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Potential Relevance of Cytoplasmic Viral Sensors and Related Regulators Involving Innate Immunity in Antiviral Response

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Background & Aims: **Clinical significance of molecules involving innate immunity in treatment response remains unclear. The aim is to elucidate the mechanisms underlying resistance to antiviral therapy and predictive usefulness of gene quantification in chronic hepatitis C (CH-C).** ***Methods:*** **We conducted a human study in 74 CH-C patients treated with pegylated interferon α-2b and ribavirin and 5 nonviral control patients. Expression of viral sensors, adaptor molecule, related ubiquitin E3-ligase, and modulators were quantified.** ***Results:*** **Hepatic RIG-I, MDA5, LGP2, ISG15, and USP18 in CH-C patients were up-regulated at 2- to 8-fold compared with non-hepatitis C virus patients with a relatively constitutive Cardif. Hepatic RIG-I, MDA5, and LGP2 were significantly up-regulated in nonvirologic responders (NVR) compared with transient (TR) or sustained virologic responders (SVR). Cardif and RNF125 were negatively correlated with RIG-I and significantly suppressed in NVR. Differences among clinical responses in RIG-I/Cardif and RIG-I/RNF125 ratios were conspicuous (NVR/TR/SVR = 1.3:0.6:0.4 and 2.3:1.3:0.8, respectively). Like viral sensors, ISG15 and USP18 were significantly up-regulated in NVR (4-fold and 2.3-fold, respectively). Multivariate and receiver operator characteristic analyses revealed higher RIG-I/Cardif ratio, ISG15, and USP18 predicted NVR. Lower Cardif in NVR was confirmed by its protein level in Western blot. Also, transcriptional responses in peripheral blood mononuclear cells to the therapy were rapid and strong except for Cardif in not only a positive (RIG-I, ISG15, and USP18) but also in a negative regulatory manner (RNF125).** ***Conclusions:*** **NVR may have adopted a different equilibrium in their innate immune response. High RIG-I/Cardif and RIG-I/RNF125 ratios and ISG15 and USP18 are useful in identifying NVR.**

Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to cirrhosis and hepatocellular carcinoma in many patients.[1] Although combination therapy with pegylated interferon (PEG-IFN) α and ribavirin is now established as the standard treatment for chronic HCV infection genotype 1b, the sustained virologic response rate in these patients is still around 50%.[2–4] Moreover, physicians have also found that 20% of patients are nonvirologic responders (NVR; those whose HCV-RNA does not become negative during 48 weeks of combination therapy).[5] Prediction of NVR status is of clinical importance because these patients have no chance of achieving a sustained virologic response even after prolonged combination therapy.[6] However, mechanisms involving resistance to PEG-IFN-α and ribavirin have not been fully elucidated, and it is difficult to predict treatment responses before initiation of PEG-IFN-α and ribavirin combination therapy.

In vitro studies have suggested that an innate immune response in viral infection is an essential part of the host antiviral defense system.[7] HCV evades the host immune response through a complex combination of processes that include signaling interference, effector modulation, and continual viral genetic variation.[8] We hypothesized that liver tissue would show a consistent difference between responders and nonresponders in expression levels of the gene involved in innate immunity and IFN signal transduction. These differences could be used to predict treatment outcomes.

The retinoic acid–inducible gene I (RIG-I), a cytoplasmic RNA helicase, and the related melanoma differentia-

Abbreviations used in this paper: CARD, Caspase-recruiting domain; Cardif, caspase-recruiting domain adaptor inducing IFN-β; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; IPS-1, IFN-β promoter stimulator I; ISG15, IFN-stimulated gene 15; PEG-IFN, pegylated interferon; MDA5, melanoma differentiation associated gene 5; MAVS, mitochondrial antiviral signaling protein; NVR, nonvirologic responders; PBMC, peripheral blood mononuclear cell; RIG-I, retinoic acid-inducible gene I; RNF125, ring-finger protein 125; ROC, receiver operator characteristic; SVR, sustained viral responder; TR, transient responder; UBP43, ubiquitin-specific protease 43; USP18, ubiquitin-specific protease 18; VISA, virus-induced signaling adaptor.

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Table 1. Patient Characteristics at Baseline According to Final Virologic Response

	SVR n = 30	TR n = 24	NVR n = 20	*P* value
Age (*y*)	52 ± 13	60 ± 8.7	60 ± 10	.04[a]
Female % (M/F)	47% (16/14)	63% (9/15)	60% (8/12)	.5[b]
Naïve & Relapser[c]/Non-responder[c]	26/4	20/4	14/6	.3[b]
BMI	24.6 ± 3.0	24.9 ± 4.4	24.0 ± 2.1	.6[a]
ALT (*IU/L*)	75 ± 57	65 ± 35	68 ± 41	1.0[a]
Hemoglobin (*g/dL*)	14.3 ± 1.6	14.1 ± 1.1	14.5 ± 1.7	.6[a]
Platelet count ($\times 10^3/\mu L$)	182 ± 62	169 ± 48	140 ± 39	.04[a]
Liver histology				
A1/A2/A3	19/8/3	14/8/1	10/10/0	.3[b]
F1/F2/F3	14/9/7	11/7/5	7/5/8	.7[b]
Viral load ($\times 10^6$ *IU/mL*)	1.6 ± 1.2	1.8 ± 1.1	1.6 ± 1.1	.8[a]
Viral decline rate ($\log_{10}$/day)				
First phase	2.1 ± 0.9	1.5 ± 0.6	0.7 ± 0.5	<.0001[a]
Second phase	0.05 ± 0.05	0.04 ± 0.02	0.006 ± 0.008	<.0001[a]

ALT, alanine aminotransferase; BMI, body mass index.
[a]*P* values were determined by Kruskal–Wallis test.
[b]*P* values were determined by chi-square test.
[c]Response to previous IFN treatment.

tion–associated gene 5 (MDA5) play essential roles in initiating the host antiviral response by detecting intracellular viral dsRNA.[9,10] Caspase-recruiting domain (CARD) adaptor inducing IFN-β (Cardif), also called IFN-β promoter stimulator 1 (IPS-1), mitochondrial antiviral signaling protein (MAVS), and virus-induced signaling adaptor (VISA), is an adaptor molecule. Cardif connects RIG-I sensing to downstream signaling, resulting in IFN-β gene activation.[11–14] On the other hand, RIG-I sensing has been shown to be negatively regulated in a dominant-negative manner by LGP2,[10,15] a helicase related to RIG-I and MDA5 lacking CARD. Interestingly, the ubiquitin ligase ring-finger protein 125 (RNF125) has been recently shown to conjugate ubiquitin to RIG-I, MDA5 as well as Cardif, which results in suppressing the functions of these proteins.[16] Furthermore, these molecules are conjugated (ISGylated) by IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein,[17] and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18), also called ubiquitin-specific protease 43 (UBP43).[18,19] Moreover, the NS3/4A protease of HCV specifically cleaves Cardif as part of its immune evasion strategy.[11,20] Therefore, the RIG-I/Cardif system and its regulatory systems have essential key functions in the innate antiviral response (see Supplementary Figure 1 online at www.gastrojournal.org). However, the clinical significance of these innate immune systems, especially in relevance to the treatment response, is unclear because findings in this field have been mainly obtained by in vitro experiments using cell lines.

The aims of this study were to elucidate the mechanisms underlying resistance to antiviral therapy in the clinical setting and to determine whether quantification of transcripts of positive and negative cytoplasmic viral sensors and related regulatory molecules involving innate immune system is useful in predicting responses to PEG-IFN-α and ribavirin combination therapy.

Patients and Methods

Patients

Among patients with biopsy-proven chronic hepatitis C hospitalized at the Musashino Red Cross Hospital, 74 patients of HCV genotype 1b with a high viral load (>100,000 IU/mL by Amplicor-HCV Monitor Assay; Roche Molecular Diagnostics Co, Tokyo, Japan) were included in the present study (Table 1). Patients with cirrhosis, autoimmune hepatitis, or alcoholic liver injury were excluded. No patient was positive for hepatitis B virus–associated antigen/antibody or anti–human immunodeficiency virus antibody. No patient received immunomodulatory therapy prior to the enrollment. Written informed consent was obtained from all the patients, and this study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Helsinki Declaration. Five patients with nonviral liver disease (2 had autoimmune hepatitis and 3 had primary biliary cirrhosis) were included in the present study as controls.

Treatment Protocol

The patients were treated for 48 weeks with subcutaneous injections of PEG-IFN-α-2b (PegIntron; Schering-Plough Corporation, Kenilworth, NJ) at a dose of 1.5 $\mu g \cdot kg^{-1} \cdot week^{-1}$. Ribavirin (Rebetol; Schering-Plough Corporation) was administered concomitantly over the 48-week period, given orally twice daily at a total daily dose of 600 mg for the patients who weighed less than 60 kg and 800 mg for the patients who weighed between 60 and 80 kg. The dose of PEG-IFN-α-2b was reduced to 0.75 $\mu g \cdot kg^{-1} \cdot week^{-1}$ when either the neutrophil count was $<750/mm^3$ or the platelet count was $<80 \times 10^3/mm^3$. The dose of ribavirin was reduced to 600 mg/day when the hemoglobin concentration decreased to <10 g/dL.

Measurement of Gene Expression in the Liver

Liver biopsy was performed immediately before starting the therapy. After extraction of total RNA from liver biopsy specimens, the messenger RNA (mRNA) expression of positive and negative cytoplasmic viral sensors (RIG-I, MDA5, and LGP2), the adaptor molecule (Cardif), related ubiquitin E3-ligase (RNF125), and the modulators of these molecules (ISG15 and USP18) was quantified by real-time quantitative polymerase chain reaction (PCR) using primers specific for target genes. In brief, total RNA was extracted by the acid-guanidinium-phenol-chloroform method using Isogen (Nippon Gene Co Ltd, Toyama, Japan) from the liver biopsy specimen, which was 0.2–0.4 cm in length and 13 gauge in diameter. Complementary DNA (cDNA) was transcribed from 2 μg total RNA template in a 140-μL reaction mixture using a SYBR RT-PCR Kit (Takara Bio Co Ltd, Otsu, Japan) with random hexamer. Real-time quantitative PCR was performed using Smart Cycler version II (Takara Bio Co Ltd) with the SYBR RT-PCR Kit (Takara Bio Co Ltd) according to the manufacturer's instructions, and intercalating SYBR Green I (Molecular Probes Inc, Eugene, Oregon) was detected. Assays were performed in duplicate, and the expression levels of target genes were normalized to expression of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene and hydroxymethylbilane synthase, which is stable in the liver, as quantified using real-time quantitative PCR as internal controls. For accurate normalization, a set of 2 housekeeping genes was used in the present study. Sequences of primer sets were as follows: RIG-I: 5′-AAAGCATGCATGGTGTTCCAGA-3′, 5′-TCATTCGTGCATGCTCACTGATAA-3′; MDA5: 5′-ACATAACAGCAACATGGGCAGTG-3′, 5′-TTTGGTAAGGCCTGAGCTGGAG-3′; LGP2: 5′-ACAGCCTTGCAAACAGTACAACCTC-3′, 5′-GTCCCAAATTTCCGGCTCAAC-3′; Cardif: 5′-GGTGCCATCCAAAGTGCCTACTA-3′, 5′-CAGCACGCCAGGCTTACTCA-3′; RNF125: 5′-AGGGCACATATTCGGACTTGTCA-3′, 5′-CGGGTATTAAACGGCAAAGTGG-3′; ISG15: 5′-AGCGAACTCATCTTTGCCAGTACA-3′, 5′-CAGCTCTGACACCGACATGGA-3′; USP18: 5′-TGGTTCTGCTTCAATGACTCCAATA-3′, 5′-TTTGGGCATTTCCATTAGCACTC-3′; GAPDH: 5′-GCACCGTCAAGGCTGAGAAC-3′, 5′-TGGTGGTGAAGACGCCAGT-3′. hydroxymethylbilane synthase: 5′-AAGCGGAGCCATGTCTGGTAAC-3′, 5′-GTACCCACGCGAATCACTCTCA-3′.

Sequential Measurement of Gene Expression in Peripheral Blood Mononuclear Cells Before and During Therapy

To understand transcriptional response of the genes to PEG-IFN-α-2b and ribavirin therapy, serial expression of RIG-I, RNF125, Cardif, ISG15, and USP18 were determined before and during treatment in peripheral blood mononuclear cells (PBMC) in 14 patients (7 were sustained viral responders [SVR] and 7 were NVR). PBMC was obtained from whole blood samples collected before and at 4, 8, 24, 48, and 168 hours after the initiation of PEG-IFN-α-2b and ribavirin combination therapy. After extraction of total RNA from the PBMC, the expression of mRNA was quantified at each specified time point using real-time quantitative PCR as described above. Gene expression levels at each time point during treatment were calculated relative to baseline expression levels measured prior to IFN treatment.

Western Blotting

Western blotting was carried out in 9 patients (5 were SVR and 4 were NVR) and 3 non-HCV control subjects as described previously.[21] Liver biopsy specimen of ~10 mg was homogenized in 100 μL Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Twenty micrograms of the homogenates were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride Western blotting membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Biosciences, Buckinghamshire, United Kingdom). The anti-VISA mouse monoclonal antibody (BioDesign, Saco, ME) and anti-β-actin antibody (Sigma Chemical Co, St. Louis, MO) were used.

HCV Dynamics in Serum

To analyze the viral dynamics, HCV RNA was quantified just before and at 4, 8, and 24 hours and 2, 7, 14, 28, 56, and 84 days after the initiation of PEG-IFN-α-2b and ribavirin combination therapy, using real-time detection PCR, as reported previously.[22] For each patient, the viral decline curve was plotted on a semilogarithmic scale, and the slopes of the exponential viral declines were calculated for each viral decline phase with a straight-line fit of the data.

Definitions of Response to Therapy

A patient negative for serum HCV RNA during the first 6 months after the completion of PEG-IFN-α-2b and ribavirin combination therapy was defined as an SVR, and a patient for whom HCV RNA became negative at the end of therapy and reappeared after completion of therapy was defined as a transient responder (TR). A patient who was positive for HCV RNA even during the course of therapy was defined as an NVR. HCV RNA was determined with the Amplicor qualitative assay (Roche Molecular Diagnostics Co, Tokyo, Japan). The detection sensitivity of this assay is approximately 50 IU/mL.

Statistical Analysis

Categorical data were compared by the χ^2 test and Fisher exact test. Distributions of continuous variables were analyzed by Mann–Whitney U test for 2 groups. Kruskal–Wallis test was used for multiple group comparisons. All tests of significance were 2-tailed, and P values $< .05$ were considered statistically significant.

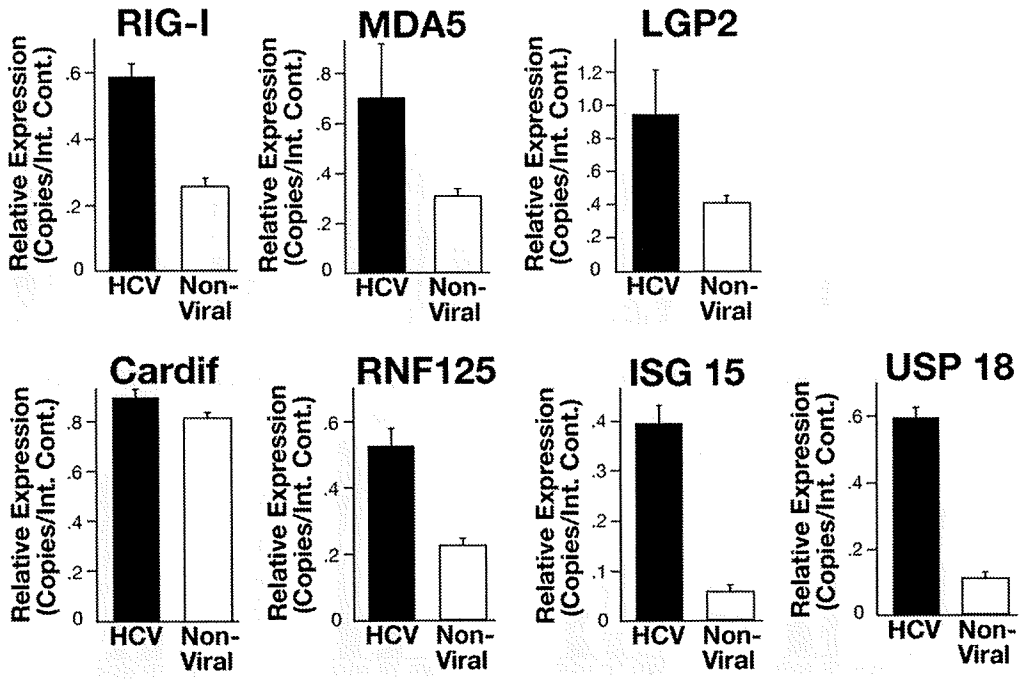

Figure 1. Comparison of hepatic gene expression levels between chronic hepatitis C patients (n = 74) and nonviral liver disease patients (n = 5). Expression levels of RIG-I, MDA5, LGP2, Cardif, RNF125, ISG15, and USP18 are shown. *Error bars* indicate the standard error. Upon HCV infection, expression of these genes except Cardif was stimulated. The *P* values determined by Mann–Whitney *U* test between 2 groups were as follows: RIG-I, *P* .02; MDA5, *P* .01; LGP2, *P* .005; Cardif, *P* .7; RNF125, *P* .06; ISG15, *P* .007; USP18, *P* .004.

Results

Patient Characteristics

According to the final virologic response, patients were classified into 3 groups: 30 were SVR, 24 were TR, and the remaining 20 were NVR, as shown in Table 1. Viral decline rates in NVR were significantly lower in both the first and second phases of HCV dynamics. It should be noted that most NVR patients exhibited no second-phase viral decline.

Data on factors that were available before starting the treatment were compared according to virologic response by univariate analysis. As shown in Table 1, only age and platelet count were associated with viral response, and no other clinical factors were predictive of NVR before initiation of the therapy.

Gene Expression Involving Innate Immunity in the Liver

First, we compared basal hepatic gene expression between the chronic hepatitis C patients (n = 74) and the nonviral liver disease patients (n = 5). As shown in Figure 1, levels of RIG-I, MDA5, LGP2, ISG15, and USP18 expression were significantly higher in the chronic hepatitis C patients than in the nonviral liver disease patients. However, there was no significant difference in levels of Cardif expression between the chronic hepatitis C and nonviral-related liver disease patients.

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, levels of gene expression were compared based on the final virologic response. As shown in Figure 2, the hepatic expression levels of RIG-I, MDA5, and LGP2 were significantly higher in NVR than in SVR and TR. In marked contrast, hepatic Cardif expression was significantly lower in the NVR group. The hepatic expression of RNF125, which is specific E3-ubiquitin ligase for RIG-I, MDA5, and Cardif, was also significantly lower in the NVR group. Because negative correlation was found between RIG-I and Cardif or RNF125 expression, we calculated the ratio of RIG-I to Cardif or RNF125 expression levels. As shown in Figure 2, the difference among the groups was conspicuous when comparison was made with the RIG-I/Cardif ratio or RIG-I/RNF125 ratio. Moreover, the RIG-I/Cardif expression ratio before treatment was negatively and significantly correlated with the exponential viral decline rate in both the first and the second phases of HCV dynamics (first phase, $r = -0.4$, $P < .0005$; second phase, $r = -0.5$, $P < .0001$). Similar correlation was found between RIG-I/RNF125 ratio and viral decline rate (first phase, $r = -0.4$, $P = .004$; second phase, $r = -0.2$, $P = .09$, data not shown).

Like RIG-I and MDA5, intrahepatic expression levels of ISG15 and USP18 were significantly higher in NVR than in SVR and TR (Figure 2). When we assessed the correlation of these 2 genes in individual patients, we found a strong and significant correlation between ISG15 and USP18 ($r^2 = 0.88$, $P < .0001$). Levels of ISG15 and USP18 expression before treatment were negatively correlated with the exponential viral decline rates calculated from

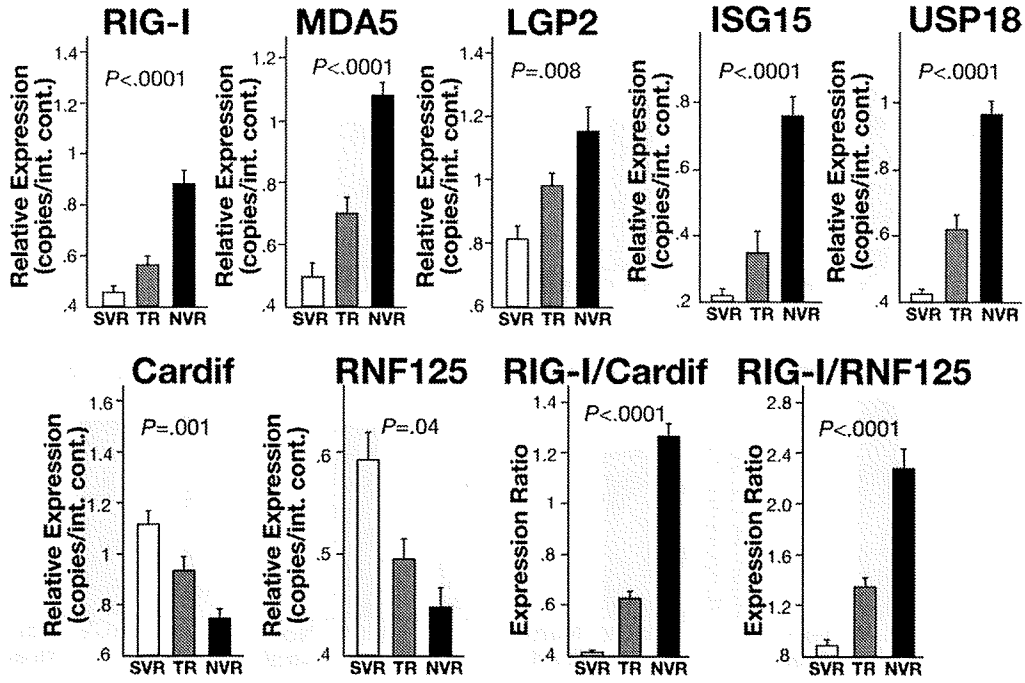

Figure 2. Comparison of hepatic gene expression levels according to final virologic outcome. Expression levels of RIG-I, MDA5, LGP2, ISG15, USP18, Cardif, RNF125, RIG-I/Cardif ratio, and RIG-I/RNF125 ratio are shown. *Open columns* indicate SVR (n = 30), *shaded columns* indicate TR (n = 24), and *solid columns* indicate NVR (n = 20). *Error bars* indicate the standard error. The *P* values were analyzed by the Kruskal–Wallis test.

the first and the second phases of HCV dynamics (ISG15, first phase, $r = -0.5$, $P < .0001$; ISG15, second phase, $r = -0.3$, $P = .02$; USP18, first phase, $r = -0.5$, $P < .0001$; USP18, second phase, $r = -0.3$, $P = .01$).

Receiver Operator Characteristic Analysis

To determine the usefulness of these gene quantifications as predictors, receiver operator characteristic (ROC) analysis was conducted (Figure 3). The area under the ROC curve for the RIG-I/Cardif ratio, ISG15, and USP18 was 0.91, 0.90, and 0.91, respectively, suggesting that quantification of these gene transcripts is of use for the prediction of NVR (Table 2). In addition, this analysis also suggested that RIG-I/Cardif ratio would be more specific for prediction of NVR, whereas ISG15 and USP18 would be more sensitive (Table 2).

Multivariate Analysis

Multivariate analysis for factors that were available before initiating therapy indicated that a higher ratio of RIG-I/Cardif and higher expression of ISG15 were independent factors that were associated with NVR (Table 3). In this analysis, USP18 was excluded because of its strong correlation with ISG15.

Protein Levels of Cardif in the Liver

Because hepatic expression of Cardif mRNA was significantly lower in NVR patients than in SVR patients,

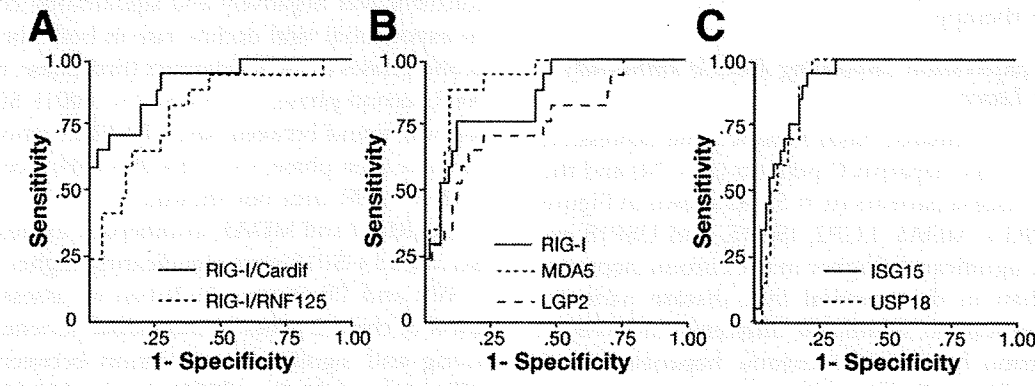

Figure 3. Receiver operator characteristic (ROC) curve for prediction of nonvirologic response. ROC curves were generated to compare (*A*) RIG-I/Cardif ratio (*solid line*) and RIG-I/RNF125 ratio (*shaded line*); (*B*) RIG-I (*solid line*), MDA5 (*shaded line*), and LGP2 (*dotted line*); and (*C*) ISG15 (*solid line*) and USP18 (*shaded line*).

Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative and Positive Predictive Values of Non-Virologic Responses

Variables	Az	95% CI	Cut-off	Sensitivity	Specificity	NPV[a]	PPV[b]
RIG-I	0.89	0.78–0.95	0.68	0.80	0.87	0.92	0.70
MDA5	0.92	0.86–0.98	0.84	0.82	0.89	0.93	0.74
LGP2	0.76	0.63–0.90	1.03	0.65	0.72	0.85	0.46
RIG-I/Cardif	0.91	0.84–0.99	0.88	0.75	0.91	0.91	0.75
RIG-I/RNF125	0.81	0.69–0.93	1.05	0.82	0.62	0.91	0.43
ISG15	0.91	0.85–0.97	0.36	0.90	0.81	0.96	0.64
USP18	0.90	0.84–0.96	0.67	0.90	0.83	0.96	0.67

[a]NPV, negative predictive value.
[b]PPV, positive predictive value.

we determined the basal protein expression levels of Cardif in the liver in NVR and SVR patients. Western blot analysis demonstrated a single Cardif product in all samples (Figure 4*A*). Similar to Cardif mRNA expression, mean Cardif expression in NVR patients was significantly lower than that in SVR (Figure 4*B*, $P = .01$). The cleavage product of Cardif, which has been reported by Loo et al,[23] was not detected in our analyses.

Transcriptional Responses to PEG-IFN-α-2b and Ribavirin Therapy in PBMC

Sequential analysis in response to PEG-IFN-α-2b and ribavirin demonstrated a rapid and strong induction of RIG-I, ISG15, and USP18 mRNA expression, which peaked 8 hours after PEG-IFN-α-2b administration (Figure 5). A greater fold change of these peak inductions was observed in SVR patients compared with NVR patients, although statistical significance was not achieved. In marked contrast, RNF125 expression profile in response to PEG-IFN-α-2b was triphasic, and consisted of (1) rapid and strong suppression peaked at 8 hours after administration, (2) increased 1.5- to 2-fold above baseline level during 24–48 hours after the administration, and (3) gradually decreased to baseline level (Figure 5). The rapid suppression and subsequent increase following PEG-IFN-α-2b administration tended to have a greater fold change in NVR patients compared with those in SVR patients. In contrast from RIG-I, ISG15, USP18, and RNF125, Cardif expression profile was relatively constitutive, and transcriptional response to PEG-IFN was weak (Figure 5).

Discussion

In the present study, we found that baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the final virologic outcome in patients with chronic hepatitis C who were treated with PEG-IFN-α-2b and ribavirin combination therapy: up-regulation of RIG-I, MDA5, LGP2, ISG15, and USP18 and lower expression of Cardif and RNF125 could predict nonresponse to subsequent treatment with PEG-IFN-α-2b and ribavirin. The positive predictive value of a high ratio of expression of RIG-I to Cardif (>0.88) for NVR was the highest at a value of 0.75, and the negative predictive values of high expression of ISG15 (>0.36/internal control) and USP18 (>0.67/internal control) were the highest at values of both 0.96. These data may be of use in predicting clinical responses to the PEG-IFN-α and ribavirin combination before initiating therapy.

Table 3. Multivariate Analysis for the Factors Associated With Non-Virologic Response

Variable	Odds ratio	95% CI	*P* value
RIG-I/Cardif Ratio (by 0.1)	1.5	1.1–2.1	.008
RIG-I/RNF125 Ratio (by 0.1)	1.2	1.0–2.5	.1
ISG15 (by 0.1/internal control)	1.5	1.1–2.0	.01
Age (by 1 y)	1.0	0.9–1.1	.6
Platelet count (by $1 \times 10^4/\mu L$)	1.2	0.9–1.5	.07

Previously, large randomized controlled trials identified several pretreatment factors associated with the final virologic outcome, such as genotype, HCV RNA level, degree of fibrosis, age, body weight, ethnicity, and steatosis.[24] However, these findings lead us to believe that predicting the final virologic response before initiating PEG-IFN-α and ribavirin is difficult. Indeed, only age and platelet count were associated with the outcome in our patients with genotype 1b and a high viral load. Currently, the final response can be gauged only after treatment has been initiated. Although an early viral response at 12 weeks suggests the eventual outcome with 60%–90% accuracy,[25] a 12-week regimen is associated with adverse effects and is expensive. Therefore, this study investigated the baseline expression of genes involving innate immunity that may have significant effects on clinical outcomes.

In the present study, we demonstrated that RIG-I and MDA5 were inducible upon HCV infection and that expression of these intrahepatic positive viral sensors was up-regulated in NVR. In vitro studies have suggested that RIG-I and MDA5 play a pivotal role in the regulation of IFN production and augment the production of IFN via an amplification circuit. These results suggest that expression of RIG-I and MDA5 and related amplification system may be up-regulated by endogenous IFN at a higher baseline level in NVR patients. However, HCV elimination by subsequent exogenous IFN is insufficient

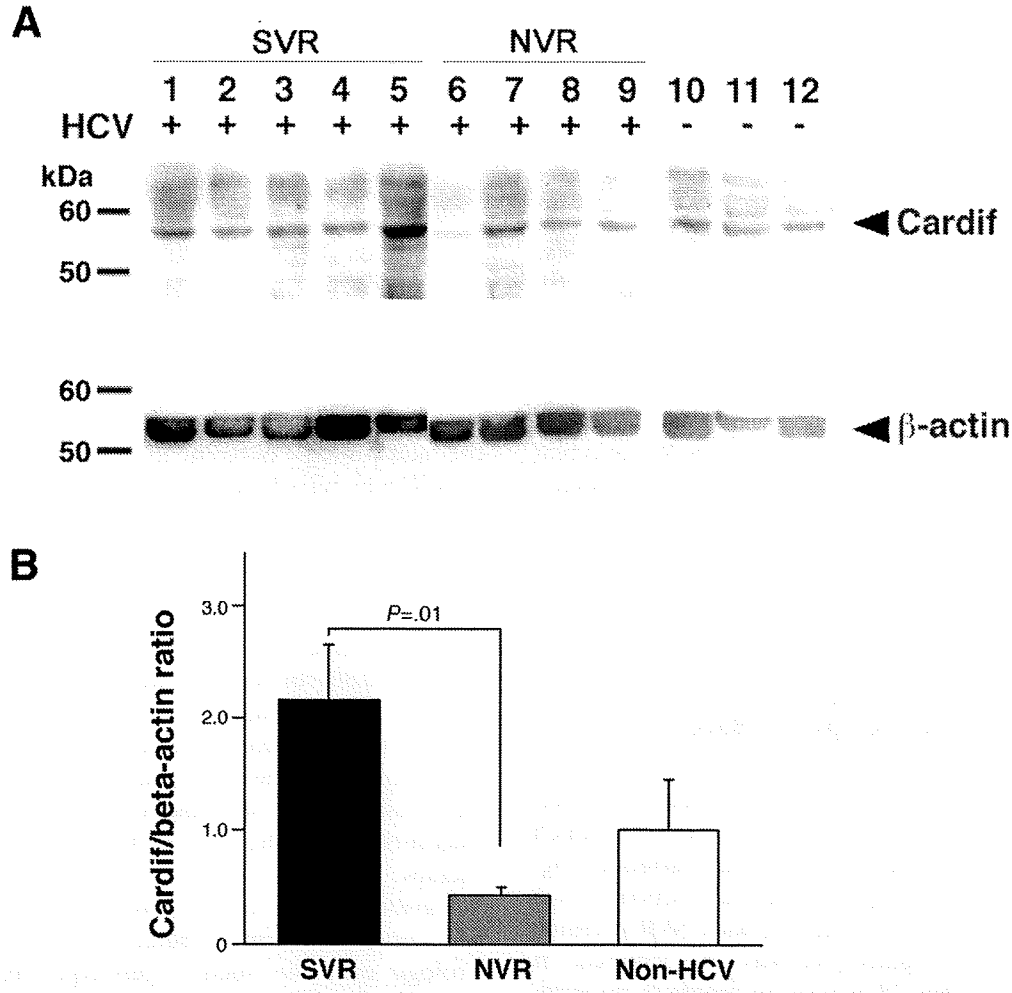

Figure 4. (*A*) Western blot analysis. Five lanes were SVR (*lanes 1–5*), 4 lanes were NVR (*lanes 6–9*), and 3 lanes were non-HCV control (*lanes 10–12*). Specific bands for Cardif and β-actin are indicated by *arrows*. (*B*) Expression level of Cardif protein normalized to β-actin in the liver biopsy specimens according to ultimate treatment response. *Error bars* indicate the standard error.

in these patients, suggesting that NVR patients may have adopted a different equilibrium in their immune response to the virus. In contrast to the expression of RIG-I and MDA5, Cardif mRNA, which was expressed in a relatively constitutive fashion, was significantly lower in NVR. Our ROC analysis highlights that lower expression of Cardif relative to that of RIG-I was one of the strongest predictors for NVR. Moreover, Western blot analysis further confirmed the down-regulation of Cardif in NVR patients, as demonstrated by its protein level. Because Cardif is one of the substantial target molecules of HCV evasion,[11,20] it is likely that Cardif expression is suppressed by HCV with resistant phenotype or is inadequate in NVR patients. Loo et al have demonstrated a Cardif cleavage product in 2 of 4 liver tissue samples of chronic HCV infection.[23] In our study, however, the Cardif cleavage product was not detected, presumably because the product could be unstable in vivo, resulting in rapid degradation. Although further studies are necessary to elucidate mechanisms of Cardif down-regulation, our findings of lower expression of Cardif in NVR suggested that the status of Cardif expression in the liver might have a significant effect on the ultimate outcome of antiviral treatment.

The antiviral effect brought by RIG-I/Cardif signaling is regulated by the coordination of negative and positive regulators. It has been shown that RNF125 functions as a negative regulator of RIG-I/Cardif signaling. RNF125 is an ubiquitin E3-ligase with activity against protein containing CARD domains, such as RIG-I, MDA5, and Cardif, and these ubiquitinated molecules undergo proteasomal degradation. In contrast, RNF125 do not have negative function against LGP2, a negative regulator of RIG-I signaling, because LGP2 lacks CARD domain. In contrast to RIG-I, RNF125 expression was rapidly suppressed by exogenous IFN; therefore, observed lower basal hepatic level of RNF125 in NVR could be explained by the suppressive effect of endogenous IFN, which may be up-regulated in NVR patients. Hence, RNF125 may constitute a negative regulatory circuit for IFN production and is responsible for responsiveness to PEG-IFN and ribavirin therapy.

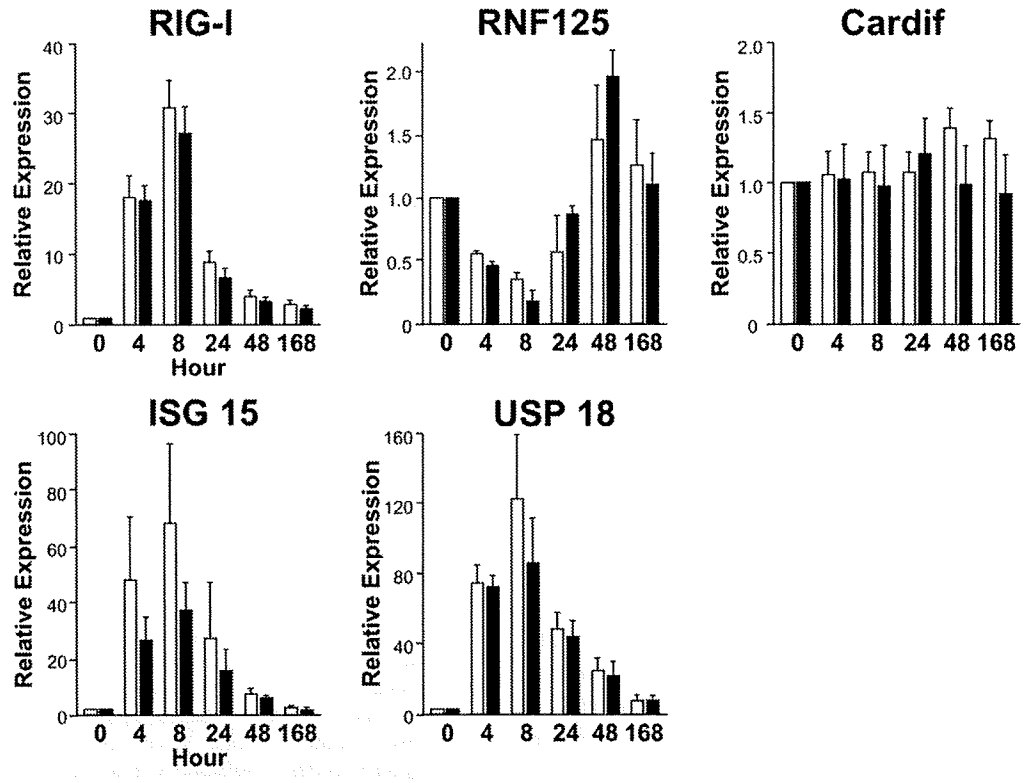

Figure 5. Transcriptional responses during PEG-IFN-α-2b and ribavirin therapy in PBMC (n = 14). *Open columns* indicate SVR (n = 7), and *solid columns* indicate NVR (n = 7). *Error bars* indicate the standard error. The *P* values determined by Mann–Whitney *U* test between 2 groups at 8 hours were as follows: RIG-I, *P* .3; RNF125, *P* .3; Cardif, *P* .7; ISG15, *P* .3; USP18, *P* .2.

It has been shown that RIG-I function is modified by ISG15 via ISGylation.[17] Consistent with our data, Chen et al identified 18 genes, including ISG15 and USP18, whose expression differed between responders and non-responders.[26] Interestingly, a recent study has shown that USP18 negatively regulates IFN signaling independently of its isopeptidase activity toward ISG15 by binding to the IFNAR2 receptor subunit and blocking the interaction between Janus kinase and the IFN receptor.[27] Moreover, the siRNA knockdown of USP18 in human cells has consistently been shown to potentiate the ability of IFN to inhibit HCV RNA replication.[28] Therefore, USP18 is suggested as a novel in vivo inhibitor of signal transduction pathways that are specifically triggered by type I IFN. Consistent with a role for USP18 in down-regulating the antiviral IFN response, we confirmed that up-regulation of USP18 was one of the factors predicting a lack of response to treatment with IFN.

The mechanism underlying the association of gene expression involving innate immunity with resistance to therapy is not well understood. Our human study with HCV patients treated by PEG-IFN and ribavirin highlights RIG-I/Cardif, RIG-I/RNF125, and ISG15/USP18, which is partly responsible for the clinical responsiveness to antiviral therapy. RIG-I signaling by viral pathogens may affect a wide variety of responses in not only innate but also acquired immunity. Our study is the first to demonstrate the potential relevance between molecules involving innate immunity and the clinical response to antiviral therapy.

In addition, sequential analysis of expression profile during PEG-IFN-α-2b and ribavirin treatment was also performed in this study. Lanford et al demonstrated transcriptional response to IFN-α in chimpanzee by genome microarray analysis, which included RIG-I, ISG15, and USP18.[29] An association of transcriptional response with early phase of virologic response has been also reported in PBMC or liver biopsy specimen.[30-32] We recently reported that the transcriptional double-stranded RNA-activated protein kinase response during treatment with PEG-IFN-α-2b and ribavirin was associated with the ultimate clinical response.[30] Similarly, the present study demonstrated a strong and rapid increase of RIG-I, ISG15, and USP18 mRNA in response to clinical PEG-IFN treatment especially in SVR patients, although few patients were available to achieve statistical significance between SVR and NVR. In marked contrast, transcriptional response of RNF125 exhibited a triphasic pattern. Rapid suppression seen in the first phase was presumably because of a negative regulatory effect of IFN. However, increase of RNF125 mRNA in the second phase, which tended to be grater in NVR, may be responsible for inhibiting RIG-I expression seen 8–48 hours after PEG-IFN-α-2b administration. Although limitations includ-

ing the use of PBMC and small sample size still deserve mention, the sequential expression profile during treatment may provide further valuable information regarding the prediction of the clinical response to the therapy and the mechanism of action of antiviral treatment.

In the present study, we have included patients with genotype 1b because it is imperative to designate a virologically homogeneous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have preliminarily studied genotype 2 patients and found that Cardif and RNF125 gene expression levels in NVR patients were significantly lower than those with SVR patients ($P = .03$ and $P = .04$, respectively) and that RIG-I/Cardif and RIG-I/RNF125 ratios were significantly higher in NVR patients ($P = .02$ and $P = .009$, respectively, see Supplementary Figure 2 online at www.gastrojournal.org). These findings suggest that the differences in gene expression profiles between SVR and NVR were almost identical to those demonstrated in patients with genotype 1b. However, the correlation between treatment responses in all the genotypes and the different status of innate immune responses needs to be explored. Further studies may be necessary to clarify this issue.

In conclusion, the results of the present study offer potentially important clinical implications for patients with chronic hepatitis C who are treated with PEG-IFN-α and ribavirin. Quantifying hepatic gene expression of the RIG-I/Cardif system, including its regulators before treatment, is useful in identifying patients who are at a higher risk for NVR. The data from these assays can provide valuable information that may influence the decision about the treatment strategy in each individual patient. Finally, this clinical human study demonstrates the potential relevance of the molecules involving innate immunity to the clinical response to therapy. Our data will help understand the pathogenesis of HCV resistance and development of new antiviral therapy targeted toward the innate immune system.

Supplementary Data

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.2008.02.019.

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HEPATOLOGY

Insulin resistance and lichen planus in patients with HCV-infectious liver diseases

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Abstract

Background and Aim: Hepatitis C virus (HCV) causes liver diseases and extrahepatic manifestations, and also contributes to insulin resistance and type 2 diabetes mellitus (DM). The aims of the present study were to examine the incidence of extrahepatic manifestations including lichen planus in HCV-infected patients and to evaluate the relationship between lichen planus and insulin resistance.

Methods: Of 9396 patients with liver diseases presenting to the study hospital, 87 patients (mean age 60.0 ± 11.5 years) with HCV-related liver diseases were identified and examined for the incidence of extrahepatic manifestations. Insulin resistance and the presence of *Helicobacter pylori* antibodies were also measured.

Results: The prevalence of DM was 21.8% (19/87), hypertension was 28.7% (25/87), thyroid dysfunction was 20.7% (18/87), and extrahepatic malignant tumor was 9.2% (8/87). The prevalence of lichen planus at oral, cutaneous, pharyngeal, and/or vulval locations was 19.5% (17/87). Characteristics of 17 patients with lichen planus (group A) were compared with 70 patients without lichen planus (group B). Prevalence of smoking history, presence of hypertension, extrahepatic malignant tumor, and insulin resistance (HOMA-IR) were significantly higher in group A than in group B. Significant differences were not observed for age, sex, body mass index, diagnosis of liver disease, alcohol consumption, presence of DM, thyroid dysfunction, liver function tests, or presence of *H. pylori* infection between the two groups.

Conclusions: Infection with HCV induces insulin resistance and may cause lichen planus. It is necessary for an HCV-infected patient to be assayed for insulin resistance, and to be checked for different extrahepatic manifestations of this infection, particularly lichen planus.

Introduction

The number of fatalities due to hepatocellular carcinoma (HCC) in Japan continues to increase, and it is estimated that this tendency will continue at least until 2015. Of the HCC cases in Japan, approximately 16% are caused by hepatitis B virus (HBV) infection and approximately 80% by hepatitis C virus (HCV) infection.[1] The average prevalence of HCV carriers in Japan is about 2%, with the absolute number estimated at 2 million.[2] The increase in HCC in Japan depends on the spread of HCV infection.[2]

Infection with HCV induces various extrahepatic manifestations as well as chronic liver diseases.[3,4] HCV infects cells or organs except hepatocytes and multiplies. Representative extrahepatic manifestations of HCV infection include lichen planus, diabetes mellitus (DM), malignant lymphoma, Sjögren's syndrome, cryoglobulinemia, and membranoproliferative glomerulonephritis. It has been reported that combined therapy using interferon and ribavirin is effective for different extrahepatic manifestations that are apt to be overlooked.[5,6]

At present, it has been shown that HCV multiplies in skin and oral mucosa leading to HCV-related lichen planus,[7,8] and that the risk of malignant transformation is higher in lichen planus with HCV infection than in lichen planus without HCV.[9] However, a mechanism for these extrahepatic manifestations has not been elucidated. Recently it was reported that there is a significant correlation between lichen planus and HCV and DM in southern Taiwan, particularly in HCV patients with elevated serum alanine aminotransferase (ALT) levels and atrophic-erosive oral lichen planus (OLP).[10] In our previous report, patients with lichen planus having DM were all found to be HCV-infected.[11]

In addition, it has been reported that DM is a risk factor for HCV-related hepatocarcinogenesis[12] and for decreased survival

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among liver cirrhosis patients.[13] In addition, the incidence of diabetes in patients having HCV-related liver cirrhosis is higher than that in patients with HBV-related liver diseases.[14]

We recently showed molecular mechanisms for HCV core-induced insulin resistance.[15] HCV core up-regulates the suppressor of cytokine signaling (SOCS) 3, and inhibits insulin signaling by down-regulation of insulin receptor substrate (IRS) -1 and IRS-2 in hepatocytes. Moreover, in an epidemiological survey, we demonstrated that a significant increase in the incidence of diabetes occurs in subjects with high titers of HCV core compared to subjects who are negative for anti-HCV antibody[16] and concluded that HCV infection induces insulin resistance, which causes an increase in the incidence of extrahepatic manifestations in HCV-infected individuals.[17]

In the current study, we surveyed the incidence of abnormal glucose tolerance in patients with or without lichen planus in a study population with HCV-related chronic liver disease, and investigated the relationship between lichen planus and insulin resistance.

Methods

Patients

A total of 105 984 consecutive patients had checkups for chronic liver disease for the first time in the Digestive Disease Center at Kurume University Hospital from April 1988 to August 2005. In the Digestive Disease Center, physicians, surgeons, radiologists, and an oral surgeon hold full-time positions. One of us (M.S.) is a hepatologist and examined 9396 of these 105 984 patients. There were 522 patients who were HCV antibody positive and who thereafter continued with regular hospital visits until April 2006.

Exclusion criteria were the following: (i) other causes of chronic liver disease or disease other than chronic HCV infection; (ii) liver disease related to HBV infection; and (iii) patients treated with interferon therapy at the time of study inclusion.

We examined the presence of extrahepatic manifestations of chronic HCV infection in 87 patients. Informed consent was obtained from all patients after the purpose and methods of the study were explained. The 87 patients were 44 men and 43 women with a mean age of 60.0 ± 11.5 years.

The patients were monitored for the presence of extrahepatic manifestations of HCV infection such as lichen planus, DM, hypertension, thyroid dysfunction, and extrahepatic malignant tumor as well as liver disease. Biochemical tests were done and insulin values, blood glucose levels, and *Helicobacter pylori* antibody were measured in patient blood samples. Life histories were taken.

Clinical examinations

Patients received oral mucosa and cutaneous medical examinations by an oral surgeon and a dermatologist. The diagnosis of OLP was made on the basis of clinical and histopathological features. Diagnosis of type 2 DM was based on the American Diabetic Association (ADA) criteria of 1997.[18] Persons in whom diabetes was diagnosed before 30 years of age and who used insulin were categorized as type 1 DM and were excluded from our study.

The following definitions of cardiovascular disease were employed. Obesity was defined as a body mass index (BMI) >25 kg/m^2 or higher. Hypertension was defined as a systolic blood pressure (SBP) of 140 mmHg or higher, or a diastolic blood pressure (DBP) of 90 mmHg or higher according to the criteria of JNC-VI of the International Hypertension Society.[19] Thyroid hormones such as FT3, FT4 and thyroid stimulating hormone were measured for all patients, and thyroid echography examination was performed for some patients. Examination of the upper gastrointestinal tract or lower digestive tract was performed on patients for whom it was deemed clinically necessary.

We also took a history of smoking and alcohol consumption.

Serological assays

Serum samples from the 87 patients were collected and tested for platelets (PLT) and for the following liver function tests: serum ALT, aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (γ-GTP), lactate dehydrogenase (LDH), total bilirubin (TBil), direct bilirubin (DBil), thymol turbidity test (TTT), zinc sulfate turbidity test (ZTT), total cholesterol (TC), total protein (TP), and albumin (Alb). Sera were also examined for the presence or absence of HCV or HBV infection. Anti-HCV was measured by a chemiluminescent enzyme immunoassay kit (Lumipulse II HCV, Fujirebio, Tokyo, Japan). HCV RNA in serum was detected using the Amplicore HCV test (Roche, Tokyo, Japan). Hepatitis B virus surface antigen (HBsAg) was assayed using a chemiluminescent immunoassay kit (Architect, HBsAg QT, Dainabot, Tokyo, Japan). Ultrasonographic examination for all patients was performed in order to investigate the shape of the liver and lesions occupying the liver. Computed tomography and liver biopsy were performed in some patients. Most patients underwent endoscopy for detection of esophagogastric varices. We used other possible predictors of liver cirrhosis progression, including serum albumin, TBil, prothrombin time, and PLT.

Plasma glucose levels were measured by a glucose oxidase method for all subjects and serum insulin levels were measured using a sandwich enzyme immunoassay kit (Eiken Chemical, Tokyo, Japan). Insulin resistance (IR) was calculated on the basis of fasting levels of plasma glucose and insulin, according to the homeostasis model assessment (HOMA-IR) method.[20] The formula for the HOMA-IR is: HOMA-IR = fasting glucose (mg/dL) × fasting insulin (μU/mL)/405.

The presence of serum IgG antibodies against *H. pylori* antibody were measured by the SRL (Tokyo) using E Plate *H. pylori* antibody produced by Eiken Chemical.

Statistical analysis

The chi-squared test and the unpaired Student *t*-test were used for statistical analyses. Differences were judged significant for $P < 0.05$ (two-tailed). This study was approved by the Institutional Review Board/Ethics Committee of our Institution.

Results

Among 87 patients with HCV-related liver diseases, the prevalence of lichen planus was 19.5% (17/87), DM was 21.8% (19/87),

Table 1 Clinical characteristics of 87 patients with HCV-related liver diseases according to presence of lichen planus (LP)

Clinical characteristic	All patients	Group A (with LP)	Group B (without LP)	*P*-value (A *vs* B)
No. subjects	87	17	70	–
Age (years)	60.0 ± 11.5	63.7 ± 10.6	59.1 ± 11.6	NS
Sex (M/F)	44/43	11/6	33/37	NS
BMI (kg/m^2)	22.8 ± 2.9	23.9 ± 2.8	22.5 ± 2.9	NS
Smoking history	32 (36.8)	10 (58.8)	22 (31.4)	0.0356
Alcohol consumption percentage	50 (57.5)	10 (58.8)	40 (57.1)	NS
Diagnosis of liver disease				
Past history of HCV infection	1	0	1	NS
Chronic hepatitis C	69	11	58	
HCV-related liver cirrhosis	9	3	6	
HCV-related HCC	8	3	5	
Comorbidities				
Diabetes mellitus	19 (21.8)	4 (23.5)	15 (21.4)	NS
Hypertension	25 (28.7)	10 (58.8)	15 (21.4)	0.0022
Thyroid dysfunction	18 (20.7)	5 (29.4)	13 (18.6)	NS
Extrahepatic malignant tumor	8 (9.2%)	5 (29.4)†	3 (4.3)‡	0.0013

Values shown as *n* (%) or mean ± SD. BMI, body mass index; F, female; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; M, male; NS, not significant.

†Tumors were: gastric cancer (two), tongue cancer (one), larynx cancer (one), and renal and colon cancer (one). ‡Tumors were: gastric cancer (one), colon cancer (one), and gallbladder cancer (one).

hypertension was 28.7% (25/87), thyroid dysfunction was 20.7% (18/87), and extrahepatic malignant tumor was 9.2% (8/87).

We compared characteristics of 17 patients who had lichen planus (group A) and 70 patients who did not have lichen planus (group B). The mean age in group A was 63.7 ± 10.6 years; there were 11 men and six women. The mean age in group B was 59.1 ± 11.6 years; there were 33 men and 37 women. Table 1 shows clinical features of groups A and B. The diagnoses of liver diseases in group A were chronic hepatitis C infection (11 patients), HCV-related liver cirrhosis (three patients), and HCV-related HCC (three patients). Those of group B were chronic hepatitis C infection (58 patients), HCV-related liver cirrhosis (six patients), HCV-related HCC (five patients) and past history of HCV infection (one patient) (Table 1).

The prevalence of smoking history ($P = 0.0356$), hypertension ($P = 0.0022$), and extrahepatic malignant tumor ($P = 0.0013$) were significantly higher in group A than in group B (Table 1). Diagnoses of extrahepatic malignant tumors in group A were: tongue cancer (one squamous cell carcinoma), larynx cancer (one squamous cell carcinoma), gastric cancer (one adenocarcinoma, one signet ring cell carcinoma), renal and colon cancer (one renal cell carcinoma). Diagnoses of extrahepatic tumor in group B were: gastric cancer (one adenocarcinoma), colon cancer (one adenocarcinoma), and gallbladder cancer (one adenocarcinoma). Significant differences were not observed for age, sex, BMI, liver disease, alcohol consumption, presence of DM, or thyroid dysfunction between these two groups.

We analyzed for differences between these two groups in liver assays, blood platelets, insulin, blood glucose, HOMA-IR, and presence of *H. pylori* infection. The laboratory data of both groups are shown in Table 2. Prevalence of insulin ($P = 0.0076$) and HOMA-IR ($P = 0.0113$) were significantly higher in group A than in group B (Table 2). Significant differences were not observed for serum AST, ALT, LDH, γGTP, TP, Alb, TBil, DBil, TTT, ZTT, TC, blood platelets, blood glucose, or presence of *H. pylori* infection between these two groups.

Seventeen patients had OLP at a total of 24 sites. The site of occurrence was: buccal mucosa in 13 (76.5%), lower lip in six (35.3%), upper lip in two (11.8%), gingiva in one (5.9%), tongue in one (5.9%), and floor of mouth in one (5.9%) (Table 3). The sites of lichen planus except oral mucosa were lower leg in four (23.5%), antebrachium in one (5.9%), skin extremities in two (11.8%), hypopharynx in one (5.9%), and vulva in one (5.9%). Biopsies of hypopharyngeal lichen planus were performed by an otolaryngologist, and of vulvar lichen planus by a gynecologist. The erosive and reticular variety, respectively, was found to be the prevalent form (Table 3).

Discussion

We performed an epidemiological survey for extrahepatic manifestations and HCC in an HCV hyperendemic area in Japan.[21,22] Anti-HCV positivity among residents of this area in 1990 was 23.6%.[23] We found that the prevalence of extrahepatic manifestations among individuals with HCV infection was higher than among those without HCV,[22] and found an association between HCV core, insulin resistance, and the development of type 2 DM.[16] Recently, we reported that insulin resistance in inhabitants who have an extrahepatic manifestation including OLP with HCV infection shows significantly greater increases than for inhabitants who have neither an extrahepatic manifestation nor HCV infection.[17] By the results of these epidemiological surveys we think that insulin resistance induced by HCV infection causes an increase in the incidence of extrahepatic manifestations in HCV-infected individuals.

In this study, we did long-term follow up for insulin resistance from the standpoint of lichen planus among patients who we identified as having HCV-related chronic liver disease at our hos-

Table 2 Laboratory data of 87 patients with HCV-related liver diseases according to presence of lichen planus (LP)

Laboratory assay	All patients	Group A (with LP)	Group B (without LP)	*P*-value (A *vs* B)
AST (IU/L)	61.1 ± 38.1	60.9 ± 33.5	61.2 ± 39.3	NS
ALT (IU/L)	68.2 ± 46.7	62.4 ± 39.6	69.6 ± 48.5	NS
LDH (IU/L)	216.8 ± 62.8	205.8 ± 72.1	219.6 ± 60.6	NS
γ-GTP (IU/L)	64.1 ± 68.4	63.5 ± 50.0	64.2 ± 72.5	NS
TP (g/dL)	7.7 ± 0.5	7.7 ± 0.5	7.7 ± 0.5	NS
Alb (g/dL)	4.1 ± 0.5	3.9 ± 0.5	4.2 ± 0.5	NS
PLT (/mm^3)	13.8 ± 5.1	12.5 ± 5.0	14.1 ± 5.09	NS
TBil (mg/dL)	1.1 ± 0.6	1.2 ± 0.9	1.0 ± 0.5	NS
DBil (mg/dL)	0.2 ± 0.2	0.2 ± 0.3	0.2 ± 0.2	NS
TTT	16.2 ± 6.7	18.4 ± 4.7	15.8 ± 7.0	NS
ZTT	20.6 ± 6.9	21.8 ± 5.8	20.3 ± 7.2	NS
TC (mg/dL)	172.3 ± 35.8	164.3 ± 41.9	174.1 ± 34.4	NS
Insulin (μU/L)	23.3 ± 42.0	47.3 ± 87.8	17.4 ± 15.4	0.0076
Blood glucose (mg/dL)	97.4 ± 30.1	103 ± 33.2	96.1 ± 29.5	NS
HOMA-IR	7.1 ± 18.8	17.4 ± 40.0	4.6 ± 6.0	0.0113
Helicobacter pylori antibody (*n* (%))	58 (66.7)	10 (58.8)	48 (68.6)	NS

Values shown as mean ± SD. Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; DBil, direct bilirubin; γ-GTP, gamma-glutamyl transpeptidase; HOMA-IR, homeostasis model assessment; LDH, lactate dehydrogenase; NS, not significant; PLT, platelets; TBil, total bilirubin; TP, total protein; TTT, thymol turbidity test; TC, total cholesterol; ZTT, zinc sulfate turbidity test.

Table 3 Location of lichen planus in 17 patients with hepatitis C virus-related liver diseases

No	Sex	Age (years)	Liver disease	Lichen planus location			Type
				Cutaneous	Oral	Other	
1	M	71	CH	Antebrachium	–	–	–
2	M	60	CH	Extremities	–	–	
3	F	70	LC	–	Gingiva	–	Erosive
4	M	72	LC	–	Lower lip	–	Reticular
5	F	64	LC	Leg	Buccal mucosa, upper lip, lower lip	–	Erosive
6	M	66	CH	Leg	Buccal mucosa, upper lip, lower lip	–	Erosive
7	M	59	CH	–	Buccal mucosa (reticular)	Pharynx (erosive)	Erosive + reticular
8	M	66	CH	Leg	Buccal mucosa, lower lip	–	Reticular
9	M	57	CH	–	Buccal mucosa	–	Reticular
10	M	50	CH	–	Buccal mucosa, tongue, lower lip	–	Erosive
11	F	77	CH	–	Buccal mucosa	–	Atrophic
12	F	75	CH	–	Buccal mucosa	–	Reticular
13	M	62	HCC	–	Buccal mucosa, lower lip	–	Erosive
14	F	83	HCC	Leg	Buccal mucosa (atrophic)	Vulva (erosive)	Atrophic + erosive
15	M	41	CH	–	Buccal mucosa	–	Reticular
16	M	58	HCC	Extremities	Buccal mucosa, floor of mouth	–	Erosive
17	F	53	CH	–	Buccal mucosa	–	Reticular

CH, chronic hepatitis C; F, female; LC, HCV-related liver cirrhosis; HCC, HCV-related hepatocellular carcinoma; M, male.

pital. Although there was no significant difference in fasting glucose levels and BMI between patients with and without lichen planus, fasting insulin levels and HOMA-IR values, an indicator of insulin resistance, were significantly higher in patients who had lichen planus than in those who did not.

In the present study, insulin levels (17.4 ± 15.4 μU/L) and HOMA-IR values (4.6 ± 6.0) in patients having HCV infection without lichen planus (group B) were higher than the normal range. Normal values for insulin are 3.06–16.9 μU/L, and for HOMA-IR are less than 2. Therefore, the significantly higher insulinemia in patients such as those in group A (among HCV infectious patients) might cause lichen planus.

In Japan, it is known that the prevalence of HCV infection in patients with lichen planus is high;[11] therefore, interferon therapy is often administered to patients with lichen planus and a persistent HCV infection. However, it has been reported that patients cannot

complete interferon therapy because of aggravation of lichen planus.[24,25] The measurement of insulin resistance as well as a search for lichen planus may be useful before performing interferon therapy. A large series of patients with OLP was evaluated for extraoral involvement by Eisen *et al.*[26] They concluded that any patient with OLP should undergo a through history and examination as part of an investigation of potential extraoral manifestations, because a high percentage of patients with OLP develop extraoral manifestations. In our 17 cases of lichen planus, cutaneous lichen planus was diagnosed in seven (41.2%), hypopharynx in one (5.9%), and vulva in one (5.9%). The simultaneous appearance of extraoral and oral lesions was noted among six (35.3%). Because the majority of OLP patients suffer from lichen planus of the genitalia,[27] clinicians should follow OLP patients with sufficient attention to the presence of extraoral manifestations.

Sikuker *et al.* evaluated an association between HCV infection and extrahepatic malignancies. Extrahepatic malignancies were found in 14.6% of anti-HCV positive patients.[28] The incidence of extrahepatic malignant tumor in our subjects was 9.2% (8/87). The insulin-like growth factor family of proteins plays a key role in cellular metabolism, differentiation, proliferation, transformation and apoptosis, during normal development and malignant growth.[29] The hyperinsulinemia that HCV infection causes may induce an extrahepatic malignant tumor as well as HCC.

Many studies have shown that *H. pylori* is involved in the pathogenesis of gastric cancer.[30] The seroprevalence of *H. pylori* is 71% in Japanese aged 50–59 years, and is 81% in those aged 60–69 years.[31] This is almost the same as the seroprevalence of our patients, which was 66.7% (58/87) overall and 82.6% (19/23) in those aged 60–69 years. Seroprevalence of *H. pylori* in our three subjects with gastric cancer was 66.7%. In our study, we did not find an association between *H. pylori* and lichen planus in patients with HCV-infectious liver diseases.

In conclusion, we investigated the association of insulin resistance and lichen planus among patients with HCV-infected chronic liver diseases. The significant factors for development of lichen planus were smoking history, presence of hypertension, extrahepatic malignant tumor, and insulin resistance (HOMA-IR). This supports our previous conclusion that insulin resistance in patients who have an extrahepatic manifestation of HCV infection increases more than insulin resistance of patients who have neither an extrahepatic manifestation nor HCV infection. HCV-infected patients with lichen planus should pay attention to the development of an extrahepatic malignancy. Cooperation with an oral surgeon and a hepatologist is vital for early diagnosis and treatment of any extrahepatic manifestations.

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Genome-wide association of *IL28B* with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C

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The recommended treatment for patients with chronic hepatitis C, pegylated interferon-α (PEG-IFN-α) plus ribavirin (RBV), does not provide sustained virologic response (SVR) in all patients. We report a genome-wide association study (GWAS) to null virological response (NVR) in the treatment of patients with hepatitis C virus (HCV) genotype 1 within a Japanese population. We found two SNPs near the gene *IL28B* on chromosome 19 to be strongly associated with NVR (rs12980275, $P = 1.93 \times 10^{-13}$, and rs8099917, 3.11×10^{-15}). We replicated these associations in an independent cohort (combined *P* values, 2.84×10^{-27} (OR = 17.7; 95% CI = 10.0–31.3) and 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3), respectively). Compared to NVR, these SNPs were also associated with SVR (rs12980275, $P = 3.99 \times 10^{-24}$, and rs8099917, $P = 1.11 \times 10^{-27}$). In further fine mapping of the region, seven SNPs (rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917 and rs7248668) located in the *IL28B* region showed the most significant associations ($P = 5.52 \times 10^{-28}$–2.68×10^{-32}; OR = 22.3–27.1). Real-time quantitative PCR assays in peripheral blood mononuclear cells showed lower *IL28B* expression levels in individuals carrying the minor alleles ($P = 0.015$).

Hepatitis C is a global health problem that affects a significant proportion of the world's population. The World Health Organization estimated that in 1999, there were 170 million HCV carriers worldwide, with 3–4 million new cases appearing each year. HCV infection affects more than 4 million people in the United States, where it represents the leading cause of cirrhosis and hepatocellular carcinoma as well as the leading cause of liver transplantation[1].The American Gastroenterological Association estimated that drugs are the largest direct costs of hepatitis C[1].

The most effective current standard of care in patients with chronic hepatitis C, a combination of PEG-IFN-α with ribavirin, does not produce SVR in all patients treated. Large-scale studies on 48-week-long PEG-IFN-α/RBV treatment in the United States and Europe showed that 42–52% of patients with HCV genotype 1 achieved SVR[2–4], and similar results were found in Japan. However, older patients (greater than 50 years of age) had a significantly lower rate of SVR due to poor adherence resulting from adverse events and laboratory-detectable abnormalities such as neutropenia and thrombocytopenia[5,6]. Specifically, various well-described side effects (such as a flu-like syndrome, hematologic abnormalities and adverse neuropsychiatric events) often necessitate dose reduction, and 10–14% of patients require premature withdrawal from interferon-based therapy[7]. To avoid these side effects in patients who will not be helped by the treatment, as well as to reduce the substantial cost of PEG-IFN-α/RBV treatment, it would be useful to be able to predict an individual's response before or early in treatment. Several viral factors, such as genotype 1, high baseline viral load, viral

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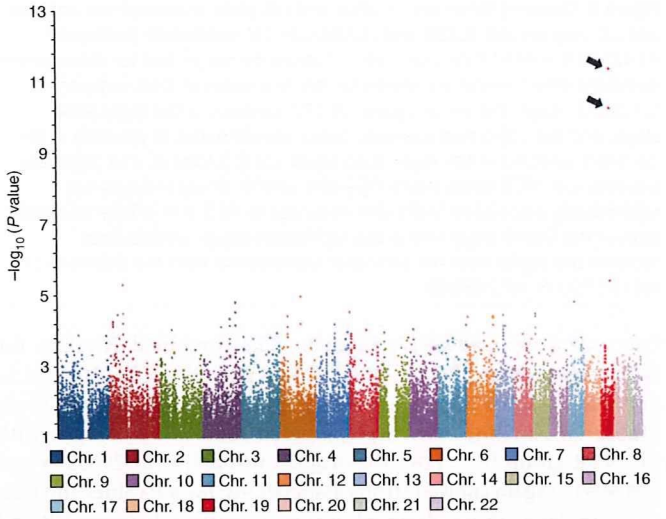

Figure 1 Genome-wide association results with PEG-IFN-α/RBV treatment in 142 Japanese patients with HCV (78 NVR and 64 VR samples). *P* values were calculated by using a χ^2 test for allele frequencies. The dots with arrows for chromosome 19 denote SNPs that showed significant genome-wide associations ($P < 8.05 \times 10^{-8}$) with response to PEG-IFN-α/RBV treatment.

kinetics during treatment, and amino acid pattern in the interferon sensitivity–determining region, have been reported to be significantly associated with the treatment outcome in a number of independent studies[8–10]. Studies have also provided strong evidence that ~20% of patients with HCV genotype 1 and 5% of patients with genotype 2 or 3 have a null response to PEG-IFN-α/RBV. No definite predictor of this resistance is currently available that make it possible to bypass the initial 12–24 weeks' treatment before deciding whether treatment should be continued. If a reliable predictor of non-response were identified for use in patients before treatment initiation, then an estimated 20%, including those who have little or no chance to achieve SVR, could be spared the side effects and cost of treatment.

Host factors, including age, sex, race, liver fibrosis and obesity, have also been reported to be associated with PEG-IFN-α/RBV therapy outcome[11,12]. However, little is known about the host genetic factors that might be associated with the response to therapy: thus far only a few candidate genes, including those encoding type I interferon receptor-1 (*IFNAR1*) and mitogen-activated protein kinase–activated protein kinase 3 (*MAPKAPK3*), have been reported to be associated with treatment response[13,14]. We describe here a GWAS for response to PEG-IFN-α/RBV treatment.

We conducted this GWAS to identify host genes associated with response to PEG-IFN-α/RBV treatment in 154 Japanese patients with HCV genotype 1 (82 with NVR and 72 with virologic response (VR), based on the selection criteria as described in Online Methods). We used the Affymetrix SNP 6.0 genome-wide SNP typing array for 900,000 SNPs. A total of 621,220 SNPs met the following criteria: (i) SNP call rate ≥95%, (ii) minor allele frequency (MAF) ≥1% and (iii) deviation from Hardy-Weinberg equilibrium (HWE) $P \geq 0.001$ in VR samples. After excluding 4 NVR and 8 VR samples that showed quality control (QC) call rates of <95%, 78 NVR and 64 VR samples were included in the association analysis. **Figure 1** shows a genome-wide view of the single-point association data based on allele frequencies. Two SNPs located close to *IL28B* on chromosome 19 showed strong associations, with a minor allele dominant model (rs12980275, $P = 1.93 \times 10^{-13}$, and rs8099917, $P = 3.11 \times 10^{-15}$, respectively), with NVR to PEG-IFN-α/RBV treatment (**Table 1**). The rs8099917 lies between *IL28B* and *IL28A*, ~8 kb downstream from *IL28B* and ~16 kb upstream from *IL28A*. These associations reached genome-wide levels of significance for both SNPs in this initial GWAS cohort (Bonferroni criterion $P < 8.05 \times 10^{-8}$ (0.05/621,220)). The frequencies of minor allele–positive patients were much higher in the NVR group than in the VR group for both SNPs (74.3% in NVR, 12.5% in VR for rs12980275; 75.6% in NVR, 9.4% in VR for rs8099917). Notably, individuals homozygous for the minor allele were observed only in the NVR group. The VR group, as compared to the NVR group, showed genotype frequencies closer to those in the healthy Japanese population[15], yet the minor allele frequencies were slightly higher in the transient virologic response (TVR) group (23.1%, 15.4%) than in the SVR group (9.8%, 7.8%) (**Table 1**). We applied the Cochrane-Armitage test on all the SNPs and found a genetic inflation factor, λ, of 1.029 for the GWAS stage (**Supplementary Fig. 1**). We also carried out principal component analysis in 142 samples for the GWAS stage together with the HapMap samples (CEU, YRI, CHB and JPT) (**Supplementary Fig. 2**); this suggested that the effect of population stratification was negligible.

Table 1 Significant association of two SNPs (rs12980275 and rs8099917) with response to PEG-IFN-α/RBV treatment

					Null responder (NVR[a], *n* = 128)			Responder (VR[a], *n* = 186)			Responder (SVR[a], *n* = 140)			NVR vs. VR		NVR vs. SVR	
dbSNP rsID	Nearest gene	MAF[b] (allele)	Allele (1/2)	Stage	11	12	22	11	12	22	11	12	22	OR (95% CI)[c]	*P* value[d]	OR (95% CI)[c]	*P* value[d]
rs12980275	*IL28B*	0.15 (G)	A/G	GWAS	20 (25.6)	54 (69.2)	4 (5.1)	56 (87.5)	8 (12.5)	0 (0.0)	46 (90.2)	5 (9.8)	0 (0.0)	20.3 (8.3–49.9)	1.93×10^{-13}	26.7 (9.3–76.5)	7.41×10^{-13}
				Replication	10 (20.0)	37 (74.0)	3 (6.0)	101 (82.8)	21 (17.2)	0 (0.0)	73 (82.0)	16 (18.0)	0 (0.0)	19.2 (8.3–44.4)	5.46×10^{-15}	18.3 (7.6–44.0)	8.37×10^{-13}
				Combined	30 (23.4)	91 (71.1)	7 (5.5)	157 (84.4)	29 (15.6)	0 (0.0)	119 (85.0)	21 (15.0)	0 (0.0)	17.7 (10.0–31.3)	2.84×10^{-27}	18.5 (10.0–34.4)	3.99×10^{-24}
rs8099917	*IL28B*	0.12 (G)	T/G	GWAS	19 (24.4)	56 (71.8)	3 (3.8)	58 (90.6)	6 (9.4)	0 (0.0)	47 (92.2)	4 (7.8)	0 (0.0)	30.0 (11.2–80.5)	3.11×10^{-15}	36.5 (11.6–114.6)	5.00×10^{-14}
				Replication	11 (22.0)	37 (74.0)	2 (4.0)	108 (88.5)	14 (11.5)	0 (0.0)	78 (87.6)	11 (12.4)	0 (0.0)	27.4 (11.5–65.3)	9.47×10^{-18}	25.1 (10.0–63.1)	1.00×10^{-14}
				Combined	30 (23.4)	93 (72.7)	5 (3.9)	166 (89.2)	20 (10.8)	0 (0.0)	125 (89.3)	15 (10.7)	0 (0.0)	27.1 (14.6–50.3)	2.68×10^{-32}	27.2 (13.9–53.4)	1.11×10^{-27}

[a]NVR, null virologic response; VR, virologic response; SVR, sustained virologic response. The 186 VRs consisted of 46 transient virologic response (TVRs) and 140 SVRs. [b]Minor allele frequency and minor allele in 184 healthy Japanese individuals[15]. The MAF of the SNPs in SVR is similar to that of TVR group, whereas that of NVR is much higher (76.6%). [c]Odds ratio for the minor allele in a dominant model. [d]*P* value by χ^2 test for the minor allele dominant model.

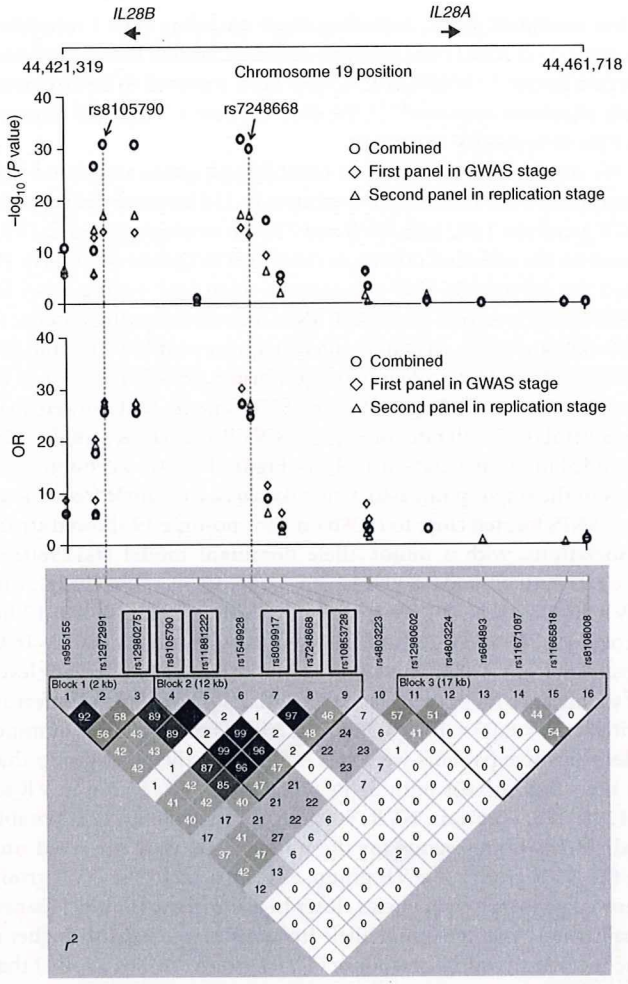

Figure 2 Genomic structure, *P* value and OR plots in association analysis and LD map around *IL28B* and *IL28A* (chr.19, nucleotide positions 44421319–44461718; build 35). *P* values by the χ^2 test for minor allele dominant effect model are shown for the first panel of 142 samples in the GWAS stage, the second panel of 172 samples in the replication stage, and the combined analysis. Below are estimates of pairwise r^2 for 16 SNPs selected in the replication study using a total of 314 Japanese patients with HCV treated with PEG-IFN-α/RBV. Boxes indicate the significantly associated SNPs with response to PEG-IFN-α/RBV treatment both in the GWAS stage and in the replication stage. Dotted lines indicate the region with the strongest associations from the positions of rs8105790 to rs7248668.

We analyzed the region of ~40 kb (chr. 19, nucleotide positions 44421319–44461718; build 35) containing the significantly associated SNPs (rs12980275 and rs8099917) using Haploview software for linkage disequilibrium (LD) and haplotype structure based on the HapMap data for individuals of Japanese ancestry. The LD blocks were analyzed using the four-gamete rule, and four blocks were observed (**Supplementary Fig. 3**). We selected 16 SNPs for both replication study and high-density association mapping, including tagging SNPs estimated on the basis of the haplotype blocks, one SNP located within *IL28B* (rs11881222) and the significantly associated SNPs from the GWAS stage (rs12980275 and rs8099917) (**Supplementary Table 1**).

To validate the results of the GWAS stage, 16 SNPs selected for the replication stage, including the original SNPs, were genotyped using the DigiTag2 assay in an independent set of 172 Japanese patients with HCV treated with PEG-IFN-α/RBV treatment (50 NVR and 122 VR samples), together with the first panel of 142 samples analyzed in the GWAS stage (**Supplementary Table 1**). The associations of the original SNPs were replicated in the replication cohort of 172 patients ($P = 5.46 \times 10^{-15}$, OR = 19.2 for rs12980275; $P = 9.47 \times 10^{-18}$, OR = 27.4 for rs8099917; **Table 1**). The combined *P* values for both stages reached 2.84×10^{-27} (OR = 17.7; 95% CI = 10.0–31.3) and 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3), respectively (**Table 1**). Notably, when we compared the SVR ($n = 140$) with the NVR group ($n = 128$), the original two SNPs (rs12980275 and rs8099917) again showed strong associations: both *P* values and ORs were similar to those observed in the comparison between VR and NVR, and the combined *P* values for both stages reached 3.99×10^{-24} (OR = 18.5; 95% CI = 10.0–34.4) and 1.11×10^{-27} (OR = 27.2; 95% CI = 13.9–53.4), respectively (**Table 1**). Comparing SVR ($n = 140$) versus NVR plus TVR ($n = 174$), we again found that these SNPs were significantly associated ($P = 1.71 \times 10^{-16}$, OR = 8.8; 95% CI 5.1–15.4 for rs12980275; $P = 1.18 \times 10^{-18}$, OR = 12.1; 95% CI 6.5–22.4 for rs8099917, **Supplementary Table 2**), suggesting that these SNPs would predict NVR as well as SVR before PEG-IFN-α/RBV therapy.

Among the newly analyzed SNPs in the replication study, six (rs12980275, rs8105790, rs11881222, rs8099917, rs7248668 and rs10853728) showed significant associations both in the GWAS stage ($P < 8.05 \times 10^{-8}$) and in the replication stage ($P < 0.0031$ (0.05/16)) after Bonferroni correction. These SNPs are located within a 15.7-kb region that includes *IL28B* (**Fig. 2** and **Supplementary Table 1**). In particular, the strongest associations with NVR were observed for four SNPs, rs8105790, rs11881222, rs8099917 and rs7248668, that are located in the downstream flanking region, the third intron and the upstream flanking region of *IL28B*. The combined *P* values for these polymorphisms were 1.98×10^{-31} (OR = 25.7; 95% CI = 13.9–47.6), 2.84×10^{-31} (OR = 25.6; 95% CI = 13.8–47.3), 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3) and 1.84×10^{-30} (OR = 24.7; 95% CI = 13.3–45.8), respectively (**Supplementary Table 1**). We then sequenced this region to identify further variants and found three SNPs (rs8103142, rs28416813 and rs4803219) located in the third exon, the first intron and the upstream flanking region of *IL28B*, and a few infrequent variations. These SNPs also showed strong associations in the combined dataset of 128 NVR and 186 VR samples ($P = 1.40 \times 10^{-29}$, OR = 26.6 for rs8103142; $P = 5.52 \times 10^{-28}$, OR = 22.3 for rs28416813; $P = 2.45 \times 10^{-29}$, OR = 23.3 for rs4803219; **Supplementary Table 3**). We also performed LD and haplotype analyses with seven SNPs. These SNPs were in strong LD, and the risk haplotype showed a level of association similar to those of individual SNPs ($P = 1.35 \times 10^{-25}$, OR = 11.1; 95% CI = 6.6–18.6) (**Table 2**). These results suggest that the association with NVR was primarily driven by one of these SNPs.

Table 2 Association analysis of response to treatment by *IL28B* haplotype

SNP							Frequencies			
rs8105790	rs11881222	rs8103142	rs28416813	rs4803219	rs8099917	rs7248668	NVR group	VR group	*P* value	OR (95% CI)
T	**A**	**T**	C	C	T	G	0.543	0.942	1.81×10^{-32}	0.1 (0.04–0.12)
C	**G**	**C**	G	T	G	A	0.387	0.054	1.35×10^{-25}	11.1 (6.6–18.6)

Association analysis of haplotypes consisting of seven SNPs with response to PEG-IFN-α/RBV treatment in 314 Japanese patients with HCV. Boldface letters: rs11881222 (third intron); rs8103142 (third exon).

Table 3 Factors associated with NVR by logistic regression model

Factors	Odds ratio	95% CI	*P* value
rs8099917 (G allele)	37.68	16.71–83.85	<0.0001
Age	1.02	0.98–1.07	0.292
Gender (Female)	3.32	1.49–7.39	0.003
Re-treatment[a]	1.12	0.55–2.33	0.750
Platelet count	0.93	0.87–1.01	0.080
Aminotransferase level	1.00	0.99–1.00	0.735
Fibrosis stage[20]	1.10	0.73–1.66	0.658
HCV-RNA level	1.01	0.99–1.02	0.139

[a]Re-treatment, non-response to previous treatment with interferon-α (plus RBV).

To examine the relative contribution of factors associated with NVR, we used a logistic regression model. One tagging SNP located within *IL28B* (minor allele of rs8099917) was the most significant factor for predicting NVR, followed by gender (**Table 3**). Clinically, viral factors such as HCV genotype and HCV RNA level are important for the outcome of PEG-IFN-α/RBV therapy. Indeed, mean HCV-RNA level was significantly lower in SVR (SVR versus TVR, $P = 0.002$; SVR versus NVR, $P = 0.016$; **Supplementary Table 4**). Mean platelet count and the proportion of mild fibrosis (F1–F2) were significantly higher in SVR than in NVR.

Real-time quantitative PCR assays in peripheral blood mononuclear cells revealed a significantly lower level of *IL28* mRNA expression in individuals with the minor alleles (**Fig. 3**), suggesting that variant(s) regulating *IL28* expression is associated with a response to PEG-IFN-α/RBV treatment. *IL28B* encodes a cytokine distantly related to type I (α and β) interferons and the interleukin (IL)-10 family. This gene and *IL28A* and *IL29* (encoding IL-28A and IL-29, respectively) are three closely related cytokine genes that encode proteins known as type III IFNs (IFN-λs) and that form a cytokine gene cluster at chromosomal region 19q13 (ref. 16). The three cytokines are induced by viral infection and have antiviral activity[16,17]. All three interact with a heterodimeric class II cytokine receptor that consists of IL-10 receptor beta (IL10Rβ) and IL-28 receptor alpha (IL28Rα, encoded by *IL28RA*)[16,17], and they may serve as an alternative to type I IFNs in providing immunity to viral infection.

Notably, a recent report showed that the strong antiviral activity evoked by treating mice with TLR3 or TLR9 agonists was significantly reduced in both *IL28RA*$^{-/-}$ and *IFNAR*$^{-/-}$ mice, indicating that IFN-λ is important in mediating antiviral protection by ligands for TLR3 and TRL9 (ref. 18). IFN-λ induced a steady increase in the expression of a subset of IFN-stimulated genes, whereas IFN-α induced the same genes with more rapid and transient kinetics[19]. Therefore, it is possible that IFN-λ induces a slower but more sustained response that is important for TLR-mediated antiviral protection. This might be one of the ways that a genetic variant regulating *IL28* expression influences the response to PEG-IFN-α/RBV treatment. Further research will be required to fully understand the specific mechanism by which a genotype might affect the response to treatment.

In conclusion, the strongest associations with NVR were observed for seven SNPs, rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917 and rs7248668, that are located in the downstream flanking region, the third intron, the third exon, the first intron and the upstream flanking region of *IL28B*. Further studies following our report of this robust genetic association to NVR may make it possible to develop a pre-treatment predictor of which individuals are likely to respond to PEG-IFN-α/RBV treatment. This would remove the need for the initial 12–24 weeks of treatment that is currently used as a basis for a clinical decision about whether treatment should be continued. That would allow better targeting of PEG-IFN-α/RBV treatment, avoiding the unpleasant side effects that commonly accompany the treatment where it is unlikely to be beneficial, and reduce overall treatment costs. Because of the small number of samples in this study, we plan to conduct a further prospective multicenter study to establish these SNPs as a clinically useful marker.

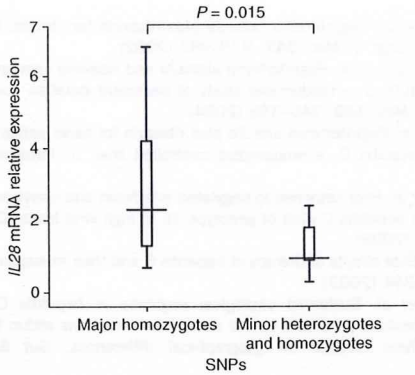

Figure 3 Quantification of *IL28* mRNA expression. The expression level of *IL28* genes was determined by real-time quantitative RT-PCR using RNA purified from peripheral blood mononuclear cells. Distribution of relative gene expression levels was compared between the individuals homozygous for major alleles ($n = 10$) and the heterozygous or homozygous individuals carrying minor alleles ($n = 10$) of rs8099917 by using the Mann-Whitney *U*-test. The bars indicate the median. All samples were obtained from HCV-infected patients before PEG-IFN-α/RBV therapy.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Study design and discussion: Y.T., N.N., N.M., K.T., M.M.; sample collection: Y.T., M.K., K.M., N.S., M.N., M.K., K.H., S.H., Y.I., E.M., E.T., S.M., Y.M., M.H., A.S., Y.H., S.N., I.S., M.I., K.I., K.Y., F.S., N.I.; genotyping: N.N.; statistical analysis: N.N., A.K., K.I.; quantitative RT-PCR: M.S.; manuscript writing: Y.T., N.N., K.T., M.M.

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