Table 6 Changes in hepatic fatty acid composition

Fatty acid	Control		p^a	Ezetimibe		p^a	p^b
	Before	After		Before	After		
C12:0 (lauric acid)	7.7±1.2	15.2±5.6	0.219	6.3±1.8	18.8±4.7	0.019	0.494
C14:0 (myristic acid)	19.9±2.5	33.0±10.1	0.228	17.6±2.2	56.6±13.0	0.014	0.148
C16:0 (palmitic acid)	185.9±23.8	303.9±118.2	0.334	169.7±22.9	583.9±176.8	0.042	0.202
C16:1n-7 (palmitoleic acid)	24.2±4.5	37.3±13.4	0.362	22.3±4.3	51.9±13.2	0.031	0.368
C17:0 (margaric acid)	4.6±0.7	3.5±0.1	0.400	5.3±0.8	16.0±4.1	0.024	0.025
C18:0 (stearic acid)	45.9±4.4	54.4±8.9	0.283	56.0±7.1	125.1±30.2	0.017	0.042
C18:1n-9 (oleic acid)	166.4±25.1	250.2±91.6	0.367	173.9±30.6	381.9±84.3	0.017	0.288
C18:2n-6 (linoleic acid)	80.4±12.3	87.9±22.5	0.556	73.9±8.5	147.3±36.1	0.035	0.066
C18:3n-6 (γ-linolenic acid)	ND	ND		ND	ND		
C18:3n-3 (α-linolenic acid)	0.6±0.4	0.0±0.0	0.171	0.6±0.4	0.0±0.0	0.178	0.981
C20:0n-6 (arachidic acid)	ND	ND		ND	ND		
C20:1n-9 (eicosenoic acid)	5.5±1.1	4.7±1.9	0.639	5.7±1.0	13.1±4.8	0.170	0.168
C20:2n-6 (eicosadienoic acid)	ND	ND		ND	ND		
C20:3n-6 (dihomo-γ-linolenic acid)	ND	ND		ND	ND		
C20:3n-9 (eicosatrienoic acid)	ND	ND		ND	ND		
C20:4n-6 (arachidonic acid)	ND	ND		ND	ND		
C20:5n-3 (eicosapentaenoic acid)	ND	ND		ND	ND		
C22:0 (behenic acid)	ND	ND		ND	ND		
C22:1n-9 (erucic acid)	14.2±2.5	11.7±2.7	0.474	16.2±2.4	19.2±1.0	0.664	0.468
C22:2n-6 (docosadienoic acid)	2.8±1.0	1.8±1.0	0.433	22.3±0.7	62.3±2.9	0.176	0.152
C22:4n-6 (docosatetraenoic acid)	ND	ND		ND	ND		
C22:5n-3 (docosapentaenoic acid)	ND	ND		ND	ND		
C22:6n-3 (docosahexaenoic acid)	13.6±3.5	7.8±3.3	0.232	14.2±3.7	48.7±19.9	0.109	0.097
C24:1 (nervonic acid)	ND	ND		ND	ND		

The data are expressed as 10^{-4} mg/mg liver, means ± SE

[a] p value for the intragroup comparison (baseline vs 6 month)

[b] p value for the intergroup comparison (changes from baseline between groups)

ND, not determined

genes involved in the L-carnitine pathway, including *CPT1A*, were coordinately downregulated. A decreased L-carnitine pathway could be associated with reduced β-oxidation of palmitic acids in mitochondria, resulting in an increase in long-chain fatty acids (lauric, myristic, palmitic, palmitoleic, margaric, stearic, oleic and linoleic acids). Unbalanced fatty acid composition could induce oxidative stress and lead to insulin resistance in the ezetimibe group. In addition, genes involved in the cholesterol and NEFA biosynthesis, including *SREBF2*, were coordinately upregulated in the ezetimibe group (Table 3), probably as a result of deceased absorption of exogenous cholesterol. Upregulation of *SREBF2* potentially represses the expression of hepatocyte nuclear factor 4, which is required for *CPT1* transcription [35]. Moreover, recent reports have demonstrated that microRNA (miR)-33, encoded by an intron of *Srebp2* [36], inhibits translation of transcripts involved in fatty acid β-oxidation, including *CPT1* [37]. miR-33 is also implicated in decreased insulin signalling by reducing insulin receptor substrate-2 [38, 39]. Hepatic gene expression profiles may, to some extent, explain hepatic fatty acid composition and impaired glycaemic control in the ezetimibe group. These novel SREBP-2-mediated pathways in the gene expression network may be relevant to a recent report that a polymorphism in the *SREBF2* predicts incidence and the severity NAFLD and the associated glucose and lipid dysmetabolism [15]. These unique hypotheses should be confirmed in future in vitro and in vivo studies.

Our study has some limitations. First, the number of patients is relatively small because the data and safety monitoring board recommended that the study intervention and enrolment be discontinued in light of the higher proportion of adverse events in the ezetimibe group than in the control group. Second, our trial was a 6 month open-label study that resulted in subtle changes in liver pathology compared with previous reports [40]. Indeed, a 6 month duration may be too short a period to expect improvement of fibrosis, which is a slowly

progressive process [40]. Third, the average serum aminotransferase levels were lower than those in previous studies [9, 10], and most of the patients had mild steatosis, fibrosis and lower NAS at baseline before ezetimibe treatment. Serum ALT levels did not decrease with ezetimibe treatment in the present study, in contrast to the significant improvement reported previously [9, 10]. And finally, secondary outcomes are always at risk of false-positive associations. Therefore, we not only presented the changes in HbA_{1c} ($p=0.001$ for ezetimibe treatment and $p=0.041$ for the intergroup difference at the end of the study), but also showed the signature of hepatic fatty acid composition and hepatic gene expression profiles that support the hypothesis that ezetimibe increases HbA_{1c} and hepatic fatty acids contents possibly through the SREBP-2–miR33 pathway. No previous studies have raised this issue, which is worth investigating. The same mechanism may underlie a statin-induced deterioration of glucose tolerance, which remains a serious concern. Furthermore, the SREBP-2–miR33 pathway may raise a concern for a safety issue of combination therapy with ezetimibe and statins because these agents may additively upregulate *SREBF2* expression [41]. Future large-scale, long-duration studies involving more severely affected patients are required to determine the definite efficacy and risks of ezetimibe in the treatment of NAFLD.

In conclusion, the present study represents the first randomised controlled clinical trial of the efficacy of ezetimibe on liver pathology, energy homeostasis, hepatic fatty acid composition and hepatic gene expression profiles in patients with NAFLD. The lipid profile and liver histology of cell ballooning and fibrosis were significantly improved by ezetimibe treatment. However, our findings suggest an increase in oxidative stress, insulin resistance and HbA_{1c} on treatment with ezetimibe, which should be taken into consideration in NAFLD patients.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement YT designed the study, recruited the patients, analysed the data and wrote the manuscript. TT designed the study, recruited the patients, interpreted the data and edited the manuscript. MH analysed the hepatic gene expression profiles. YK performed the statistical analyses. YZ analysed all the biopsies. KK, HM and TO recruited the patients and collected the clinical information. HS, KA and TY performed the liver biopsies and histological examinations. MN performed the DNA chip experiments. KY and EM analysed the hepatic fatty acid compositions. SK initiated and organised the study. All authors contributed to the acquisition, analysis and interpretation of data and the drafting and editing of the manuscript. All of the authors approved the final version of the manuscript.

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Hepatic Interferon-Stimulated Genes Are Differentially Regulated in the Liver of Chronic Hepatitis C Patients With Different Interleukin-28B Genotypes

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Pretreatment up-regulation of hepatic interferon (IFN)-stimulated genes (ISGs) has a stronger association with the treatment-resistant interleukin (IL)28B minor genotype (MI; TG/GG at rs8099917) than with the treatment-sensitive IL28B major genotype (MA; TT at rs8099917). We compared the expression of ISGs in the liver and blood of 146 patients with chronic hepatitis C who received pegylated IFN and ribavirin combination therapy. Gene expression profiles in the liver and blood of 85 patients were analyzed using an Affymetrix GeneChip (Affymetrix, Santa Clara, CA). ISG expression was correlated between the liver and blood of the MA patients, whereas no correlation was observed in the MI patients. This loss of correlation was the result of the impaired infiltration of immune cells into the liver lobules of MI patients, as demonstrated by regional gene expression analysis in liver lobules and portal areas using laser capture microdissection and immunohistochemical staining. Despite having lower levels of immune cells, hepatic ISGs were up-regulated in the liver of MI patients and they were found to be regulated by multiple factors, namely, IL28A/B, IFN-λ4, and wingless-related MMTV integration site 5A (WNT5A). Interestingly, WNT5A induced the expression of ISGs, but also increased hepatitis C virus replication by inducing the expression of the stress granule protein, GTPase-activating protein (SH3 domain)-binding protein 1 (G3BP1), in the Huh-7 cell line. In the liver, the expression of WNT5A and its receptor, frizzled family receptor 5, was significantly correlated with G3BP1. *Conclusions:* Immune cells were lost and induced the expression of other inflammatory mediators, such as WNT5A, in the liver of IL28B minor genotype patients. This might be related to the high level of hepatic ISG expression in these patients and the treatment-resistant phenotype of the IL28B minor genotype. (HEPATOLOGY 2014;59:828-838)

Interferon (IFN) and ribavirin (RBV) combination therapy has been a popular modality for treating patients with chronic hepatitis C (CHC); however, ~50% of patients usually relapse, particularly those with hepatitis C virus (HCV) genotype 1b and a high viral load.[1] The recently developed direct-acting antiviral drug, telaprevir, combined with pegylated (Peg)-IFN plus RBV, significantly improved sustained virologic response (SVR) rates; however, the SVR rate was not satisfactory (29%-33%) in patients who had no

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCL, CC chemokine ligand; CHC, chronic hepatitis C; CLLs, cells in liver lobules; CPAs, cells in portal areas; CXCL10/IP-10, chemokine (C-X-C motif) ligand 10/interferon-gamma-induced protein 10; CXCR3, chemokine (C-X-C motif) receptor 3; DCs, dendritic cells; DVL, disheveled; FZD5, frizzled family receptor 5; G3BP1, GTPase-activating protein (SH3 domain)-binding protein 1; GGT, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IFN, interferon; IHC, immunohistochemical; IL, interleukin; ISGs, interferon-stimulated genes; JFH-1, Japanese fulminant hepatitis type 1; LCM, laser capture microdissection; MA, major genotype; MAd, major genotype, down-regulated; MAu, major genotype, up-regulated; MI, minor genotype; Mx, myxovirus (influenza virus) resistance; NK, natural killer; OAS2, 2′-5′-oligoadenylate synthetase 2; PALT, portal-tract-associated lymphoid tissue; Peg_IFN, pegylated IFN; RBV, ribavirin; RTD-PCR, real-time detection polymerase chain reaction; SG, stress granule; siRNA, small interfering RNA; SVR, sustained virologic response; WNT5A, wingless-related MMTV integration site 5A.

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response to previous therapy.[2] Therefore, IFN responsiveness is still an essential clinical determinant for treatment response to triple (Peg-IFN+RBV+DAA) therapy.

A recent landmark genome-wide association study identified a polymorphism in the interleukin (IL)28B, IFN-λ3) gene that was associated with either a sensitive (major genotype; MA) or resistant (minor genotype; MI) treatment response to Peg-IFN and RBV combination therapy and was characterized by either up- (-u) or down-regulation (-d) of interferon-stimulated genes (ISGs).[3-5] However, the underlying mechanism for the association of this polymorphism and treatment response has not been clarified. Previously, we showed that up-regulation of the pretreatment expression of hepatic ISGs was associated with an unfavorable treatment outcome and was closely related to the treatment-resistant IL28B genotype (TG or GG at rs8099917).[6] It could be speculated that the pretreatment activation of ISGs would repress additional induction of ISGs after treatment with exogenous IFN. However, it is unknown how hepatic ISGs are up-regulated in treatment-resistant CHC patients and why patients with high levels of ISG expression cannot eliminate HCV. Therefore, other mechanisms should be involved in the unfavorable treatment outcome of patients with the treatment-resistant IL28B genotype.

In the present study, we performed gene expression profiling in the liver and blood and compared the expression of ISGs between them. Furthermore, ISG expression in liver lobules and portal areas was analyzed separately using a laser capture microdissection (LCM) method. Finally, we identified an immune factor that is up-regulated in patients with the treatment-resistant IL28B genotype and mediates favorable signaling for HCV replication.

Materials and Methods

Patients. We analyzed 168 patients with CHC who had received Peg-IFN-α2b (Schering-Plough K.K., Tokyo, Japan) and RBV combination therapy for 48 weeks at the Graduate School of Medicine, Kanazawa University Hospital, Japan and its related hospitals, as reported previously (Table 1 and Supporting Table 1).[6]

Preparation of Liver Tissue and Blood Samples. A liver biopsy was performed on samples from 168 patients, and blood samples were obtained from 146 of these patients before starting therapy (Table 1 and Supporting Table 1). Detailed procedures are described in the Supporting Materials and Methods.

Affymetrix GeneChip Analysis. Liver tissue samples from 91 patients and blood samples from 85 patients were analyzed using an Affymetrix GeneChip (Affymetrix, Santa Clara, CA). LCM analysis was performed in 5 MAu, MAd, and MI patients. Affymetrix GeneChip analysis and LCM were performed, as described previously.[6,7] Detailed procedures are described in the Supporting Materials and Methods.

Hierarchical Clustering and Pathway Analysis of GeneChip Data. GeneChip data analysis was performed using BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.htm), as described previously.[7] Pathway analysis was performed using MetaCore (Thomson Reuters, New York, NY). Detailed procedures are described in the Supporting Materials and Methods.

Quantitative Real-Time Detection Polymerase Chain Reaction, Cell Lines, Cell Migration Assay, Vector Preparation, HCV Replication Analysis, and Statistical Analysis. These procedures are described in detail in the Supplemental Material and Methods.

Results

Differential ISG Expression in Liver and Blood of Patients With Different IL28B Genotypes. Previously, we showed that pretreatment up-regulation of hepatic ISGs was associated with an unfavorable treatment outcome and was closely related to the treatment-resistant IL28B MI (TG or GG at rs8099917).[6] To examine whether expression of hepatic ISGs would reflect the expression of blood ISGs, we compared ISG expression between the liver and blood. We utilized three ISGs (interferon-induced protein 44 [IFI44], interferon-induced protein with

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Additional Supporting Information may be found in the online version of this article.

Table 1. Clinical Characteristics of 146 Patients Whose Liver and Blood Samples Were Analyzed by RT-PCR

	Major (MA)				Minor (MI)		
Clinical Category	Major ISG Up (MAu)		Major ISG Down (MAd)				*P* Value
No. of patients	n = 42		n = 68		n = 36		NA
Age and sex							
Age (years)	55	(30-72)	56	(31-72)	55	(30-73)	NS
Sex (M vs. F)	27 vs. 15		34 vs. 34		19 vs. 17		NS
Treatment responses							
SVR/TR/NR	24/12/6		30/33/6		6/7/23*		MAu vs. MI < 0.0001, MAd vs. MI < 0.0001
IL28B genotype (TT vs. TG+GG)	TT		TT		TG/GG (31/5)		NA
Liver factors							
F stage (1/2/3/4)	14/13/11/4		30/20/11/7		14/8/10/4		NS
A grade (A0-1 vs. A2-3)	16 vs. 26		37 vs. 31		20 vs. 16		NS
ISGs (Mx1, IFI44, IFIT1)	3.83*	(2.14-9.48)	1.30*	(0.36-2.08)	5.52*	(0.86-17.3)	MAu vs. MAd < 0.0001, MAu vs. MI < 0.0001, MAd vs. MI < 0.0001
IL28A/B	41.3*	(4-151)	11.7*	(1-53)	22.7*	(3-93)	MAu vs. MAd < 0.0001, MAu vs. MI = 0.0004, MAd vs. MI = 0.031
Blood factors							
ISGs (Mx1, IFI44, IFIT1)	11.1*	(2.78-24.9)	4.76	(0.41-20.6)	5.64	(0.71-2.8)	MAu vs. MAd < 0.0001, MAu vs. MI < 0.0001
IL28A/B	1.6	(0.1-7.7)	1.3	(0.2-6.4)	1.3	(0.3-3.6)	NS
Laboratory parameters							
HCV-RNA (KIU/mL)	2,430	(160-5,000)	2,692	(140-5,000)	1,854*	(126-5,000)	MAd vs. MI = 0.017
BMI (kg/m2)	24	(18.7-31.9)	24	(16.3-34.7)	22.8	(19.1-30.5)	NS
AST (IU/L)	86*	(22-258)	54	(18-192)	64	(21-178)	MAu vs. MAd = 0.0008
ALT (IU/L)	112*	(17-376)	75	(16-345)	79	(18-236)	MAu vs. MAd = 0.023
γ-GTP (IU/L)	99*	(21-392)	47	(4-367)	74	(20-298)	MAu vs. MAd = 0.0003
WBC (/mm3)	4,761	(2,100-8,100)	4,982	(2,800-9,100)	4,823	(2,500-8,200)	NS
Hb (g/dL)	14.1	(11.4-16.7)	14.1	(9.3-16.9)	13.9	(11.2-16.4)	NS
PLT (× 104/mm3)	15.2	(9.2-27.8)	16.8	(7-39.4)	16.3	(9-27.8)	NS
TG (mg/dL)	112	(42-248)	102	(42-260)	136*	(30-323)	MAd vs. MI = 0.02
T-Chol (mg/dL)	162	(90-221)	169	(107-229)	167	(81-237)	NS
LDL-Chol (mg/dL)	77	(36-123)	83*	(42-134)	72	(29-107)	MAd vs. MI = 0.04
HDL-Chol (mg/dL)	40	(18-67)	43	(27-71)	47*	(27-82)	NS
Viral factors							
ISDR mutations ≦ 1 vs. ≧ 2	23 vs. 19*		51 vs. 17		26 vs. 10		MAu vs. MAd = 0.02
Core aa 70 (wild-type vs. mutant)	24 vs. 18		42 vs. 22		16 vs. 20*		MAd vs. MI = 0.02

*$P < 0.05$.

Abbreviations: BMI, body mass index; ALT, alanine aminotransferase; WBC, leukocytes; Hb, hemoglobin; PLT, platelets; TG, triglycerides; T-chol, total cholesterol; LDL-chol, low density lipoprotein cholesterol; HDL-chol, high density lipoprotein cholesterol; NA, not applicable; NS, not significant.

tetratricopeptide repeats 1 [IFIT1], and myxovirus (influenza virus) resistance [Mx1]) with a high dynamic range, comparable relative expression, and good predictive performance.[6] Mean values of the three ISGs detected by real-time detection polymerase chain reaction (RTD-PCR) in 168 liver tissue samples (Supporting Table 1) showed a significant up-regulation of their expression in nonresponder or treatment-resistant IL28B MI (TG/GG; rs8099917) patients, compared to responder (SVR+TR) or treatment-sensitive IL28B MA (TT; rs8099917) patients, as reported previously (Fig. 1A and Supporting Fig. 1A).[6] However, ISG expression in 146 blood samples (Table 1) showed no difference between responders and nonresponders or the IL28B major and minor genotypes (Fig. 1B and Supporting Fig. 1B). To explore these findings further, gene expression profiling using Affymetrix GeneChips was performed on liver and blood samples from 85 patients (Supporting Tables 2 and 3), and the expression of 37 representative ISGs[6] was compared (Fig. 1C-E). MA patients were divided into two groups according to their ISG expression pattern in the liver: MAu and MAd. MI patients expressed ISGs at a higher level than MAu patients. Interestingly, ISG expression in MA patients showed a similar expression pattern in liver and blood, and ISGs were up-regulated in MAu patients and down-regulated in the MAd patients. However, MI patients showed a different ISG expression pattern in liver and blood, where ISGs were up-regulated in the liver, but down-regulated in the blood (Fig. 1C). The correlation of the mean values of the three ISGs (IFI44, IFIT1, and Mx1) between liver and blood from 146 patients demonstrated a significant correlation between values in MA patients (Fig. 1D), whereas no correlation was observed in MI patients (Fig. 1E). Interestingly, ISG expression correlated significantly between liver and blood of responders, but not of nonresponders, in MA and MI patients (Supporting Fig. 1C-F). These results indicate that the correlation of ISG expression in the liver and blood is an important predictor of treatment response.

Clinical Characteristics of IL28B MA Patients With Up- and Down-Regulated ISGs and IL28B MI Patients. From the expression pattern of ISGs and mean values of the three ISGs (IFI44, IFIT1, and Mx1), we could use receiver operating characteristic curve analysis to set a threshold of 2.1-fold to differentiate MAu and MAd patients. Following this criterion, 42 MAu, 68 MAd, and 36 MI patients (total, 146) were grouped (Table 1). Hepatic ISG expression was highest in MI patients, whereas blood ISG expression was highest in MAu patients. Conversely, hepatic IL28A/B (IFN-λ2/3) expression was highest in MAu patients, whereas blood IL28A/B expression showed no difference among the three groups. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (GGT) levels were significantly higher in MAu patients than in MAd patients. Interestingly, serum ALT levels were significantly correlated with ISG expression in MA patients, but not in MI patients (Supporting Fig. 2E,F).

Gene expression profiling in peripheral immune cells showed the presence of active inflammation in MAu patients, whereas the inactive or remissive phase of inflammation was observed in MAd patients. In contrast, monophasic and intermediate inflammation existed in MI patients (Supporting Fig. 3).

Reduced Number of Immune Cells in the Liver Lobules of IL28B MI Patients. To examine the discordant expression of ISGs in liver and blood of MI patients, we performed LCM to collect cells in liver lobules (CLLs) and cells in portal areas (CPAs) separately from each of five liver biopsied samples from MAu, MAd, and MI patients (Fig. 2A). Interestingly, the ISG expression pattern in CLLs from MA patients was similar to that of CPAs, and ISGs were up-regulated in MAu patients and down-regulated in MAd patients. ISG expression in CLLs from the MI patients was different to that in CPAs, and ISGs were up-regulated in CLLs, but down-regulated in CPAs (Fig. 2A). We hypothesized that the discordance of ISG expression between CLLs and CPAs in MI patients might be the result of the lower number of immune cells that infiltrated the liver lobules of these patients. To prove this hypothesis, immunohistochemical (IHC) staining was performed (Fig. 2B). IHC staining showed that IFI44 was strongly expressed in the cytoplasm and nucleus of CLLs from MI patients, whereas it was intermediately expressed in MAu patients and weakly expressed in MAd patients. Interestingly, IFI44 was strongly expressed in CPAs of MAu patients and weakly expressed in CPAs of MAd patients, showing a correlation between expression in CLLs and CPAs of MA patients, whereas ILI44 expression was relatively weak in CPAs, compared with CLLs, in MI patients (Fig. 2B). In the same section of the specimens, there were less CD163-positive monocytes and macrophages in MI patients than in MAu and MAd patients. Similarly, there were fewer CD8-positive T cells in MI patients than in MAu and MAd patients (Fig. 2B). Semiquantitative evaluation of CD163- and CD8-positive lymphocytes in liver lobules showed a significantly lower number of cells in

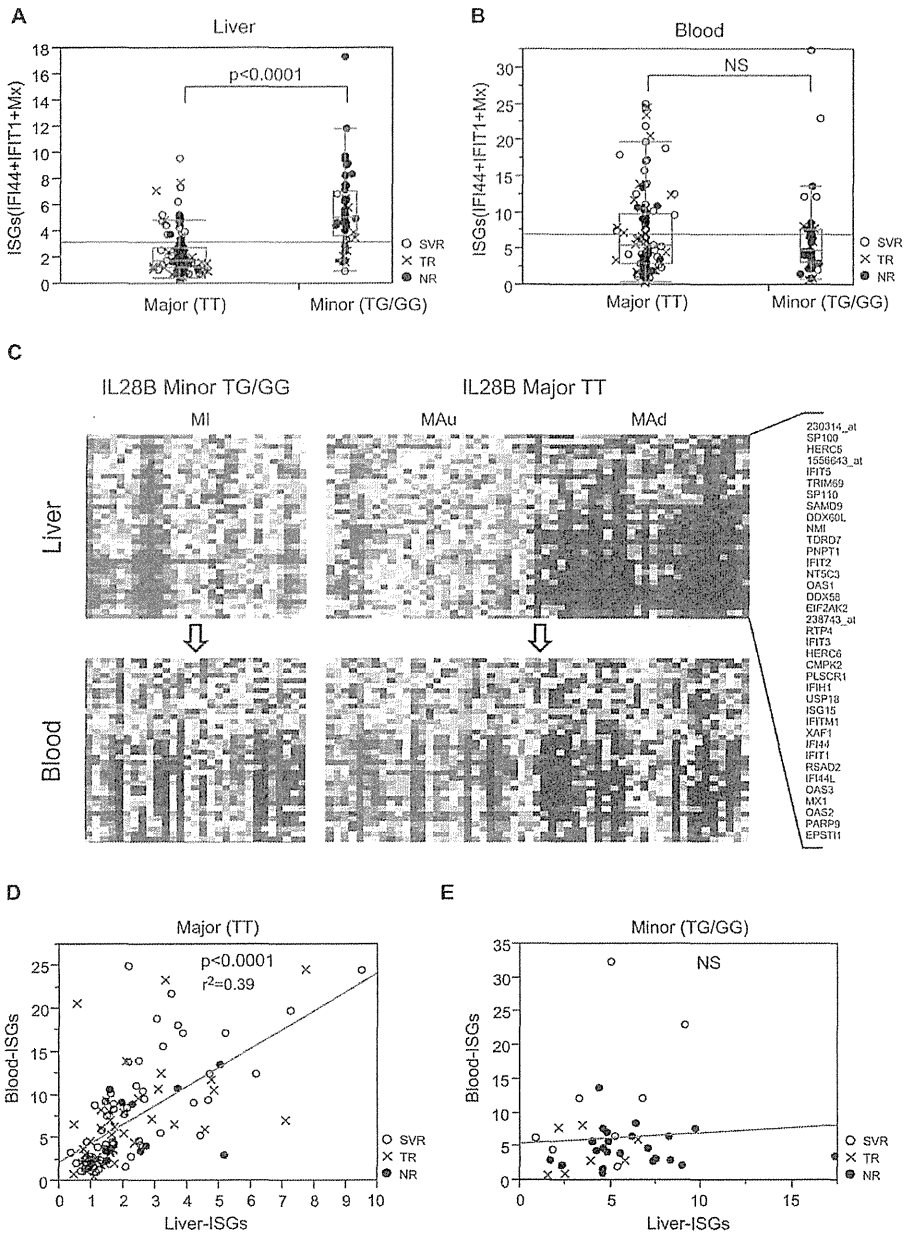

Fig. 1. Comparison of ISG expression in liver and blood of patients with different IL28B genotypes. (A and B) RTD-PCR results of mean ISG expression (IFI44-IFIT1+Mx1) in liver (A) and blood (B) of IL28B major (MAu/Mad) and minor (MI) genotype patients. (C) One-way hierarchical clustering analysis of 85 patients using 37 representative ISGs derived from liver (upper) and blood (lower). (D and E) Correlation of mean ISG expression (IFI44-IFIT1+Mx1) in liver and blood of IL28B major (MA; D) and minor (MI; E) genotype patients.

MI patients than in MAu and MAd patients (Supporting Fig. 4A,B). To support these findings, we examined the expression of 24 surface markers of immune cells in CLL, including dendritic cells (DCs), natural killer (NK) cells, macrophages, T cells, B cells, and granulocytes (Supporting Fig. 5A). The expression of immune cell-surface markers was repressed in MI patients, compared to MAu and MAd patients. Furthermore, whole-liver expression profiling in 85 patients showed the reduced expression of these surface markers in MI patients, compared to MAu and MAd patients (Supporting Fig. 5B). These results indicated that fewer immune cells had infiltrated the liver lobules of MI patients.

In addition to these findings, various chemokines, such as CC chemokine ligand (CCL)19, CCL21, CCL5, and chemokine (C-X-C motif) ligand (CXCL)13, which are important regulators for the recruitment of DCs, NK cells, T cells, and B cells in the liver, were significantly down-regulated in MI patients, compared to MAd and MAu patients (Supporting Fig. 4C-F).

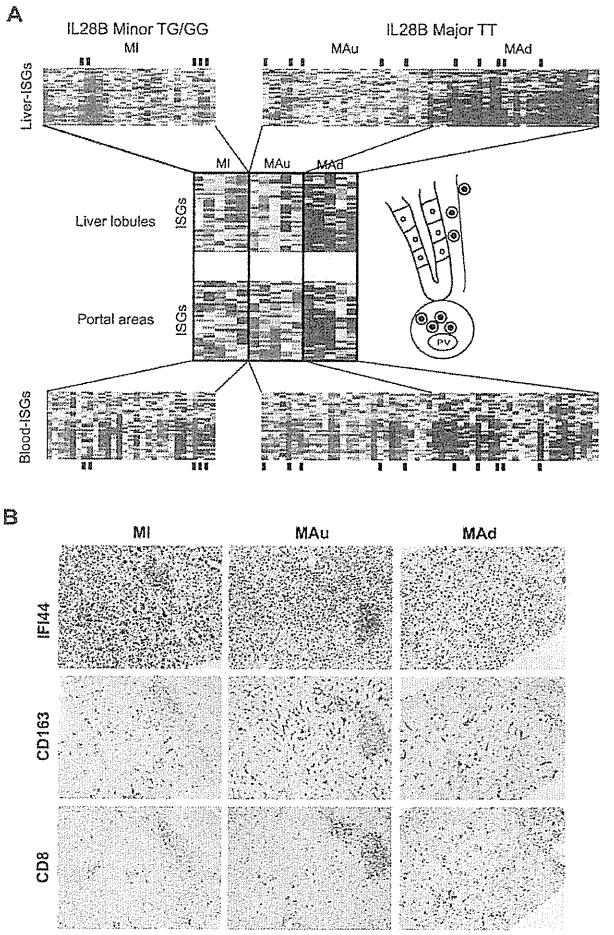

Fig. 2. LCM and IHC staining of biopsied liver specimens. (A) Comparison of the ISG expression pattern of whole liver (upper), CLLs (upper middle), CPAs (lower middle), and blood (bottom). CLLs and CPAs were obtained from 5 MI, MAu, and MAd patients, who are indicated by small black bars. (B) IHC staining of IFI44, CD163, and CD8 in MI, MAu, and MAd patients.

Hepatic ISG Expression Is Significantly Correlated With IL28A/B, but not IFN-α or IFN-β. The lower number of immune cells in the liver lobules of MI patients implies that reduced levels of IFN are produced from DCs, macrophages, and so on. These findings prompted us to examine the relationship between hepatic ISGs and IFN-α, IFN-β, IL29/IFN-λ1, and IL28A/B in CHC patients. Hepatic ISG expression was significantly correlated with IL28A/B, but not IFN-β (Fig. 3A-C) or IFN-α (data not shown) in MAu, MAd, and MI patients. Expression of IL29 was correlated with hepatic ISG expression only in MAu patients. These results indicate that hepatic ISGs would be mainly induced by IL28A/B in CHC patients. Interestingly, the correlation between hepatic ISGs and IL28A/B was strongest in MA patients ($P < 0.0001$ in MAu; $P = 0.0006$ in MAd), whereas rather a weak correlation was observed in MI patients ($P = 0.015$). Moreover, the ratio of hepatic ISGs to IL28A/B was larger in MI patients than in MA patients (S = 0.061 in MI; S = 0.028 in MAu; S = 0.020 in MAd), suggesting the presence of additional factors that can induce expression of ISGs in MI patients. Therefore, we evaluated the expression of the recently discovered IFN-λ4 in MI patients. Interestingly, there was a significant correlation between hepatic ISG and IFN-λ4 expression ($P = 0.0003$; Fig. 3C).

Wingless-Related MMTV Integration Site 5A and Its Receptor, Frizzled Receptor 5, Are Significantly Up-Regulated in the Liver of Patients With the IL28B MI. IFN-λ4 is a promising factor to induce ISG expression in MI patients,[8] and the functional relevance of IFN-λ4 for the pathogenesis of CHC is under investigation. We searched for other factors that could induce ISG expression in MI patients. A closer observation of gene expression profiling in CLLs obtained by LCM demonstrated that WNT signaling was specifically up-regulated in MI patients

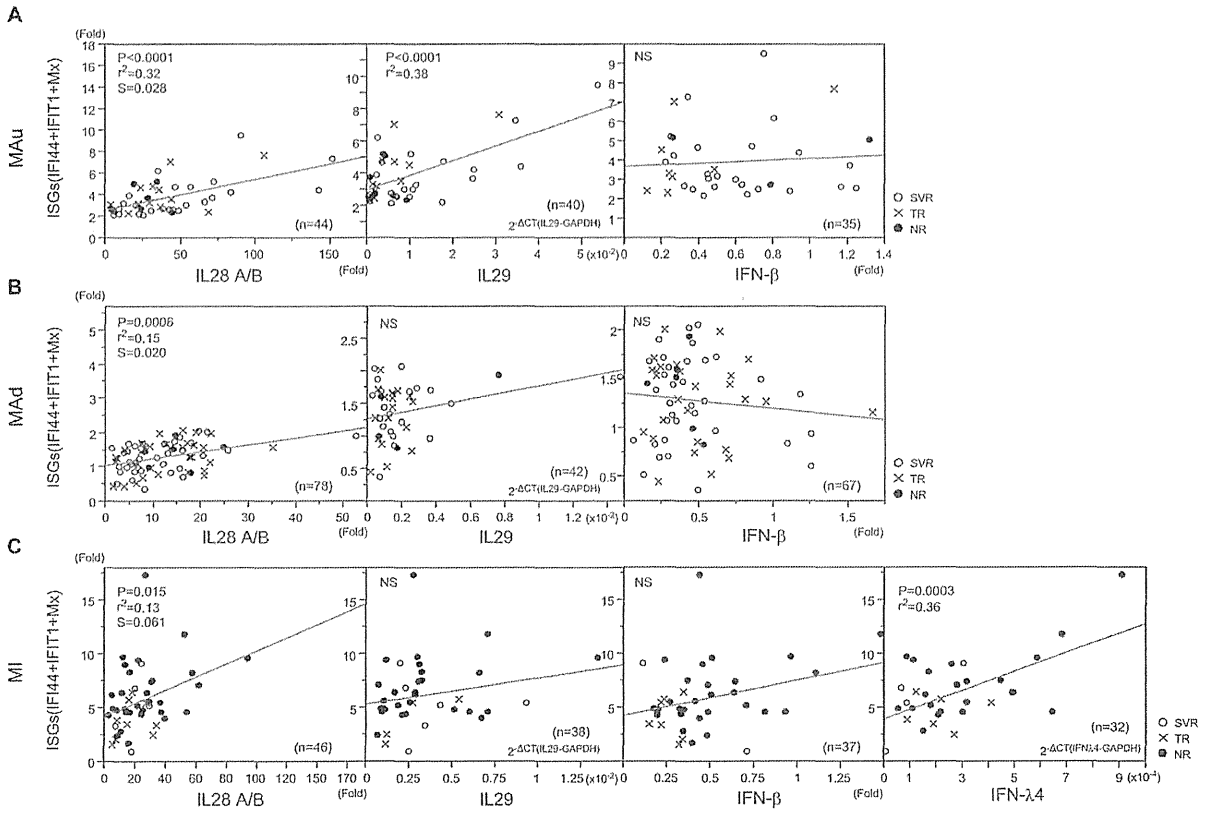

Fig. 3. Correlation analysis of hepatic ISGs and IL28A/B, IL29, IFN-β, and IFN-λ4. Correlation of mean ISG (IFI44+IFIT1+Mx1) and IL28A/B, IL29, IFN-β, and IFN-λ4 expression was evaluated in MAu (A), MAd (B), and MI (C) patients. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Supporting Fig. 6). Further observation enabled us to identify that the WNT ligand, wingless-related MMTV integration site 5A (WNT5A), and its receptor, frizzled receptor 5 (FZD5), were up-regulated in MI patients. RTD-PCR results on 168 liver-biopsied samples confirmed the significant up-regulation of WNT5A and FZD5 in MI patients, compared to MAu and MAd patients (Fig. 4A,B). Interestingly, WNT5A expression was negatively correlated with chemokine expression (Supporting Fig. 7). IHC staining showed up-regulation of FZD5 in liver lobules of MI patients, but not in MAu or MAd patients (Fig. 4C). WNT5A expression was significantly correlated with hepatic ISG expression in MI and MAd patients (Fig. 4D). Interestingly, we found a weak, but significant, correlation between WNT5A and IFN-λ4 expression in MI patients (Fig. 4E).

WNT5A Induces ISG Expression, but Stimulates HCV Replication in Huh-7 Cells. To examine the functional relevance of up-regulated expression of WNT5A in MI patients, we first evaluated expression levels of WNT5A and ISGs (2′-5′-oligoadenylate synthetase 2 [OAS2], Mx1, IFI44, and IFIT1) in two immortalized human hepatocyte cell lines, THLE-5b and TTNT cells (Supporting Materials and Methods), and one human hepatoma cell line, Huh-7 cells (Supporting Fig. 8A,B). WNT5A was moderately expressed in THLE-5b and TTNT cells, whereas its expression in Huh-7 cells was minimal. Interestingly, ISG expression in these cells correlated well with expression of WNT5A (Supporting Fig. 8B). Small interfering RNA (siRNA) to WNT5A efficiently repressed WNT5A expression to ~20% of the control in THLE-5b cells, and in this condition, ISG expression was significantly decreased to 30%-50% of the control (Supporting Fig. 8C). Conversely, transduction of WNT5A using a lentivirus expression system in Huh-7 cells significantly increased OAS2 expression (Supporting Fig. 8D), as well as Mx1 and IFIT1 expression (data not shown), in the presence and absence of HCV infection. Surprisingly, HCV replication, as determined using Gaussia luciferase activity, increased in WNT5A-transduced cells (Supporting Fig. 8E). Furthermore, WNT5A-transduced cells supported more HCV replication than

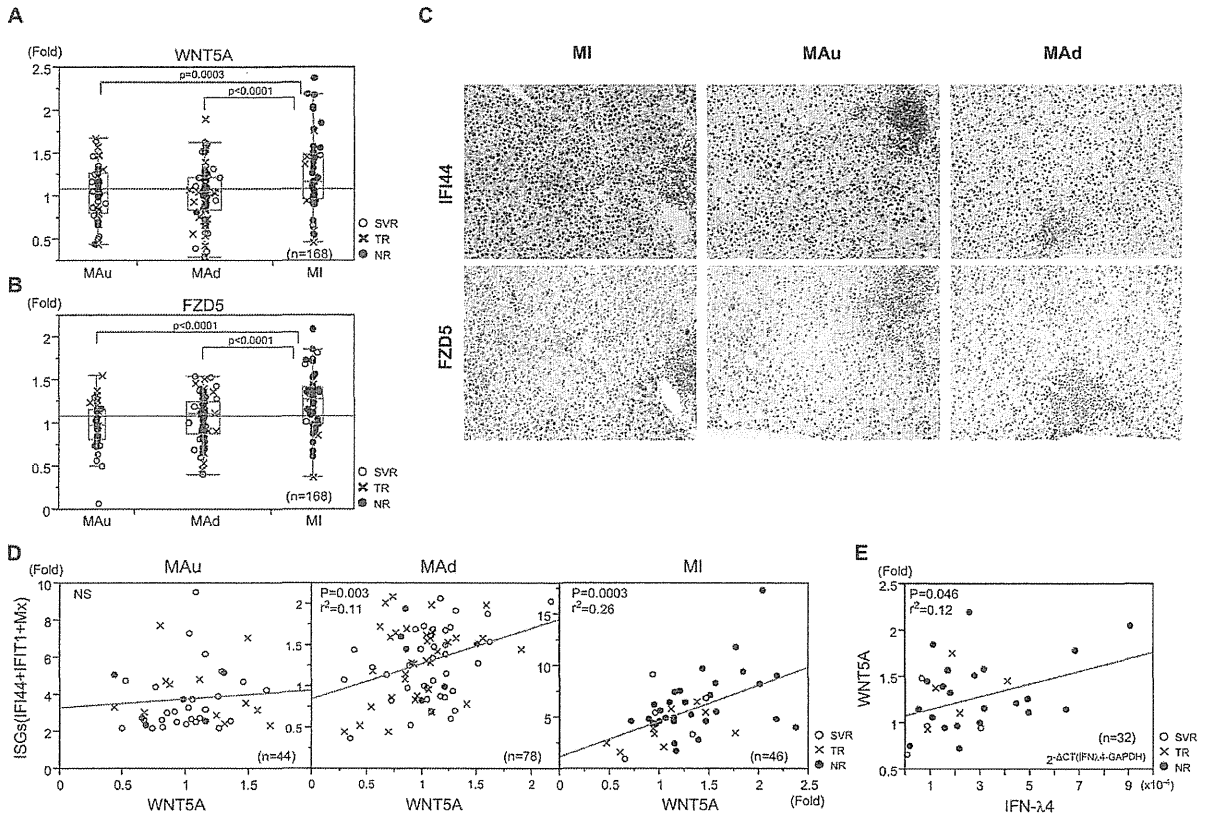

Fig. 4. WNT5A and FZD5 are up-regulated in IL28B MI patients. (A) RTD-PCR results of WNT5A expression in liver of MAu, MAd, and MI patients. (B) RTD-PCR results of FZD5 expression in liver of MAu, MAd, and MI patients. (C) IHC staining of IFI44 and FZD5 expression in liver of MAu, MAd, and MI patients. (D) Correlation of mean ISG (IFI44+IFIT1+Mx1) and WNT5A expression in liver of MAu, MAd, and MI patients. (E) Correlation of WNT5A and IFN-λ4 expression in liver of MI patients.

nontransduced cells under IFN treatment (Supporting Fig. 8F).

WNT5A-FZD5 Signaling Induces the Expression of the Stress Granule Protein, GTPase-Activating Protein (SH3 Domain)-Binding Protein 1, Which Supports HCV Replication. These findings were further confirmed by using Huh-7 cells that were continuously infected with Japanese fulminant hepatitis type 1 (JFH-1; Huh7-JFH1), which is a genotype 2a HCV isolate.[9] Interestingly, expression of WNT5A in Huh7-JFH1 cells was significantly up-regulated, compared with uninfected Huh-7 cells, and showed an equivalent expression level with THLE-5b cells (Fig. 5A). siRNA to WNT5A efficiently repressed WNT5A expression to ~20% of the control, and in this condition, ISG expression (IFI44 was not expressed in Huh-7 cells), HCV RNA, and infectivity were repressed to 25%-65%, 60%, and 40% of the control, respectively (Fig. 5B and Supporting Fig. 9A). Interestingly, CXCL13 expression was significantly increased in this condition. We evaluated the expression of GTPase-activating protein (SH3 domain)-binding protein 1 (G3BP1), a recently recognized stress granule (SG) protein that supports HCV infection and replication.[10] Expression of G3BP1 was repressed to 60% of the control by knocking down WNT5A. Conversely, overexpression of WNT5A in Huh7-JFH1 cells significantly decreased CXCL13 expression and increased HCV RNA, infectivity, and G3BP1 expression (Fig. 5C and Supporting Fig. 9B). A recent report demonstrated that G3BP1 is a disheveled (DVL)-associated protein that regulates WNT signaling downstream of the FZD receptor.[11] Knocking down FZD5 in Huh7-JFH1 cells significantly reduced the expression of DVL1-3, G3BP1, Mx1, and IFIT1 as well as HCV infectivity (Supporting Fig. 9C,D). Interestingly, G3BP1 expression was significantly up-regulated in liver of MI patients (Fig. 5D). Furthermore, G3BP1 expression was significantly correlated with WNT5A expression in liver of the CHC patients (Fig. 5E). More dramatically, a strong correlation was observed between expression of FZD5 and G3BP1 in liver of CHC patients (Fig. 5F).

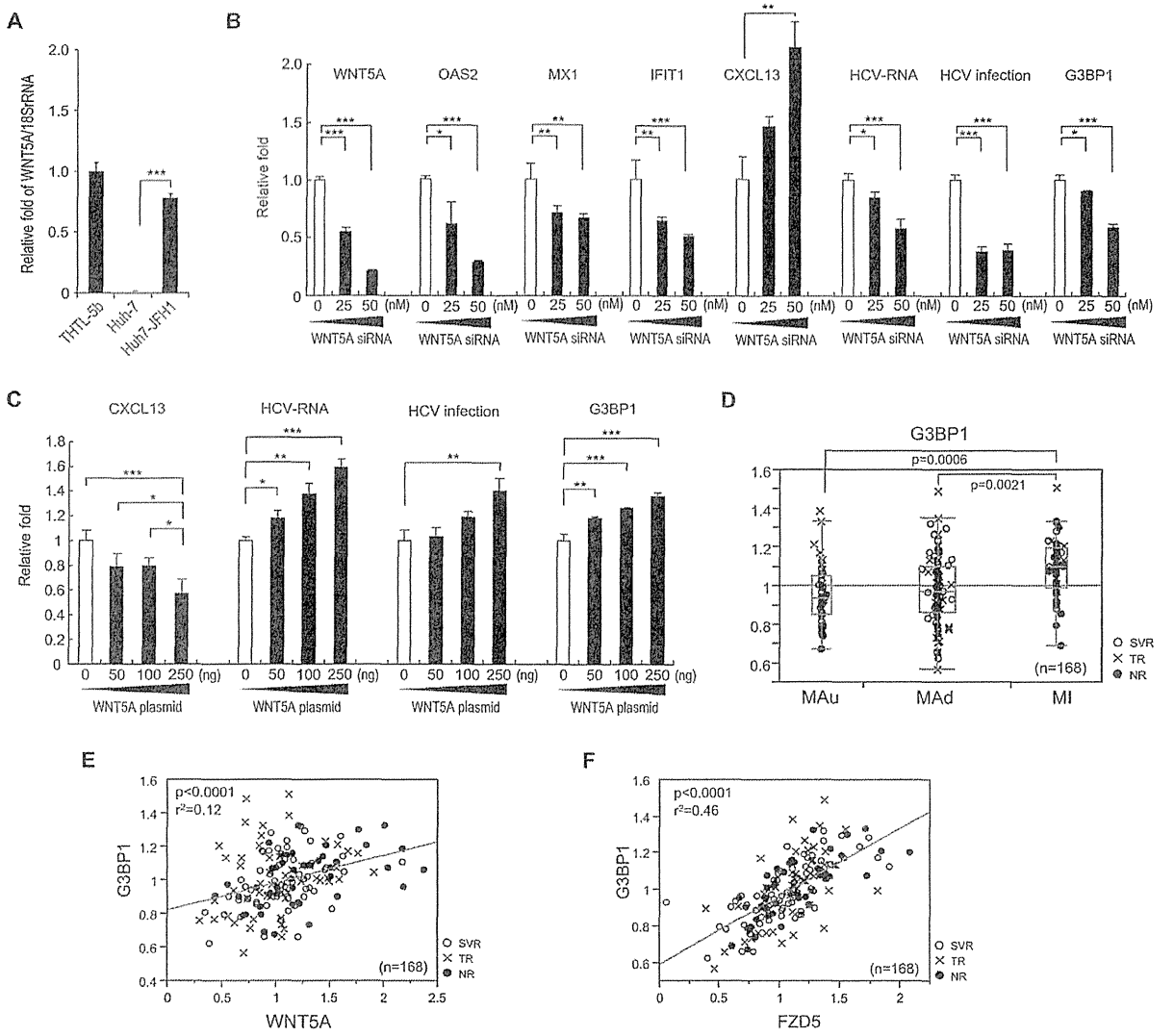

Fig. 5. Relationship between WNT5A and FZD5 signaling and the SG protein, G3BP1. (A) WNT5A expression in THLE-5b, Huh-7, and Huh7-JFH1 cells. (B) Knocking down WNT5A and changes of OAS2, Mx1, IFIT1, CXCL13, and G3BP1 expression, HCV RNA, and infectivity in Huh7-JFH1 cells. (C) Overexpression of WNT5A after transfection with pCMV-WNT5A and decrease in CXCL13 expression and increase in HCV RNA, infectivity, and G3BP1 expression. (A-C) Experiments were performed in duplicate and repeated three times (n = 6). Values are the means ± standard error. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$. (D) RTD-PCR results for G3BP1 expression in liver of MAu, MAd, and MI patients. (E) Correlation of WNT5A and G3BP1 expression in the liver. (F) Correlation of FZD5 and G3BP1 expression in the liver. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Discussion

The underlying mechanism for the association of the IL28B genotype with treatment responses to IFN-based therapy for HCV has not yet been clarified. We and others have shown that pretreatment up-regulation of hepatic ISGs was associated with an unfavorable treatment outcome[7,12,13] and was closely related to treatment-resistant MI IL28B, compared with treatment-sensitive MA IL28B.[6]

By comparing ISG expression in liver and blood, we found that their expression was correlated in MA patients, but not in MI patients. LCM analysis of ISG expression in CLLs and CPAs showed the loss of the correlation between CLLs and CPAs in MI patients (Fig. 2A). This might be the result of the impaired migration of immune cells into liver lobules that was demonstrated by decreased expression of immune cell-surface markers in CLLs by LCM (Supporting Fig. 5A) and IHC staining (Fig. 2B). Lymphocyte accumulation in the portal area (portal-tract-associated lymphoid tissue; PALT) might be involved in extravasation of lymphocytes from vessels in the portal area, but

others demonstrated that DCs appeared in the sinusoidal wall and passed through the space of Disse to PALT, where the draining lymphatic duct is located.[14] There should be an active movement of immune cells between liver lobules and PALT, as reflected by the correlation of ISG expression in CLLs and CPAs in the MA patients of this study.

ISGs were reportedly up-regulated in hepatocytes of treatment-resistant IL28B genotype patients, but were up-regulated in Kupffer cells of treatment-sensitive genotype patients.[15] Our results confirmed these findings; however, we also showed that expression of various immune cell-surface markers, such as those on DCs, NK cells, macrophages, T cells, B cells, and granulocytes, was lower in MI than in MA patients (Supporting Fig. 5). In addition, we showed that expression of various chemokines was also repressed in MI patients, compared to MA patients (Supporting Fig. 4C-F).

Up-regulation of pretreatment chemokine (C-X-C motif) ligand 10/interferon-gamma-induced protein 10 (CXCL10/IP-10) serum levels is also associated with an unfavorable treatment outcome.[16] CXCL10 expression in the liver was significantly correlated with hepatic ISG expression and was higher in nonresponders than in responders (Supporting Fig. 10). Our results support the usefulness of serum CXCL10 for prediction of treatment outcome. Chemokine (C-X-C motif) receptor 3 (CXCR3) expression, a receptor for CXCL10, was inversely correlated with hepatic ISG expression and was significantly lower in MI than in MA patients (Supporting Fig. 10).

The lower number of immune cells in the liver lobules of MI patients would imply the reduced production of IFN from DCs, macrophages, and so on. Correlation analysis showed that hepatic ISGs were mainly associated with type III IFNs (IL28A/B and IL29), but not type I IFNs (IFN-α or IFN-β), although a significant association with IL29 was only observed in MA patients with up-regulated ISGs. This might be related to the high serum ALT levels in MAu patients (Fig. 3). Closer examination of hepatic ISGs and IL28A/B suggested that factors other than IL28A/B might regulate ISG expression in MI patients. During the preparation of this study, IFN-λ4 was newly identified to be expressed in hepatocytes from treatment-resistant IL28B genotype patients.[8] Interestingly, we found a significant correlation between hepatic ISGs and IFN-λ4 in MI patients (Fig. 3C). Moreover, a closer examination of gene expression profiling in MI patients enabled us to detect up-regulation of the noncanonical WNT ligand, WNT5A. RTD-PCR analysis of 168 patients confirmed up-regulation of WNT5A and its receptor, FZD5, in MI patients. Importantly, WNT5A expression was significantly correlated with hepatic ISG expression in MI patients. A recent report showed that WNT5A induces expression of ISGs, increases sensitivity of keratinocytes to IFN-α,[17] and might be involved in the immune response to influenza virus infection.[18] Therefore, we examined the role of WNT5A in hepatocytes. Interestingly, expression of WNT5A and ISGs was well correlated, and knocking down WNT5A using siRNA reduced expression of ISGs in THLE-5b cells (Supporting Fig. 8). Conversely, transduction of Huh-7 cells with WNT5A using a lentivirus system increased expression of ISGs. Despite the increase in ISG expression, WNT5A did not suppress HCV replication, but rather increased it in Huh-7 cells (Supporting Fig. 8). These results were also confirmed by using Huh-7 cells continuously infected with JFH-1. By knocking down or overexpressing WNT5A in Huh7-JFH1 cells, we showed that HCV-RNA was positively regulated by WNT5A (Fig. 5B,C).

WNT5A and its receptor, FZD5, mediate noncanonical WNT signaling, such as planar cell polarity and the WNT-Ca^{2+}-signaling pathway through G proteins. WNT5A reportedly inhibits B- and T-cell development by counteracting canonical WNT signaling.[19] We found that G3BP1, an SG assembly factor, was up-regulated by WNT5A (Fig. 5C). SGs were reportedly formed by endoplasmic reticulum stress, followed by HCV infection, and localized around lipid droplets with HCV replication complexes.[10] G3BP1 contributes to SG formation and increases HCV replication and infection in Huh-7 cells.[10] Moreover, a recent report demonstrated that G3BP1 is a DVL-associated protein that regulates WNT signaling downstream of the FZD receptor.[11] In this study, repression of WNT5A or FZD5 significantly reduced expression of DVL1-3, G3BP1, Mx1, and IFIT1 as well as HCV infectivity in Huh7-JFH1 cells (Fig. 5 and Supporting Fig. 9).

Importantly, we found a significant correlation between WNT5A and G3BP1 expression in liver tissue samples (Fig. 5E). We also found a significant correlation between FZD5 and G3BP1 expression in liver tissue samples (Fig. 5F). Thus, up-regulated noncanonical WNT5A-FZD5 signaling participates in the induction of ISG expression, but preserves HCV replication and infection in hepatocytes by increasing levels of the SG protein, G3BP1. These findings may explain the pathophysiological state of the treatment-resistant phenotype in MI patients.

In this study, we demonstrated impaired immune cell infiltration of the liver in treatment-resistant IL28B genotype patients, and we also demonstrated

that up-regulation of hepatic ISGs in treatment-resistant IL28B genotype patients was mediated by multiple factors, including IL28A/B, IFN-λ4, and WNT5A. We found a significant negative correlation between WNT5A and various chemokines in liver of CHC patients (Supporting Fig. 7). Interestingly, WNT5A directly repressed one of these chemokines, CXCL13, a B-lymphocyte chemoattractant, in HCV-infected hepatocytes. These results indicate that loss of immune cells from the liver may be associated with the induction of other inflammatory factors, such as WNT5A, in MI patients, although we did not identify which cells express WNT5A. Further studies are needed to explore their functional relevance in the pathogenesis of CHC.

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Ultradeep Sequencing Study of Chronic Hepatitis C Virus Genotype 1 Infection in Patients Treated with Daclatasvir, Peginterferon, and Ribavirin

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Direct-acting antivirals (DAAs) are either part of the current standard of care or are in advanced clinical development for the treatment of patients chronically infected with hepatitis C virus (HCV) genotype 1, but concern exists with respect to the patients who fail these regimens with emergent drug-resistant variants. In the present study, ultradeep sequencing was performed to analyze resistance to daclatasvir (DCV), which is a highly selective nonstructural protein 5A (NS5A) inhibitor. Eight patients with HCV genotype 1b, who were either treatment naive or prior nonresponders to pegylated interferon plus ribavirin (Rebetol; Schering-Plough) (PEG-IFN/RBV) therapy, were treated with DCV combined with PEG-IFN alpha-2b (Pegintron; Schering-Plough, Kenilworth, NJ) and RBV. To identify the cause of viral breakthrough, the preexistence and emergence of DCV-resistant variants at NS5A amino acids were analyzed by ultradeep sequencing. Sustained virological response (SVR) was achieved in 6 of 8 patients (75%), with viral breakthrough occurring in the other 2 patients (25%). DCV-resistant variant Y93H preexisted as a minor population at higher frequencies (0.1% to 0.5%) in patients who achieved SVR. In patients with viral breakthrough, DCV-resistant variant mixtures emerged at NS5A-31 over time that persisted posttreatment with Y93H. Although enrichment of DCV-resistant variants was detected, the preexistence of a minor population of the variant did not appear to be associated with virologic response in patients treated with DCV/PEG-IFN/RBV. Ultradeep sequencing results shed light on the complexity of DCV-resistant quasispecies emerging over time, suggesting that multiple resistance pathways are possible within a patient who does not rapidly respond to a DCV-containing regimen. (This study has been registered at ClinicalTrials.gov under registration no. NCT01016912.)

Chronic hepatitis C virus (HCV) infection is one of the most serious global health problems preceding development of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (1, 2, 3, 4, 5). To prevent the development of advanced liver disease, including HCC, pegylated interferon (PEG-IFN)-based therapies have been administered to patients with chronic HCV infection. Eradication of HCV using PEG-IFN combined with ribavirin (Rebetol; Schering-Plough) (PEG-IFN/RBV) has been shown to result in remarkable biochemical and histological improvements in the liver (6, 7). However, patients infected with HCV genotype 1 have experienced a poor response to this therapy as observed by sustained virological response (SVR) rates of only 40% to 50% (8, 9, 10). Recently, new antiviral agents targeting the HCV nonstructural protein 3/4A (NS3/4A) protease activity, telaprevir (TVR) and simeprevir, were approved in several countries as an add-on to PEG-IFN and RBV (triple therapy) for treating patients infected with HCV genotype 1. The triple therapy significantly improved SVR rates in this patient population (11, 12). However, many severe adverse effects such as skin rash, anemia, and renal dysfunction have been reported which often prevent successful continuation of this triple therapy (12).

To improve safety and effectiveness of anti-HCV therapy, a number of selective inhibitors targeting HCV proteins, otherwise known as direct-acting antivirals (DAAs), are currently under development. Daclatasvir (DCV; BMS-790052) is a first-in-class, highly selective nonstructural protein 5A (NS5A) inhibitor with picomolar potency and broad genotypic coverage (13, 14, 15). NS5A is an RNA binding multifunctional viral protein and is essential for viral proliferation by interacting with other HCV nonstructural proteins and cellular proteins (16, 17, 18). In a phase 2a study, a higher SVR rate was observed by adding DCV to the PEG-IFN alpha-2a plus RBV regimen (19).

Although DAAs are expected to improve the antiviral effect of PEG-IFN/RBV against HCV genotype 1, drug resistance is still considered a concern. Emergence of drug resistance is often associated with viral rebound and subsequent virologic failure. In the case of the DCV NS5A inhibitor, the emergence of substitutions at the NS5A drug target has been reported (19). In patients infected with HCV genotype 1, one of the most predominant genotypes in

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TABLE 1 Clinical characteristics of 8 patients treated with combination therapy with daclatasvir, PEG-IFN alpha-2b, and RBV for 24 weeks against chronic HCV genotype 1b infection[a]

Case	Age (yr)	Sex	Previous interferon treatment	*IL28B*	HCV RNA (log IU/ml)	No. of platelets ($\times 10^3/\mu l$)	Hepatic fibrosis stage	DCV (mg/day)	Efficacy
1	67	F	Naive	TT	7.1	262	ND	60	SVR
2	42	F	Partial	TG	5.5	146	F2	60	SVR
3	55	F	Naive	TT	5.1	181	F2	60	SVR
4	61	M	Naive	TT	7.1	225	F1	60	SVR
5	39	F	Naive	TG	6.4	207	F1	10	SVR
6	59	M	Partial	TG	7.1	178	F2	10	SVR
7	59	F	Null	TG	7.6	158	ND	10	Breakthrough
8	70	F	Null	GG	7.0	167	F2	60	Breakthrough

[a] *IL28B*, rs8099917 genotype; DCV, daclatasvir; M, male; F, female; SVR, sustained virological response; Partial, partial responder; Null, null responder; ND, not determined. The hepatic fibrosis stage was determined by liver biopsy analysis according to New Inuyama Classification as follows: F1, fibrous portal expansion; F2, bridging fibrosis.

the world, NS5A amino acid (aa) positions 31 and 93 have been shown to be susceptible to substitution or enrichment (19). Double-amino-acid substitutions in the NS5A region, such as L31M (substitution from leucine to methionine) plus Y93H (from tyrosine to histidine) or L31V (leucine to valine) plus Y93H, conferred high resistance to DCV in an *in vitro* HCV replication system (19).

Recently, ultradeep sequencing has been used as a sensitive technique for characterizing resistance variants (20, 21, 22, 23). In the present study, ultradeep sequencing was performed using sera from 8 Japanese chronic hepatitis C patients who participated in a clinical phase 2a trial using DCV, PEG-IFN alpha-2b (Pegintron; Schering-Plough, Kenilworth, NJ), and RBV to analyze the association between preexisting DCV-resistant variants and clinical antiviral responses.

MATERIALS AND METHODS

Study design. This study was a phase 2a, double-blind, placebo-controlled trial (clinicaltrials.gov identifier NCT01016912) for evaluating the antiviral activity and safety of DCV combined with PEG-IFN alpha-2b and RBV in treatment-naive patients and nonresponders to the standard of care with HCV genotype 1. Written informed consent was obtained from all patients. The study was approved by institutional review boards at each site and conducted in compliance with the Declaration of Helsinki, good clinical practice guidelines, and local regulatory requirements.

Patients. Ten patients who met the following inclusion and exclusion criteria participated in the clinical trial. However, two were excluded from the following analysis because they were assigned to a placebo cohort group and treated with PEG-IFN plus RBV combination therapy without DCV. Inclusion and exclusion criteria for this clinical trial used the following parameters. (i) The patient age was between 20 and 75 years. (ii) The patients had been infected with HCV genotype 1 for at least 6 months, and the serum HCV RNA level was $>10^5$ IU/ml. (iii) Eligible patients had had no evidence of cirrhosis diagnosed by laparoscopy, imaging, or liver biopsy analysis within 2 years. (iv) Eligible patients consisted of three groups: *(a)* treatment-naive patients with no history of anti-HCV therapy, including interferon therapy; *(b)* null responders who had failed to achieve a 2 $\log_{10}$ HCV-RNA decrease in previous interferon therapy lasting 12 weeks or longer; and *(c)* partial responders who had failed to achieved undetectable RNA but had achieved a greater than 2 $\log_{10}$ HCV-RNA decrease in previous interferon therapy lasting 12 weeks or longer. (iv) The patients had no history of hepatocellular carcinoma, coinfection with hepatitis B virus or human immunodeficiency virus, other chronic liver disease, or evidence of hepatic decompensation. (vi) Patients were also excluded if they had other severe or unstable conditions or evidence of organ dysfunction in excess of that consistent with the age of the patient, were unable to tolerate interferon and oral medication or had conditions that could impact absorption of the study drug, or had been exposed to any investigational drug within 4 weeks of study participation or had any previous exposure to inhibitors of NS5A. (vii) Laboratory findings that excluded participation were alanine aminotransferase (ALT) $>$ 5 times the upper limit of normal (ULN); total bilirubin $\geq$ 2 mg/dl; direct bilirubin $>$ 1.5$\times$ ULN; international normalized ratio of prothrombin time $\geq$ 1.7; albumin $\leq$ 3.5 g/dl; hemoglobin $<$ 9.0 g/dl; white blood cells $<$ 1,500/mm^3; absolute neutrophil count $<$ 750/mm^3; platelets $<$ 50,000/mm^3; or creatinine $>$ 1.8$\times$ ULN.

Treatment protocol. All patients received a combination of DCV, PEG-IFN alpha-2b, and RBV for 24 weeks. Patients subcutaneously received PEG-IFN alpha-2b at a dosage of 1.5 mg/kg of body weight/week and were administered ribavirin orally according to their body weight (600 mg for $<$ 60 kg, 800 mg for 60 to 80 kg, 1,000 mg for $>$80 kg). Patients were randomly assigned to receive DCV at 10 mg or 60 mg once daily for 24 weeks. DCV was provided by Bristol-Myers Squibb, which conducted this clinical trial. When viral breakthrough occurred, treatment was discontinued with the patient's consent.

Determination of IL28B genotypes. The *IL28B* SNP genotype (rs8099917) was determined using TaqMan predesigned single nucleotide polymorphism (SNP) genotyping assays as described previously (24).

Assessment of virological responses. Plasma was collected at baseline and at the following fixed time points: weeks 1, 2, 4, 6, 8, and 12 and then every 4 weeks during treatment. HCV RNA was determined at a central laboratory using a Roche Cobas TaqMan HCV Auto assay (Roche Diagnostics KK, Tokyo, Japan) (lower limit of quantitation [LLOQ], 15 IU/ml). Sustained viral response (SVR) occurred if HCV RNA became continuously undetectable by qualitative PCR assay and ALT levels normalized for 24 weeks after the end of treatment. Viral breakthrough was defined as an increase of $\geq$1 $\log_{10}$ IU/ml from nadir at more than one time point or HCV RNA $\geq$ 15 IU/ml after declining to below that level.

Detection of drug-resistant substitutions by ultradeep sequencing. HCV RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), and the reverse-transcriptase reaction was performed using a random primer and Moloney murine leukemia virus (MMLV) reverse transcriptase. Briefly, the NS5A region in HCV genome was amplified by nested PCR using the specific primers 5′-TGGCTCCA GTCCAAACTCCT G-3′, 5′-GGGAATGTTCCATGCCACGTG-3′, 5′-T GGAACATTCCCCATCAACGC-3′, and 5′-CCAACCAGGTACTGATT GAGC-3′, and the amplified fragment distributions were assessed using an Agilent BioAnalyzer 2100 platform. The fragments were modified by the use of a Multiplexing Sample Preparation kit (Illumina), and sequence analysis was performed by the use of a Illumina Genome Analyzer. Imaging analysis and base calling were performed using Illumina Pipeline software with default settings as previously reported (20). The N-terminal domain of NS5A, which includes L31 and Y93, was analyzed. This technique revealed an average coverage depth of over 1,000 sequence reads per base pair in the unique regions of the genome. Read mapping to a refer-

TABLE 2 Threshold assessment introduced by error in ultradeep sequencing analysis at NS5A amino acids 31 and 93, determined by a basal experiment using a wild-type HCV-expressing plasmid as a control[a]

Position	Total no. of reads	Frequencies (%)	Error rate (%)
aa 31	1,284,644	L (99.27), S/F/V (0.073)	0.073
aa 93	512,323	Y (99.44), H/C (0.056)	0.056

[a] Substituted amino acids are shown by standard single-letter codes. Amino acid substitutions were defined as those occurring at a rate of more than 0.1% among the total reads. This frequency is expected to be sufficient to overcome the error threshold of the sequencing platform used in this study.

ence sequence was performed using Bowtie (25). Because of the short 36-nucleotide read length, mapping hypervariable regions with multiple closely spaced variants against a reference sequence yields poor coverage. Alternative reference sequences were included to improve coverage in variable regions.

RESULTS

Characteristics of patients and treatment efficacy. Eight patients were treated with DCV, PEG-IFN alpha-2b, and RBV triple therapy. To compare dosing effects of DCV, 3 patients were administered 10 mg/day of DCV and the remaining 5 patients were administered 60 mg/day of DCV. As shown in Table 1, subjects included 2 males and 6 females, with a median age of 59. All subjects were infected with HCV genotype 1b. SVR was achieved in 6 of 8 patients (75%), and viral breakthrough occurred in the remaining 2 patients (25%).

Detection of drug-resistant HCV variants prior triple therapy. To analyze the differences in antiviral effects, ultradeep sequencing was performed on pretreatment serum samples from 7 of the 8 patients; sample from patient case 3 was not assessed. To account for errors introduced by RT-PCR as well as errors inherent in the PCR technology as reported (26), we used a minimum variant frequency threshold of 0.1% of the total reads, referring to our basal experiments using a HCV-expressing plasmid as a control (Table 2). At aa 31 in NS5A, 866,032 reads (496,711 to 1,432,680) on average were obtained, and no significant DCV-resistant variants were detected in any of the 7 patient samples examined (Table 3). At NS5A aa 93, 154,093 reads (49,349 to 289,481) on average were obtained, and DCV-resistant variants (Y93H) were detected in 4 patients (cases 1, 2, 4, and 5). Other NS5A regions relating to low resistance, including aa 28, aa 30, aa 32, and aa 92, were also analyzed prior to the treatment. The preexistence of these amino acid substitutions was less related to treatment efficacy (Table 3).

Virological response. The serum HCV RNA titers in 6 patients (cases 1 to 6) who achieved SVR are shown in Fig. 1. In cases 1, 2, 4, and 5, despite the presence of DCV-resistant variants (Y93H), serum HCV RNA levels were below the detectable limit between weeks 1 and 4 of treatment and remained undetectable, resulting in the patients achieving SVR. In contrast, the serum HCV RNA titers of 2 patients (cases 7 and 8) rebounded at week 4 or 6 of treatment and returned to pretreatment levels (Fig. 2A and 3A). Interestingly, no significant DCV-resistant variants were detected prior to treatment in these 2 patients.

To analyze the mechanism of viral breakthrough, ultradeep sequencing of the NS5A N-terminal region was performed using patient sera at several time points, and the percentages of drug-

TABLE 3 Ultradeep sequencing analysis of NS5A amino acids 28, 30, 31, 32, and 93 in 7 patients prior to the start of combination therapy with daclatasvir, PEG-IFN alpha-2b, and RBV[a]

Case	aa 28			aa 30			aa 31			aa 32			aa 92			aa 93		
	Total no. of reads	WT (L) (%)	Variant(s) (%)	Total no. of reads	WT (R) (%)	Variant(s) (%)	Total no. of reads	WT (L) (%)	Variant	Total no. of reads	WT (P) (%)	Variant(s) (%)	Total no. of reads	WT (A) (%)	Variant	Total no. of reads	WT (Y) (%)	Variant
1	1,430,702	—	V (84.3), M (15.6), I (0.1)	1,432,739	—	Q (99.3), L (0.7)	1,432,680	100	—	1,432,501	99.8	L (0.1), Q (0.1)	289,588	99.9	T (0.1)	289,481	99.9	H (0.1)
2	726,522	—	M (98.2), V (1.5), I (0.3)	729,514	—	Q (100)	729,642	100	—	729,572	100	—	123,468	99.9	K (0.1)	123,510	99.6	H (0.4)
4	496,643	100	—	496,730	100	—	496,711	100	—	496,660	100	—	49,389	98.6	T (1.4)	49,349	99.6	H (0.4)
5	1,327,588	100	—	1,327,743	100	—	1,327,703	100	—	1,327,685	100	—	105,928	99.8	V (0.2)	105,963	99.5	H (0.5)
6	900,736	100	—	900,846	100	—	900,816	100	—	900,932	100	—	116,298	100	—	116,279	100	—
7	695,962	100	—	697,367	100	—	697,275	100	—	697,215	100	—	222,020	99.9	T (0.1)	221,916	100	—
8	477,238	—	M (99.5), V (0.3), I (0.2)	477,351	0.2	Q (99.7), L (0.1)	477,400	100	—	477,275	100	—	172,210	100	—	172,156	100	—

[a] Substituted amino acids are shown by standard single-letter codes. Dashes indicate amino acid substitutions in less than 0.1% of the total reads. WT, wild type.

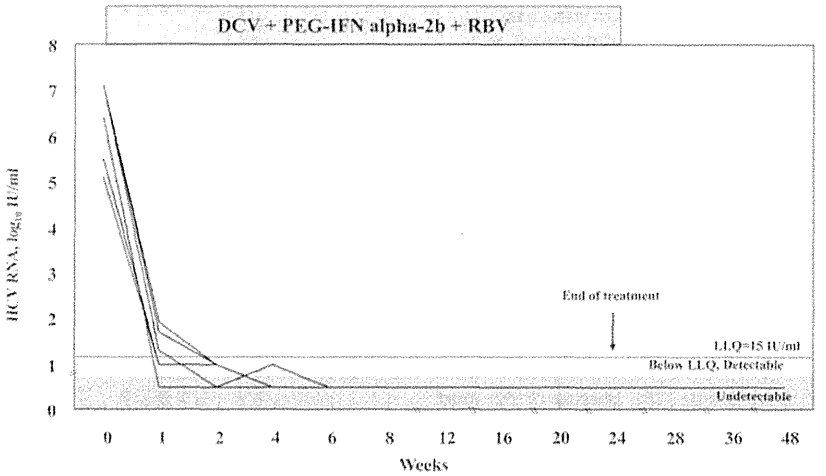

FIG 1 Plasma HCV RNA levels of 6 patients who achieved SVR during combination therapy with daclatasvir, PEG-IFN alpha-2b, and RBV for 24 weeks followed by 24 weeks posttreatment. LLQ, lower limit of quantitation (15 IU/ml).

resistant variants at aa 31 and aa 93 were compared. In case 7, according to the results of ultradeep sequencing, 100% of the total reads showed a wild-type amino acid sequence (leucine) at aa 31, and 100% of the total reads showed the wild type (tyrosine) at aa 93 before the treatment (Fig. 2B). However, the proportion of the wild type at aa 31 at week 10 of treatment was predominantly replaced by DCV-resistant variants L31I (92.8%) and L31M (4.9%), and enrichment of the L31I and L31M variants was observed during triple therapy. The level of detection of these variants was maintained 16 weeks after the end of treatment. In addition, although a variant at aa 93 could not be identified before treatment, the Y93H variant also appeared (32.5%) at week 10 of treatment. The Y93H variant, which is known to be associated with DCV resistance, persisted (32.5%) 16 weeks after the end of treatment.

In patient case 8, DCV-resistant variants were not detected prior to treatment (Table 3). Surprisingly, L31V and L31M were rapidly enriched and comprised more than 98% of the clonal sequences at week 1 of treatment (Fig. 3B). At the same time, the Y93H variant also started to outgrow the wild-type sequence and was detected in up to 35.5% of the sequences during the course of therapy. The proportions of resistance variants at aa 31 and aa 93 did not decrease after discontinuation of the therapy and persisted at similar levels 16 weeks after the end of therapy.

According to these results, viral breakthrough was induced by the selection of DCV-resistant variants that included substitutions at L31I/V/M and Y93H. These DCV-resistant variants persisted at high frequency after discontinuation of the triple therapy.

DISCUSSION

Treatment of chronic hepatitis C has drastically improved since the introduction of PEG-IFN and RBV combination therapy. However, only approximately 40% to 50% of patients infected with a high titer of HCV genotype 1 are able to achieve SVR (27). To improve the effectiveness of anti-HCV therapy, a number of DAAs targeting HCV-related proteins, such as NS3/4A protease or NS5B polymerase, are under development. DCV is one of the DAAs under development and is a first-in-class NS5A inhibitor with picomolar potency and broad genotypic coverage (13, 14, 15). In a proof-of-concept clinical study, 90% of patients with HCV genotype 1b infection treated with the dual oral combination of DCV plus asunaprevir achieved SVR (28, 29, 30). Based on these reports, DCV is expected to be a specific agent against chronic hepatitis C. In the present study, triple therapy using DCV, PEG-IFN alpha-2b, and RBV was administered to patients with HCV genotype 1b infection. As shown in Table 1, all patients had HCV RNA titers $> 5 \log_{10}$ IU/ml, 5 of 8 patients had unfavorable *IL28B* (rs8099917) genotypes (TG or GG), and 4 of 8 patients were prior partial or null responders to previous treatment with PEG-IFN plus RBV combination therapy. Based on this clinical background, the study patients were predicted to be difficult to treat using conventional PEG-IFN plus RBV combination therapy. However, HCV RNA titers reduced rapidly with the DCV triple therapy, and 75% of patients were able to achieve SVR. Although these clinical results were obtained from a small number of subjects in the clinical trial and at one hospital, these results suggest that DCV is likely to improve the outcome of the anti-HCV treatment in combination with PEG-IFN plus RBV therapy.

Resistance has been shown to emerge with different classes of DAA regimens. The reason that treatment of some of these patients fails, however, remains unclear. Prior to antiviral treatment with DAAs, amino acid substitutions in HCV-related proteins that confer resistance to DAAs can preexist. Enrichment of variants during therapy has been reported, although monitoring the changes using ultradeep sequencing is not so common. HCV is an error-prone RNA virus where mutations frequently occur throughout the HCV genome (31, 32, 33), and drug-resistant variants are sometimes present as a minor population in patients who have never been treated with DAAs (34). Of the sequenced HCV clones, samples from patient cases 1, 2, 4, and 5 had DCV-resistant variants at frequencies ranging from 0.1% to 0.5% (Table 3). Interestingly, viral breakthrough did not occur during triple therapy in these cases despite the preexistence of a higher proportion of DCV-resistant variants. Viral breakthrough occurred in patient cases 7 and 8, where drug-resistant variants had not been detected prior to treatments. Consequently, several clinical factors were compared to identify additional factors that may be associated with viral breakthrough. There were no differences in HCV RNA

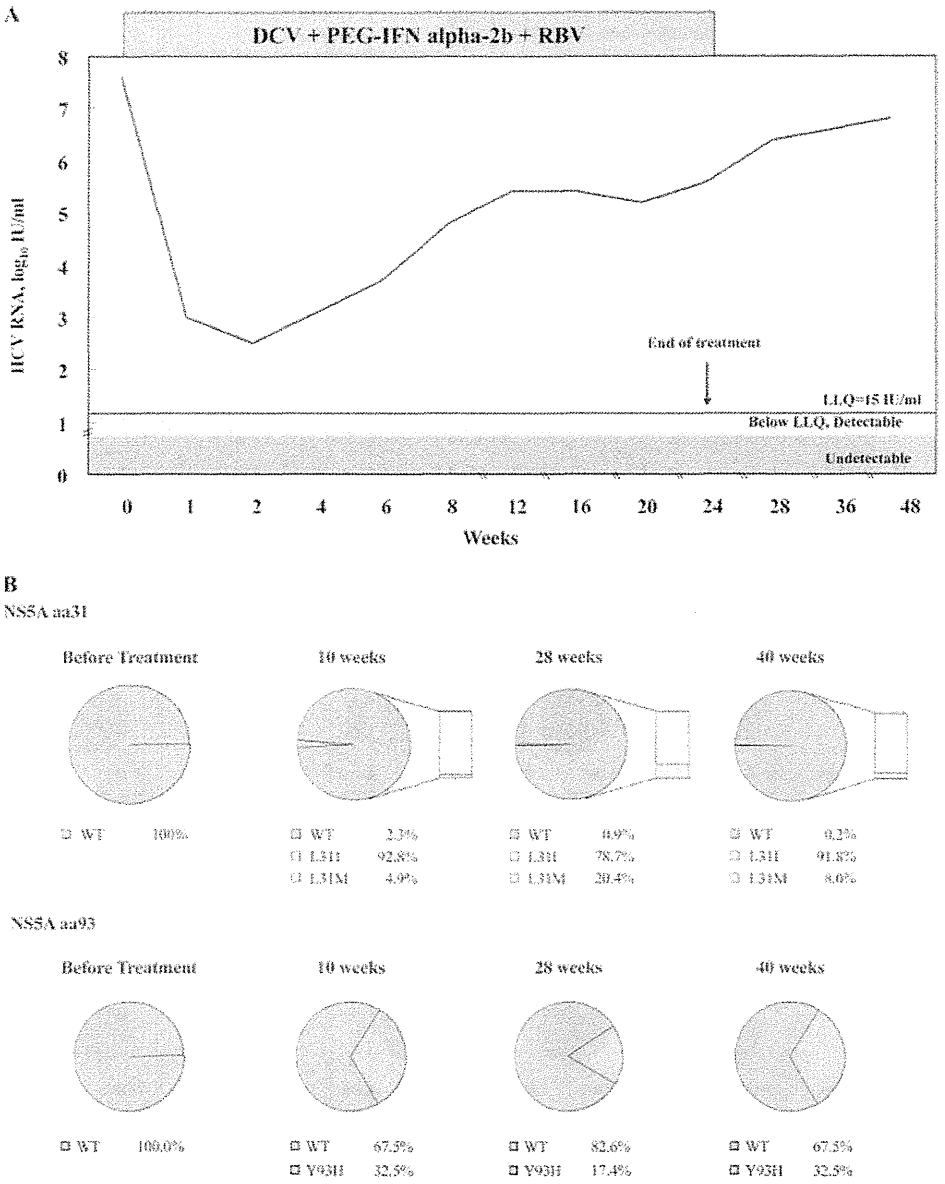

FIG 2 Clinical course of case 7 with viral breakthrough during combination therapy. (A) Plasma HCV RNA levels. (B) Time course of the amino acid frequency at L31 and Y93 in the NS5A region by ultradeep sequencing. WT, wild type; LLQ, lower limit of quantitation (15 IU/ml).

levels or baseline clinical characteristics (Table 1). However, the two patients with viral breakthrough both had unfavorable *IL28B* genotypes (TG or GG) and were null responders to prior PEG-IFN plus RBV combination therapy. In previous studies using a human hepatocyte chimeric mice model, TVR-resistant populations remained highly susceptible to IFN treatment (20). Since the two patients experiencing viral breakthrough in this study were prior null responders to IFN, there is a possibility that they could respond to a quadruple therapy using IFN as a component of the treatment. Patient cases 1, 2, 4, and 5 achieved SVR despite the detection of higher proportions of DCV-resistant variants before treatment initiation with DCV, PEG-IFN, and RBV. It is possible that the preexistence of DCV-resistant variants might have a greater impact on virologic response in patients considered to be refractory to IFN, such as those with a poor response to previous IFN therapy, although that could not be concluded from this study given that the 2 failures had no significant DCV-resistant variants before treatment.

Recent studies have demonstrated that levels of enriched drug-resistant variants gradually decline after DAA treatment is discontinued and that most HCV variants are eventually replaced by baseline sequence posttreatment (20). In patient case 7, although DCV-resistant variants had not been detected prior treatment, more than 90% of HCV sequences were replaced by sequences encoding L31I/M and Y93H at week 10 of therapy. These drug-resistant variants were still detected at high proportions 16 weeks after cessation of treatment. Similarly, in patient case 8, more than 99% of HCV sequences had already been replaced by the L31I/

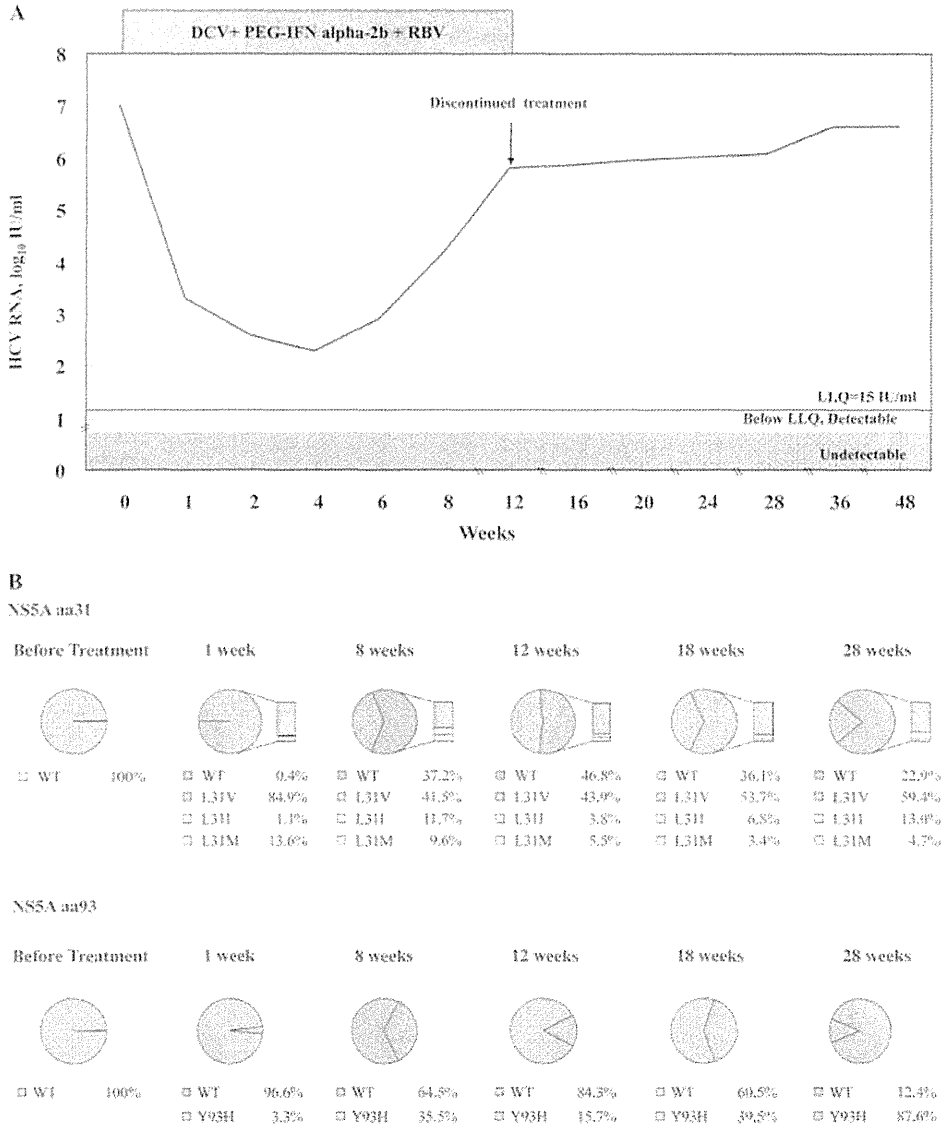

FIG 3 Clinical course of case 8 with viral breakthrough during combination therapy. (A) Plasma HCV RNA levels. (B) Time course of the amino acid frequency at L31 and Y93 in the NS5A region by ultradeep sequencing. LLQ, lower limit of quantitation (15 IU/ml).

V/M variant at week 1, and a high proportion of these variants persisted until the last posttreatment time point, 16 weeks after treatment. These results suggest that drug-resistant variants can be rapidly enriched during the early phase of DAA therapy. Because ultradeep sequencing using this Illumina technology yields only 36 nucleotide fragments, it is not clear whether or not the mutations that encode the L31IM/V and Y93H substitutions exist in the same genomic RNA strand. However, based on the frequency of the mutations, at least some of these are likely to exist on the same genomic RNA strand. Only 8 patients could be assessed in this study; however, rapid selection of DAA-resistant variants during combination treatment has been previously observed (20). Interestingly, both patient 7 and patient 8 had higher viral loads at week 1 of treatment (≥1,000 IU/ml) than the other patients within the group. Viral load response at week 1 may therefore be more of a predictor of the emergence of resistance and virologic outcome than preexisting minor populations of NS5A resistance-associated polymorphisms.

Ultradeep sequencing analysis revealed that the DCV-resistant variants were maintained at a high frequency after cessation of the treatment. It has been reported that drug-resistant variants have reduced replication capacity and are easily replaced by the wild type (20). However, the present results, in agreement with other studies (19), suggest that NS5A aa 31 or aa 93 resistance variants are fit and possibly comparable to the wild type in fitness. With respect to viral fitness, a L31M/V plus Y93H double-substitution variant was reported to reduce DCV susceptibility (4,227/8,336-fold change, respectively) with impaired replication (36%/30% per the wild type, respectively) in the HCV genotype 1b replicon (35). Although it was reported that second-site replacements at NS5A restore efficient replication in HCV genotype 2a *in vitro* (13), there is not sufficient evidence about third-site replacements at NS5A that can restore replica-