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ABSTRACT

Background & Aim: 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 PEG-IFN alpha-2b and ribavirin and 5 non-viral 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-HCV patients with a relatively constitutive Cardif. Hepatic RIG-I, MDA5, and LGP2 were significantly up-regulated in non-virological responders (NVR) compared with transient (TR) or sustained virological 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 ratio 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 ROC 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 PBMC to the therapy were rapid and strong except for Cardif in not only positive (RIG-I, ISG15 and USP18) but also in negative regulatory manner (RNF125).

Conclusion: 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 liver cirrhosis and hepatocellular carcinoma in many patients (1). Although combination therapy with pegylated interferon (PEG-IFN) alpha and ribavirin is now established as the standard treatment for chronic HCV infection genotype 1b, the sustained virological response rate in these patients is still around 50% (2–4). Moreover, physicians have also found that 20% of patients are non-virological 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 virological response even after prolonged combination therapy (6). However, mechanisms involving resistance to PEG-IFN alpha and ribavirin have not been fully elucidated, and it is difficult to predict treatment responses before initiation of PEG-IFN alpha 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 non-responders 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 differentiation associated gene 5 (MDA5) play essential roles in initiating the host antiviral response by detecting intracellular viral dsRNA (9–11). Caspase-recruiting

domain (CARD) adaptor inducing IFN-beta (Cardif), also called IFN-beta 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 beta gene activation (12–15). On the other hand, RIG-I sensing has been shown to be negatively regulated in a dominant-negative manner by LGP2 (10, 16), 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 (17). Further, these molecules are ISGylated by IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein (18), and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18), also called ubiquitin-specific protease 43 (UBP43) (19, 20). Moreover, the NS3/4A protease of HCV specifically cleaves Cardif as part of its immune evasion strategy (12, 21). Therefore, the RIG-I/Cardif system and its regulatory systems have essential key functions in the innate antiviral response (Supplemental Figure 1). 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 aim of this study was 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 alpha and ribavirin combination therapy.

MATERIALS 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 KIU/mL by Amplicor-HCV Monitor Assay; Roche Molecular Diagnostics Co., Tokyo, Japan) were included in the present study (Table 1). Patients with liver 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 non-viral liver disease (two had autoimmune hepatitis and three 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 alpha-2b (PegIntron®, Schering-Plough Corporation, Kenilworth, NJ, USA) at a dose of 1.5 $\mu g\ kg^{-1}\ week^{-1}$. 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 alpha-2b was

reduced to 0.75 μg kg^{-1} $week^{-1}$ when either the neutrophil count was <750/mm^3 or the platelet count was <80 × 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 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 PCR using primers specific for target genes. In brief, total RNA was extracted by the acid-guanidium–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. cDNA was transcribed from 2 μg of 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 (HMBS), which is stable in the liver, as quantified using real-time quantitative PCR as internal controls. For accurate normalization, a set of two 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'-ATGGTGGTGAAGACGCCAGT-3'.

HMBS: 5'-AAGCGGAGCCATGTCTGGTAAC-3',

5'-GTACCCACGCGAATCACTCTCA-3'.

Sequential measurement of gene expression in PBMC before and during therapy

To understand transcriptional response of the genes to PEG-IFN alpha-2b and ribavirin therapy, serial expression of RIG-I, RNF125, Cardif, ISG15, and USP18 were determined before and during treatment in peripheral blood mononuclear cell (PBMC) in 14 patients (7 were 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 alpha-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 nine patients (five were SVR and four were NVR) and three non-HCV control subjects as described previously (22). Liver biopsy specimen of ~10 mg was homogenized in 100 μL of Complete Lysis-M™ (Roche Applied Science, Penzberg, Germany). Twenty micrograms of the homogenates were separated by SDS-PAGE and blotted onto a PVDF 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, UK). The anti-VISA mouse monoclonal antibody (BioDesign, Saco, ME) and anti-beta-actin antibody (Sigma, 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 alpha-2b and ribavirin combination therapy, using real-time detection PCR, as reported previously (23). 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 six months after the completion of PEG-IFN alpha-2b and ribavirin combination therapy was defined as a sustained viral responder (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 a non-virological responder (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 chi-square test and Fisher's exact test. Distributions of continuous variables were analyzed by Mann–Whitney U-test for two groups. Kruskal–Wallis test was used for multiple group comparisons. All tests of significance were two-tailed, and p values < 0.05 were considered statistically significant.

RESULTS

Patient characteristics

According to the final virological response, patients were classified into three 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 virological 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 non-viral 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 non-viral liver disease patients. However, there was no significant difference in levels of Cardif expression between the chronic hepatitis C and non-viral 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 virological response. As shown in Figure 2, the hepatic expression level 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 second phases of HCV dynamics (first phase, $r = -0.4$, $p < 0.0005$; second phase, $r = -0.5$, $p < 0.0001$). Similar correlation was found between RIG-I/RNF125 ratio and viral decline rate (first phase, $r = -0.4$, $p = 0.004$; second phase, $r = -0.2$, $p = 0.09$, data were 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 two genes in individual patients, we found a strong and significant correlation between ISG15 and USP18 ($r^2 = 0.88$, $p < 0.0001$). Levels of ISG15 and USP18 expression before treatment were negatively correlated with the exponential viral decline rates calculated from the first and second phases of HCV dynamics (ISG15, first phase, $r = -0.5$, $p < 0.0001$; ISG15, second phase, $r = -0.3$, $p = 0.02$; USP18, first phase, $r = -0.5$, $p < 0.0001$; USP18, second phase, $r = -0.3$, $p = 0.01$).

Receiver operator characteristic (ROC) 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 (Az) 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, 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 4A). Similar to Cardif mRNA expression, mean Cardif expression in NVR patients was significantly lower than that in SVR (Figure 4B, $p = 0.01$). The cleavage product of Cardif, which has been reported by Loo et al. (24), was not detected in our analyses.

Transcriptional responses to PEG-IFN alpha-2b and ribavirin therapy in PBMC

Sequential analysis in response to PEG-IFN alpha-2b and ribavirin demonstrated a rapid and strong induction of RIG-I, ISG15, and USP18 mRNA expression, which peaked 8 hours after PEG-IFN-alpha-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-alpha-2b was triphasic, and consisted of rapid and strong suppression peaked at 8 hours after administration, and increasing 1.5- to 2-fold above baseline level during 24–48 hours after the administration and gradually decreasing to baseline level (Figure 5). The rapid suppression and subsequent increase following PEG-IFN alpha-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 virological outcome in patients with chronic hepatitis C who were treated with PEG-IFN alpha-2b and ribavirin combination therapy: up-regulation of RIG-I, MDA5, LGP2, ISG15, and USP18, and lower expression of Cardif and RNF125 could predict non-response to subsequent treatment with PEG-IFN alpha-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 alpha and ribavirin combination before initiating therapy.

Previously, large randomized controlled trials identified several pretreatment factors associated with the final virological outcome, such as genotype, HCV RNA level, degree of fibrosis, age, body weight, ethnicity, and steatosis (25). However, these findings lead us to believe that predicting the final virological response before initiating PEG-IFN alpha 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 (26), a 12-week regimen is associated with side 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 expression of these intrahepatic positive viral sensors was upregulated 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 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 lower expression of Cardif

relative to that of RIG-I was one of the strongest predictor 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 (12, 21), 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 two of four liver tissue samples of chronic HCV infection (24). 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 anti-viral 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 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.

It has been shown that RIG-I function is modified by ISG15 via ISGylation (18). Consistent with our data, Chen et al. identified 18 genes, including ISG15 and USP18, whose expression differed between responders and non-responders (27). Interestingly, a recent study has shown that USP18 negatively regulates IFN signaling independently of its isopeptidase activity towards ISG15 by binding to the IFNAR2 receptor subunit and blocking the interaction between Janus kinase (JAK) and the IFN receptor (28). Moreover, the siRNA knockdown of USP18 in human cells has consistently been shown to potentiate the ability of IFN to inhibit HCV RNA replication (29). 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 alpha-2b and ribavirin was also performed in this study. Lanford et al. demonstrated transcriptional response to IFN alpha in chimpanzee by genome microarray analysis, which included RIG-I, ISG15, and USP18 (30). An association of transcriptional response with early phase of virological response has been also reported in PBMC or liver biopsy specimen (31-33). We recently reported that the transcriptional double-stranded RNA-activated protein kinase (PKR) response during treatment with PEG-IFN alpha-2b and ribavirin was associated with the ultimate clinical response (31). 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 in 8–48 hours after PEG-IFN alpha-2b administration. Although limitations including 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 homogenous 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 ($p = 0.03$ and 0.04, respectively), and that RIG-I/Cardif and RIG-I/RNF125 ratios were significantly higher in NVR patients ($p = 0.02$ and 0.009, respectively, *see the* Supplementary Figure 2). 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 alpha 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.

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