, and rs7248668 had a significant association with treatment response (odds ratio: 24.7–27.1, $P = 1.84 \times 10^{-30}$–2.68×10^{-32}). These SNPs are located in block 2 of the IL28B haplotype and show significant linkage disequilibrium in the HapMap data.[12] Ge et al[11] reported a different SNP (rs12979860) that was located between rs11881222 and rs8099917. The nucleotide sequence of rs12979860 was determined by direct sequencing, and the results are shown in Table 3. There was a strong association of rs12979860 and the other 4 SNPs indicating that this SNP was located within the same haplotype block. We confirmed these findings in multiple samples from Japanese patients (data not shown).

We selected rs8099917 for further study and evaluated it using TaqMan Pre-Designed SNP Genotyping Assays. The G nucleotide of rs8099917 was associated with a poor response to treatment (minor allele), whereas the T was associated with a fair response to treatment (major allele).[12] Out of 91 patients (Supplementary Table 3), the proportion of major homozygotes (TT), heterozygotes (TG), and minor homozygotes (GG) were 66% (60/91), 30% (27/91), and 4% (4/91), respectively (Table 4); 86% (51/60) of the major genotype (TT) patients had SVR or TR, whereas 65% (20/31) with the minor genotypes (TG or GG) had NR ($P < .001$).

Interestingly, hepatic gene expression profiles revealed that patients with the minor genotype showed higher expression of hepatic ISGs, whereas patients with the major genotype showed lower expression of hepatic ISGs (Figures 2 and 3). To examine further the relationship of the genetic variation in IL28B and its expression levels, we evaluated the expression of IL28B in the liver by RTD-PCR (Figure 3). IL28B expression was approximately 10-fold less than the expression of ISGs. Although IL28B expression tended to be higher in some patients with the major genotype, there was no significant difference in IL28B expression in the liver between the major and minor genotypes (Figure 3*A*). Nevertheless, the expression of ISGs was clearly high in patients with the minor genotype ($P < .0001$) (Figure 3*B*). IL28 activates signal tranducers and activators of transcription 1 (STAT1) through downstream signaling from a heterodimeric class II cytokine receptor that consists of IL-10 receptor β (IL-10Rβ) and IL-28 receptor α (IL-28Rα).[18,19] Therefore, we examined the correlation between the expression of IL28B and ISGs. IL28B expression correlated with the expression of ISGs ($r = 0.44$, $P < .001$); however, the correlation was different according to the SNP genotype. We observed a steep-slope correlation for the minor genotype and a slow-slope correlation for the major genotype (Figure 3*C* and *D*). Interestingly, 4 minor homozygotic (GG) patients showed a steeper correlation than the heterozygotes (TG) (Figure 3*D*). Thus, the IL28B polymorphism might differentially regulate the expression of ISGs in the liver, leading to the different treatment outcomes.

We performed univariate and multivariate analyses to identify the clinical factors associated with the major and minor genotypes (Table 4). Univariate analysis showed that higher hepatic ISGs and lower body mass index were significantly associated with the minor genotype; however, multivariate analysis showed that only hepatic ISGs (≥3.5) were associated with the minor genotype ($P < .001$; OR, 18.1; 95% confidence interval: 3.95–113). We further compared the predictive capacity of multivariate models using the expression of hepatic ISGs (<3.5 vs ≥3.5) or the IL28B genotype (major vs minor) (Supplementary Table 6). The predictive performance and fitness of the multivariate model using the IL28B genotype was superior to that using the expression of hepatic ISGs. However, when these factors were included in the same model, the expression of hepatic ISGs was still useful for the predictive model independent of the IL28B genotype (Supplementary Table 6).

Table 3. Clinical Characteristics of 32 Patients Genotyped by GWAS and 5 SNPs in Strong Linkage Disequilibrium With IL28B,[11] Including rs12979860

Patient No.	Response	Age, y	Sex	F stage	ISGs	IL28B	RefSNP (chr pos) Minor allele	rs8105790 (44424341) C	rs11881222 (44426763) G	rs12979860 (44430627) T	rs8099917 (44435005) G	rs7248668 (44435661) A
1	SVR	42	M	1	4.20	83.8		TT	AA	CC	TT	GG
2	SVR	59	M	1	2.62	45.5		TT	AA	CC	TT	GG
3	SVR	41	F	1	1.54	1.3		TT	AA	CC	TT	GG
4	TR	57	M	1	3.18	21.7		TT	AA	CC	TT	GG
5	TR	68	F	1	1.43	20.3		TT	AA	CC	TT	GG
6	SVR	44	M	1	0.97	4.6		TT	AA	CC	TT	GG
7	SVR	61	M	2	2.15	6.1		TT	AA	CC	TT	GG
8	SVR	50	M	2	3.25	66.4		TT	AA	CC	TT	GG
9	SVR	49	M	2	1.25	ND		TT	AA	CC	TT	GG
10	TR	59	F	2	1.29	17.4		TT	AA	CC	TT	GG
11	SVR	48	F	2	1.00	90.2		TT	AA	CC	TT	GG
12	TR	65	F	2	2.86	36.4		TT	AA	CC	TT	GG
13	NR	34	M	3	0.82	17.8		TT	AA	CC	TT	GG
14	SVR	55	M	3	0.83	13.8		TT	AA	CC	TT	GG
15	TR	68	M	3	0.75	20.6		TT	AA	CC	TT	GG
16	SVR	64	M	3	0.94	15.7		TT	AA	CC	TT	GG
17	SVR	67	F	3	1.50	25.7		TT	AA	CC	TT	GG
18	SVR	48	M	4	1.69	7.9		TT	AA	CC	TT	GG
19	NR	66	F	1	4.57	16.5		TC	AG	CT	TG	GA
20	SVR	52	F	1	5.23	29.3		TC	AG	CT	TG	GA
21	NR	55	F	1	8.25	57.2		TC	AG	CT	TG	GA
22	SVR	49	F	1	5.36	ND		TC	AG	CT	TG	GA
23	TR	44	M	1	2.08	7.0		TC	AG	CT	TG	GA
24	NR	63	M	1	2.77	10.5		TC	AG	CT	TG	GA
25	NR	61	F	2	3.98	39.1		TC	AG	CT	TG	GA
26	NR	42	M	2	4.89	5.9		TC	AG	CT	TG	GA
27	SVR	49	M	3	3.31	6.9		TC	AG	CT	TG	GA
28	TR	71	F	3	5.53	27.3		TC	AG	CT	TG	GA
29	TR	63	M	3	3.40	33.5		TC	AG	CT	TG	GA
30	NR	70	F	3	4.78	8.1		TC	AG	CT	TG	GA
31	TR	62	F	3	3.53	14.0		TC	AG	CT	TG	GA
32	NR	56	M	4	7.37	30.8		CC	GG	TT	GG	AA

NOTE. The Pearson correlation of the r^2 estimates for adjacent pairs; rs8099917 vs rs8105790, rs8099917 vs rs11881222, rs8099917 vs rs12979860, and rs8099917 vs rs7248668 = 0.99, 0.99, 0.98, and 0.97, respectively.
IL28B, interleukin 28B; GWAS, genome-wide association studies; ISGs, interferon stimulated genes; SNP, single nucleotide polymorphism; SVR, sustained viral response; TR, transient response; NR, no response; M, male; F, female.

To examine further the different hepatic gene expression of patients with the major or minor genotypes, pathway analysis of differentially expressed genes between the 2 groups was performed. By comparing the expression of hepatic genes between patients with the major and minor genotypes, 1359 differentially expressed genes were identified ($P < .01$; 711 genes were up-regulated with the minor genotype, and 648 genes were up-regulated with the major genotype). Pathway analysis of these genes demonstrated that signaling pathways related to interferon action, apoptosis, and Wnt signaling were up-regulated in the liver of patients with the minor genotype, whereas B-cell-, dendritic cell-, and natural killer cell-related genes were up-regulated in the liver of patients with the major genotype (Supplementary Figure 3). These results suggest that IL28B may be involved in innate and adaptive immune responses and that different antiviral signaling pathways might be involved in the liver of patients with different SNPs.

Discussion

Multiple viral and host factors may be related to the treatment response to Peg-IFN and RBV combination therapy. For the viral factors, a higher number of aa substitutions in the ISDR of nonstructural 5A region was strongly associated with a favorable response to IFN-α monotherapy in patients with genotype-1 HCV.[4]

Besides viral factors, host factors such as age, gender, fibrotic stage of the liver, and the presence of steatosis and insulin resistance were associated with the treatment outcome.[20] Analysis of hepatic gene expression demonstrated that the up-regulation of ISGs in the liver before treatment may be related to a poor treatment response.[6–9] To reveal the underlying mechanism of treatment resistance, 2 reports compared gene expression profiling in the liver before and during therapy and showed that patients with up-regulated ISGs in the liver prior to treatment failed to further induce ISGs following the ad-

Table 4. Comparison of Clinical Factors Between Patients With Major (TT) and Minor (TG+GG) Alleles

Clinical category	TT		TG+GG		Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
No. of patients	n = 60		n = 31			—	
Treatment response							
SVR+TR vs NR	51 vs 9		11 vs 20		<.001	—	
Age and gender							
Age, *y*	56	(30–69)	56	(30–71)	.843	—	
Sex (M vs F)	39 vs 21		19 vs 12		.518	—	
Liver factors							
F stage (F1-2 vs F3-4)	36 vs 24		23 vs 17		.905	—	
A grade (A0-1 vs A2-3)	27 vs 33		20 vs 11		.075	—	
ISGs (Mx, IFI44, IFIT1) (<3.5 vs ≥3.5)	46 vs 14		5 vs 26		<.001	18.1 (3.95–113)	<.001
Laboratory parameters							
HCV-RNA (*KIU/mL*)	2055	(160–5000)	1970	(126–5000)	.602	—	
BMI (kg/m^2)	24.5	(16.3–40.5)	22.9	(19.1–26.6)	.006	—	.077
AST (*IU/L*)	59	(22–258)	54	(21–283)	.227	—	
ALT (*IU/L*)	75	(24–376)	60	(18–236)	.077	—	
γ-GTP (*IU/L*)	61	(4–392)	53	(20–229)	.517	—	.167
WBC ($/mm^3$)	4450	(2100–11,100)	4600	(2500–8200)	.947	—	
Hb (*g/dL*)	14.2	(11.4–16.7)	14.5	(11.2–17.2)	.606	—	
PLT ($\times 10^4/mm^3$)	15.4	(7–39.4)	16.2	(9.2–27.7)	.832	—	
TG (*mg/dL*)	98	(58–248)	131	(30–303)	.053	—	.055
T-Chol (*mg/dL*)	172	(115–222)	168	(129–237)	.910	—	
LDL-Chol (*mg/dL*)	84	(42–123)	69	(51–107)	.052	—	.055
HDL-Chol (*mg/dL*)	44	(18–72)	45	(29–77)	.218	—	
FBS (*mg/dL*)	95	(59–291)	96	(66–206)	.849	—	
Insulin (*μU/mL*)	7.5	(0.7–23.2)	9.2	(2–23.2)	.195	—	
HOMA-IR	1.3	(0.3–11.7)	1.2	(0.4–9.6)	.339	—	
Viral factors							
ISDR mutations (≤1 vs ≥2)	38 vs 22		23 vs 7		.194	—	.083
Treatment factors							
Total dose administrated							
Peg-IFN (*μg*)	3960	(1500–7200)	3840	(1920–5760)	.377	—	
RBV (*g*)	203	(26–336)	201	(106–268)	.777	—	
Achieved administration rate							
Peg-IFN (%)							
≥80%	41		17		.207	—	
<80%	19		14				
RBV (%)							
≥80%	34		19		.671	—	
<80%	26		12				
Achievement of EVR	40/60 (62%)		9/31 (29%)		<.001	—	

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; EVR, early virologic response; γ-GTP, γ-glutamyl transpeptidase; ISDR, interferon sensitivity determining region; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); WBC, leukocytes; HOMA-IR, homeostasis model assessment of insulin resistance; Hb, hemoglobin; RBV, ribavirin; PLT, platelets; TG, triglycerides; TR, transient response; T-chol, total cholesterol; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; FBS, fasting blood sugar; CI, confidence interval.

ministration of IFN and could not eliminate HCV.[6,7] We performed a similar analysis and observed that these findings were more evident in liver lobular cells than in infiltrating lymphocytes in the portal area (submitted for publication). Thus, both viral and host factors might be closely related to the treatment response to Peg-IFN and RBV combination therapy. However, the clinical relevance and relationships of these factors have not been fully evaluated. In this study, we validated the clinical significance of the expression of hepatic ISGs on treatment outcome using a relatively large cohort of patients and compared its significance with other viral and host factors. To compare the patients with SVR, TR, and NR, we assessed the overall plausibility of each group using Fisher C statistic,[16] and patients with SVR and TR were grouped together for further analysis.

We examined hepatic gene expression in 91 of 168 patients using the Affymetrix genechip. Expression profiling using 37 representative ISGs (see Materials and Methods), which were selected from gene expression profiling comparing pretreatment and under treatment liver, differentiated 2 groups of

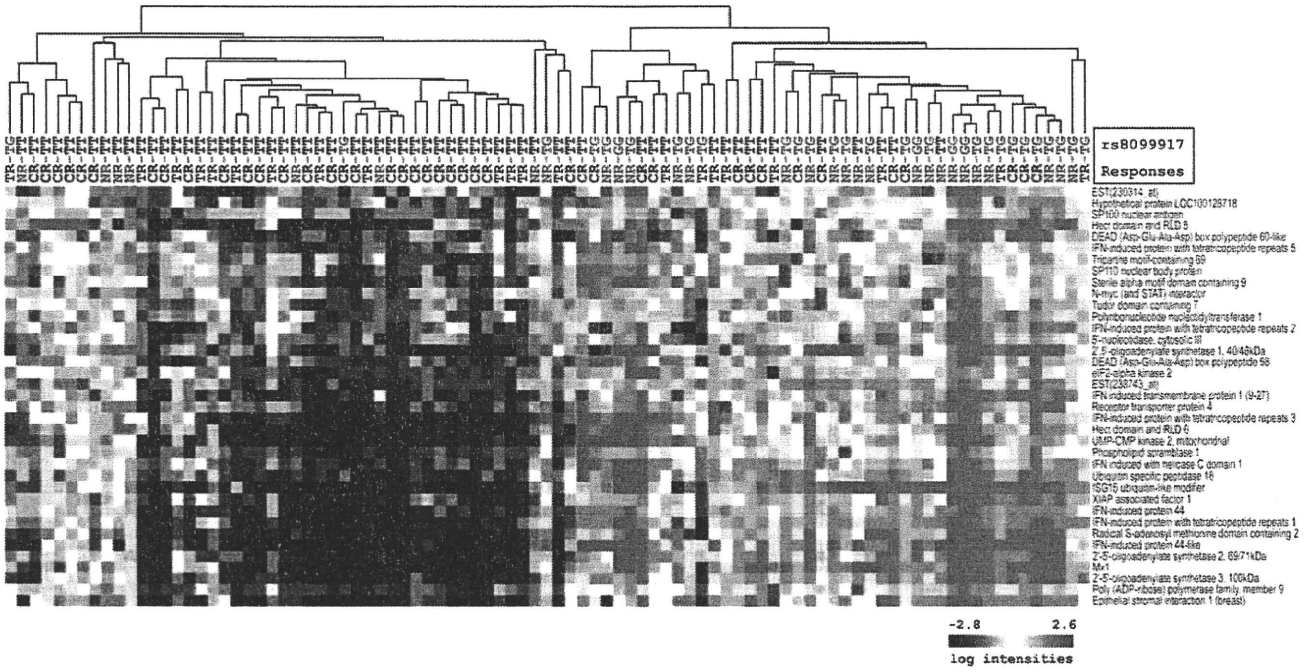

Figure 2. Hierarchical clustering analysis of 91 patients with the defined genotype of IL28B. Responses to therapy (SVR, TR, and NR) and IL28B genotype (TT, TG, or GG) are shown. The structure of the dendrogram and heat map is the same as in Figure 1.

patients: the Up-ISG and Down-ISG groups (Figure 1). The proportion of patients with NR to treatment was significantly higher in the Up-ISGs group.

Multivariate analysis showed that hepatic ISGs (<3.5), fibrosis stage (F1-F2), and ISDR mutations (≥2) significantly contributed to the outcome for the SVR+TR group (Table 1). Discriminate analysis using variables selected by multivariable analysis predicted the SVR+TR patients with 82% accuracy and NR patients with 79% accuracy. However, the accuracy decreased to 67% for SVR+TR patients and 53% for NR patients when the expression of hepatic ISGs was removed from the variables (data not shown). Interestingly, the expression of hepatic ISGs was strongly correlated with γ-GTP and weakly correlated with insulin resistance. A recent study describing the association between insulin resistance and poor treatment outcome might be partially explained by this observation.[20]

In this study, we utilized 3 ISGs (Mx1, IFI44, and IFIT1) out of 15 validated by RTD-PCR. The expression values of these ISGs were higher than those of other ISGs (Supplementary Figure 1*A*). We averaged these ISGs and set the cut-off value as 3.5 from the ROC curve (Supplementary Figure 1*B*). The sensitivity, specificity, and positive and negative predictive values on the likelihood of achieving SVR+TR using this cut-off value were 82% (103/125), 72% (31/43), 90% (103/115), and 58% (31/53), respectively. The results were compared with those observed for the 15 ISGs (Supplementary Table 5). These results showed that the 3.5 cut-off value for Mx1, IFI44, and IFIT1 would be valuable for clinical use.

Despite the importance of the expression of hepatic ISGs, viral factors may also allow us to predict the outcome of treatment. Multivariate analysis showed that ISDR mutations (≥2) independently contributed to the treatment outcome, although univariate analysis did not show significance (P = .07); therefore, ISDR might be uniquely and differentially involved in treatment resistance.

What causes the differences in the expression of hepatic ISGs? In parallel to the gene expression analysis, a GWAS was applied to identify genomic loci associated with treatment response, and a polymorphism in IL28B was found to predict hepatitis C treatment-induced viral clearance.[10–12] To examine the relationship between the genetic variation of IL28B and hepatic gene expression, we determined the IL28B polymorphism in 91 patents (Table 3). The patients with the minor genotype (TG or GG) had an increased expression of hepatic ISGs compared with the patients with major genotype (TT) (Figures 2 and 3). In European-Americans, the proportion of major homozygotes is 39% (CC at rs1297986), 49% for heterozygotes (TC), and 12% for minor homozygotes (TT).[11] Although the proportion of minor homozygotes was much less in this study (GG, 4%), as reported in a previous study in Japan,[12] more patients are required for proper evaluation. It is interesting that the expression of hepatic ISGs in minor homozygotes (GG) was higher than in heterozygotes (TG) in this study.

The results clearly showed that the differences in the expression of hepatic ISGs before treatment are associated with the IL28B polymorphism and results in different treatment outcomes. Although we could not detect significant differences in the expression levels of IL28B depending on the different SNP, some patients with the major genotype showed a higher expression of IL28B. Because IL28B expression was approximately 10-fold less than the expression of ISGs, the lower

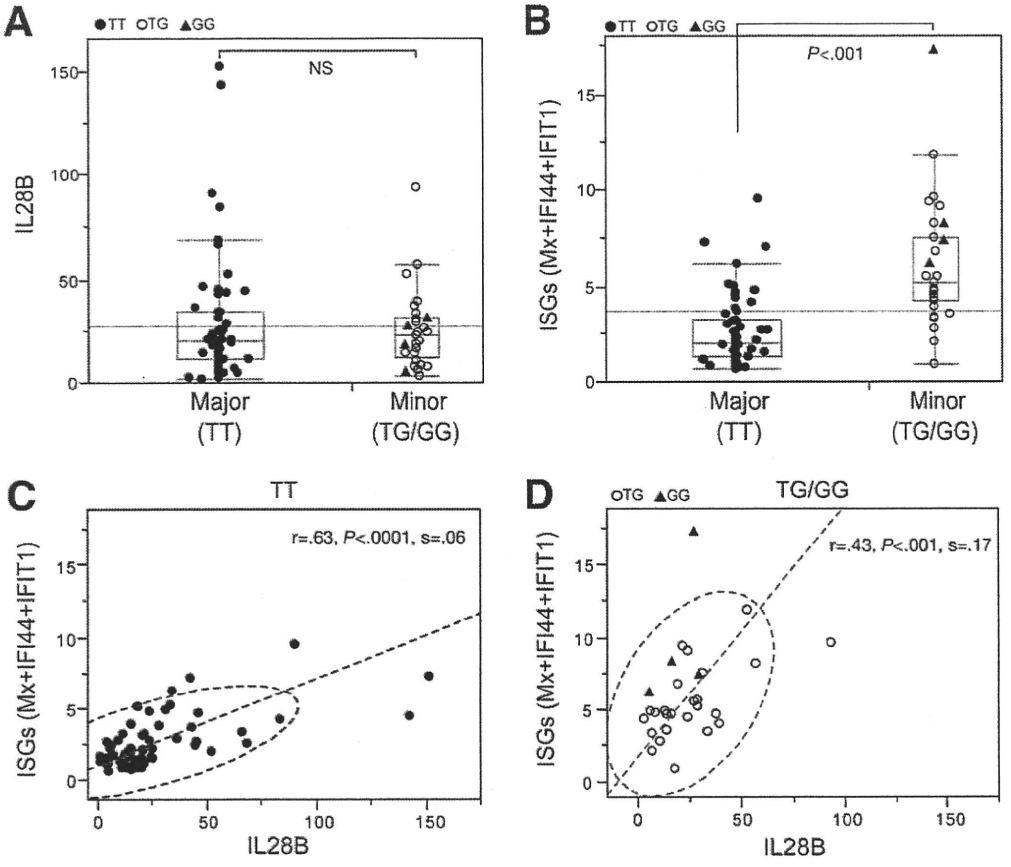

Figure 3. (*A*) IL28 expression in the liver of 91 patients with the major (TT) or minor (TG or GG) genotype (rs8099917). (*B*) Expression of ISGs in the liver of patients with the major (TT) or minor (TG or GG) genotype (rs8099917). (*C*) Relationship between IL28 and ISGs in the liver of patients with the major (TT) genotype (rs8099917). (*D*) Relationship between IL28 and ISGs in the liver of patients with the minor (TG or GG) genotype (rs8099917).

expression of IL28B may be a reason for the decreased ability to distinguish differences in its expression. Another possibility may be the specificity of the IL28B primers used in this study; because IL28B shares a 98.2% nucleotide sequence homology with IL28A, IL28B specific primers are not available.[21] When the expression of IL28B and hepatic ISGs were compared, a significant correlation was observed, and, interestingly, IL28B and ISGs derived from different SNPs were correlated in a different way (Figure 3*C* and *D*). It appeared that hepatic ISGs were more induced by the reduced amounts of IL28B in patients with the minor genotype. The mechanism behind these findings has yet to be determined; however, IL28B interacts with a heterodimeric class II cytokine receptor that consists of IL-10 receptor β (IL-10Rβ) and IL-28 receptor α (IL-28Rα).[18,19] It is possible that IL28B could mediate antiviral signaling through IL-10 signaling as well as STAT1 activation. The Th 2 dominant signaling of IL28B may modulate signaling pathways in livers with CH-C and contributes to the different expression of ISGs. Another possibility may be that the cell origin of hepatic ISGs is different. A recent study revealed cell-type specific ISG expression in macrophages and hepatocytes, which could be related to the IFN response.[22] As more of the B-cell-, dendritic cell-, and natural killer cell-related genes were up-regulated in the liver of patients with the major genotype, ISGs could be expressed by these cells, whereas they are expressed by hepatocytes in the liver of patients with the minor genotype. It is known that the induction of ISGs in lymphocytes is lower than that in hepatocytes. The precise mechanism should be investigated further as a different regulatory mechanism for the expression of ISGs may be present.

In conclusion, we presented the clinical relevance of the expression of hepatic ISGs for the treatment outcome of Peg-IFN and RBV combination therapy. The different expressions of hepatic ISGs before treatment might be due to polymorphisms in IL28B. Further studies are required to clarify the detailed pathways of IL28B and hepatic gene expression through molecular biologic and immunologic aspects.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.04.049.

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Participating investigators are listed in Appendix 1.

Conflicts of interest

The authors disclose no conflicts.

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Ms.wjg/2010/027402 Case report

Endoscopic naso-pancreatic drainage for the treatment of pancreatic fistula occurring after living donor liver transplantation

Running title: Endoscopic naso-pancreatic drainage for pancreatic fistula after liver transplantation

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ABSTRACT

Pancreatic fistula is a quite rare complication in patients who undergo living donor liver transplantation (LDLT). However, in the cases that show pancreatic

fistula, the limited volume of the graft and the resultant inadequate liver function may complicate the management of the fistula. As a result, pancreatic fistula may result in the death of the patient. We present 2 cases in which endoscopic treatment was effective against pancreatic fistulas that developed after LDLT. In case 1, a 61-year-old woman underwent LDLT for primary biliary cirrhosis. Because of a portal venous thrombus caused by a splenorenal shunt, the patient underwent portal vein reconstruction, and a splenorenal shunt was ligated on postoperative day (POD) 7. The main pancreatic duct was injured during the manipulation to achieve hemostasis, thereby necessitating open drainage. However, discharge of pancreatic fluid continued even after POD 300. Endoscopic naso-pancreatic drainage (ENPD) was performed, and this procedure resulted in a remarkable decrease in drain output. The refractory pancreatic fistula healed on day 40 after ENPD. In case 2, a 58-year-old man underwent LDLT for cirrhosis caused by the hepatitis C virus. When the portal vein was exposed during thrombectomy, the pancreatic head was injured, which led to the formation of a pancreatic fistula. Conservative therapy was ineffective; therefore, ENPD was performed. The pancreatic fistula healed on day 38 after ENPD. The findings in these 2 cases show that endoscopic drainage

of the main pancreatic duct is a less invasive and effective treatment for pancreatic fistulas that develop after LDLT.

Key words: pancreatic fistula, endoscopic treatment, living donor liver transplantation, complications.

INTRODUCTION

The incidence of complications associated with living donor liver transplantation (LDLT) is known to be greater than that associated with deceased donor liver transplantation (DDLT)[1-4]. In the patients who undergo LDLT, the incidence of complications, including mild complications, during the perioperative period can be as high as 82.8%. In particular, the rate of development of biliary complications after LDLT is twice of that after DDLT[1]. Moreover, patients who undergo LDLT often have inadequate liver function because of the limited volume of the graft. Therefore, the management of complications is very difficult, and the mortality rate in critical cases is high.

Compared to other abdominal surgery, pancreatic fistula is a quite rare complication after LDLT[1-5], but it is theoretically possible because LDLT