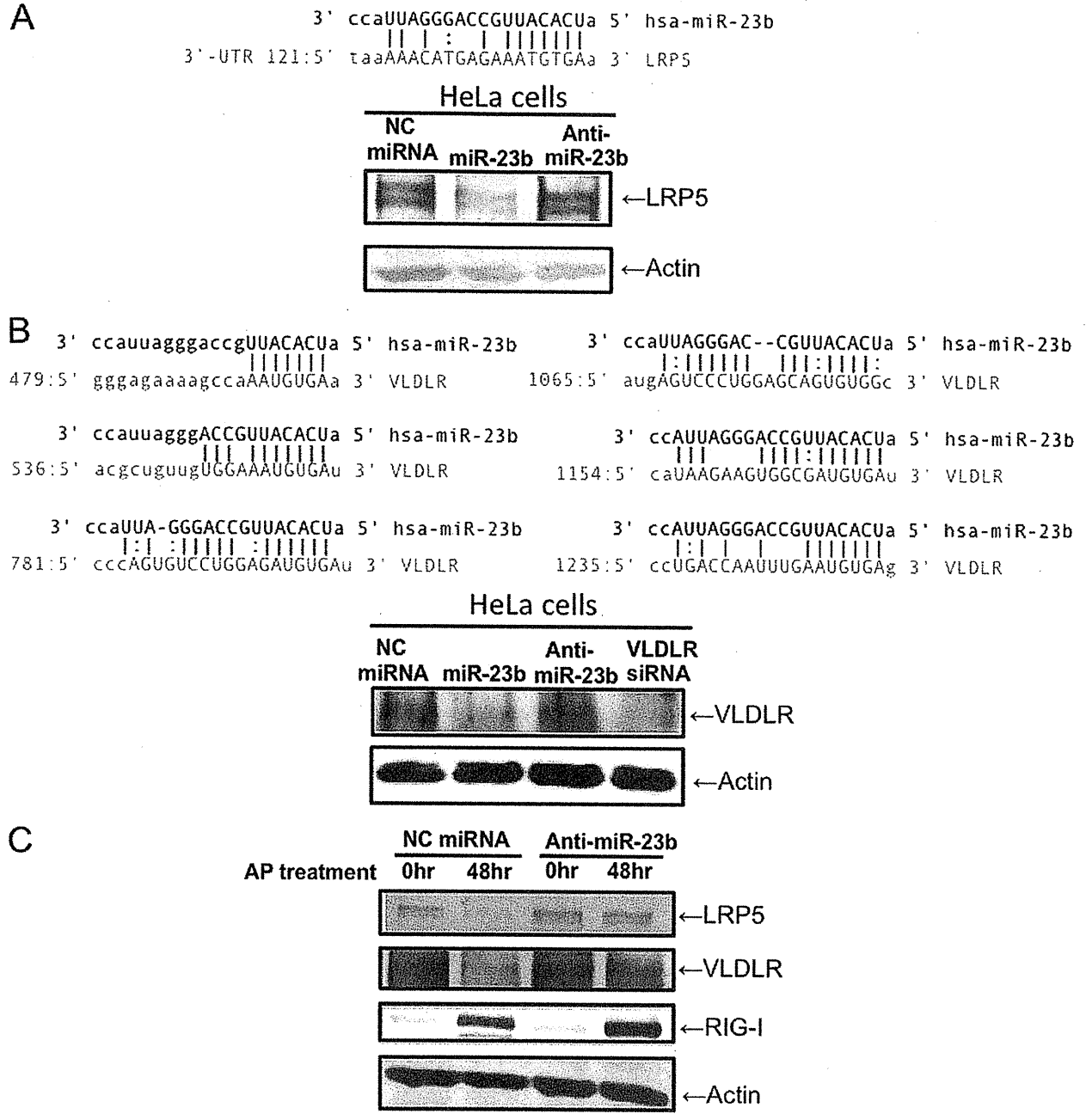

FIGURE 5. **miR-23b targets LRP5 and VLDLR.** *A* and *B*, the results of the search for target sequences of miR-23b (miRBase Target Data base). Candidate sequences in LRP5 (*A*) and VLDLR (*B*) mRNA are shown. HeLa cells were transfected with NC miRNA, miR-23b, or anti-miR-23b for 48 h and LRP5 (*A*) and VLDLR (*B*) were detected by Western blotting. *C*, HeLa FK/RIG cells were transfected with either NC miRNA or anti-miR-23b for 24 h and AP20187 was treated for 48 h. LRP5 and VLDLR were detected by Western blotting.

knockdown of VLDLR and LRP5. The protein level of VLDLR is slightly increased in cells transfected with LRP5 siRNA by an unknown mechanism (Fig. 6*A*). The higher RV1B replication in cells transfected with LRP5 siRNA (Fig. 6*C*) could be explained by the increased expression of its receptor, VLDLR. These results suggest that VLDLR but not LRP5 is critical for viral growth of RV1B.

miR-23b Did Not Influence Intracellular Replication of RV1B—The above results suggest that miR-23b blocks entry of RV1B by inhibiting expression of the receptor VLDLR. Because there are several reports that viral genomic RNA is directly targeted by host miRNA (37, 38), we investigated whether RV1B replication initiated by transfection of infectious viral genomic RNA is affected by miR-23b. HeLa cells were co-transfected with viral RNA and miRNA (Fig. 7), and the cells were harvested to examine the level of miR-23b and viral RNA at 3 and 9 h post-infection. High levels of miR-23b were detected in miR-23b-transfected but not NC miRNA-transfected cells, suggesting that the miR-23b was efficiently incorporated, and remained in the cells at 9 h after transfection (Fig. 7*A*). In the cells transfected with

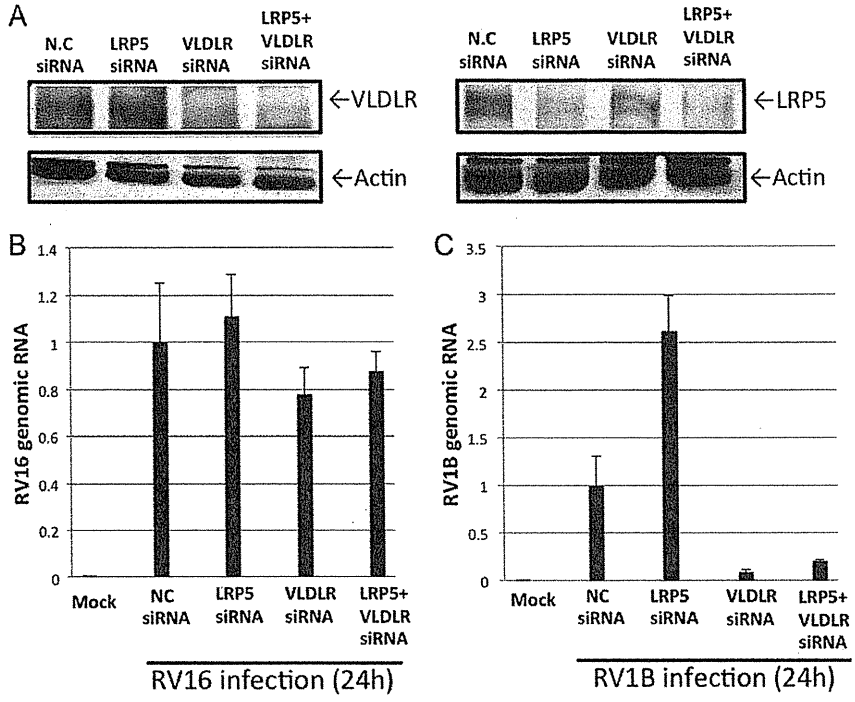

FIGURE 6. **Knockdown of VLDLR blocks accumulation of viral RNA in RV1B-infected cells.** HeLa cells were transfected with control siRNA or siRNA targeting VLDLR or LRP5 as indicated. *A–C*, at 48 h after the transfection, cells were mock infected or infected with RV16 or RV1B for an additional 24 h. VLDLR and LRP5 were detected by Western blotting in mock-infected cells (*A*). RNA levels of RV16 (*B*) or RV1B (*C*) were determined by real-time PCR.

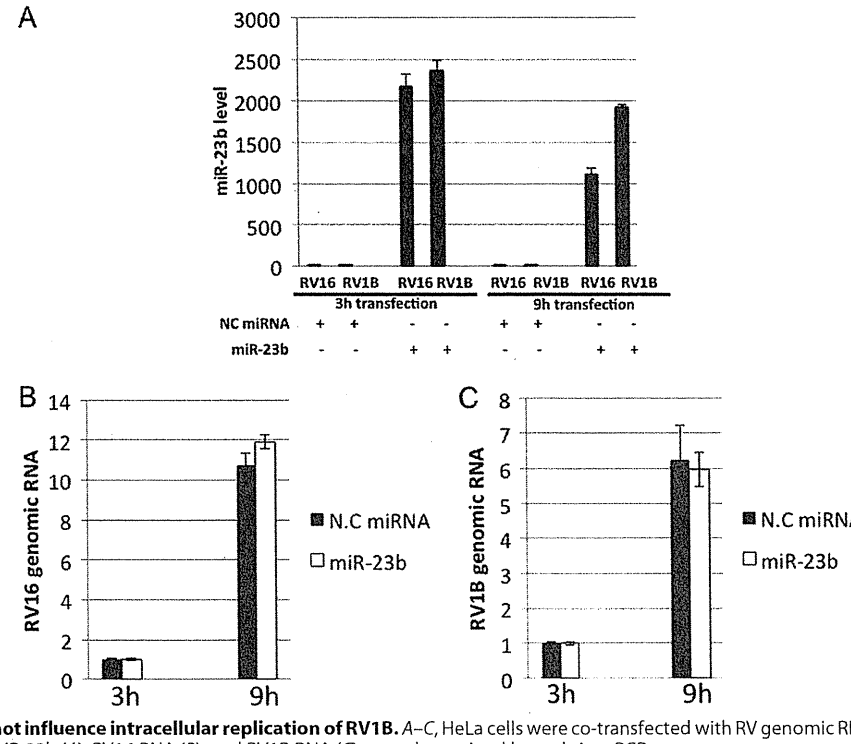

FIGURE 7. **miR-23b did not influence intracellular replication of RV1B.** *A–C*, HeLa cells were co-transfected with RV genomic RNA and NC miRNA or miR-23b for 3 and 9 h. Levels of miR-23b (*A*), RV16 RNA (*B*), and RV1B RNA (*C*) were determined by real-time PCR.

RV16 or RV1B genomic RNAs, although increasing amounts of viral RNAs were detected, no inhibitory effect of miR-23b was observed (Fig. 7, *B* and *C*). These results suggest that miR-23b inhibits infection of RV1B by down-regulating the expression of its major receptor, VLDLR, rather than impairment of the viral replication.

DISCUSSION

It has been well established that IFN treatment activates a Janus kinase-signal transducer activator of transcription (STAT) pathway resulting in activation of a variety of ISGs (39–41). Some ISGs encode proteins, collectively known as antiviral proteins, which directly inhibit viral replication. In this report, we demonstrated an alternative mechanism of inducing an antiviral state, that is, reducing the level of a protein essential for viral infection via activating a gene encoding miRNA. Thus innate immune responses restrict viral replication either by adding antiviral proteins or by removing proteins necessary for viral infection.

In addition to the regulation of host genes, miRNAs targeting viral RNA genomes have been reported. Pedersen and colleagues (38) reported that treatment of hepatic cells with IFN-β resulted in the production of at least eight miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431, and miR-448) that perfectly complement hepatitis C virus mRNAs. These findings suggest that the mammalian immune system utilizes miRNA to combat viral infections via multiple mechanisms.

We examined 900 miRNAs using a microarray and found that 37 and 28 miRNAs were up- and down-regulated, respectively (Table 1). The results show a marked regulation of miRNA expression upon viral infection (3–4%). In this report, we focused on miR-23b, because its possible target genes encode cell-surface proteins that are known to be viral receptors. Overexpression of miR-23b and anti-miR-23b resulted in repressed and enhanced production of RV1B, respectively. Although miR-23b targets both LRP5 and VLDLR, our analyses revealed the down-regulation of VLDLR to be responsible for the inhibition of RV1B.

Among the minor group rhinovirus there are 12 types of RV (RV1A, RV1B, RV2, RV44, RV47, RV49, RV23, RV25, RV29, RV30, RV31, and RV62). Considering that these minor group RVs commonly utilize VLDLR for their entry, miR-23b should exhibit an antiviral effect on these 12 types RVs. Importantly, the minor group RVs are shown to cause disease more often than the major group RV (42), suggesting that down-regulation of VLDLR by miR-23b is of significance for host defense to the minor group of RVs. Because the binding of viruses to the host cell is the initial step for viral entry, transient down-regulation of cell surface molecules could be an effective strategy to avoid viral transmission.

Artificial activation of RIG-I or infection by SeV and RV resulted in the accumulation of miR-23b with a peak at 6 and 9 h, respectively (Fig. 2). IFN-β treatment also induced the accumulation of miR-23b albeit with slower kinetics and a peak around 24 h. These results suggest that the expression of miR-23b is regulated by similar mechanisms to that of some ISGs, which are stimulated by both IRF-3/IRF-7 and IFN-stimulated gene factor 3 (ISGF3), the trimeric complex of STAT1, STAT2, and IRF-9. As reported previously, NF-κB may participate in the production of miR-23b inducted by viral infection or RIG-I stimulation (32). Because type I IFN is produced and secreted in the initial stages of viral infection, IFN induces the accumulation of miR-23b in uninfected cells, thereby protecting them from initial infection.

In summary, our study presents evidence that RIG-I-mediated signaling up-regulates the expression of 37 miRNAs, one of which, miR-23b, strongly inhibits minor group rhinoviruses through down-regulation of VLDLR, which functions as a receptor for entry into the cell. This finding provides a novel perspective for RIG-I-mediated antiviral effects.

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Dysregulation of IFN System Can Lead to Poor Response to Pegylated Interferon and Ribavirin Therapy in Chronic Hepatitis C

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Abstract

Background: Despite being expensive, the standard combination of pegylated interferon (Peg-IFN)- α and ribavirin used to treat chronic hepatitis C (CH) results in a moderate clearance rate and a plethora of side effects. This makes it necessary to predict patient outcome so as to improve the accuracy of treatment. Although the antiviral mechanism of genetically altered IL28B is unknown, IL28B polymorphism is considered a good predictor of IFN combination treatment outcome.

Methodology: Using microarray, we quantified the expression profile of 237 IFN related genes in 87 CH liver biopsy specimens to clarify the relationship between IFN pathway and viral elimination, and to predict patients' clinical outcome. In 72 out of 87 patients we also analyzed IL28B polymorphism (rs8099917).

Principal Findings: Five IFN related-genes (IFI27, IFI 44, ISG15, MX1, and OAS1) had expression levels significantly higher in nonresponders (NR) than in normal liver (NL) and sustained virological responders (SVR); this high expression was also frequently seen in cases with the minor (TG or GG) IL28B genotype. The expression pattern of 31 IFN related-genes also differed significantly between NR and NL. We predicted drug response in NR with 86.1% accuracy by diagonal linear discriminant analysis (DLDA).

Conclusion: IFN system dysregulation before treatment was associated with poor IFN therapy response. Determining IFN related-gene expression pattern based on patients' response to combination therapy, allowed us to predict drug response with high accuracy. This method can be applied to establishing novel antiviral therapies and strategies for patients using a more individual approach.

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Introduction

Hepatitis C virus (HCV) infection affects more than 3% of the world population. Without suitable treatment, chronic hepatitis C (CH) frequently leads to the development of chronic liver diseases such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC) [1]. The current standard treatment for CH is a combination of pegylated-IFN (Peg-IFN)- α and ribavirin (hereafter CH combination therapy). Over a 15-year observation period, the rate of hepatocarcinogenesis was found to be significantly lower in sustained viral responders (SVR) and relapse (R) patients than in non responders (NR) and interferon (IFN) untreated patients [2]. However, CH combination therapy achieves a sustained virological response in 50–55% of patients with HCV genotype 1b infection [3]. Consequently, this creates a pressing need to develop alternative strategies for treating CH.

IFN Type-I and III play various important immunomodulatory roles in both innate immune and acquired immune responses. Four main effector pathways of the IFN-mediated antiviral response have been recognized by gene targeting studies: the Mx GTPase pathway, the 2′, 5′-oligoadenylate-synthetase-directed ribonuclease L (OASL) pathway, the protein kinase R (PKR) pathway and the interferon stimulated gene (ISG) 15 ubiquitin-like pathway. These effector-pathways individually block viral

transcription, degrade viral RNA, inhibit translation and modify protein function to control all steps of viral replication [4–5].

IFN treatment for CH usually results in a high incidence of side effects; therefore, it is important to adjust IFN treatment accurately using a prediction method. Viral factors (HCV genotype, pretreatment viral load, and sequence of HCV gene core and NS5A), [6–7] host factors (obesity, cirrhosis, ethnic background, serum cytokine levels, liver fibrosis grades) [8], and treatment factors (adequate course of treatment, adherence to the treatment, management of side effects) [9] has been utilized in prior research to predict the outcome of combination therapy. Hepatic microRNA expression pattern before anti-viral treatment has also been utilized as a prediction biomarker of drug response in CH [10], while other studies have shown that there is a possible association between two SNPs near the gene interleukin 28B (IL28B) on chromosome 19 and lack of response to combination therapy [11–13].

In this study, we evaluated the IFN related gene expression profiles in CH patients before administering CH combination treatment. After the anti-viral therapy, patients were classified according to their clinical outcome: sustained viral response (SVR), relapse (R), and non responder (NR). It was observed that in the NR group, the expression level of some IFN related genes was significantly higher than that in normal liver (NL) groups, and that the expression level of the other IFN related genes was significantly lower than in NL. Moreover, the significantly high expression of IFN related genes was associated with low response to combination therapy. This suggests that dysregulation of the IFN system can be related to cases of CH combination therapy failure.

Results

In order to provide specific information with less data analysis, we developed a custom-made focused DNA microarray called Genopal (Mitsubishi Rayon, Tokyo, Japan) using genes that target human innate-immunity. Based on the results from the expression profiles, we carefully selected 237 gene probes (materials and methods) by activating RIG-I with Agilent DNA microarray. A microarray platform was used to establish IFN-related gene expression profiles in the specimens collected from the 87 CH and 5 NL samples (Table 1). The results of the analysis of these genes using the DNA chip strongly correlated with those obtained by real-time PCR (Pearson's correlation coefficient $R2 = 0.996$, $P<0.0001$; data not shown).

IFN related genes associated with the final response to combination therapy

We determined unique IFN gene expression patterns for liver specimens with or without HCV based on the final virological response to the combination therapy. The expression level of 66 genes significantly differed among NR, R, SVR, and normal liver (NL) groups (Figure 1). To clearly identify the IFN-related genes associated with the clinical outcome, we extracted genes that showed significant differences ($p<0.05$). It was observed that the expression level of 5 genes (myxovirus (influenza virus) resistance 1 (MX1), 2″,5″-oligoadenylate synthetase 1 (OAS1), ISG15 ubiquitin-like modifier (ISG15), interferon, alpha-inducible protein 27 (IFI27), and interferon, alpha-inducible protein 44 (IFI44)) were significantly higher in NR than in SVR samples (Table 2). The expression levels of 3 genes (MX1, IFI27, and ISG15) were significantly higher in NR than in R samples (Table 2). We also analyzed the IFN-related genes expression pattern according to the grade of inflammation or stage of fibrosis, however, no significant differences was observed between the two (data not shown).

Table 1. Clinical characteristics of patients.

Characteristics	SVR (n = 38)	R (n = 26)	NR (n = 23)	NL (n = 5)
Age	56.7±10.3	61.3±8.6	60.8±7.8	57.2±9.5
Male (%)	28 (61%)	11 (39%)	9 (36%)	3(60%)
Weight (kg)	59.5±8.9	57.2±10.3	55.7±7.2	ND
HCV RNA ($\times10^6$ copies/ml)	2.00±2.07	1.79±1.02	1.55±0.95	ND
Fibrosis stage				
F 0	1	1	1	
F 1	29	13	10	
F 2	9	7	5	
F 3	6	4	6	
F 4	0	0	1	
WBC($\times10^3$/mm^3)	5.42±1.63	5.23±1.25	4.69±1.13	ND
Hemoglobin (g/dl)	14.3±1.14	13.5±1.35	13.6±1.09	ND
Platelet ($\times10^4$/mm^3)	16.7±5.3	16.6±4.0	15.0±5.7	ND
AST (IU/L)	59.2±51.0	48.7±30.1	57.4±29.7	ND
ALT (IU/L)	80.8±93.7	49.3±29.6	69.1±44.4	ND
γGTP (IU/L)	60.3±74.2	41.2±29.7	76.2±60.2	ND
ALP (IU/L)	255±74.0	246±71.3	314±144	ND
Total bilirubin (mg/dl)	0.66±0.22	0.73±0.31	0.69±0.19	ND
Albumin (g/dl)	4.20±0.34	4.14±0.25	4.02±0.48	ND

Abbreviations; NR, non-virological responder; R, relapse; SVR, sustained virological responder; AST, aspartate aminotransferase;ALT, alanine aminotransferase; WBC, white blood cell; ALP, alkaline phosphatase; γGTP, gamma-glutamyl transpeptidase; ND, not detected.

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Comparison of IFN related genes between CH and NL

We also compared the gene expression pattern in NR and NL. After extracting genes with a fold change $<1/3$, $3<$ and p-value<0.05, we found that the expression level of 6 genes (growth arrest and DNA-damage-inducible, beta (GADD45B), hairy and enhancer of split 1 (HES1), B-cell CLL/lymphoma 3 (BCL3), signal transducer and activator of transcription 3 (STAT3), suppressor of cytokine signaling 3 (SOCS3), and DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (DDX11)) was significantly lower in NR than in NL. The expression level of SOCS3 and DDX11 in NR was significantly lower than in SVR. The expression level of 25 genes were significantly higher in NR than in NL. The expression levels of most of these genes were significantly higher in NR than in SVR, but the expression level of tumor necrosis factor (ligand) superfamily, member 10 (TRAIL), major histocompatibility complex, class I, C (HLA–C), major histocompatibility complex, class I, B (HLA–B), and chemokine (C-X-C motif) ligand 10 (CXCL10 (IP10)) were similar in NR and SVR samples (Table 3).

Validation of the microarray result by real-time qPCR

The five genes (ISG15, MX1, OAS1, IFI27 and IFI44) with the largest difference in fold change between NR and SVR groups were chosen to confirm the microarray results using real-time

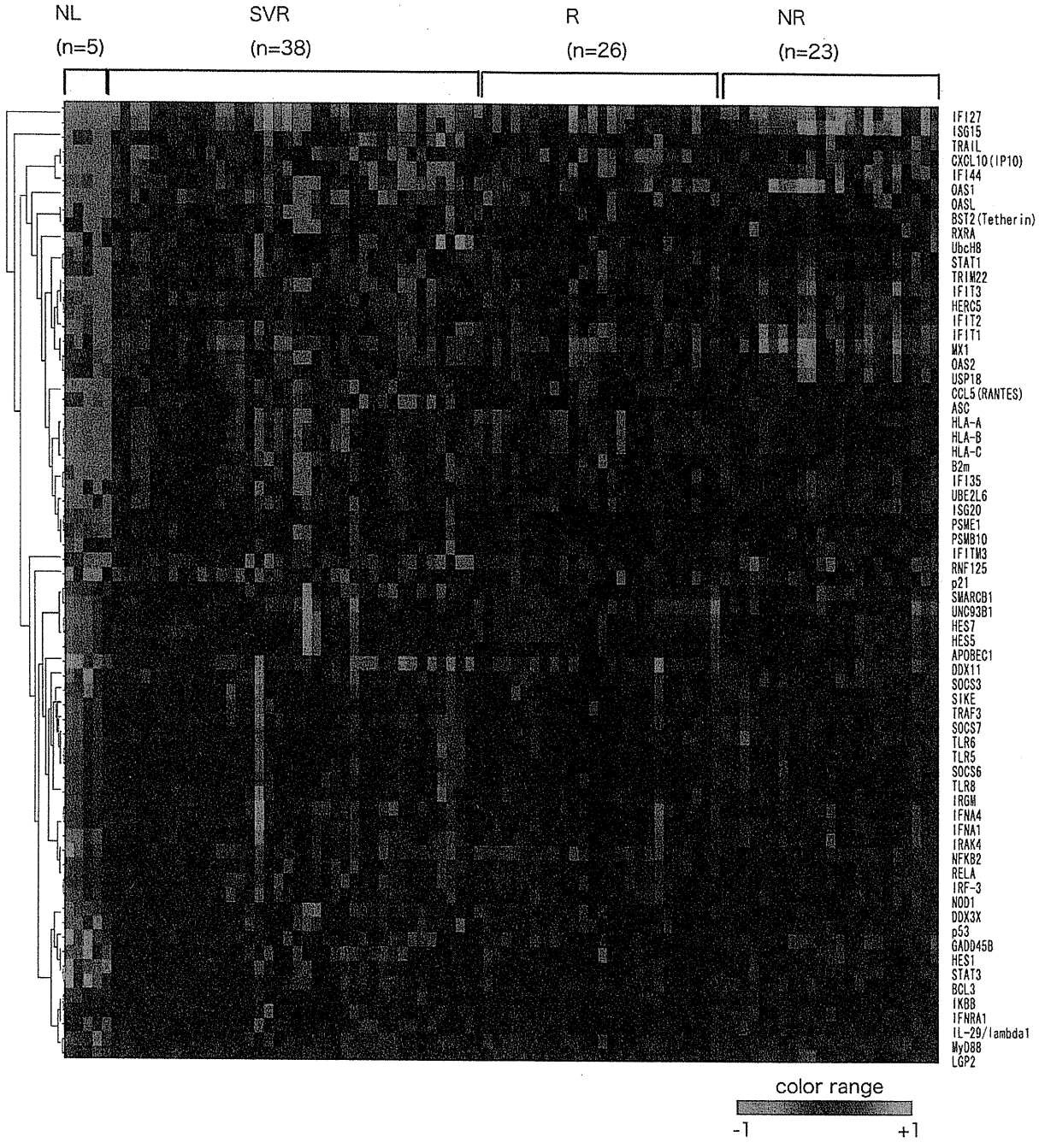

Figure 1. Clustering of IFN related gene expression. Clustering of CH patients according to the expression profiles of the 66 genes that showed significant differences among SVR, R, NR, and NL. Vertical bars represent the IFN related genes and the horizontal bars represent the samples. Green bars reflect down-regulated genes and red bars up-regulated genes.
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qPCR. The result from real-time qPCR supported the results from the microarray analysis (Figure S1).

Prediction of the clinical outcome by DLDA

We attempted to simulate the clinical outcome of the CH combination therapy using diagonal linear discriminant analysis (DLDA). Patients were randomly divided into TS (training set) and VS (validation set) (Table 4) in the order in which their samples were obtained. Samples within each group were then classified as NR or non-NR (SVR+R). DLDA showed that the accuracy, sensitivity, specificity, positive and negative predictive value of these two classifications were 86.1%, 87.5%, 81.8%, 93.3%, and

Table 2. Extracted genes related to the clinical outcome with a fold change greater than or equal to 1.5 between two groups (NR/SVR, NR/R) ($p<0.05$).

Accession No.	gene	symbol	fold change (NR/SVR)	p-value
NM_006417.4	interferon, alpha-inducible protein 44	IFI44	2.13	2.01E-03
NM_005532.3	interferon, alpha-inducible protein 27	IFI27*	2.37	2.01E-03
NM_016816.2	2",5"-oligoadenylate synthetase 1, 40/46kDa, transcript variant 1	OAS1	2.51	1.36E-02
NM_005101.2	ISG15 ubiquitin-like modifier	ISG15*	2.68	1.18E-03
NM_002462.2	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1*	2.71	1.57E-03
Accession No.	gene	symbol	fold change (NR/R)	p-value
NM_002462.2	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1*	2.27	1.11E-03
NM_005532.3	interferon, alpha-inducible protein 27	IFI27*	2.33	1.69E-03
NM_005101.2	ISG15 ubiquitin-like modifier	ISG15*	2.5	1.11E-03

Asterisk deposits extracted genes that are common to both SVR and NR and to NR and R.
doi:10.1371/journal.pone.0019799.t002

69.2% respectively (Table 5). Additionally, we attempted to predict (1) SVR and nonSVR (R+NR), and (2) SVR, R, and NR by DLDA. The accuracy with which patients were classified as SVR and nonSVR, was 56.8% and as SVR, R, and NR was 56.9%.

Genetic variation of IL28B is correlated with the expression of IFN related genes

To examine the relationship between the genetic variation of IL28B and IFN related gene expression, we determined the IL28B polymorphism in 72 patients (Table 6). Patients with the minor genotype of IL28B displayed higher levels of hepatic ISGs expression, whereas patients with the major genotype showed significantly lower expression levels (Figure 2A). In order to further widen our understanding of the above relationship, we significantly identified individual genetic variations in IL28B at the clinical outcome (Figure 2B). We then individually compared the expression level of several IFN-lambda related genes at the clinical outcome with the genetic variation of IL28B. The expression level of interleukin 28A (IL28A), IL28B, interleukin 29 (IL29), interleukin 10 receptor, beta (IL10RB), signal transducer and activator of transcription 1 (STAT1), STAT5A, and tyrosine kinase 2 (TYK2) in IL28B genotype minor allele and major allele did not differ; however, the expression level of STAT5A and IRF9 was significantly higher in IL28B minor allele cases than in major allele (Figure 3A). The expression levels of these nine genes did not significantly differ among the clinical outcomes (NR, R, and SVR) (Figure 3B).

Finally, in regards to genes which contribute to IFN production (interferon regulatory factor 7 (IRF7), interleukin-1 receptor-associated kinase 1 (IRAK1), myeloid differentiation primary response gene (MyD88), and toll-like receptor 7 (TLR7)) there was not much difference in their expression level prior to CH combination treatment and their expression level at the clinical outcome (Figure 4A) [14]. Unlike IRF7 and MyD88, there was no significant difference in the expression level of IRAK1 and TLR7 according to the IL28B genetic variation (Figure 4B). When we attempted to predict NR and nonNR by using ISG genes with and without IL28B polymorphism using DLDA by using 72 patients (36 patients for training set, 36 patients for validation set). DLDA with IFN related gene and IL28B polymorphism showed that the accuracy, sensitivity, specificity, positive and negative predictive value of these two classifications were 83.3%, 85.1%, 77.8%, 92.0%, 63.6%, respectively (Table 7). DLDA with IFN related gene only showed that the accuracy, sensitivity, specificity, positive and negative predictive value were 83.3%, 81.5%, 88.9%, 95.7%, 61.5%, respectively (Table 8).

Discussion

Our comprehensive analysis identified 66 genes with expression levels that consistently differed depending on the drug response of 87 CH patients and 5 normal liver specimens (Figure 1). Comparing the gene expression pattern in NR and NL showed the expression levels of 31 genes were significantly different (Table 3). In addition, most genes with expression levels in NR that were higher or lower than in NL, also differed between NR and SVR. Therefore, it is possible that innate immunity in the early period of HCV infection strongly influences IFN reaction.

HCV infection induces the impairment of cell subset number and the function of plasmacytoid dendritic cells (PDC) and natural killer cells [15]. The amount of PDC, which are the most potent producers of antiviral Type-I and III IFN [16], decreased in patients' peripheral blood [17], however, PDC was trapped in the HCV infected liver tissue. Therapeutic non-responders had increased PDC migration to inflammatory chemokines before therapy, compared with therapeutic responders [18]. This situation resulted in elevated expressions of IFN-related genes in the CH samples and was associated with their inability to eliminate the virus [19].

Inadequate expression of IFN related genes has been associated with several diseases. High expression of ISG can induce a refractory state in IFN therapy [20] and impaired IFN production leads to high risk of HCV-related hepatocarcinogenesis [21]. Lymphocyte IFN signaling was less responsive in patients with breast cancer, melanoma, and gastrointestinal cancer and these defects may represent a common cancer-associated mechanism of immune dysfunction. Alternately, since immunotherapeutic strategies require functional immune activation, such impaired IFN signaling may hinder therapeutic approaches designed to stimulate anti-tumor immunity [22]. In this way, the dysregulation of the IFN system can influence the progression of diseases and decrease curative effects.

Table 3. List of genes that had significantly different expression levels in NR and NL (fold change <1/3, 3<, and p<0.05).

symbol	NR/NL (fold change)	NR/NL (t-test)	NR/SVR (fold change)	NR/SVR (t-test)
GADD45B	0.20	1.14E-02	1.01	NS
HES1	0.26	1.26E-03	0.97	NS
BCL3	0.26	1.84E-02	1.02	NS
STAT3	0.26	5.81E-04	0.97	NS
SOCS3	0.27	7.96E-03	0.68	2.15E-02
DDX11	0.28	4.33E-05	0.59	9.52E-03
TRIM22	3.06	2.91E-03	1.37	7.97E-03
ASC	3.19	1.35E-03	1.33	4.07E-03
UBE2L6	3.32	1.06E-02	1.41	1.01E-03
STAT1	3.38	6.04E-04	1.33	1.86E-02
ISG20	3.64	2.42E-04	1.42	2.37E-03
TRAIL	3.81	2.08E-02	0.78	NS
OAS2	4.02	2.91E-03	1.89	1.07E-04
IFIT2	4.60	1.48E-03	1.56	8.34E-05
BST2(Tetherin)	5.14	8.17E-03	1.49	5.67E-04
IFI35	5.29	1.35E-03	1.63	2.37E-05
HERC5	5.32	1.16E-03	1.68	4.07E-05
MX1	6.21	1.33E-03	2.94	8.46E-07
HLA-C	6.49	6.34E-04	1.21	NS
CCL5(RANTES)	6.73	5.48E-04	1.25	3.77E-02
HLA-B	6.84	4.91E-04	1.22	NS
OAS1	7.80	5.52E-04	2.75	1.92E-04
HLA-A	8.49	5.92E-05	1.41	9.08E-04
B2m	9.09	7.78E-04	1.25	1.89E-02
IFIT1	9.42	1.86E-03	2.11	1.41E-05
OASL	10.38	3.97E-06	1.48	1.24E-02
IFIT3	10.45	4.33E-05	2.11	5.63E-06
CXCL10(IP10)	15.67	8.89E-07	1.28	NS
IFI44	17.00	9.40E-05	2.22	4.83E-06
ISG15	21.12	1.05E-04	2.85	3.99E-05
IFI27	43.74	1.80E-05	2.56	5.62E-05

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Genes which participate in IFN production (TLR7, MyD88, IRAK1, and IRF7) did not show any significant difference in their expression level prior to CH combination therapy, and their level at the clinical outcome (Figure 4A and 4B). However, the gene expression pattern of down-stream IFN pathway genes (IFI27, IFI44, ISG15, MX1, and OAS1) was significantly different among SVR, R, and NR (Table 2). IFN is usually up-regulated in HCV infected cells; however in some cases, the mechanism that controls IFN becomes abnormal, and the expression levels of IFN and ISG remain high without any curative effect [23]. The ISG family was generally up-regulated in NR compared to SVR [24–27] and this high expression of ISG related genes was associated with poor response to IFN therapy in previous, as well as in this present study. ISG15 has been linked to innate immune response to viruses and to cellular response to IFN. Although over-expression of ISG15 enhances the antiviral activity of IFN in vitro in acute infection [28], in chronic infection, extended pre-activation of IFN induced genes leads to dysregulation of the IFN system.

CH therapy is still imperfect at present and therefore suitable prediction methods are necessary to avoid adverse effects. Treatment failure using CH combination therapy is associated with up-regulation of a specific set of IFN-responsive genes thereby making it possible to predict non-response to exogenous therapy [29]. Early gene expression during anti-HCV therapy may elucidate important molecular pathways that might be influencing the probability of achieving a virological response [30]. Our study supports this fact by demonstrating that CH and NL differ fundamentally in their innate response to CH combination therapy.

IFN related gene expression suggests novel aspects of HCV pathogenesis, and form the basis for a subset of genes that can predict treatment response before initiation of combination therapy. After proper external validation, these gene sets may provide the basis for a diagnostic biomarker that can determine early on whether a patient treated with combination therapy is likely to be NR or not. In this respect, what sets our analysis apart is the effect of using DLDA to predict final response with high accuracy in NR and non-NR groups. This prediction showed that the expectation in NR (proportion of actual non-NR versus the predicted number of non-NR) was 93.3% and overall accuracy was 86.1%. In prior report, Dill et al. successfully predicted SVR, but were unable to predict R and NR with high accuracy [31]. In our experiments on the other hand, we predicted NR with high accuracy but were unable to do so for SVR and R. Possible causes for differences between our results and those received by Dill et al. may be (1) the differences in the races of subjects; European patients vs. Japanese patients in our study, (2) the composition of genotype; genotype 1 and 4 vs. genotype 1b in our study, and (3) the difference of the ISG genes extracted.

Genome-wide association studies have described alleic variants near the IL28B gene that are associated with treatment response and with spontaneous clearance of HCV [11–13]. In order to clarify the relationship between IL28B polymorphism and drug response, we compared the expression level of IFN-lambda related gene at the clinical outcome with any genetic variation in IL28B. The expression of hepatic ISG and related genes was strongly associated with treatment response and genetic variation of IL28B [32]. Classification of the patients into SVR and NR revealed that ISG expression was conditionally independent of the IL28B genotype. In CH patients in Europe, the expression pattern of genes induced by IFN more accurately predicts CH combination treatment clinical outcome than polymorphism of IL28B [31]. We observed that curative effect prediction using IFN gene expression pattern resulted in high level of accuracy, however, IFN with IL28B or IFN alone resulted in approximately similar levels of accuracy, therefore, the polymorphism of IL28B did not contribute significantly to our prediction. These findings are accordance with Dill et al. results (Table 7). There was an increased expression in NR compared to SVR irrespective of the IL28B genotype. However, there was no significant difference in their expression at the clinical outcome or in the genetic variation of IL28B (Figure 3A and 3B). Genetic variation of IL28B polymorphism is effective in predicting curative effect; however, the reason for this is not fully understood.

In conclusion, comprehensive analysis of IFN related gene showed that dysregulation of the IFN system might be related to treatment failure and that IFN related gene expression before treatment can enable accurate prediction of CH combination therapy clinical outcome. By focusing the full course of treatment on only those patients who have the highest likelihood of achieving

Table 4. Characteristics of the training and validation set.

	non NR (SVR+R) group	non NR (SVR+R) group		NR group	NR group	
	average (training set)	average (validation set)	p-value	average (training set)	average (validation set)	p-value
No.	32	32		12	11	
Age	59.3	57.1	0.38	60.6	61.7	0.74
HCVRNA ($\times 10^6$ IU/ml)	1.77	2.08	0.48	1.51	1.52	0.97
AST (IU/L)	44.6	65.3	0.06	55.3	56.9	0.89
ALT (IU/L)	50	87.3	0.05	67.7	66.8	0.96
WBC($\times 10^3$/mm^3)	5220	5440	0.57	4610	4860	0.6
Platelet ($\times 10^4$/mm^3)	15.8	17.6	0.15	15	15.2	0.95
Total bilirubin (mg/dl)	0.71	0.69	0.78	0.68	0.68	0.92
weight	58.1	59.2	0.67	57	53.8	0.28
ALP (IU/L)	251	249	0.92	298	326	0.64
gGTP (IU/L)	48	57.4	0.54	73.3	73.8	0.98
Hemoglobin (g/dl)	13.9	14.1	0.53	13.7	13.5	0.78
Albumin (g/dl)	4.15	4.21	0.41	4.11	3.98	0.52

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SVR, clinicians could potentially reduce the side effects and costs associated with these regimens and provide a more personalized approach to treating CH patients.

Materials and Methods

Patients and sample preparation

Eighty seven CH patients with HCV genotype 1b in the Department of Gastroenterology at the Ogaki Municipal Hospital were enrolled between 2004 and 2006 (Table 1). Patients with autoimmune hepatitis, alcohol-induced liver injury, and patients positive for hepatitis B virus associated antigen/antibody or anti-human immunodeficiency virus antibody were excluded. None of the patients had received IFN therapy or immunomodulatory therapy prior to enrollment. Five normal liver specimens were obtained by surgical resection. Three of these were obtained from Osaka City University Hospital and were taken from gall bladder cancer, cholangiocarcinoma, and hemangioma patients whose liver tissue were normal based on histological, virological and blood examination of their liver function. The remaining two normal liver samples were obtained from the Liver Transplantation Unit of Kyoto University Hospital.

Patients' serum HCV RNA was quantified before IFN treatment using Amplicor-HCV Monitor Assay (Roche Molecular Diagnostics Co., Tokyo, Japan). Histological grading and staging of liver biopsy specimens from the CH patients were performed according to the Metavir classification system. Pretreatment blood samples were analyzed to determine the level of aspartate aminotransferase, alanine aminotransferase (ALT), total bilirubin, alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γGTP), white blood cell (WBC), platelets, and hemoglobin. Written informed consent was obtained from all patients or their guardians and provided to the Ethics Committee of the Graduate School of Kyoto University, Osaka City University and Ogaki Municipal Hospital, who approved this study in accordance with the Helsinki Declaration.

Treatment protocol

For all enrolled patients, treatment with PegIFN- α-2b (Schering-Plough Corporation, Kenilworth, NJ, USA) and ribavirin (Schering-Plough) was initiated at the beginning of the 1st week and lasted for 48 weeks. PegIFN was administrated at a dose of 1.5 μg kg/week and ribavirin was administrated at the dose recommended by the manufacturer.

Definition of drug response to therapy

The patients were classified into the following three groups at the completion of follow-up period (24 weeks): (1) sustained virological responder (SVR): a patient who was negative for serum HCV RNA during the 24 weeks following the completion of the

Table 5. Quality of NR-prediction by DLDA.

		Predicted		
		NR	nonNR(SVR+R)	Total
Diagnosed	NR	9	2	11
	nonNR(SVR+R)	4	28	32
	Total	13	30	43

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Table 6. Result of the IL28B polymorphism (rs8099917).

		rs8099917		
		TT	TG	GG
outcome	NR	7	12	1
	Relapse	18	3	0
	SVR	30	1	0
	Total	55	16	1

doi:10.1371/journal.pone.0019799.t006

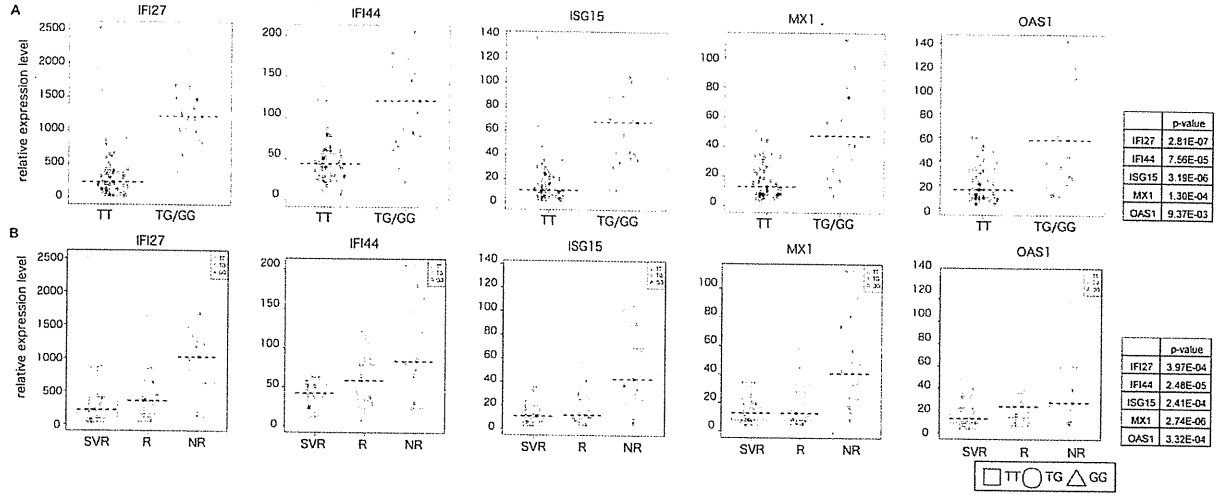

Figure 2. The relationship among the expression of IFN-related genes, IL28B polymorphism and clinical outcome. (A) The relationship between expression of ISG and five related genes (MX1, OAS1, ISG15, IFI27, and IFI44) in the liver of CH patients and IL28B with the major (TT) or minor (TG or GG) genotype (rs8099917) is shown. The p-value of the relationship between gene expression level and IL28B genotype is also depicted. (B) The relationship among the expression level of the above five genes, clinical outcome, and IL28 genotype in individual cases. Red square, green circle, and blue rectangle represent TT, TG, and GG in IL28B genotype, respectively. The p value was calculated from a linear regression employing outcome as an explanatory variable (in which SVR, R and NR are encoded to 0, 1 and 2 respectively) and expression level as the response variable. We tested the null hypothesis that the coefficient of the outcome is 0. Summary table of the p-value is also shown. NS shows no significant difference.
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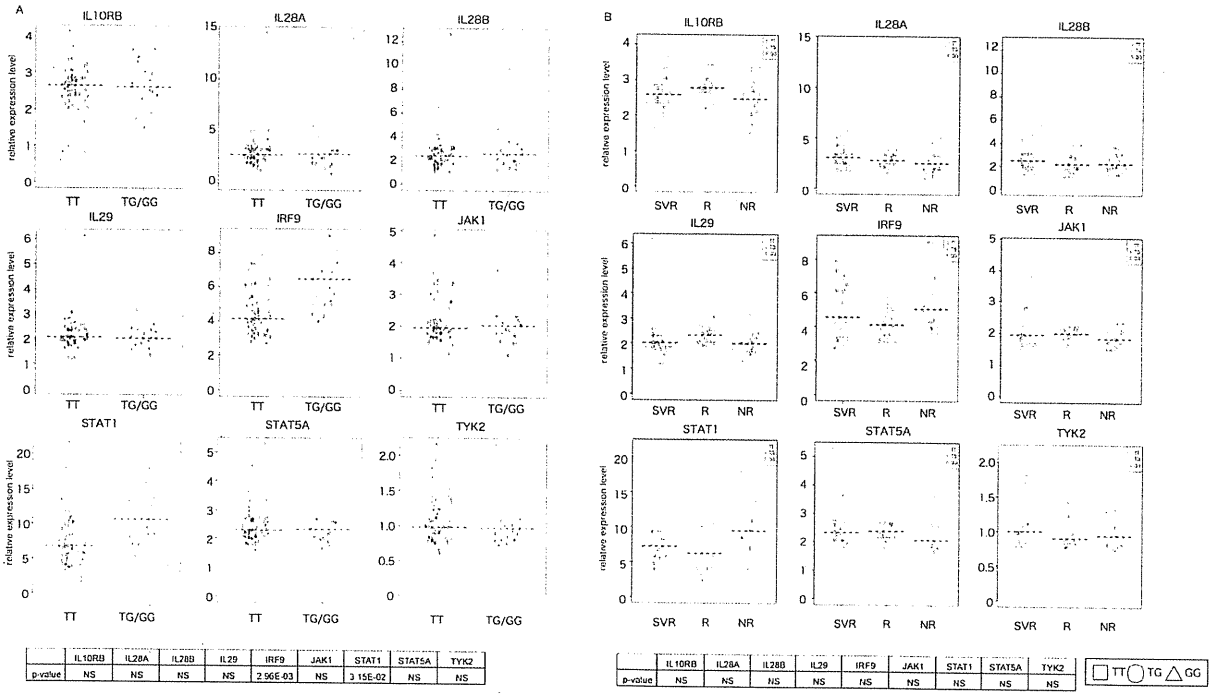

Figure 3. The relationship among the expression of IFN lambda-related genes, IL28B polymorphism and clinical outcome. (A) The relationship between the expression level of IFN lambda related genes (TYK2, STAT5A, STAT1, IL10RB, IL29, IL28A, IL28B, JAK1, and IRF9) in the liver of CH patients and IL28B with genotype. The p-value of the relationship between gene expression level and IL28B genotype is also presented. (B) The relationship among IFN lambda related genes, clinical outcome, and IL28 genotype in individual cases. Summary table of the p-value is also shown. NS was not significantly different.
doi:10.1371/journal.pone.0019799.g003

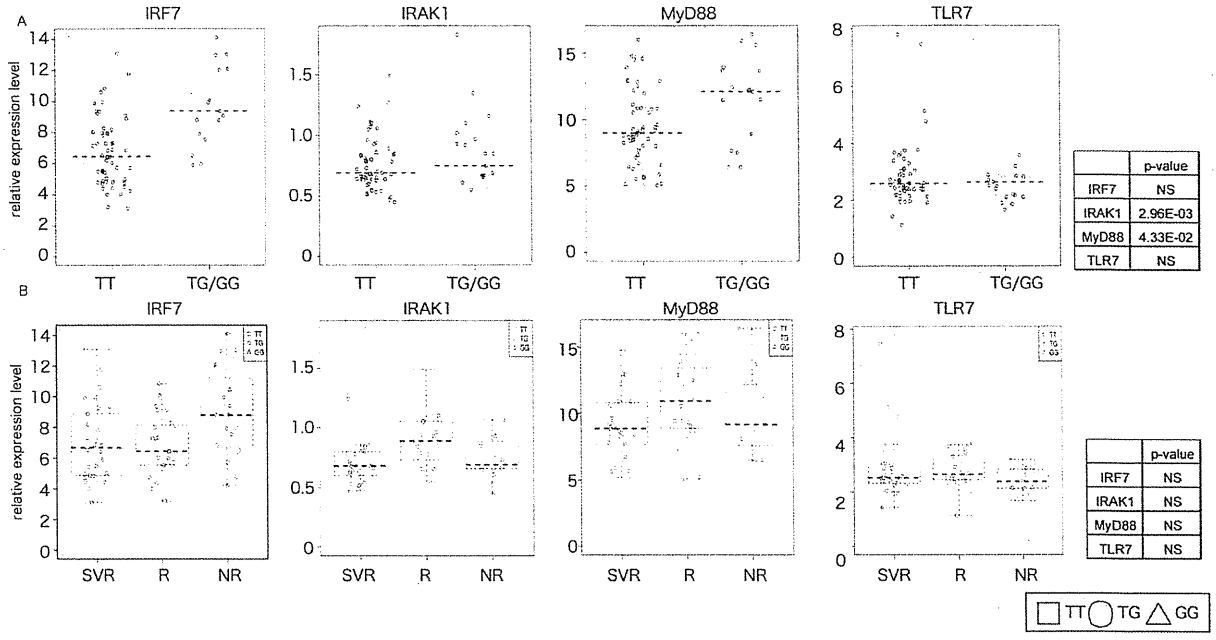

Figure 4. The relationship between the expression level of genes which participate in IFN production (TLR7, MyD88, IRAK1, and IRF7) in the liver of CH patients and IL28B genotype. (A) The relationship between IFN early response genes and clinical outcome is shown. A summary table of the p-value is also presented. NS shows no significant difference. (B) The relationship between IFN early response genes and IL28 genotype is shown. The p-value is also presented.
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combination therapy; (2) relapse (R): a patient whose serum HCV RNA was negative by the end of the combination therapy but reappeared during the 24 week observation period; and (3) non responder (NR): a patient who was positive for serum HCV RNA during the entire course of the combination therapy (Figure 5). No patients were withdrawn from the study due to side effects or any other reason.

RNA preparation and real-time qPCR

Total RNA from tissue samples was prepared using a mirVana miRNA extraction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. cDNA was synthesized by Transcriptor High Fidelity cDNA synthesis Kit (Roche, Basel, Switzerland). Total RNA (2 μg) in 11 μl of nuclease free water was added to 1 μl of 50 μM random hexamer and denatured for 10 min at 65°C. The denatured RNA mixture was added to 4 μl of 5x reverse transcriptase buffer, 2 μl of 10 mM dNTP, 0.5 μl of 40 U/ml RNase inhibitor, and 0.5 μl of reverse transcriptase (FastStart Universal SYBR Green Master (Roche) in a total volume of 20 μl. cDNA synthesis was performed for 30 min at 50°C, and enzyme denaturation for 5 min at 85°C. Chromo 4 detector (Bio-Rad, Hercules, CA, USA) was used to detect mRNA expression. Assays were performed in triplicate, and the expression levels of target genes were normalized to that of the ß-actin gene, as quantified using real-time qPCR as internal controls. Nucleotide sequences of primers were as follows: IFI27 (sense) 5′-ctaggccacggaattaaccc-3′, IFI27 (anti-sense) 5′-gactgcagagtagc-cacaag-3′, IFI44 (sense) 5′-gcatgtaacgcatcaggctt-3′, IFI44 (anti-sense) 5′-ccacaccagcgtttaccaac-3′, ISG15 (sense) 5′-ctttgccagta-caggagctt-3′, ISG15 (anti-sense) 5′-gcccttgttattcctcacca-3′, MX1 (sense) 5′-aatcagcctgctgacattgg-3′, MX1 (anti-sense) 5′-gtgatgagctcgctggtaag-3′, OAS1 (sense) 5′-gtgcgctcagcttcgtactg-3′, OAS1 (anti-sense) 5′-actaggcggatgaggctctt-3′, and β-actin (sense) 5′-ccactggcatcgtgatggac-3′, β-actin (anti-sense) 5′-tcattgccaatggtgatgacct-3′.

Table 7. Quality of NR-prediction by DLDA with IFN related gene and IL28B polymorphism A.IFN+IL28B.

		Predicted		
		NR	**nonNR**	**Total**
Diagnosed	NR	7	2	9
	nonNR	4	23	27
	Total	11	25	36

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Table 8. Quality of NR-prediction by DLDA with IFN related gene only.

		Predicted		
		NR	**nonNR**	**Total**
Diagnosed	NR	8	1	9
	nonNR	5	22	27
	Total	13	23	36

doi:10.1371/journal.pone.0019799.t008

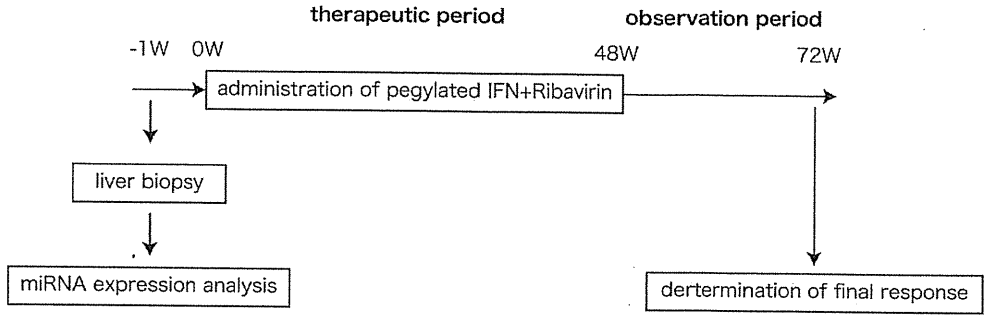

Figure 5. Study design and time line of response to combination therapy. The time frame of liver biopsy, microarray analysis, therapeutic period, observation period after combination therapy, and the judging of clinical outcome is shown.
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cDNA microarray

RNA was amplified and biotinylated using the MessageAmp-Biotin Enhanced Kit (Ambion). DNA oligonucleotide probes were synthesized onto a DNA microarray chip called Genopal (Mitsubishi Rayon) in order to detect the 237 genes (200 genes on Chip1 and 37 genes on Chip2) related to the innate immune response. Hybridization was carried out overnight at 65°C using Genopal in an hybridization buffer[0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20]. After hybridization, Genopal was washed with hybridization buffer twice at 65°C for 20 min followed by washing in 0.12 M Tris-HCl/0.12 M NaCl at 65°C for 10 min. Genopal was then labeled with streptavidin-Cy5 (GE Healthcare Bioscience, Tokyo, Japan). The fluorescent labeled-Genopal was washed for 5 min four times with hybridization buffer at RT and scanned at multiple exposure times ranging from 0 to 40s by DNA microarray reader (Yokogawa Electric Co, Tokyo, Japan). Intensity values with the best exposure condition for each spot were selected. The data presented here have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE20119: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=xlmbxyyumcwkeba&acc=GSE20119. All data are MIAME compliant, and are also registered with GEO.

Statistical analysis

To identify the genes that varied significantly among NR, R, SVR and NL groups, one-way ANOVA and Turkey's post hoc tests were used to assess each of the 237 IFN related-genes on the arrays. Benjamini-Hochberg correction for multiple hypotheses testing was applied to all tests. P values <0.05 were considered statistically significant.

Method of predicting prognosis

The patients were randomly divided into two groups: one was used as a TS and the other VS to calculate the prediction discriminant. A prognosis signature (PS) was defined in terms of the expression levels of the six genes that differed significantly between NR and non-NR groups using post hoc analysis (IFI27, IFI44, interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), ISG15, MX1, OAS1). A prognosis predictor (PP) was computed by applying a diagonal linear DLDA to the TS [33] and then using it to predict the prognoses of the VS. The predicted and actual prognoses of VS patients were compared to obtain the following five measures of prognosis prediction performance: accuracy (proportion of correctly predicted prognoses), sensitivity (proportion of correctly predicted non-NR), specificity (proportion of correctly predicted NR), PPV (proportion of actual non-NR versus predicted non-NR) and NPV (proportion of actual NR versus predicted NR).

Genetic Variation of IL28B Polymorphism

Genotypes rs8099917 was determined in 72 out of 87 patients by Taqman SNP assays (Applied Biosystems) using a pre-designed and functionally tested probe (ABI assay ID (C_11710096_10). The experiment was carried out according to the manufacturer's instruction.

Supporting Information

Figure S1 Real-time qPCR validation of the five IFN related genes. Each column represents the relative amount of mRNAs normalized to expression level of β-actin. The data shown are means+SD of three independent experiments. Asterisk was indicated to the significant difference at $p<0.05$.
(TIF)

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Author Contributions

Conceived and designed the experiments: KS YM. Performed the experiments: KO SM T. Kawaguchi YM. Analyzed the data: T. Kawaguchi MT MK. Contributed reagents/materials/analysis tools: HT T. Kumada. Wrote the paper: HT KU T. Kawaguchi FM TF YM.

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Retinoic Acid-Inducible Gene-I-Like Receptors

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Retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) form a family of DExD/H box RNA helicases. RIG-I-like receptors (RLRs) are expressed ubiquitously at low levels, and their expression is induced by treatment with type I interferon (IFN) or a viral infection. RLRs function as sensors for the detection of viral RNA (such as double-stranded RNA) in the cytoplasm to initiate antiviral responses by producing type I and type III IFNs. Unlike Toll-like receptors, which sense exogenous pathogen-associated molecular patterns, RLRs detect cytoplasmic viral RNA. Because RLRs are IFN-inducible viral sensors, they are critical in amplifying antiviral responses.

Retinoic Acid-Inducible Gene-I-Like Receptor as Interferon Stimulated Genes

Although retinoic acid-inducible gene-I (RIG)-like receptor (RLRs) are detectable in all tissues, treatment with type I interferon (IFN) induces their expression at the mRNA and protein level (Kang and others 2004; Yoneyama and others 2004, 2005). Viral infections induce RLR expression in type I IFN receptor-deficient cells, suggesting the existence of a direct mechanism. This is consistent with the observation that such cells efficiently produce IFN in response to a viral infection (Yoneyama and others 1998). Similar to protein kinase RNA-dependent (PKR) and oligoadenylate synthetase (OAS), whose enzymatic activities require dsRNA, RLR also requires agonist RNA for signaling to initiate IFN production (see below). However, unlike PKR and OAS, no direct role of RLR in inhibiting viral replication has been reported.

Structure of RLRs

The function of RIG-I was discovered by screening an expression cDNA library (Yoneyama and others 2004). The cDNA clone initially obtained encoded the N-terminal portion of RIG-I. This partial clone encompassed the signaling domain with 2 repeats of the Caspase activation and recruitment domain (CARD, Fig. 1). Forced expression of the N-terminal portion resulted in the activation of type I IFN genes without any viral infection. However, the full-length protein exhibits little activity, suggesting autorepression. The largest portion of RIG-I is the helicase domain containing helicase motifs conserved in other DExH/D helicases. The C-terminal region consists of a single structural domain termed the RNA recognition domain because this portion is responsible for binding dsRNA (Cui and others 2008; Takahasi and others 2008). The repression function was mapped to a region partially overlapping the RNA-binding domain (Saito and others 2007). MDA5 contains these 3 domains; however, its RNA recognition domain exhibits significantly low affinity for dsRNA as well as little repression (Saito and others 2007; Takahasi and others 2009). Laboratory of genetics and physiology 2 (LGP2) lacks the N-terminal tandem CARD but retains the RNA-binding and repressive functions (Yoneyama and others 2005; Komuro and Horvath 2006; Takahasi and others 2009).

RLR as the Cytoplasmic Sensor for Viral Infections

An antiviral function of RLR was identified by the generation of knockout mice. RIG-I-deficient mice and cells exhibited impaired IFN production in response to different viruses (Table 1) (Kato and others 2005, 2006). However, RIG-I deficiency had little influence on infections of *Picornaviridae* (Kato and others 2006). MDA5 appeared to be critical for sensing this family to produce IFN, suggesting that retention of these nonredundant has physiological significance (Gitlin and others 2006; Kato and others 2006). As expected, infections of some viruses are sensed by both RIG-I and MDA5 (Fredericksen and others 2008; Kato and others 2008; Loo and others 2008). Further, DNA viruses activate IFN genes through the activation of RIG-I or MDA5, presumably sensing RNA species produced during viral replication (Ablasser and others 2009; Chiu and others 2009; Choi and others 2009). *Legionella pneumophila*, a bacterium that replicates intracellularly, induces IFN production through the activation of RIG-I (Monroe and others 2009).

LGP2, which lacks a signaling domain, blocks the IFN production induced by viral infections when overexpressed (Rothenfusser and others 2005; Yoneyama and others

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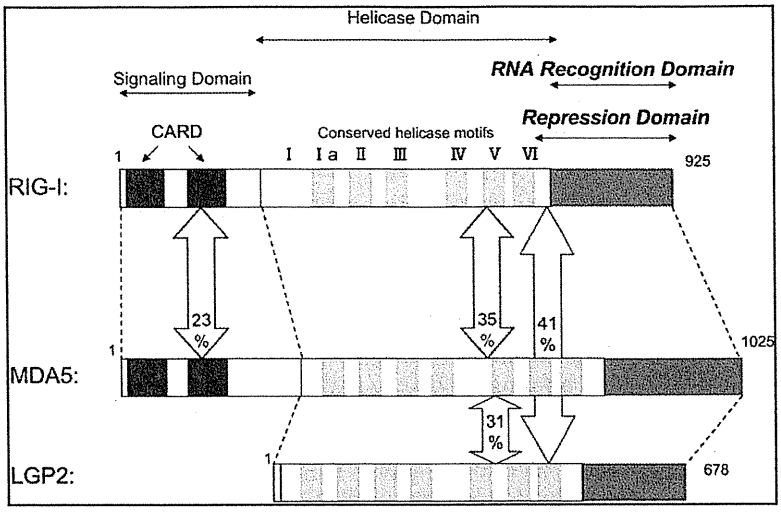

FIG. 1. Structure of RLRs. Schematic representation of RLR domains. The Caspase activation and recruitment domain (CARD), Helicase domain, and RNA recognition domain are indicated. Conserved helicase motifs (I to VI) are shown. The repression domain (hRIG-I: aa. 735–925; hLGP2: aa. 476–678) and RNA recognition domain (hRIG-I: aa. 799–925; hMDA5: aa. 896–1,025; hLGP2: aa. 546–678) are shown. Conservation of the amino acid sequence in the indicated region among human RLRs is indicated in percentage terms. RIG-I, retinoic acid inducible gene-I; MDA5, melanoma differentiation-associated 5; LGP, laboratory of genetics and physiology; RLR, RIG-I-like receptor.

2005). However, LGP2-knockout mice or cells exhibited diminished IFN production upon EMCV infection, suggesting functional cooperation between LGP2 and MDA5 (Venkataraman and others 2007; Satoh and others 2010). Upon infection by VSV, LGP2-deficient dendritic cells exhibited reduced IFN production (Satoh and others 2010); however, a different knockout line exhibited the opposite effect (Venkataraman and others 2007). This could be due to a different virus strain (Indiana strain wild-type VS M protein mutant) or cell type (fibroblast VS cDC). LGP2 deficiency did not

TABLE 1. VIRUS-SPECIFIC RECOGNITION BY RETINOIC ACID-INDUCIBLE GENE-I-LIKE RECEPTORS

Family	*Genome*	*Type species*	*RLR*	*Reference*
Picornaviridae	(+)ssRNA	Encephalomyocarditis virus	MDA5	Kato and others (2006), Gitlin and others (2006)
		Theiler's virus		
		Mengo virus		
Caliciviridae		Murine norovirus-1		McCartney and others (2008)
Coronaviridae		Murine hepatitis virus		Roth-Cross and others (2008)
Flaviviridae		West Nile virus	MDA5/RIG-1	Fredericksen and others (2008)
		Dengue virus		Loo and others (2008)
		Japanese encephalitis virus		Kato and others (2006)
		Hepatitis C virus		Saito and others (2008)
Orthomyxoviridae	(−)ssRNA	Influenza A virus	RIG-I	Kato and others (2006)
Paramyxoviridae		Newcastle disease virus		Kato and others (2006)
		Sendai virus		Kato and others (2006)
Rhabdoviridae		Vesicular stomatitis virus		Kato and others (2006)
Reoviridae	dsRNA	Reovirus	MDA5/RIG-1	Kato and others (2008), Loo and others (2008)
Poxviridae	dsDNA	Vaccinia virus	MDA5	Pichlmair and others (2009)
		Myxoma virus	RIG-I	Wang and others (2008)
Herpesviridae		Epstein-Barr virus	RIG-I	Samanta and others (2006)

Summary of the specificity with which RLRs recognize a variety of viruses. Family and type of genomic nucleic acids are indicated.

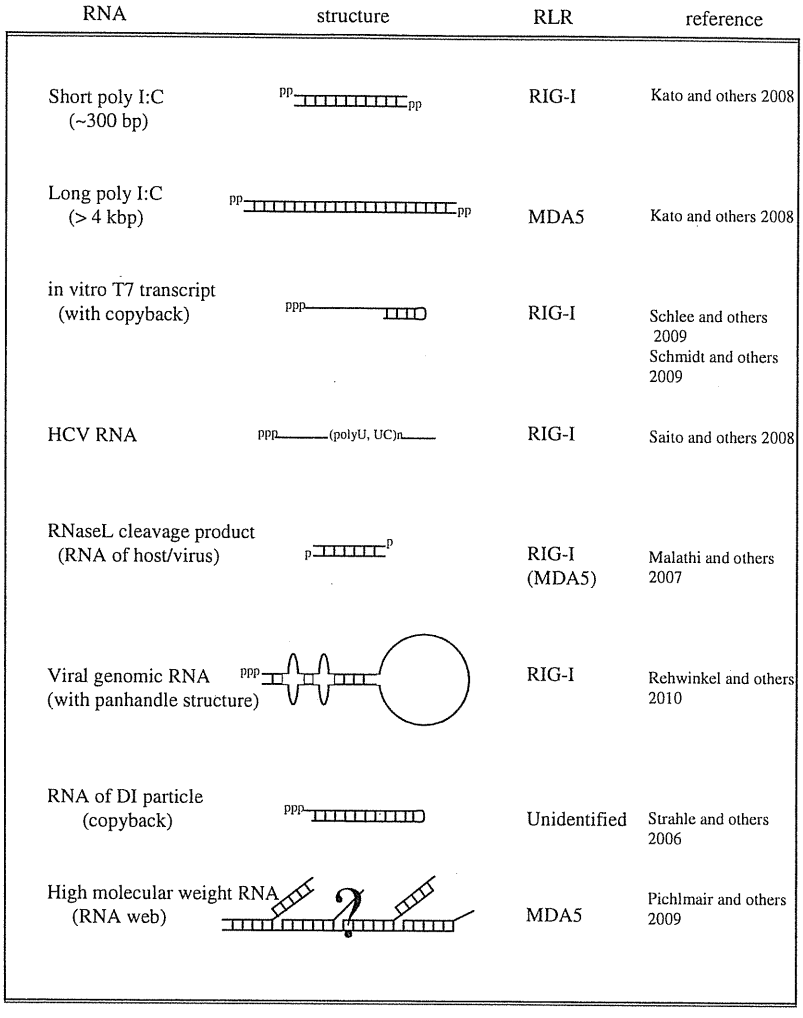

FIG. 2. Viral RNA patterns and RLR recognition. Synthetic and viral RNA patterns and the RLRs recognizing them are summarized. The schematic structure of each RNA pattern is indicated. The precise structure of RNA web is unknown.

influence the IFN production triggered by an influenza A virus infection (Satoh and others 2010), suggesting that LGP2 also functions in a virus-specific manner.

The viral specificity described above is determined by RNA species produced by different viruses and/or inhibitory molecules encoded by different viruses (below). Figure 2 summarizes the relationship between the structure of RNA and the sensors. Commercial poly I:C activates MDA5 but not RIG-I (Kato and others 2006). Double-stranded RNA produced by annealing complementary T7 transcripts preferentially activates RIG-I (Kato and others 2006). The length of dsRNA is one of the determinants for this specificity: short (~300 bp) and long (>4 kbp) poly I:C activate RIG-I and MDA5, respectively (Kato and others 2008). Although it was reported that 5′-ppp-containing single-stranded RNA is the ligand for RIG-I (Hornung and others 2006; Pichlmair and others 2006), later it was discovered that RNA produced by *in vitro* transcription contains a partial double-stranded structure due to copyback activity of the phage polymerase (Schlee and others 2009; Schmidt and others 2009). Importantly, chemically synthesized single-stranded RNA with 5′-ppp is inactive toward RIG-I. It is proposed that RIG-I recognizes most efficiently 5′-ppp-containing RNA with a partial double-stranded structure (Schlee and others 2009). Natural RNA of this category includes viral genomic panhandle RNA such as of influenza A virus (Rehwinkel and others 2010) and copyback RNA contained in defective interfering (DI) particles of Sendai virus (Strahle and others 2006). Mapping of HCV RNA revealed that a poly (rU) tract is responsible for activating RIG-I (Saito and others 2008). In this case, 5′-ppp is indispensable. It is implied that host RNaseL activated by the antiviral response participates in producing the RLR ligand (dsRNA with 3′ monophosphate) by digesting host or viral RNA (Malathi and others 2007). EMCV and Vaccinia virus produce high molecular RNA (RNA web) with a single- and double-stranded structure, and this RNA complex is responsible for the activation of MDA5 (Pichlmair and others 2009); however, the molecular mechanism by which MDA5 but not RIG-I recognizes this complex is unknown.

Viral Countermeasures for RLR-Mediated Signaling

Viruses have acquired inhibitory molecules to block antiviral responses and secure their survival. Often nonstructural,

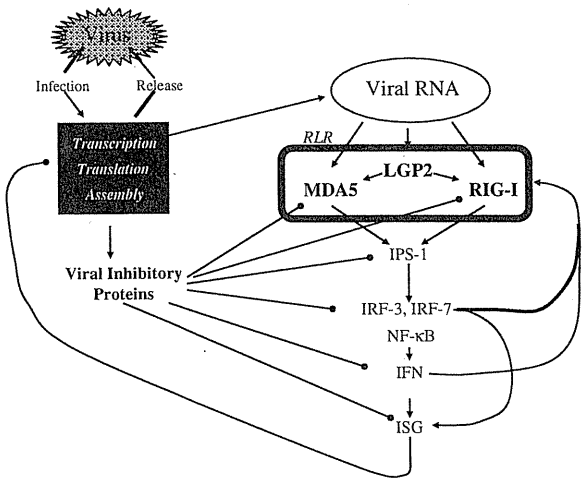

FIG. 3. RLR-mediated signaling and its regulation. Viral replication and viral RNA-induced innate immune signaling are summarized (see text). IPS-1, interferon promoter stimulator 1; ISG, interferon stimulated gene.

accessory proteins confer such activities. NS-1 of the influenza A virus inhibits production of IFN by blocking RIG-I's function (Guo and others 2007; Opitz and others 2007). The NS3/4A complex of the hepatitis C virus inactivates interferon promoter stimulator 1 (IPS-1), an immediate downstream signaling adaptor of RIG-I, through serine protease activity (Li and others 2005). The V protein of *Paramyxoviridae* selectively blocks MDA5 (Andrejeva and others 2004). In addition, replication-competent viruses have acquired means to counteract the host immune system, and the specificity of the inhibitors also contributes to the virus specificity of RLR.

Targets of RLR Signaling and Signal Amplification Mediated by RLR

As mentioned, RLR requires agonist RNA to trigger signals, which result in the activation of several classes of genes. The first category is the genes activated by viral infection or dsRNA transfection, but not by IFN treatment. Genes of this group include those of type I and type III IFN (Onoguchi and others 2007). The second category is the genes secondarily activated by secreted IFN. Genes of this category are activated with delayed kinetics and dependent on functional IFN receptors (Sadler and Williams 2008). The third category is the genes activated by both virus and RNA as well as by the secreted IFN (Mossman and others 2001).

Figure 3 summarizes viral replication and signal amplification mediated by RLRs. The infection initiates transcription of the viral genome within the cells. Eventually, viral structural and nonstructural proteins are translated and accumulate. The viral genome and structural proteins assemble to form new virions to expand the infection. Viral RNA with unusual structures such as those described in Fig. 2 are recognized by RLR, resulting in the signal to produce type I and type III IFN. The signal directly activates some of the interferon stimulated genes (ISGs) and the secreted IFN secondarily activates other ISGs to establish an antiviral state. Because RLRs are ISGs, the signaling is amplified. However, viruses encode inhibitory proteins to counteract the components of the IFN signaling system, and the initial balance between viral replication and host antiviral response determines the outcome of the infection.

Author Disclosure Statement

No competing financial interests exist.

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RIG-I-like receptors: cytoplasmic sensors for non-self RNA

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Summary: Viral infection results in the generation of non-self RNA species in the cells, which is recognized by retinoic acid inducible gene-I-like receptors (RLRs), and initiates innate antiviral responses, including the production of proinflammatory cytokines and type I interferon. In this review, we summarize reports on virus-specificity of RLRs, structures of non-self RNA patterns, structural biology of RLRs, and the signaling adapter molecules involved in antiviral innate immunity.

Keywords: *antiviral innate immunity, type I interferon, RIG-I-like receptors, non-self RNA*

RIG-I-like receptors (RLRs)

Pattern recognition receptors (PRRs) are essential in innate immunity. Major PRRs are Toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), and nucleotide oligomerization domain (Nod)-like receptors (NLRs) (1–3). TLR3, TLR7, TLR8, and TLR9 detect viral nucleic acids in endosome (4–8). These receptors essentially sense extracellular molecules incorporated into endosome by endocytosis. These sensors detect viral nucleic acids, released from virus-infected cells, and activate subsequent immune reactions. RLRs detect replicating viruses in cytoplasm, particularly at early phase of viral infection. Some of the NLRs sense viral infection to initiate inflammatory responses. Activation of innate immune responses lead to the induction of type I and III interferon (IFN) and inflammatory cytokines, whose antiviral activity blocks viral replication and facilitate the activation of antigen-presenting cells to activate antigen-specific immunity to eradicate the viral pathogens.

RLRs include RIG-I, MDA5, and LGP2, all of which contain a DExD/H box helicase domain (9, 10). The helicase domain retains catalytic activity to unwind double stranded RNA (dsRNA) in an adenosine triphosphate (ATP) hydrolysis-dependent