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.
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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 allelic 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 average (training set)	non NR (SVR+R) group average (validation set)	p-value	NR group average (training set)	NR group 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 (AST), 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	Predicted 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

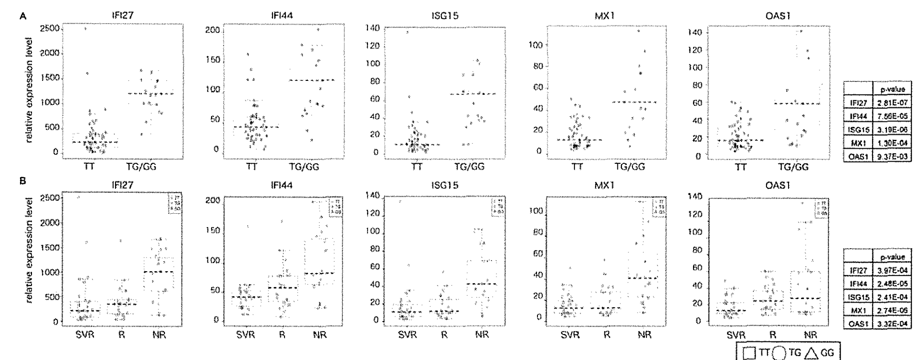

Figure 2. The relationship among the expression of IFN-related genes, IL28B polymorphism and clinical outcome. (A) The relationship between expression of ISG and five related genes (MX1, OAS1, ISG15, IFI27, and IFI44) in the liver of CH patients and IL28B with the major (TT) or minor (TG or GG) genotype (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.
doi:10.1371/journal.pone.0019799.g002

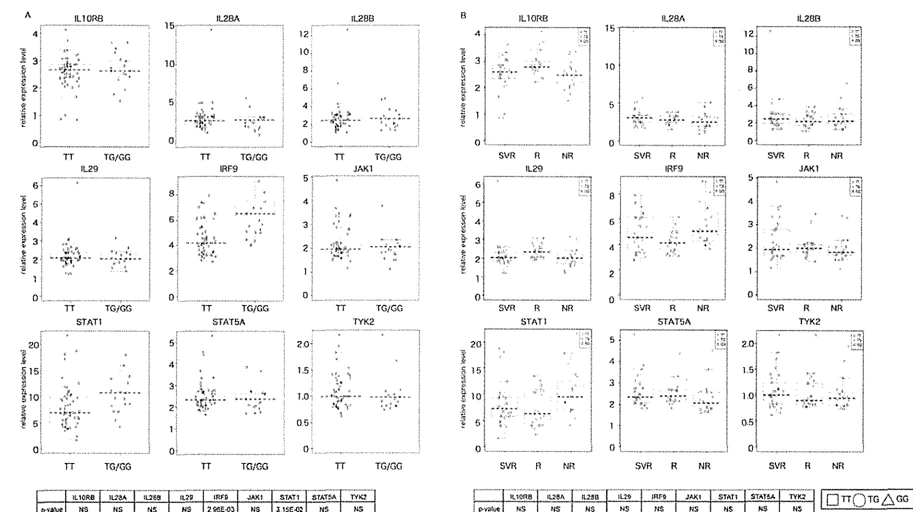

Figure 3. The relationship among the expression of IFN lambda-related genes, IL28B polymorphism and clinical outcome. (A) The relationship between the expression level of IFN lambda related genes (TYK2, STAT5A, STAT1, IL10RB, IL29, IL28A, IL28B, JAK1, and IRF9) in the liver of CH patients and IL28B with 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

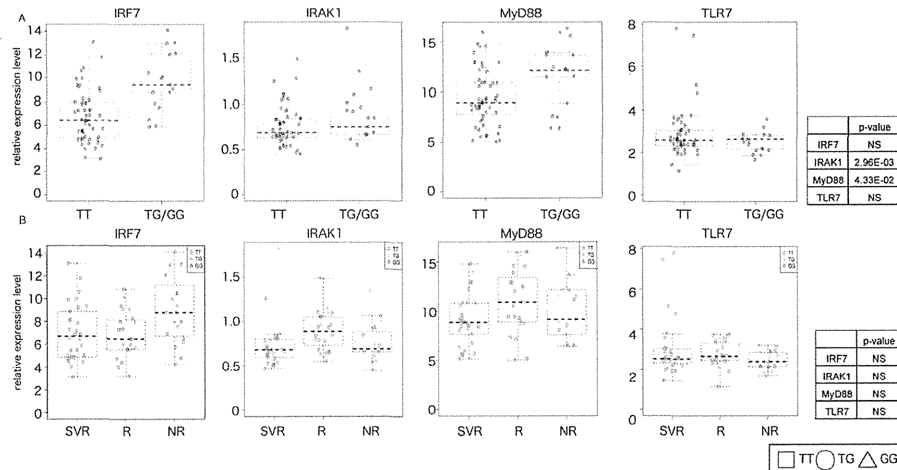

Figure 4. The relationship between the expression level of genes which participate in IFN production (TLR7, MyD88, IRAK1, and IRF7) in the liver of CH patients and IL28B genotype. (A) The relationship between IFN early response genes and clinical outcome is shown. A summary table of the p-value is also 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′-gactgcagagtagccacaag-3′, IFI44 (sense) 5′-gcatgtaacgcatcaggctt-3′, IFI44 (anti-sense) 5′-ccacaccagcgtttaccaac-3′, ISG15 (sense) 5′-ctttgccagtacaggagctt-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

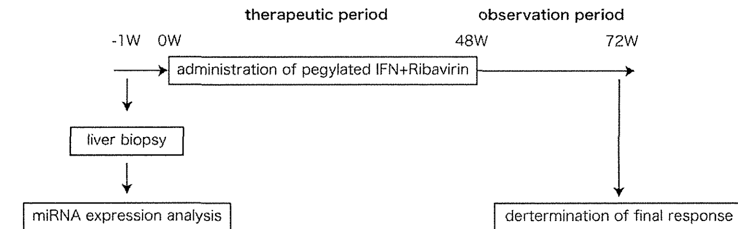

Figure 5. Study design and time line of response to combination therapy. The time frame of liver biopsy, microarray analysis, therapeutic period, observation period after combination therapy, and the judging of clinical outcome is shown.
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cDNA microarray

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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Antiviral Combination Therapy With Peginterferon and Ribavirin Does not Induce a Therapeutically Resistant Mutation in the HCV Core Region Regardless of Genetic Polymorphism Near the *IL28B* Gene

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An association has been reported between genetic polymorphism near *IL28B* gene and the prevalence of mutation of hepatitis C virus (HCV) core region residue 70, both of which have been associated with a lack of virologic response to antiviral combination therapy with peginterferon (PEG-IFN) and ribavirin. This study investigated whether PEG-IFN/ribavirin combination therapy induces amino acid (AA) mutation at residue 70 of HCV and whether genetic polymorphism near *IL28B* gene affects it. AA substitutions at residue 70 of the HCV core region were measured and compared before and after combination therapy in 65 non-responders and 88 relapsers to the combination therapy. In three patients in whom both wild-type AA (arginine) and mutant-type AA (glutamine or histidine) were detected at residue 70 before treatment, only mutant-type AA was identified after treatment. In two patients who had wild-type AA solely before treatment, both wild-type and mutant-type AAs were identified at residue 70 after treatment. In five patients, in whom the AA had changed at residue 70 between before and after treatment, four patients carried the TT genotype at a polymorphic locus (rs8099917) near the *IL28B* gene and one carried the TG/GG genotype. No difference was found in the prevalence of this change of AA at residue 70 between the TT and the TG/GG genotype. Antiviral combination therapy with PEG-IFN and ribavirin does not appear to induce mutation of HCV core region residue 70 regardless of genetic polymorphism near the *IL28B* gene in Japanese patients infected with HCV genotype 1b. ***J. Med. Virol. 83:1559–1564, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis C; peginterferon and ribavirin; amino acid substitution of HCV core region residue 70; genetic polymorphisms near the *IL28B* gene; mutation; non-sustained virologic responder

INTRODUCTION

Hepatitis C virus (HCV) causes chronic infection that can result in chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [Niederau et al., 1998; Kenny-Walsh, 1999]. The current standard therapy for patients with chronic HCV infection is the combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although the current treatment regimen has markedly increased the rate of patients with sustained virologic response, which indicates the eradication of HCV, only approximately 50% of patients infected with HCV genotype 1 achieve a sustained virologic response.

Many studies have investigated the potential baseline host- or virus-related factors that are associated

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with the lack of virologic response to IFN-based antiviral therapy. As a host-related factor, recent studies reported that genetic polymorphisms near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19 are strongly associated with a resistance to the combination therapy in patients infected with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. Patients having the TT genotype at a polymorphic locus (rs8099917) near the *IL28B* gene show a favorable response to the combination therapy with PEG-IFN and ribavirin, whereas patients having the GG genotype or those who are TG heterozygote show a resistance to the therapy. As for virus-related factors, amino acid (AA) mutations at residue 70 in the HCV core region have been reported to be associated strongly with a resistance to PEG-IFN/ribavirin combination therapy in patients infected with HCV genotype 1b [Akuta et al., 2005, 2007a; Donlin et al., 2007]. Patients with the mutant-type AA (glutamine or histidine) at residue 70 in the HCV core region show a resistance to the combination therapy in comparison to those with the wild-type AA (arginine) at this residue. These host- and virus-related factors are both associated with the outcome of the combination therapy with PEG-IFN and ribavirin independently in a previous report [Hayes et al., 2011].

A previous study reported that the percentage of patients with the mutant-type AA at residue 70 of the HCV core region increases with the progression of chronic hepatitis, suggesting that the mutation of AA at residue 70 (from arginine to glutamine or histidine) occurs in the natural course of chronic HCV infection [Kobayashi et al., 2010a]. Several recent studies have reported a higher prevalence of the mutant-type AA at residue 70 in patients who have the TG/GG genotype of genetic polymorphism of rs8099917 near the *IL28B* gene, which is associated with an unfavorable response to the combination therapy with PEG-IFN and ribavirin, than in patients who have the TT genotype [Abe et al., 2010; Kobayashi et al., 2010b]. These reports suggest that the mutation of AA residue 70 of the HCV core region may occur more frequently in patients with the TG/GG genotype. Especially, the induction of this mutation may occur easily in patients who underwent PEG-IFN/ribavirin combination therapy and failed to clear HCV (non-sustained virologic response), wherein HCV obtained a resistance to combination therapy.

Mutation at HCV core region residue 70 has reportedly been associated with a hepatocarcinogenesis and an insulin resistance [Akuta et al., 2007b, 2009; Nakamoto et al., 2010]. In addition, a recent study reported that patients who have both the TG/GG genotype of rs8099917 near the *IL28B* gene and the mutant-type AA at residue 70 of the HCV core region have shown further resistance even to the triple therapy with telaprevir, PEG-IFN, and ribavirin [Akuta et al., 2010]. It is, therefore, important to clarify whether PEG-IFN/ribavirin combination therapy induces the mutation of the HCV core region residue 70 in patients who failed to eradicate HCV, and whether genetic polymorphism near the *IL28B* gene are correlated with this mutation. If so, some patients should not undergo the current standard combination therapy in order to prevent the acquisition of the resistance (i.e., mutation at residue 70).

The present study investigated the effects of the combination therapy with PEG-IFN and ribavirin and genetic polymorphisms near the *IL28B* gene on the mutation of HCV core region residue 70 in patients who failed to achieve a sustained virologic reponse.

PATIENTS AND METHODS

Patients and Treatment

Three hundred and forty six patients with chronic hepatitis C who had been infected with HCV genotype 1b (as assessed by amplification of core-gene sequences with polymerase chain reaction (PCR) using genotype-specific primers [Ohno et al., 1997]) and pretreatment HCV-RNA level of $>100 \times 10^3$ IU/ml [as assessed by a quantitative PCR assay (Amplicor GT-HCV Monitor, Version 2.0; Roche Molecular Systems, Pleasanton, CA)] underwent antiviral combination therapy with PEG-IFN and ribavirin between January, 2007 and December, 2009 at the Ogaki Municipal Hospital or the Nagoya University Hospital. Of these patients, 19 patients dropped out and their outcome could not be defined. Among the remaining 327 patients, 274 patients who gave written informed consent for genetic analyses were enrolled to the study (Fig. 1). No patients were coinfected with hepatitis B virus or human immunodeficiency virus.

All patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough) daily. The initial doses of PEG-IFN and ribavirin and the dose reductions were according to the manufacturer's recommendations. All patients were scheduled to undergo 48 weeks of the treatment. Some patients had an extended treatment duration of up to 72 weeks. In some patients, the treatment was discontinued before 48 weeks because they had a low likelihood of achieving a sustained virologic response, when serum HCV-RNA was positive 24 weeks after starting the therapy. The outcomes of the combination therapy were classified as a sustained virologic response when serum HCV-RNA became undetectable during the treatment and remained undetectable for 6 months after the treatment ended (i.e., eradication of HCV), a relapse when the serum HCV-RNA became undetectable during the treatment period but returned detectable after the treatment, and no-response when the serum HCV-RNA remained detectable during and after the treatment period.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of the Ogaki Municipal Hospital and the Nagoya University School of Medicine. Written

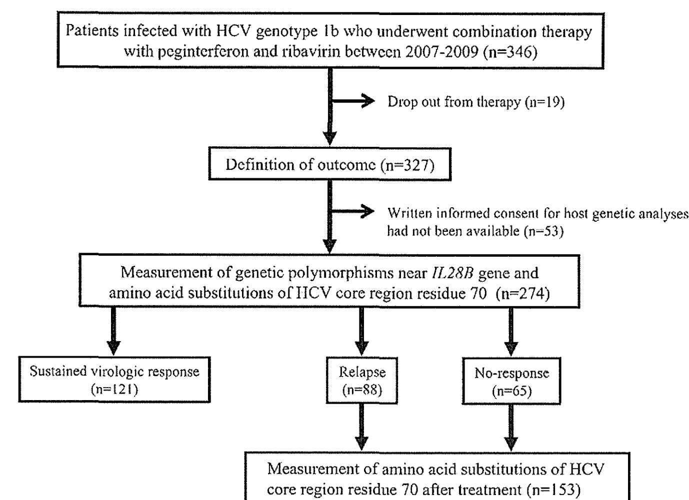

Fig. 1. Schematic representation of the study design.

informed consent was obtained from all patients prior to the study for the measurement of genetic polymorphism of rs8099917 near *IL28B* gene and AA substitution of HCV core region residue 70, and for the use of the laboratory data.

Measurements of Genetic Polymorphism Near the *IL28B* Gene and Amino Acid Substitution of the HCV Core Region Residue 70

Genotyping of polymorphisms of the rs8099917 locus near the *IL28B* gene was carried out in all 274 patients using a Taqman SNP assay (Applied Biosystems, Foster City, CA) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C_11710096_10, Applied Biosystems).

The AA at residue 70 of the core region of HCV was measured before the treatment in all patients. In patients who failed to achieve a sustained virologic response, that is, patients who showed a relapse or no-response, the AA identity was measured at residue 70 after the treatment and compared pre- to post-treatment AA identity at this residue (Fig. 1). The AA at residue 70 after the treatment was measured in serum samples obtained at the end of treatment in patients who showed no-response. In patients with a relapse, it was measured in serum samples obtained upon the reappearance of HCV-RNA after the completion of the therapy. The AA identity was analyzed by direct nucleotide sequencing according to a previous report [Akuta et al., 2007c]. The primer pairs used for PCR for direct sequencing the HCV core region were 5′-GCCATAGTGGTCTGCGGAAC-3′ (outer, sense primer), 5′-GGAGCAGTCCTTCGTGACATG-3′ (outer, antisense primer), 5′-GCTAGCCGAGTAGTGTT-3′ (inner, sense primer), and 5′-GGAGCAGTCCTTCGTGACATG-3′ (inner, antisense primer).

Statistical Analysis

The chi-square test was used to analyze the differences in percentages between groups.

RESULTS

Patient Characteristics and the Outcome of the Combination Therapy

The characteristics of study patients are shown in Table I. The study patients comprised 139 males (50.7%) and 135 females (49.3%), with a mean age of 58.0 ± 10.4 years. The grade of liver fibrosis according to the METAVIR score [The French METAVIR Cooperative Study Group, 1994] was F0 in 31 patients (11.6%), F1 in 122 patients (45.9%), F2 in 75 patients (28.2%), and F3 in 38 patients (14.3%). Analysis of the genetic polymorphism of the rs8099917 near the *IL28B* gene indicated 202 patients (73.7%) had the TT genotype, three patients (1.1%) had the GG genotype, and the remaining 69 patients (25.2%) were TG heterozygous. Before the treatment, 204 patients (74.4%)

TABLE I. Baseline Characteristics of the Study Patients (n = 274)

Age (years)	55.9 ± 11.2
Sex (female/male)	135 (49.3)/139 (50.7)
Body weight (kg)	58.0 ± 10.4
Alanine aminotransferase (IU/L)	64.5 ± 56.3
Aspartate aminotransferase (IU/L)	53.7 ± 42.2
Gamma-glutamyl transpeptidase (IU)	49.7 ± 48.5
Alkaline phosphatase (IU/L)	267.9 ± 100.6
Albumin (g/dl)	4.07 ± 0.38
Total bilirubin (mg/dl)	0.79 ± 0.30
White blood cell count (/μl)	4933 ± 1331
Hemoglobin (g/dl)	14.0 ± 1.4
Platelet count ($\times 10^3$/μl)	164 ± 50
Liver histology-activity (A0/A1/A2/A3)[a]	2 (0.7)/147 (55.3)/99 (37.2)/18 (6.8)
Liver histology-fibrosis (F0/F1/F2/F3)[a]	31 (11.6)/122 (45.9)/75 (28.2)/38 (14.3)
HCV-RNA concentration ($\log_{10}$ IU/ml)[b]	6.34 ± 0.54
Genetic polymorphisms near the *IL28B* gene (TT/TG/GG)[b]	202 (73.7)/69 (25.2)/6 (2.2)
Amino acid at HCV core 70 (wild type/mutant type/both)[c]	204 (74.4)/64 (23.4)/6 (2.2)
Response (SVR/relapse/NR)	121 (44.2)/88 (32.1)/65 (23.7)

HCV, hepatitis C virus; SVR, sustained virologic response; NR, no-response.
Percentages are shown in parentheses.
[a]Liver biopsy was not performed in eight patients.
[b]rs8099917 genetic polymorphism
[c]Before the treatment.

carried HCV with the wild-type AA at residue 70 of the HCV core region, 64 patients (23.4%) carried the mutant-type AA at residue 70, and both the wild-type AA and the mutant-type AA were identified at residue 70 in the remaining six patients (3.5%).

As a final outcome, 121 patients (44.2%) achieved a sustained virologic response, 88 patients (32.1%) relapsed, and the remaining 65 patients (23.7%) showed no-response (Fig. 1). Treatment was discontinued before 48 weeks in 11 of 65 patients who showed no-response because HCV-RNA remained detectable in serum 24 weeks after starting the therapy. The identity of the AA 70 of the core region of HCV was determined after the treatment in serum obtained at the discontinuation of the therapy in these 11 patients. Table II shows the association between the genetic polymorphisms of the rs8099917 near the *IL28B* gene, the AA substitutions of the HCV core region residue 70, and the outcome of the combination therapy. The wild-type AA was more frequently identified at residue 70 in patients with the TT genotype in comparison to those with the TG/GG genotype (82.2% vs. 52.8%, $P < 0.0001$). The rate of a sustained virologic response was significantly higher in patients with the TT genotype than those with the TG/GG genotype (107 of 202 patients, 53.0% vs. 14 of 72 patients, 19.4%, $P < 0.0001$), as well as being higher in patients carrying HCV with the wild-type AA at residue 70 of the core region than those with the mutant-type AA at this residue (101 of 204 patients, 49.5% vs. 19 of 64 patients, 29.7%, $P = 0.0083$, one patient had both the wild-type and the mutant-type AAs).

Comparison of the Amino Acid at Residue 70 of the HCV Core Region Before and After the Combination Therapy in Patients Who Showed a Relapse or No-Response

Table III shows the comparison of the AA at residue 70 of the HCV core region before and after the combination therapy in patients who showed a relapse or no-response, according to the genetic polymorphisms of the rs8099917 near the *IL28B* gene. In three of five

TABLE II. Association Between the Genetic Polymorphisms Near the *IL28B* Gene, the Amino Acid at the HCV Core Region Residue 70, and the Final Outcome of Peginterferon/Ribavirin Combination Therapy

Genetic polymorphism of rs8099917 near *IL28B* gene	Amino acid at residue 70 of the HCV core region		
	Wild type (n = 204)	Mutant type (n = 64)	Wild type + mutant type (n = 6)
TT (n = 202)	166 (92/60/14)	31 (14/9/8)	5 (1/2/2)
TG/GG (n = 72)	38 (9/9/20)	33 (5/7/21)	1 (0/1/0)

Outcomes of the combination therapy with peginterferon and ribavirin are shown in parentheses as sustained virologic response/relapse/no-response.

TABLE III. Amino Acid Substitutions of HCV Core Region Residue 70 Before and After the Combination Therapy With Peginterferon and Ribavirin in No-Responders or Relapsers

Amino acid at HCV core region residue 70	After treatment		
Before treatment	Wild type	Wild + Mutant	Mutant type
(A) Genetic polymorphisms near the *IL28B* gene (rs8099917): TT (n = 91)			
No-responders (n = 24)			
Wild type (n = 14)	13	1	0
Wild + mutant (n = 2)	0	0	2
Mutant type (n = 8)	0	0	8
Relapsers (n = 71)			
Wild type (n = 60)	60	0	0
Wild + mutant (n = 2)	0	1	1
Mutant type (n = 9)	0	0	9
(B) Genetic polymorphisms near the *IL28B* gene (rs8099917): TG/GG (n = 57)			
No-responders (n = 41)			
Wild type (n = 20)	19	1	0
Wild + mutant (n = 0)	0	0	0
Mutant type (n = 21)	0	0	21
Relapsers (n = 17)			
Wild type (n = 9)	9	0	0
Wild + mutant (n = 1)	0	1	0
Mutant type (n = 7)	0	0	7

HCV, hepatitis C virus.

patients in whom both the wild-type and mutant-type AAs had been identified at residue 70 of the HCV core region before treatment, only the mutant-type AA was identified at this residue after the treatment. All three of these patients (two no-responders and one relapser) had the TT genotype of the rs8099917. Both the wild-type and mutant-type AAs were identified at residue 70 after the treatment in two no-responders in whom only the wild-type AA had been identified before the treatment. One of them had the TT genotype at the rs 8099917 and the other patient was TG heterozygous. No change in the HCV core region residue 70 was found after the treatment in patients with the mutant-type AA at this residue before the treatment.

DISCUSSION

The present study investigated whether the combination therapy with PEG-IFN and ribavirin causes the mutation of residue 70 of the HCV core region, and whether the genetic polymorphisms of the rs8099917 locus near the *IL28B* gene influence this mutation. It is thought to be important to verify this issue, because it may be advisable to avoid the treatment of patients who have the TG/GG genotypes by the combination therapy with PEG-IFN and ribavirin so as to avoid an acquisition of the further resistance to emerging new therapies against HCV, as well as to avoid a potential enhancement of hepatocarcinogenesis.

The mutation of the AA at residue 70 was not observed before and after the treatment in all patients who had failed to achieve a sustained virologic response. The mutant-type AA was identified solely at residue 70 after the treatment in three patients who had both the wild-type and the mutant-type AAs at residue 70 before the treatment. This could be due to the selection of HCV strains with the mutant-type AA at residue 70 by the combination therapy with PEG-IFN and ribavirin, as reported previously [Kurbanov et al., 2010]. In two patients who carried only the wild-type AA before the treatment, the HCV with the mutant-type AA at residue 70 was also detected with the persistence of the wild-type AA at this residue after the treatment. The very minor HCV strain with the mutant-type AA at residue 70 which were not detected before the treatment may have been detected after the treatment due to the reduction of HCV with the wild-type AA at residue 70 by the combination therapy. Indeed, HCV with the mutant-type AA at core region residue 70 was not detectable in serum 6 months after the end of the combination therapy, suggesting that it returned to being a very minor population (data not shown). These two phenomena were observed in patients with both the TT genotype of the rs8099917, that is associated with a favorable response to the combination therapy and those with the TG/GG genotypes that is associated with an unfavorable response, without difference in the prevalence according to the genetic polymorphisms at the rs8099917 near the *IL28B* gene.

In conclusion, PEG-IFN/ribavirin combination therapy does not appear to induce the mutation of the AA at the HCV core region residue 70 regardless of the genetic polymorphism near the *IL28B* gene in Japanese patients infected with HCV genotype 1b. The combination therapy can be attempted regardless of the genetic polymorphisms near the *IL28B* gene in

treatment-naïve patients without the anxiety for the acquisition of the further resistance to the antiviral therapy. However, future studies should be undertaken to confirm the absence of the mutation at residue 70 of the HCV core region induced by the combination therapy with PEG-IFN and ribavirin. In addition, the effect of the genetic polymorphisms near the *IL28B* gene on the mutation of the AA at the HCV core region residue 70 should be investigated in the long-term observation of the natural course of chronic hepatitis C.

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Impact of Genetic Polymorphisms Near the *IL28B* Gene and Amino Acid Substitutions in the Hepatitis C Virus Core Region on Interferon Sensitivity/Resistance in Patients With Chronic Hepatitis C

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It has been reported that genetic polymorphisms near the *IL28B* gene or amino acid substitutions in hepatitis C virus (HCV) core protein are associated with the clinical outcome of peginterferon (PEG-IFN) and ribavirin combination therapy. The impact of these factors on the pure sensitivity/resistance to interferon was evaluated. Changes in the HCV RNA levels 24, 48, 72, and 120 hr after administering a single dose of standard interferon (IFN) were measured in 156 HCV-infected patients. The changes were compared based on the genetic polymorphisms near the *IL28B* gene or amino acid substitutions in the HCV core region. Among patients with HCV genotype 1b, there were differences in the reduction and subsequent increase in HCV RNA levels after administering IFN based on rs8099917 genetic polymorphisms. Amino acid substitutions at residue 70 were associated with differences in the changes in HCV RNA levels only in patients with TG/GG genotype. Multivariate analyses showed that genetic polymorphisms near the *IL28B* gene was the sole independent factor that was associated with the reduction in HCV RNA levels after administering IFN and the final response to the combination therapy. Among patients infected with HCV genotype 2a or 2b, there were no differences in the changes in HCV RNA levels based on the genetic polymorphisms near the *IL28B* gene. In HCV genotype 1b, genetic variations near the *IL28B* gene affected the sensitivity/resistance to IFN strongly. Genetic polymorphisms near the *IL28B* gene did not affect the sensitivity/resistance to IFN in HCV genotype 2. ***J. Med. Virol. 83:1203–1211, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis C; genetic polymorphism near the *IL28B* gene; amino acid substitution at residue 70 of the HCV core region; resistance to interferon

INTRODUCTION

Hepatitis C virus (HCV) causes a chronic infection that can result in chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [Niederau et al., 1998]. The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although the current treatment regimen has markedly increased the rate of patients who achieve a sustained virologic response, which is an eradication of HCV, only approximately 50% of patients infected with HCV genotype 1 achieved sustained virologic response.

Many studies have examined baseline host- or virus-related factors that affect potentially the outcome of IFN-based antiviral therapy. Recently, several studies reported that genetic polymorphisms near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19, which encodes IFN-λ-3, affect the virologic response to a 48-week regimen of PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch

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et al., 2010]. In addition, a recent report showed the effects of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during PEG-IFN and ribavirin combination therapy in this patient population [Thompson et al., 2010].

Amino acid substitutions at residue 70 in the HCV core region of patients with HCV 1b have been identified as a virus-related factor that affects the virologic response to combination therapy with PEG-IFN and ribavirin [Akuta et al., 2005, 2007a; Donlin et al., 2007]. Additional studies have showed the effects of this factor on the dynamics of HCV during combination therapy [Akuta et al., 2007b; Toyoda et al., 2010a].

Although several studies have shown a strong association between these factors and the final outcome of PEG-IFN and ribavirin combination therapy or the HCV viral dynamics during combination therapy, the mechanisms that contribute to these associations have not been identified. Is the effect of these factors on the antiviral efficacy of the combination therapy with PEG-IFN and ribavirin related mainly to the pure sensitivity/resistance to IFN or ribavirin? Or does these factors affect HCV replication?

The aim of the present study was to investigate the difference in the sensitivity/resistance to IFN based on these factors. Therefore, the changes in HCV RNA levels after administering a single dose of standard IFN were measured and then these results were compared to the genetic polymorphisms near the *IL28B* gene and amino acid substitutions at residue 70 of the HCV core region.

PATIENTS AND METHODS

Patients

In a previous study, a single dose of standard IFN were administered to 208 patients infected with HCV and the changes in HCV RNA levels were measured 24, 48, 72, and 120 hr after administration in order to investigate the pure sensitivity/resistance to IFN [Toyoda et al., 2009, 2010b]. These patients had pretreatment HCV RNA levels of $>100 \times 10^3$ IU/ml as determined by a quantitative polymerase chain reaction (PCR) assay (Amplicor GT-HCV Monitor, Version 2.0; Roche Molecular Systems, Pleasanton, CA), and were not coinfected with hepatitis B virus or human immunodeficiency virus. None of the patients abused alcohol or were intravenous drug users. Among these 208 patients, 156 patients who had provided written informed consent to use their laboratory data and undergo host genetic analyses were enrolled to the present study. The study protocol was in compliance with the Helsinki Declaration and was approved by the hospital ethics committee.

Single Administration of Standard Interferon and Measurement of Changes in Serum HCV RNA Levels to Evaluate the Sensitivity/Resistance to Interferon

All patients received a single dose of standard IFN-alpha 2b at least 2 weeks before starting the combination therapy with PEG-IFN and ribavirin (Fig. 1). The patients received an injection of six mega-units of standard IFN-alpha 2b (Intron A; Schering–Plough). The HCV RNA levels were measured before and 24, 48, 72, and 120 hr after IFN was administered, and the changes in HCV RNA levels were calculated and compared to the HCV RNA levels before administration.

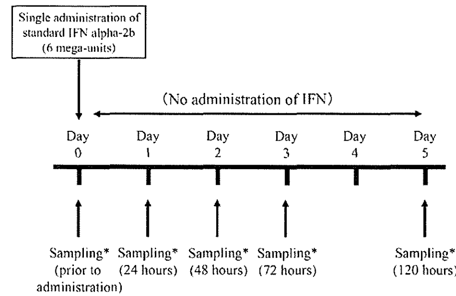

Fig. 1. Schematic representation of administration of standard IFN-alpha and measurements of HCV RNA levels. The serum HCV RNA levels were measured before, and 24, 48, 72, and 120 hr after administration of a singe dose of 6 mega-units of standard IFN-alpha. IFN, interferon; Sampling*, sampling of serum samples to measure HCV RNA levels.

Antiviral Combination Therapy With Peginterferon and Ribavirin

After conducting the single administration examination for standard IFN, all patients started PEG-IFN and ribavirin combination therapy after at least a 2-week interval. The initial doses of PEG-IFN and ribavirin and the dose reductions were according to the manufacturer's recommendations. Patients with HCV genotype 1b were scheduled to receive a 48-week treatment regimen, and those with genotype 2a or 2b were scheduled to receive a 24-week regimen. The outcomes of the combination therapy were classified as a sustained virologic response when serum HCV RNA became undetectable during the treatment and remained undetectable for 6 months after the treatment ended (i.e., eradication of HCV), a relapse when the serum HCV RNA became undetectable during the treatment period but was detectable after treatment, and no response when the serum HCV RNA remained detectable during and after treatment.

Examination of the Serum HCV RNA Levels, Genetic Polymorphisms Near the *IL28B* Gene, and Amino Acid Substitutions at Residue 70 of the HCV Core

The HCV genotype was determined by PCR amplifying the core gene sequences using genotype-specific primers [Ohno et al., 1997]. The HCV RNA levels in serum samples were measured using a real-time PCR-based quantitation method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Molecular Systems; lower limit of quantitation: 1.7 $\log_{10}$ IU/ml, lower limit of detection: 1.0 $\log_{10}$ IU/ml). The HCV RNA levels before the administration of a single dose of IFN and before PEG-IFN and ribavirin combination therapy were also examined using the same method on stored serum samples.

Genotyping of rs8099917 polymorphisms near the *IL28B* gene was performed using the TaqMan SNP assays (Applied Biosystems, Foster City, CA) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C_11710096_10, Applied Biosystems).

Amino acid 70 of the HCV core region was analyzed by direct nucleotide sequencing as previously described [Akuta et al., 2007c]. The PCR primer pairs for direct sequencing of the HCV core region were as follows:

5′-GCCATAGTGGTCTGCGGAAC-3′ (outer, sense primer),

5′-GGAGCAGTCCTTCGTGACATG-3′ (outer, antisense primer),

5′-GCTAGCCGAGTAGTGTT-3′ (inner, sense primer), and

5′-GGAGCAGTCCTTCGTGACATG-3′ (inner, antisense primer).

Statistical Analyses

Quantitative values are reported as the means ± SD. Between-group differences were analyzed by a chi-square test. Differences in the quantitative values of two groups were analyzed by the Mann–Whitney *U*-test. Univariate and multivariate analyses using a logistic regression model were performed to identify factors that were associated with a decrease in serum HCV RNA levels at 24 hr after administering standard IFN, including age, sex, body weight, serum alanine aminotransferase activity, serum aspartate aminotransferase activity, serum gamma-glutamyl transpeptidase levels, serum alkaline phosphatase values, serum albumin levels, total serum bilirubin values, white blood cell counts, hemoglobin, platelet counts, hepatitis activity grade (A0 and A1 vs. A2 and A3), liver fibrosis grade (F0 and F1 vs. F2 and F3), pretreatment HCV RNA levels, genetic polymorphisms near the *IL28B* gene, and amino acid substitutions at residue 70 of the HCV core region (arginine vs. glutamine). All *P*-values were two-tailed, and $P < 0.05$ was considered as significant statistically.

RESULTS

Patient Characteristics and Combination Therapy

The patient characteristics are shown in Table I. The patients included 69 males (44.2%) and 87 females (55.8%) with a mean age of 58.4 ± 9.3 years. The grade of liver fibrosis according to the METAVIR score [The French Cooperative METAVIR Study Group, 1994] was F0 in 8 patients (5.1%), F1 in 97 patients (62.2%), F2 in 35 patients (22.4%), and F3 in 16 patients (10.3%). One hundred one patients (64.8%) were infected with HCV genotype 1b, 42 patients (26.9%) were infected with HCV genotype 2a, and the remaining 13 patients (8.3%) were infected with HCV genotype 2b. An analysis of genetic

TABLE I. Baseline Characteristics of Patients Infected With HCV Genotype 1b and Those With Genotype 2a/2b (n = 156)

	Genotype 1b (n = 101)	Genotype 2a/2b (n = 55)
Age (years)	59.0 ± 8.1	57.1 ± 11.0
Sex (female/male)	51 (50.5)/50 (49.5)	36 (65.5)/19 (34.5)
Body weight (kg)	58.5 ± 9.5	57.8 ± 8.8
Alanine aminotransferase (IU/L)	60.9 ± 63.9	47.6 ± 51.1
Aspartate aminotransferase (IU/L)	50.9 ± 41.2	40.3 ± 36.9
Gamma-glutamyl transpeptidase (IU)	49.0 ± 44.3	41.2 ± 68.6
Alkaline phosphatase (IU/L)	261.3 ± 81.8	281.6 ± 155.8
Albumin (g/dl)	4.18 ± 0.34	4.25 ± 0.35
Total bilirubin (mg/dl)	0.65 ± 0.24	0.62 ± 0.24
White blood cell count (/μl)	5169 ± 1338	5029 ± 1442
Hemoglobin (g/dl)	14.2 ± 1.2	13.8 ± 1.6
Platelet count ($\times 10^3$/μl)	166 ± 49	201 ± 57
Liver histology-activity (A0/A1/A2/A3)	2 (2.0)/64 (63.4)/25 (24.7)/10 (9.9)	1 (1.8)/42 (76.4)/9 (16.4)/3 (5.4)
Liver histology-fibrosis (F0/F1/F2/F3)	4 (4.0)/59 (58.4)/25 (24.7)/13 (12.9)	4 (7.3)/38 (69.1)/10 (18.2)/3 (5.4)
HCV RNA levels ($\log_{10}$ IU/ml)[a]	6.10 ± 0.41	6.04 ± 0.57
Amino acid at HCV core 70 (arginine/glutamine)[b]	71 (70.3)/30 (29.7)	—
Genetic polymorphisms near the *IL28B* gene (TT/TG/GG)[c]	76 (75.2)/24 (23.8)/1 (1.0)	45 (81.8)/10 (18.2)/0
Response (SVR/relapse/NR)[d]	38 (38.8)/38 (38.8)/22 (22.4)	41 (78.9)/10 (19.2)/1 (1.9)

HCV, hepatitis C virus; SVR, sustained virologic response; NR, no response
Percentages are shown in parentheses.
[a]Before the administration of standard interferon.
[b]Analyzed only in HCV genotype 1b-infected patients.
[c]rs8099917 genetic polymorphism.
[d]Six patients (three patients with HCV genotype 1b and three patients with genotype 2a) discontinued treatment.

polymorphisms near the *IL28B* gene indicated that 121 patients had a TT genotype, 1 patient had a GG genotype, and the remaining 34 patients were TG heterozygous. There were no differences in the distribution of the genetic polymorphisms near the *IL28B* gene between patients infected with HCV genotype 1b and those infected with HCV genotype 2a or 2b. An analysis of the amino acid substitutions at residue 70 of the HCV core region in HCV genotype 1b-infected patients showed that 71 and 30 patients arginine and glutamine at this residue, respectively.

Although all patients started PEG-IFN and ribavirin combination therapy after receiving single administration examination of standard IFN, six patients (three patients with genotype 1b and three patients with genotype 2a) discontinued the therapy because of adverse effects (depression in three, severe general fatigue in one, delirium in one, retinopathy in one, and thrombocytopenia in one).

Changes in the Serum HCV RNA Levels After Administering a Single Dose of Standard Interferon-Alpha to Assess the Sensitivity/Resistance to Interferon in Patients Infected With HCV Genotype 1b

Figure 2 shows the changes in the serum HCV RNA levels after a single dose of standard IFN-alpha in patients infected with HCV genotype 1b based on both the genetic polymorphisms near the *IL28B* gene (left panel) and amino acid substitutions at residue 70 of the HCV core region (right panel). Compared to the pretreatment levels, patients with the TT genotype had a more marked reduction in HCV RNA levels than patients with the TG or GG genotype, and this reduction was more marked in patients with arginine than glutamine at residue 70 of the HCV core region. The differences in the reduction in the HCV RNA levels 24 hr after IFN administration were more pronounced based on the genetic polymorphisms near the *IL28B* gene than the amino acid at residue 70 of the HCV core region. These differences were decreased at 48 and 72 hr after IFN administration and disappeared at 120 hr in the case of the TT genotype versus the TG/GG genotype. In contrast, the differences in the reduction in HCV RNA levels based on whether patients had an arginine or glutamine at residue 70 of the HCV core were maintained at 48, 72, and 120 hr after IFN administration.

Univariate and multivariate analyses were conducted for factors that are associated with <0.8 $\log_{10}$ decrease in HCV RNA levels 24 hr after administering standard interferon alpha, which was associated strongly with virologic no-response to PEG-IFN and ribavirin combination therapy in our previous study [Toyoda et al., 2010b]. Also in the present study, 38 of 82 patients (46.3%) with reductions in serum HCV RNA levels >0.8 $\log_{10}$ achieved a sustained virologic response. In contrast, of the 16 patients with reduction in serum HCV RNA levels ≤0.8 $\log_{10}$, none achieved a sustained virologic response ($P = 0.0014$). A univariate analysis indicated that pretreatment gamma-glutamyl transpeptidase, genetic polymorphisms near the *IL28B* gene, and amino acid substitutions at residue 70 of the HCV core region were associated significantly with a reduction in HCV RNA levels 24 hr after the administration of standard IFN, and pretreatment total bilirubin tended to be

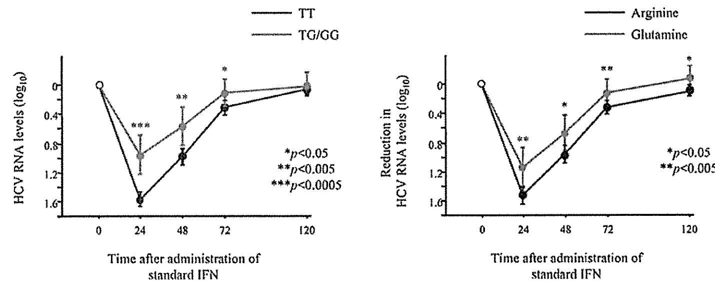

Fig. 2. Changes in HCV RNA levels after administering standard IFN to patients with the TT genotype compared to the TG/GG genotype near the *IL28B* gene (**left panel**), and in patients with an arginine compared to a glutamine at residue 70 of the HCV core region (**right panel**). The decrease in HCV RNA levels for the TT and TG/GG genotypes was 1.56 ± 0.46 $\log_{10}$ IU/ml versus 0.95 ± 0.66 $\log_{10}$ IU/ml ($P < 0.0001$) at 24 hr, 0.98 ± 0.50 $\log_{10}$ IU/ml versus 0.56 ± 0.62 $\log_{10}$ IU/ml ($P = 0.0002$) at 48 hr, 0.31 ± 0.42 $\log_{10}$ IU/ml versus 0.11 ± 0.44 $\log_{10}$ IU/ml ($P = 0.0238$) at 72 hr, and 0.06 ± 0.36 $\log_{10}$ IU/ml versus −0.01 ± 0.41 $\log_{10}$ IU/ml ($P = 0.3856$) at 120 hr after administration of IFN. The decrease in HCV RNA levels for arginine and glutamine was 1.53 ± 0.47 $\log_{10}$ IU/ml versus 1.14 ± 0.71 $\log_{10}$ IU/ml ($P = 0.0013$) at 24 hr, 0.96 ± 0.50 $\log_{10}$ IU/ml versus 0.67 ± 0.65 $\log_{10}$ IU/ml ($P = 0.0058$) at 48 hr, 0.32 ± 0.39 $\log_{10}$ IU/ml versus 0.12 ± 0.49 $\log_{10}$ IU/ml ($P = 0.0043$) at 72 hr, and 0.09 ± 0.32 $\log_{10}$ IU/ml versus −0.08 ± 0.32 $\log_{10}$ IU/ml ($P = 0.0289$) at 120 hr after administration of IFN.

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associated with this reduction. A multivariate analysis showed that only genetic polymorphisms near the *IL28B* gene was associated independently with this reduction (Table II).

When patients were stratified according to the TT or TG/GG genotype, we found that there was a significant difference in the reduction in HCV RNA levels 24 hr after IFN administration in patients with TG/GG genotype based on whether the patients had arginine or glutamine at residue 70 of the HCV core (Fig. 3, right panel). However, there were no differences in patients with the TT genotype (Fig. 3, left panel).

Outcome to Combination Therapy With Peginterferon and Ribavirin in Patients Infected With HCV Genotype 1b

As for the final therapeutic outcome, 79 patients (52.7%) achieved a sustained virologic response, 48 patients (32.0%) relapsed, and the remaining 23 patients (15.3%) had no-response. Among 74 patients with the TT genotype of rs8099917 polymorphism near the *IL28B* gene, 36 (48.6%) achieved a sustained virologic response, whereas 2 of 24 patients (8.3%) with TG/GG achieved it. Among 69 patients with arginine at residue 70 of the HCV core region, 32 (46.4%) patients achieved a sustained virologic response, whereas 6 of 29 patients (20.7%) with glutamine at this residue achieved it. The rate of sustained virologic response was significantly higher in patients with the TT genotype ($P = 0.0010$) and in patients with arginine at residue 70 ($P = 0.0312$). When genotype of rs8099917 polymorphism and amino acid at residue 70 of the HCV core region were combined, the rate of sustained virologic response was highest in patients bearing the TT genotype and arginine (50.0%), followed by those with the TT genotype and glutamine (42.8%), those with the TG/GG genotype and arginine (22.2%), and those with the TG/GG genotype and glutamine in this order. None of 15 patients bearing both the TG/GG genotype and glutamine achieved a sustained virologic response.

Univariate and multivariate analyses were conducted for factors that are associated with sustained virologic response to the combination therapy with PEG-IFN and ribavirin. A univariate analysis indicated that pretreatment albumin and platelet counts, genetic polymorphisms near the *IL28B* gene, and amino acid substitutions at residue 70 of the HCV core region were associated significantly with sustained virologic response. A multivariate analysis showed that genetic polymorphisms near the *IL28B* gene and pretreatment platelet counts were associated independently with this reduction (Table III).

Changes in Serum HCV RNA Levels After Administering a Single Dose of Standard Interferon-Alpha to Assess the Sensitivity/Resistance to Interferon in Patients Infected With HCV Genotype 2

Figure 4 shows the changes in the serum HCV RNA levels after a single dose of standard IFN was administered to patients with HCV genotype 2a or 2b based on the genetic polymorphisms near the *IL28B* gene (left panel) and the subtype of HCV genotype 2 (right panel). There was a more marked reduction in HCV RNA levels after IFN administration in patients infected with HCV subtype 2a than in those infected

TABLE II. Univariate and Multivariate Analyses of Factors Associated With <0.8 $\log_{10}$ Decrease in HCV RNA Levels 24 hr After Administering Standard Interferon-Alpha

	Univariate analysis	Multivariate analysis	Odds ratio (95% confidence interval)
Age (years)	0.8801	—	
Sex (female/male)	0.9656	—	
Body weight (kg)	0.7199	—	
Alanine aminotransferase (IU/L)	0.9223	—	
Aspartate aminotransferase (IU/L)	0.7110	—	
Gamma-glutamyl transpeptidase (IU)	0.0290	0.2445	
Alkaline phosphatase (IU/L)	0.3261	—	
Albumin (g/dl)	0.4481	—	
Total bilirubin (mg/dl)	0.0582	0.7530	
White blood cell count (/μl)	0.9814	—	
Hemoglobin (g/dl)	0.6485	—	
Platelet count ($\times 10^3$/μl)	0.3020	—	
Liver histology-activity (A0–1/A2–3)	0.8062	—	
Liver histology-fibrosis (F0–1/F2–3)	0.7220	—	
HCV RNA levels ($\log_{10}$ IU/ml)[a]	0.1954	—	
Genetic polymorphisms near the IL28B gene (TT/TG + GG)[b]	<0.0001	0.0005	15.0446 (3.5533–81.5225)
Amino acid at residue 70 of the HCV core region (arginine/glutamine)	0.0007	0.0983	

HCV, hepatitis C virus.
[a]Before the administration of standard interferon.
[b]rs8099917 genetic polymorphism.

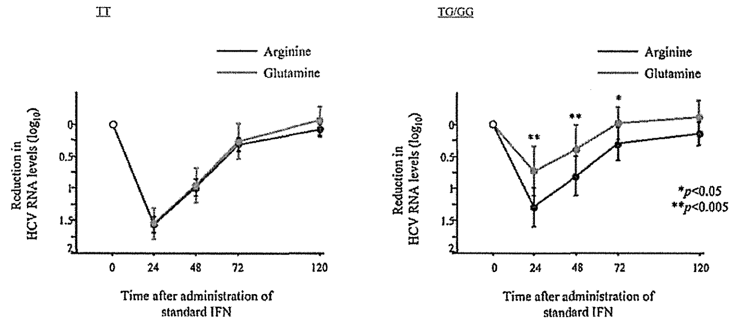

Fig. 3. Changes in HCV RNA levels after administering standard IFN in patients with an arginine compared to a glutamine at residue 70 of the HCV core region in patients with the TT genotype (**left panel**) and in those with the TG/GG genotype (**right panel**) near the *IL28B* gene. The decrease in HCV RNA levels for arginine and glutamine was 1.57 ± 0.47 $\log_{10}$ IU/ml versus 1.54 ± 0.44 $\log_{10}$ IU/ml ($P = 0.6292$) at 24 hr, 0.98 ± 0.51 $\log_{10}$ IU/ml versus 0.95 ± 0.47 $\log_{10}$ IU/ml ($P = 0.6810$) at 48 hr, 0.32 ± 0.40 $\log_{10}$ IU/ml versus 0.26 ± 0.50 $\log_{10}$ IU/ml ($P = 0.2745$) at 72 hr, and 0.08 ± 0.34 $\log_{10}$ IU/ml versus −0.05 ± 0.43 $\log_{10}$ IU/ml ($P = 0.2230$) at 120 hr after administration of IFN in patients with the TT genotype. The decrease in HCV RNA levels for arginine and glutamine was 1.29 ± 0.42 $\log_{10}$ IU/ml versus 0.73 ± 0.70 $\log_{10}$ IU/ml ($P = 0.0043$) at 24 hr, 0.81 ± 0.42 $\log_{10}$ IU/ml versus 0.39 ± 0.69 $\log_{10}$ IU/ml ($P = 0.0047$) at 48 hr, 0.30 ± 0.36 $\log_{10}$ IU/ml versus −0.02 ± 0.46 $\log_{10}$ IU/ml ($P = 0.0327$) at 72 hr, and 0.14 ± 0.25 $\log_{10}$ IU/ml versus −0.11 ± 0.46 $\log_{10}$ IU/ml ($P = 0.0672$) at 120 hr after administration of IFN in patients with the TG/GG genotype.

with HCV subtype 2b. In contrast, there were no differences in the reduction in the HCV RNA levels between patients with the TT genotype and the TG/GG genotype. The final outcome of PEG-IFN and ribavirin combination therapy was not different based on either the genetic polymorphisms near the *IL28B* gene or the HCV subtype (data not shown).

DISCUSSION

In the present study, the impact of rs8099917 genetic polymorphisms near the *IL28B* gene on the sensitivity/resistance to IFN was investigated by analyzing the association between genetic polymorphisms and changes in HCV RNA levels after administering a single dose of standard IFN. A previous study by Thompson et al. [2010] reported that genetic polymorphisms near the *IL28B* gene was associated strongly with early viral kinetics during the combination therapy with PEG-IFN and ribavirin. However, the viral response in their study reflected the response of HCV to both PEG-IFN and ribavirin that were administered in combination, and the response did not represent a pure sensitivity/resistance to IFN in the absence of ribavirin. In a previous study, the decrease in HCV RNA levels 24 hr after administering standard IFN was investigated and the decrease was shown to be associated strongly with the outcome of PEG-IFN and ribavirin combination therapy [Toyoda et al., 2010b]. In the present study, a difference in the decrease in HCV RNA levels 24 hr after administering standard IFN was observed in patients with the TT genotype compared to those with the TG/GG genotype. The rs8099917 genetic polymorphisms near the *IL28B* gene was an only independent factor that was associated with a decrease in HCV RNA levels 24 hr after IFN administration. This finding indicates that genetic polymorphisms near the *IL28B* gene affect the pure sensitivity/resistance to IFN in patients infected with HCV genotype 1b.

In the absence of subsequent IFN administration, HCV RNA levels increased after 24 hr and were restored to the pretreatment levels in both patients with the TT genotype and those with the TG/GG genotype. The differences in HCV RNA levels between patients with the TT and TG/GG genotypes decreased rapidly, and there were no differences in HCV RNA levels 120 hr after administering standard IFN. Based on this finding, rs8099917 genetic polymorphisms do not appear to affect HCV replication.

A difference in the decrease in HCV RNA levels 24 hr after administering a single dose of standard IFN was observed also based on amino acid substitutions at residue 70 of the HCV core region, although this difference was less marked compared to the differences associated with the genetic polymorphisms. In contrast to the differences associated with these genetic polymorphisms, the differences associated with the amino acid substitutions in the HCV core region were maintained until 120 hr after IFN administration. Therefore, the effects on HCV of host genetic polymorphisms and amino acid substitutions in the core protein of infected HCV during administration of IFN or PEG-IFN may be by a different mechanism.

When patients were stratified according to the TT genotype or the TG/GG genotype and the changes in HCV RNA levels 24 hr after IFN administration were compared, there were no differences between patients with arginine and those with glutamine at residue 70 of the HCV core among patients with the TT genotype. However, there was a difference in the changes in HCV RNA levels among patients with the TG/GG genotype. Therefore, genetic polymorphisms near the *IL28B* gene are a strong factor that affects the reduction in HCV RNA levels and amino acid substitutions at residue 70 of the HCV core region have an effect only in patients with the TG/GG genotype. These findings are consistent with the rate at which patients achieved a sustained virologic response as a final outcome and a result of the multivariate analysis for sustained virologic response.

In a previous study by Hayes et al. [2011], genetic polymorphisms near the *IL28B* gene, amino acid substitutions at residue 70 of the HCV core region, and

TABLE III. Univariate and Multivariate Analyses of Factors Associated With Sustained Virologic Response to the Combination Therapy With Peginterferon and Ribavirin

	Univariate analysis	Multivariate analysis	Odds ratio (95% confidence interval)
Age (years)	0.6173	—	
Sex (female/male)	1.0000	—	
Body weight (kg)	0.3904	—	
Alanine aminotransferase (IU/L)	0.3630	—	
Aspartate aminotransferase (IU/L)	0.4537	—	
Gamma-glutamyl transpeptidase (IU)	0.2782	—	
Alkaline phosphatase (IU/L)	0.2500	—	
Albumin (g/dl)	0.0473	0.1203	
Total bilirubin (mg/dl)	0.9748	—	
White blood cell count (/μl)	0.4362	—	
Hemoglobin (g/dl)	0.5580	—	
Platelet count ($\times 10^3/\mu$l)	0.0445	0.0408	14.9668 (1.2103–230.4323)
Liver histology-activity (A0–1/A2–3)	0.8789	—	
Liver histology-fibrosis (F0–1/F2–3)	0.1119	—	
HCV RNA levels ($\log_{10}$ IU/ml)[a]	0.9591	—	
Genetic polymorphisms near the IL28B gene (TT/TG + GG)[b]	0.0025	0.0020	0.06233 (0.00780–0.29468)
Amino acid at residue 70 of the HCV core region (arginine/glutamine)	0.0207	0.5067	

HCV, hepatitis C virus.
[a]Before the administration of standard interferon.
[b]rs8099917 genetic polymorphism.

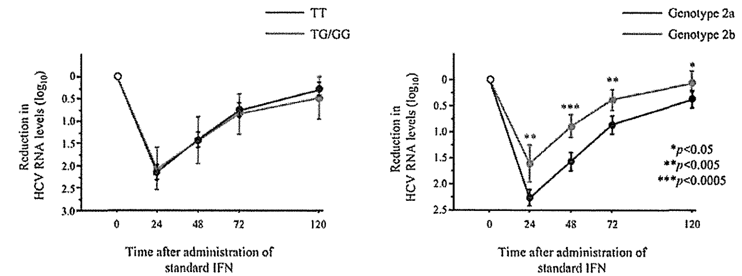

Fig. 4. Changes in HCV RNA levels after administering standard IFN to patients with the TT genotype compared to the TG/GG genotype near the *IL28B* gene (**left panel**) and in patients infected with HCV genotype 2a compared to HCV genotype 2b (**right panel**). The decrease in HCV RNA levels for the TT and TG/GG genotypes was 2.12 ± 0.58 $\log_{10}$ IU/ml versus 2.07 ± 0.66 $\log_{10}$ IU/ml ($P = 0.9652$) at 24 hr, 1.40 ± 0.56 $\log_{10}$ IU/ml versus 1.43 ± 0.74 $\log_{10}$ IU/ml ($P = 0.8872$) at 48 hr, 0.73 ± 0.53 $\log_{10}$ IU/ml versus 0.85 ± 0.64 $\log_{10}$ IU/ml ($P = 0.6005$) at 72 hr, and 0.47 ± 1.53 $\log_{10}$ IU/ml versus 0.48 ± 0.67 $\log_{10}$ IU/ml ($P = 0.6372$) at 120 hr after administration of IFN. The decrease in HCV RNA levels in patients infected with HCV genotype 2a and 2b was 2.27 ± 0.51 $\log_{10}$ IU/ml versus 1.60 ± 0.59 $\log_{10}$ IU/ml ($P = 0.0007$) at 24 hr, 1.57 ± 0.55 $\log_{10}$ IU/ml versus 0.89 ± 0.36 $\log_{10}$ IU/ml ($P = 0.0002$) at 48 hr, 0.86 ± 0.55 $\log_{10}$ IU/ml versus 0.38 ± 0.33 $\log_{10}$ IU/ml ($P = 0.0012$) at 72 hr, and 0.60 ± 1.58 $\log_{10}$ IU/ml versus 0.04 ± 0.33 $\log_{10}$ IU/ml ($P = 0.0354$) at 120 hr after administration of IFN.

mutations in the interferon sensitivity-determining region of HCV NS5A region were evaluated as a predictor of response to the combination therapy with PEG-IFN and ribavirin in 817 Japanese patients with chronic HCV genotype 1b infection. They reported that genetic polymorphisms near the *IL28B* gene and amino acid substitutions at residue 70 of the HCV core contributed independently to a sustained virologic response to the combination therapy, indicating the different effects of these two factors on the response to PEG-IFN and ribavirin combination therapy. In contrast, amino acid substitutions at residue 70 of the HCV core region failed to be an independent predictor by multivariate analysis in the present study. This discrepancy may be simply due to a small number of patients in our study population. Indeed, the difference of changes in the serum HCV RNA levels after administering a single dose of standard IFN in the present study also indicated the different mechanism of resistance to IFN between genetic polymorphisms near the *IL28B* gene and amino acid substitutions at HCV residue 70.

In patients infected with HCV genotype 2a or 2b, there were no differences in the changes in HCV RNA levels after a single dose of standard IFN based on genetic polymorphisms near the *IL28B* gene. Rather, there was a significant difference in the reduction in HCV RNA levels in patients infected with HCV genotype 2a compared to those infected with genotype 2b, as our previous report [Toyoda et al., 2009]. The genetic polymorphisms near the *IL28B* gene appeared to have few effects on the reduction in HCV RNA levels after IFN administration in patients infected with HCV genotype 2.

There are several limitations on this study. The data were based on Japanese patients infected with HCV genotype 1b, because there are so few patients infected with HCV genotype 1a in Japan. Therefore, these results should be confirmed in patients of other ethnicities and patients infected with HCV genotype 1a. In addition, the number of patients was small in comparison to previous studies. This was because of the difficulty to conduct the examination of single administration of standard IFN and measurement of changes in serum HCV RNA levels. As a result, only 25 patients with HCV genotype 1b were bearing minor allele of polymorphisms near the *IL28B* gene (GG genotype or TG heterozygote); 10 had arginine and 15 had glutamine at residue 70 of the HCV core region. Finally, only standard IFN-alpha 2b and PEG-IFN-alpha 2b were used in this study. Results may differ with the use of IFN/PEG-IFN alpha-2a, as the pharmacokinetics of PEG-IFN are different between PEG-IFN alpha-2b and PEG-IFN alpha-2a.

In conclusion, rs8099917 genetic polymorphisms near the *IL28B* gene are associated with the sensitivity/resistance to IFN in patients infected with HCV genotype 1b. In addition, amino acid substitutions at residue 70 of the HCV core region are related to the sensitivity/resistance to IFN only in patients with the TG/GG genotype. These associations were not seen in patients infected with HCV genotype 2.

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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 ($x10^6$ 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($\times 10^3/mm^3$)	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 ($\times 10^4/mm^3$)	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

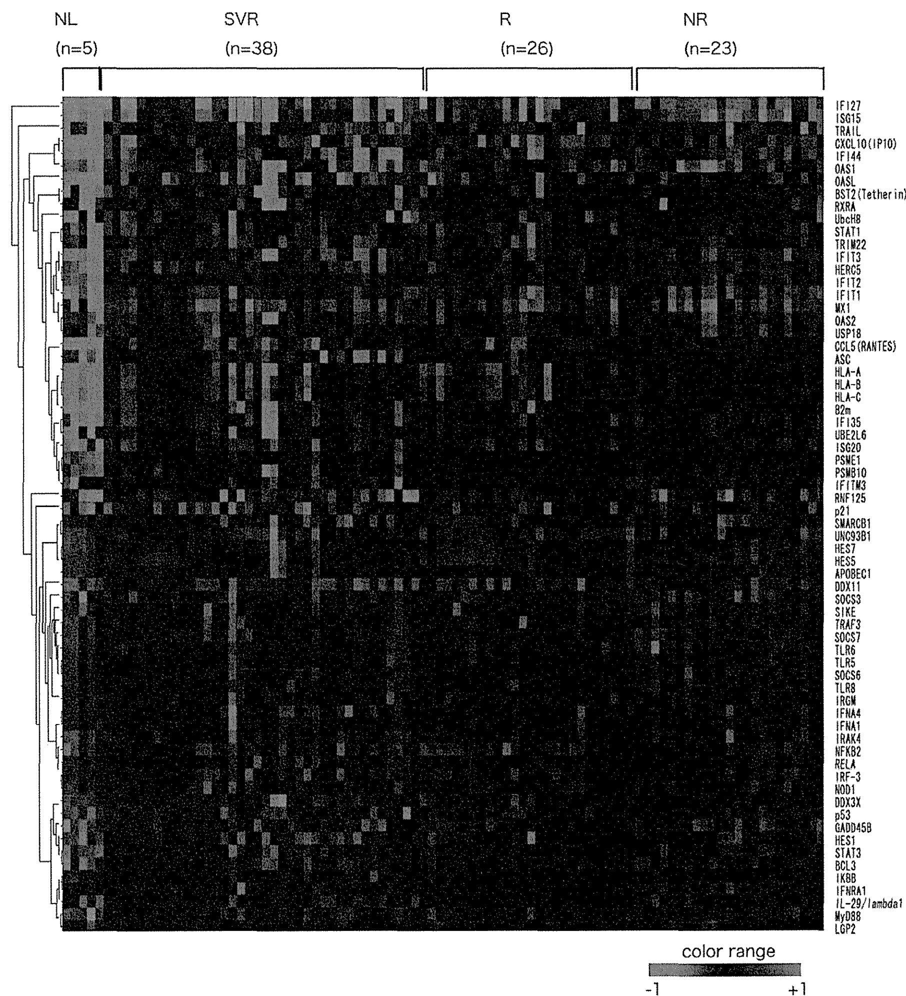

Figure 1. Clustering of IFN related gene expression. Clustering of CH patients according to the expression profiles of the 66 genes that showed significant differences among SVR, R, NR, and NL. *Vertical bars represent the IFN related genes and the horizontal bars represent the samples.* Green bars reflect 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.
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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 allelic 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.

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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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 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

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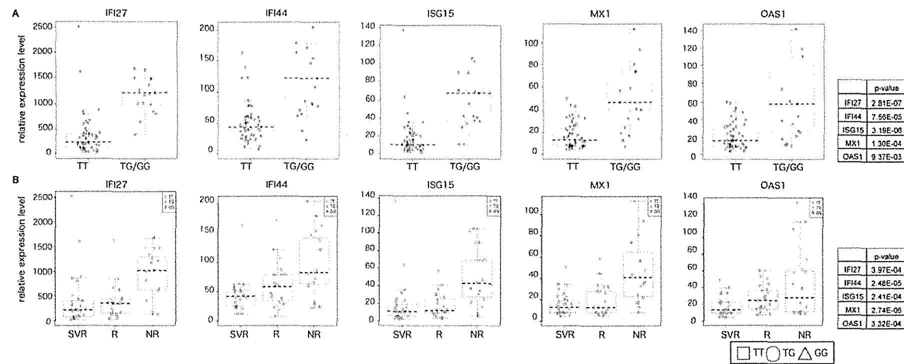

Figure 2. The relationship among the expression of IFN-related genes, IL28B polymorphism and clinical outcome. (A) The relationship between expression of ISG and five related genes (MX1, OAS1, ISG15, IFI27, and IFI44) in the liver of CH patients and IL28B with the major (TT) or minor (TG or GG) genotype (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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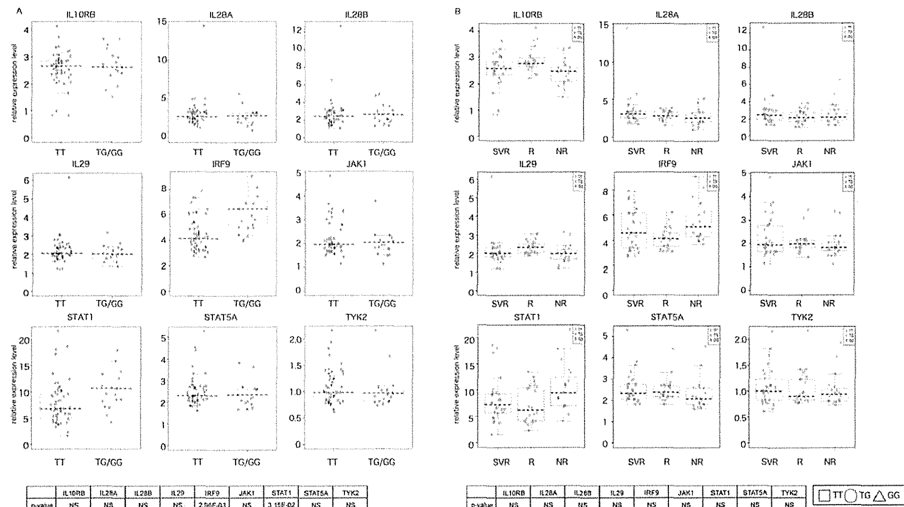

Figure 3. The relationship among the expression of IFN lambda-related genes, IL28B polymorphism and clinical outcome. (A) The relationship between the expression level of IFN lambda related genes (TYK2, STAT5A, STAT1, IL10RB, IL29, IL28A, IL28B, JAK1, and IRF9) in the liver of CH patients and IL28B with 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

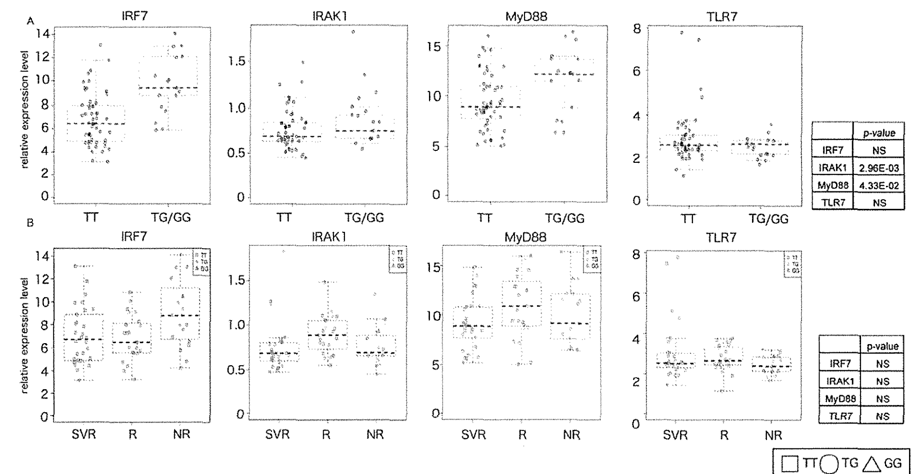

Figure 4. The relationship between the expression level of genes which participate in IFN production (TLR7, MyD88, IRAK1, and IRF7) in the liver of CH patients and IL28B genotype. (A) The relationship between IFN early response genes and clinical outcome is shown. A summary table of the p-value is also 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′-gactgcagagtagccacaag-3′, IFI44 (sense) 5′-gcatgtaacgcatcaggctt-3′, IFI44 (anti-sense) 5′-ccacaccagcgttaccaac-3′, ISG15 (sense) 5′-ctttgccagtacaggagctt-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

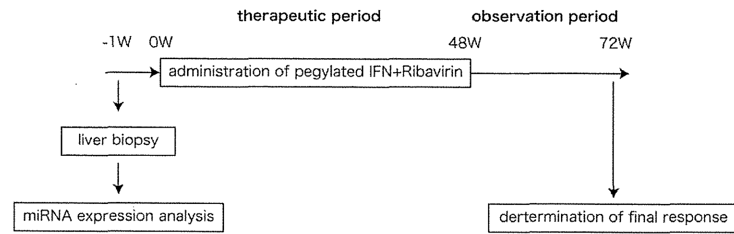

Figure 5. Study design and time line of response to combination therapy. The time frame of liver biopsy, microarray analysis, therapeutic period, observation period after combination therapy, and the judging of clinical outcome is shown.
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cDNA microarray

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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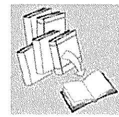
REVIEW



Antiviral responses induced by the TLR3 pathway

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SUMMARY

Antiviral responses are successively induced in virus-infected animals, and include primary innate immune responses such as type I interferon (IFN) and cytokine production, secondary natural killer (NK) cell responses, and final cytotoxic T lymphocyte (CTL) responses and antibody production. The endosomal Toll-like receptors (TLRs) and cytoplasmic RIG-I-like receptors (RLRs), which recognize viral nucleic acids, are responsible for virus-induced type I IFN production. RLRs are expressed in most tissues and cells and are primarily implicated in innate immune responses against various viruses through type I IFN production, whereas nucleic acid-sensing TLRs, TLRs 3, 7, 8 and 9, are expressed on the endosomal membrane of dendritic cells (DCs) and play distinct roles in antiviral immunity. TLR3 recognizes viral double-stranded RNA taken up into the endosome and serves to protect the host against viral infection by the induction of a range of responses including type I IFN production and DC-mediated activation of NK cells and CTLs, although the deteriorative role of TLR3 has also been reported in some virus infections. Here, we review the current knowledge on the role of TLR3 during viral infection, and the current understanding of the TLR3-signalling cascade that operates via the adaptor protein TICAM-1 (also called TRIF). Copyright © 2011 John Wiley & Sons, Ltd.

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INTRODUCTION

Mammalian cells possess several defense strategies against viral infection, of which, the type I interferon (IFN) system is most important for innate and adaptive antiviral responses [1,2]. Type I IFN induces an antiviral state in uninfected host cells by upregulating IFN-stimulated genes (ISGs) through IFN-α/β receptor signalling, and also activates innate and adaptive immune cells, such as dendritic cells (DCs), natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) [3]. Intrinsic double-stranded RNA (dsRNA) sensors, dsRNA-binding protein kinase R and 2′–5′ oligoadenylate synthetase, are both ISGs, which trigger the shutdown of protein translation and induce RNA degradation within virus-infected cells, respectively [4,5]. Recent progressive studies have demonstrated that the endosomal Toll-like receptors (TLRs) and cytoplasmic retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) are responsible for virus-induced type I IFN production [6–8]. These receptors recognize viral nucleic acids and induce type I IFN, inflammatory cytokine and chemokine production and DC maturation. TLR3 recognizes virus-derived dsRNA and its synthetic analogue, polyriboinosinic:polyribocytidylic acid (poly (I:C)) [9–11]. dsRNA is found in some virus particles as a viral genome and can be generated

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Abbreviations:
CT, C-terminal; CTL, cytotoxic T lymphocytes; CVB3, coxsackievirus group B serotype 3; dsRNA, double-stranded RNA; DC, dendritic cell; DUBA, deubiquitinating enzyme A; ECD, ectodomain; EMCV, encephalomyocarditis virus; HCV, hepatitis C virus; HSV-1, herpes simplex virus-1; IAV, influenza A virus; IFN, interferon; INAM, IRF-3-dependent NK-activating molecule; ISG, IFN-stimulated gene; LRR, leucine-rich repeat; MCMV, murine cytomegarovirus; MDA5, melanoma differentiation associated gene 5; NAK, NF-κB activating kinase; NAP1, NAK-associated protein 1; NK, natural killer; NT, N-terminal; NTD, N-terminal domain of TICAM-1; pDC, plasmacytoid DC; poly(I:C), polyriboinosinic:polyribocytidylic acid; PVR, poliovirus receptor; RIG-I, retinoic acid inducible gene-I; RIP1, receptor-interacting protein 1; ss, single-stranded; TBK1, TANK-binding kinase 1; TICAM-1, TIR-containing adaptor molecule-1; TIR, Toll-IL-1 receptor; TLR, Toll-like receptor; RLR, RIG-I-like receptor; WNV, West Nile virus.

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during the process of positive-stranded RNA virus and DNA virus replication [12]. TLR7 and TLR8 recognize virus-derived single-stranded (ss) RNA, while TLR9 recognizes non-methylated CpG-containing DNA that is found in some microbes [13–15]. Since these TLRs localize to the endosomal membranes of myeloid or plasmacytoid DCs (pDCs), they appear to detect extracellular viral nucleic acids released from infected cells or virus particles. However, the mechanism by which TLRs encounter virus-derived nucleic acids in endosomes remains to be determined. Interestingly, a recent report showed that TLR7-mediated IFN-α secretion by pDCs in response to ssRNA virus infection requires the transport of cytosolic viral RNA into the lysosome via the process of autophagy [16]. Whether this autophagy-dependent viral recognition is applicable to TLRs 3, 8 and 9 remains unclear.

By contrast, RLRs are expressed in most tissues and cells and detect viral nucleic acids in the cytoplasm. RIG-I recognizes viral RNA genomes bearing 5′-triphosphates and panhandle structures and also short-length dsRNAs [17–21], while melanoma differentiation-associated gene 5 (MDA5) detects long-length dsRNAs and poly(I:C) [22]. Studies using gene-disrupted mice and cells revealed that RIG-I is essential for the detection of various negative-stranded RNA viruses including influenza A virus (IAV), Sendai virus and vesicular stomatitis virus and a positive-stranded RNA virus, hepatitis C virus (HCV), whereas MDA5 plays a key role in sensing encephalomyocarditis virus, a member of *Picornavilridae* family [23–26]. Thus, multiple innate immune pathways are implicated in dsRNA responses and each pathway plays a distinct role in antiviral responses. In this review, we focus on TLR3, whose antiviral function has been controversial, but recent studies have demonstrated the critical role of the TLR3–TICAM-1 pathway in antiviral responses and the induction of adaptive immunity.

Expression and subcellular localization of TLR3

Human TLR3 mRNA has been detected in various tissues including the placenta, pancreas, lung, liver, heart and brain [27]. Interestingly, in the human central nervous system, TLR3 is expressed constitutively in neurons, astrocytes and microglia, suggesting a role in the response to viruses causing encephalopathy [28–30]. In immune cells, only myeloid DCs and macrophages express TLR3. The pDCs, which express TLR7 and TLR9 and secrete large amounts of IFN-α in response to viral infection, do not express TLR3 [31–35]. TLR3 is also expressed in fibroblasts and a variety of epithelial cells, including airway, corneal, cervical, biliary and intestinal cells [10,36–38], which are target sites of virus infection. TLR3 localizes both on the cell surface and endosomes in fibroblasts, macrophages and some of epithelial cell lines. Cell surface-expressed TLR3 participates in dsRNA recognition, as shown by the finding that an anti-human TLR3 monoclonal antibody (mAb) (TLR3.7) inhibits poly(I:C)-induced IFN-β production by fibroblasts [10]. By contrast, myeloid DCs only express TLR3 intracellularly [35]. Subcellular localization analysis showed that endogenous human TLR3 localizes to the early endosome but not to late endosomes/lysosomes in HeLa cells [39], while transfected human TLR3 predominantly localizes to multivesicular bodies in the mouse B-cell line Ba/F3, in which TLR3 was stably expressed at high levels. In any case, TLR3 signalling arises in the endosomal compartment, requiring endosomal maturation [35]. The 'linker' region consisting of 26 a.a. between the transmembrane domain and the Toll-IL-1 receptor (TIR) domain of TLR3, determines intracellular localization of TLR3 [40,41]. An unidentified molecule associating with the linker region may regulate the endosomal retention of TLR3 in myeloid DCs.

Notably, TLR3 expression is upregulated by viral infection and the exogenous addition of poly(I:C) or type I IFN [42]. The IFN-responsive element is located at approximately −30 bp in the human *TLR3* promoter region [43,44].

Recognition of dsRNA by TLR3

TLR3 recognizes dsRNA through its ectodomain (ECD), which induces receptor dimerization required for adaptor-mediated signal transduction [45]. TLR3 consists of an ECD formed by 23 leucine-rich repeats (LRRs) and N- and C-terminal flanking regions, known as the LRR N-terminal (LRR-NT) and C-terminal (LRR-CT) regions, the transmembrane domain and the cytoplasmic TIR domain [46] (Figure 1A). TLR3–ECD possesses 15 putative carbohydrate-binding motifs. Structural analyses

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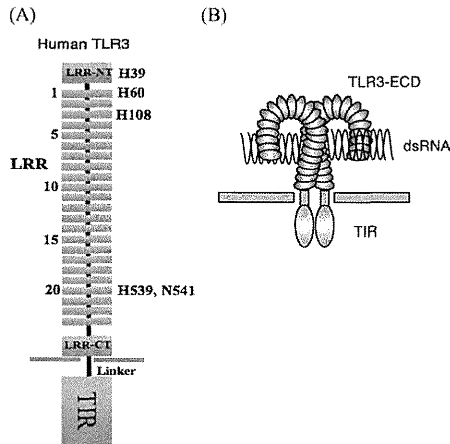

Figure 1. (A) Schematic structure of human TLR3. TLR3 is a type I transmembrane protein of 904 a.a. TLR3 consists of an ECD formed by 23 LRRs and N- and C-terminal flanking regions (LRR-NT and LRR-CT), the transmembrane domain, cytoplasmic linker region and the TIR domain. H539 and N541 in TLR3-LRR20, H39 in the LRR-NT, H60 in LRR1 and H108 in LRR3 are essential for dsRNA-binding. (B) Model of the dsRNA–TLR3-signalling complex. dsRNA interacts with both an N- and a C-terminal binding site on the glycan-free surface of each TLR3–ECD, which are located on opposite sides of the dsRNA (53).

of human TLR3–ECD revealed that the LRRs form a large horseshoe-shaped solenoid of which one face is largely masked by carbohydrate, while the other face is unglycosylated [47,48]. By point mutation analysis, Bell *et al.* [49] demonstrated that the His539 and Asn541 residues in TLR3–LRR20, located on the glycan-free lateral face, are critical amino acids for dsRNA binding and signalling. Wild-type TLR3–ECD protein directly binds poly(I:C) at pH7.6, while mutant proteins H539E and N541A fail to bind poly(I:C). Based on the observation that an acidic pH (pH 6.0 and below) is required for TLR3 recognition of dsRNA, the N-terminal conserved histidine residues, His39 in the LRR-NT, His60 in LRR1 and His108 in LRR3, were identified as a second binding site for dsRNA [50,51]. Protonation of these imidazole groups under acidic conditions, such as those found in endosomes, appears to generate an ionic interaction between the histidine residues and the negatively charged phosphate backbone of dsRNA.

In addition, Leonard *et al.* [52], showed that TLR3–ECD binds as a dimer to 40–50 bp length of dsRNA, and multiple TLR3–ECD dimers bind to long dsRNA strands. Binding affinities increase with both buffer acidity and dsRNA length. At the pH within early endosomes (~6.0–6.5), >90-bp length of dsRNA is required to form a stable complex with TLR3. However, at the pH within late endosomes (~5.5 and below), 40–50-bp length of dsRNA forms stable complex with dimeric TLR3, suggesting that dsRNA-induced TLR3-mediated signalling depends on the length of the dsRNA and the TLR3 localization site [52]. Finally, structural analysis of the complex of two mouse TLR3–ECDs and one 46-bp dsRNA oligonucleotide revealed that dsRNA interacts with both an N- and a C-terminal binding site on the glycan-free surface of each mTLR3–ECD, which are located on opposite sides of the dsRNA [53] (Figure 1B). The dsRNA in the complex retains a typical A-form DNA-like structure. dsRNA has been predicted to adopt a right-handed A-form helix with 11 bp per helical turn and a 28 Å helical pitch [54]. Therefore, two helical turns would fit between the N- and C-terminal binding sites of TLR3 [53]. In addition, the two LRR-CT domains are brought into proximity and form a series of protein–protein interactions, which facilitate the dimerization of the cytoplasmic TIR domain. Funami *et al.* [40], reported that the Phe732, Leu742 and Gly743 residues in the TLR3 cytoplasmic linker region are essential for TLR3 signalling, suggesting that the linker region controls the dimerization of the TLR3–TIR domain.

TLR3–TICAM-1-signalling pathway

TLR3 mediates signalling via an adaptor protein, TIR-containing adaptor molecule-1 (TICAM-1; also called TRIF) [55,56] (Figure 2A). TICAM-1 activates the transcription factors IRF-3, NF-κB and AP-1, leading to the induction of type I IFN, cytokine/chemokine production and DC maturation, which then enables the activation of NK cells and CTLs. TLR3 also associates with c-Src tyrosine kinase on endosomes in response to dsRNA [57]. The Src kinase inhibitor markedly inhibits dsRNA-elicited phosphorylation of Akt, a downstream target of phosphatidylinositol 3-kinase (PI3-K). In addition, PI3-K is required for full phosphorylation and activation of IRF-3 by dsRNA [58]. The precise role of c-Src in IRF-3 activation via the PI3-K-Akt pathway requires further elucidation.

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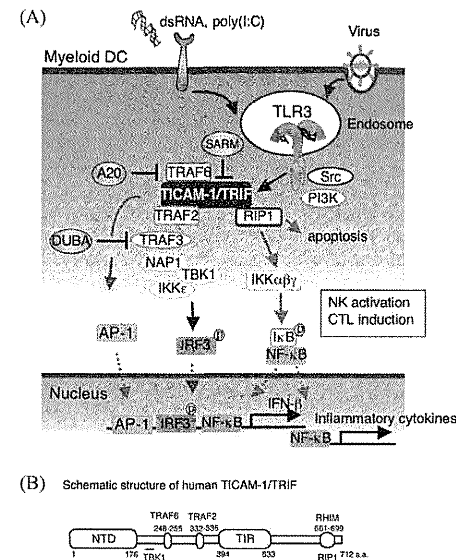

Figure 2. (A) TLR3–TICAM-1-signalling pathway. In myeloid DCs, TLR3 is expressed in the endosomal compartments and recognizes extracellular viral dsRNA and its synthetic analogue poly(I:C). Once TLR3 is dimerized by dsRNA, it recruits the adaptor protein TICAM-1/TRIF that activates the transcription factors, IRF3, NF-κB and AP-1. RIP1 associates with TICAM-1 via the PHIM domain in the C-terminal region and acts as an NF-κB activator and apoptosis mediator in TICAM-1-mediated signalling. TRAF3 and NAP1 participate in the recruitment and activation of the IRF-3 kinases TBK1 and IKKε. Phosphorylated IRF-3 translocates into the nucleus and together with NF-κB and AP-1 induces IFN-β gene transcription. The TICAM-1-mediated AP-1 activation pathway is unclear. (B) Schematic structure of human TICAM-1/TRIF. N-terminal domain (NTD) (1–176 a.a.), TIR domain (394–533 a.a.), RHIM domain (661–699 a.a.), TRAF6-binding site (248–256 a.a.), TRAF2-binding site (332–336 a.a.) and TBK1-binding site (under line) are shown.

TICAM-1 consists of an N-terminal region, a TIR domain and a C-terminal region (Figure 2B). The TIR domain of TICAM-1 is essential for binding to the TIR domain of TLR3 and also to the TLR4 adaptor TICAM-2 (also called TRIF-related adaptor molecule) [59,60]. TICAM-1 is expressed at a low level in most tissues and cells and is diffusely localized in the cytoplasm of resting cells [39]. When endosomal TLR3 is activated by dsRNA, TICAM-1 transiently co-localizes with TLR3, then dissociates from the receptor and forms speckled structures that co-localize with downstream-signalling molecules [39]. Homo-oligomerization through the Pro434 residue in the TIR domain and the C-terminal region is essential for TICAM-1-mediated activation of NF-κB and IRF-3 [61]. Once TICAM-1 is oligomerized, the serine-threonine kinases, TANK-binding kinase 1 (TBK1; also called NAK or T2K) and IkB kinase-related kinase-ε (IKK-ε; also called IKK-i), are activated and phosphorylate IRF-3 [62,63]. The ubiquitin ligase of the TRAF family members, TRAF2, TRAF3 and TRAF6, are downstream-signalling molecules of TICAM-1. TRAF2 and TRAF6 directly bind to the N-terminal region of TICAM-1 [64,65] (Figure 2B). The Lys63-linked autoubiquitination of TRAF3 is required for IRF-3 activation [66,67]. Furthermore, NF-κB-activating kinase (NAK)-associated protein 1 (NAP1) participates in the recruitment of IRF-3 kinases to the N-terminal region of TICAM-1 [68]. Although both TRAF3 and NAP1 associate with oligomerized TICAM-1 and serve as a critical link between TICAM-1 and downstream IRF-3 kinases, there is no evidence that they bind directly to TICAM-1. Interestingly, recent reports showed that direct binding of TBK1 to TICAM-1 is necessary for IRF-3 activation [69]. The Leu194 residue in the N-terminal region is critical for TBK1 binding to TICAM-1. In addition, the Ser189, Arg195 and Ser196 residues are involved in TBK1–TICAM-1 binding.

The N-terminal 176 a.a. of TICAM-1 form a protease-resistant structural domain, designated NTD (Figure 2B). Because the crucial amino acids for TRAF2-, TRAF6- and TBK1-binding reside between the NTD and the TIR domain, naive TICAM-1 may have a closed conformation that covers these binding sites. Indeed, protein–protein interaction analysis revealed that the NTD interacts with the N-terminus of TICAM-1–TIR [69]. Thus, the NTD folds into the TIR domain structure to maintain the naive conformation of TICAM-1. Upon stimulation of TLR3 or TLR4, TICAM-1 oligomerizes through the TIR domain and the C-terminal region, possibly breaking the intramolecular association and inducing a conformational change that allows TBK1 access to TICAM-1.

Whereas the N-terminal region is crucial for TICAM-1-mediated IRF-3 activation, the C-terminal region of TICAM-1 is involved in NF-κB activation and apoptosis. Receptor-interacting protein 1 (RIP1), a kinase containing a death domain, associates with

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TICAM-1 via the RIP homotypic interaction motif domain in the C-terminal region and acts as an NF-κB inducer and apoptosis mediator in TICAM-1-mediated signalling [70–72]. TRAF6 has also been implicated in NF-κB activation by TICAM-1 in a cell-type-dependent manner [64,73].

TLR3–TICAM-1-mediated signalling is negatively regulated by a fifth TIR adaptor protein SARM [74]. SARM and TICAM-1 have been shown to interact and SARM strongly suppresses NF-κB activation, as well as IRF-3 activation by TICAM-1. Moreover, deubiquitinating enzyme A (DUBA) negatively regulates TLR3-mediated type I IFN production. DUBA selectively cleaves the Lys63-linked polyubiquitin chains on TRAF3, resulting in its dissociation from the downstream-signalling molecules [75]. In addition, the ubiquitin-modifying enzyme A20 inhibits TICAM-1-mediated NF-κB activation by deubiquitinating TRAF6 [76]. However, the precise mechanisms by which TRAF3 and TRAF6 are ubiquitinated and their interaction with downstream-signalling molecules are unknown.

Antiviral function of TLR3

The role of TLR3 in viral infection is complex (Table 1). Studies in TLR3-deficient ($TLR3^{-/-}$) mice showed that the immune response to different viruses, including lymphocytic choriomeningitis virus (an ambisense RNA virus), vesicular stomatitis virus (a negative-stranded RNA virus), murine cytomegarovirus (MCMV, a dsDNA virus) and reovirus (a dsRNA virus), was unaffected in these mutant mice compared with wild-type mice [77].

By contrast, Hardarson *et al.* [78] reported that TLR3 is important in host defense against encephalomyocarditis virus (EMCV, a positive sense ssRNA virus belonging to the *Picornaviridae* family). When mice were inoculated intraperitoneally with 50 plaque-forming units EMCV, $TLR3^{-/-}$ mice were more susceptible to EMCV infection and had a significantly high viral load in the heart compared with wild-type mice. Opposing to these data, Kato *et al.* [24] showed that MDA5 but not TLR3 plays an important role in host defense against EMCV infection, when mice were infected with 100 plaque-forming units EMCV intraperitoneally. It is unclear why these different results were obtained from similar EMCV infection studies.

Table 1. The role of TLR3 in antiviral responses

	References
Protection	
Flaviviridae [+, ss]	
West Nile virus (WNV)	[84]
Picornaviridae [+, ss]	
Encephalomyocarditis virus (EMCV)	[78]
Poliovirus	[79,80]
Coxsackievirus group B serotype 3 (CVB3)	[82]
Herpesviridae [dsDNA]	
Murine cytomegarovirus (MCMV)	[90]
Herpes simplex virus 1 (HSV-1)	[101]
Deterioration	
Flaviviridae [+, ss]	
West Nile virus (WNV)	[83]
Orthomyxoviridae [−, ss]	
Influenza A virus (IAV)	[88]
Bunyaviridae [−, ss]	
Phlebovirus	[89]

More recently, the essential role of the TLR3–TICAM-1 pathway in protection from poliovirus infection, a virus belonging to the *Picornaviridae* family, has been demonstrated [79,80]. Poliovirus receptor (PVR)-transgenic/TICAM-1-deficient mice are more susceptible than PVR-transgenic mice to intraperitoneal or intravenous inoculation with a low titre of poliovirus [79,80]. Forty-eight hours after infection, virus titres in serum dramatically increased and mortality greatly decreased compared with PVR-transgenic or PVR-transgenic/IPS-1 (RLR adaptor)-deficient mice. It is well known that in cultured mammalian cells, poliovirus infection results in inhibition of cellular protein synthesis so-called 'shut-off' event [81]. Therefore, mRNA upregulation of RIG-I and MDA5 by type I IFN does not link to protein synthesis at an early stage of virus infection. Thus,

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it appears that the inhibitory effects of viral multiplication on host cells depend on the TLR3–TICAM-1 pathway, but not the RLR-IPS-1 pathway.

In addition, Negishi *et al.* [82] showed that $TLR3^{-/-}$ mice are more vulnerable to coxsackievirus group B serotype 3 (CVB3, a virus belonging to the *Picornaviridae* family) than wild-type mice, in terms of higher mortality and acute myocarditis. The expression of IL-12p40, IL-1β and IFN-γ mRNAs, but not IFN-β mRNA, was impaired in the hearts of CVB3-infected TLR3-deficient mice compared with those of wild-type mice infected with CVB3. By contrast, expression of TLR3 by transgene protects mice from lethal CVB3 infection and hepatitis even in the absence of type I IFN signalling. Antibody blocking studies revealed that TLR3–TICAM-1-dependent type II IFN (IFN-γ) production is critical for host defense against CVB3 infection [82].

Remarkably, Wang *et al.* [83] demonstrated that TLR3 is involved in the viral pathogenesis of West Nile virus (WNV, a positive-stranded RNA virus). $TLR3^{-/-}$ mice showed impaired cytokine production and enhanced viral loads in the periphery, whereas in the brain, the viral load, inflammatory responses and neuropathology were reduced compared with wild-type mice [83]. TLR3-mediated peripheral inflammatory cytokine production is critical for disruption of the blood–brain barrier, which facilitates viral entry into the brain causing lethal encephalitis. Therefore, $TLR3^{-/-}$ mice are more resistant to lethal WNV infection. In contrast, Daffis *et al.* [84] reported the protective role of TLR3 in sublethal WNV infection. The absence of TLR3 enhances WNV mortality in mice and increases viral burden in the brain after inoculation with the pathogenic New York strain of WNV, although there are little differences in WNV-specific antibody responses, $CD8^+$ T-cell activation, blood–brain barrier permeability and IFN-α/β induction in draining lymph nodes and serum, between wild-type and $TLR3^{-/-}$ mice [84]. The reason why TLR3 shows the opposite function against WNV infection remains to be determined.

In other RNA viral infections such as respiratory syncytial virus, IAV and phlebovirus (all negative-stranded RNA viruses), TLR3-dependent inflammatory cytokine and chemokine production also appears to affect virus-induced pathology and host survival [85–89]. $TLR3^{-/-}$ mice infected with IAV exhibited reduced inflammatory mediators, leading to increased survival [88]. It is notable that experimental conditions using high viral doses may lead to the over-production of inflammatory cytokines and chemokines. However, what type of TLR3-expressing cells that respond to virus-derived dsRNA *in vivo* has not been shown in these studies.

Cellular immunity induced by the TLR3–TICAM-1 pathway

In addition to type I IFNs, CTLs and NK cells are also principal effector cells in antiviral immunity. The contribution of TLR3 to antiviral responses has been shown in MCMV infection [90], during which virus clearance is partly dependent on NK cell activation. $TLR3^{-/-}$ mice are hypersusceptible to MCMV infection. Cytokine (type I IFN, IL-12p40 and IFN-γ) production, and NK cell and NKT cell activation are impaired in $TLR3^{-/-}$ mice compared with wild-type mice.

Selective TLR3 expression in myeloid DCs but not in pDCs raises the possibility that TLR3 also plays a key role in the antiviral response by induction of adaptive immune responses rather than primary IFN-α/β production (Table 2). Myeloid DCs are the most effective professional antigen-presenting cells, possessing several antigen processing and transporting pathways [91,92]. One of the most notable features of myeloid DCs is the cross-presentation of exogenous antigens to $CD8^+$ T cells. This pathway is important for effective host CTL induction against viruses that do not directly infect DCs. Among the myeloid DC subsets, the splenic $CD8\alpha^+$ DC subset in mice and the $CD141(BDCA3)^+DNGR\text{-}1(CLEC9A)^+$ DC subset in humans highly express TLR3 and display a superior capacity for cross-presenting apoptotic and necrotic cell antigens after TLR3 stimulation [93–97]. Using TLR3-deficient mice, Schultz *et al.* [98] clearly showed that TLR3 plays an important role in cross-priming. Mouse $CD8\alpha^+$ DCs are activated by phagocytosis of apoptotic bodies from virally infected cells or cells containing poly(I:C) in a TLR3-dependent manner. Furthermore, immunization with virally infected cells or cells containing poly(I:C), both carrying ovalbumin antigen, induces ovalbumin-specific $CD8^+$ T-cell responses, which are largely dependent on TLR3-expressing DCs [98]. In many cases, virally infected cells produce IFN-α/β which activates DCs to

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Table 2. Expression of nucleic acid-sensing TLRs in DC subsets

	DC subset	TLR3	TLR7	TLR8	TLR9	References
Human	Myeloid DC					
	MoDC	+	−	+	−	[31–35]
	$CD11c^+CD1c^+DC$	+	−	+	−	[34,35,94,95]
	$CD141^+CLEC9A^+DC$	++	−	+	−	[94,95]
	Plasmacytoid DC	−	+	−	+	[34,35]
Mouse	Myeloid DC					
	BMDC	+	−	−	+	[95]
	$CD8\alpha^+$ DC	++	−	−	+	[93,95]
	Plasmacytoid DC	−	+	−	+	[93,95]

MoDC, monocyte-derived immature dendritic cells; BMDC, bone marrow-derived DC.

promote $CD8^+$ T-cell cross-priming [99]. Thus, both TLR3- and IFN-α/β-mediated signalling are likely implicated in licensing DCs for the cross-priming of $CD8^+$ T cells.

In humans, Ebihara *et al.* [100] demonstrated the role of TLR3, expressed in myeloid DCs, in the immune response to HCV infection. The JFH1 strain of HCV does not directly infect or stimulate myeloid DCs to activate T cells and NK cells, but instead the phagocytosis of HCV-infected apoptotic cells that contain HCV-derived dsRNA and their interaction with the TLR3 pathway in myeloid DCs, plays a critical role in DC maturation and activation of T and NK cells [100]. In addition, Jongbloed *et al.* [94] reported that $CD141^+$ DCs are able to cross-present viral antigens from human cytomegalovirus-infected necrotic fibroblasts. Physiologically, TLR3 in a DC subset specialized for antigen presentation appears to encounter viral dsRNAs in the endosome by uptake of apoptotic or necrotic virus-infected cells and signals for cross-presentation of viral antigens. Furthermore, a dominant-negative TLR3 allele was found in children with herpes simplex virus 1 (HSV-1) encephalitis [101]. TLR3 is expressed in the central nervous system, where it is required to control HSV-1. Interestingly, recent paper demonstrated that mouse $CD8\alpha^+$ DCs and human $CD141^+$ DCs are major producers of IFN-λ in response to poly(I:C), which depends on TLR3 [102]. Thus, TLR3 plays a role in the antiviral response, dependent on the viral genome structure, the route of virus entry into cells, the TLR3-expressing cell type that encounters viral dsRNA, and the properties of the host anti-viral effector functions.

Application of the TLR3 ligand to adjuvant vaccine therapy

Selective expression of TLR3 in myeloid DCs, especially human $CD141^+$ DCs and mouse $CD8\alpha^+$ DC subsets, is the advantage in employing TLR3 ligands as adjuvant. In addition to the TLR3-dependent CTL activation described above, DC-mediated NK cell activation is also important for the adjuvancy of TLR3 ligands. Akazawa *et al.* [103] showed that the TLR3–TICAM-1 pathway is essential for poly(I:C)-induced NK-cell-mediated tumour regression in a syngeneic mouse tumour implant model. Remarkably, production of IFN-α is not impaired in TICAM-$1^{-/-}$ mice compared with wild-type mice after *in vivo* poly(I:C) injection or *in vitro* bone marrow-derived DC (BMDC) stimulation, whereas IL-12 production is completely dependent on TICAM-1, consistent with other reports [22,104]. Furthermore, NK cell activation requires cell–cell contact with BMDCs preactivated by poly(I:C) but not IFN-α or IL-12. Thus, the TLR3–TICAM-1 pathway in myeloid DCs facilitates the DC–NK cell interaction following NK cell activation. TICAM-1–IRF3-dependent expression of a novel molecule, namely IRF-3-dependent NK-activating molecule (INAM), in myeloid DCs is required for NK activation [104]. Poly(I:C)-induced MDA5-dependent myeloid DC activation is also implicated in NK cell activation [105,106].

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However, several issues remain unresolved including a suitable transport system for TLR3 ligands. Poly(I:C) injected intraperitoneally in mice activates both TLR3 and MDA5, indicating that extracellular poly(I:C) is delivered to endosomal TLR3 and further to cytosolic MDA5 in murine cells. A recent study demonstrated that CD14 enhances poly(I:C)-mediated TLR3 activation in bone marrow-derived macrophages by directly binding to poly(I:C) and mediating cellular uptake of poly(I:C) [107]. The internalized poly(I:C) then colocalizes with CD14 and TLR3. Since the extracellular domain of CD14 consists of LRRs [108], CD14 may associate with TLR3 and transfer poly(I:C) to TLR3 in macrophage endosomes. In the case of CD14-negative myeloid DCs, extracellular dsRNA must be internalized with the putative uptake receptor. Indeed, it has been demonstrated by our group and others that poly(I:C) is internalized into human monocyte-derived immature DCs and mouse BMDCs via clathrin-dependent endocytosis, and B- and C-type oligodeoxynucleotides share the uptake receptor with poly(I:C) [109]. Notably, among various synthetic dsRNAs, poly(I:C) is preferentially internalized and activates TLR3 in myeloid DCs. By contrast, *in vitro*-transcribed dsRNAs of various lengths (50–1000 bp) cannot be internalized into myeloid DCs [110]. Thus, uptake of TLR3 ligands largely depends on the dsRNA structure recognized by the uptake receptor expressed on myeloid DCs.

The dsRNA structure and the targeting approach of dsRNA to the endosomal TLR3 in the appropriate DC subset, are important factors involved in generating innate and adaptive immune responses by TLR3 ligands. Gowen *et al.* [111] showed that poly(C_{12}U) induces IFN-β in a TLR3-dependent and MDA5-independent manner, and exhibits protective anti-viral effects in mice. Identification of the putative dsRNA uptake receptor is crucial for analysing the intracellular transport of dsRNA. Furthermore, clarification of the differences between the RIG-I/MDA5-mediated and TLR3–TICAM-1-mediated signalling pathways is important for assessment of dsRNA-induced immune responses.

Concluding remarks

The protective role of TLR3 in virus infection is now becoming clear from experiments using an infectious mouse model or TLR3-transgenic mice. Since both CVB3 and poliovirus belong to the *Picornaviridae* family, along with encephalomyocarditis virus that is recognized by MDA5, distinct virus properties rather than virus genome structure appear to determine which RNA sensors act in antiviral defense in host cells. The molecular mechanism behind the anti-viral function of TLR3 *in vivo* and the identification of TLR3–TICAM-1-mediated signalling cascades distinct from those of RIG-I/MDA5, are important factors for understanding the role of RNA-sensors in the host defense system. In addition, characterization of a new myeloid DC subset that expresses a high level of TLR3 and has a high capacity to present antigen from apoptotic and necrotic cells after TLR3 activation, may provide insight into the role of TLR3 in the activation of NK cells and CTLs in viral infection. This, in turn, may advance the development of TLR3-related vaccine adjuvants effective against tumours and/or infectious diseases.

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