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Hepatic ISG Expression Is Associated With Genetic Variation in Interleukin 28B and the Outcome of IFN Therapy for Chronic Hepatitis C

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See related article, Younossi and Stepanova, on page 718 in *CGH*.

BACKGROUND & AIMS: Multiple viral and host factors are related to the treatment response to pegylated-interferon and ribavirin combination therapy; however, the clinical relevance and relationship of these factors have not yet been fully evaluated. **METHODS:** We studied 168 patients with chronic hepatitis C who received pegylated-interferon and ribavirin combination therapy. Gene expression profiles in the livers of 91 patients were analyzed using an Affymetrix genechip (Affymetrix, Santa Clara, CA). The expression of interferon-stimulated genes (ISGs) was evaluated in all samples by real-time polymerase chain reaction. Genetic variation in interleukin 28B (IL28B; rs8099917) was determined in 91 patients. **RESULTS:** Gene expression profiling of the liver differentiated patients into 2 groups: patients with up-regulated ISGs and patients with down-regulated ISGs. A high proportion of patients with no response to treatment was found in the up-regulated ISGs group (P = .002). Multivariate logistic regression analysis showed that ISGs (<3.5) (odds ratio [OR], 16.2; $P < .001$), fibrosis stage (F1-F2) (OR, 4.18; P = .003), and ISDR mutation (≥2) (OR, 5.09; P = .003) were strongly associated with the viral response. The IL28B polymorphism of 91 patients showed that 66% were major homozygotes (TT), 30% were heterozygotes (TG), and 4% were minor homozygotes (GG). Interestingly, hepatic ISGs were associated with the IL28B polymorphism (OR, 18.1; $P < .001$), and its expression was significantly higher in patients with the minor genotype (TG or GG) than in those with the major genotype (TT). **CONCLUSIONS: The expression of hepatic ISGs is strongly associated with treatment response and genetic variation of IL28B. The differential role of host and viral factors as predicting factors may also be present.**

Keywords: Pegylated Interferon, Ribavirin; Gene Expression; Single Nucleotide Polymorphism.

A human liver infected with hepatitis C virus (HCV) develops chronic hepatitis, cirrhosis, and, in some instances, hepatocellular carcinoma.[1] Interferon (IFN) and ribavirin (RBV) combination therapy is a popular modality for treating patients with chronic hepatitis C (CH-C); approximately 50% of patients usually relapse, particularly those with HCV genotype 1b and a high viral load.[2,3] Therefore, it is beneficial to predict the response of patients with the 1b genotype and a high viral load to pegylated-IFN (Peg-IFN) and RBV combination therapy before starting treatment because therapy can be long, costly, and have many adverse effects. Amino acid (aa) substitutions in the interferon sensitivity determining region (ISDR), located in the HCV nonstructural region 5A, are useful for predicting the response of patients with genotype 1b to IFN therapy.[4] However, viral factors alone do not sufficiently predict the outcome of treatment in every case.[5]

In addition to viral factors, hepatic gene expression before and during IFN treatment has been examined to determine host factors associated with the response to treatment.[6,7] Interferon-stimulated genes (ISGs) up-regulated in the liver prior to treatment might be related to the poor induction of ISGs and the impaired eradication of HCV during treatment.[6–9] This may be because the ISGs have already been maximally induced before treat-

Abbreviations used in this paper: **aa, amino acid; AST, aspartate aminotransferase; cDNA, complementary DNA; CH-C, chronic hepatitis C; Down-ISGs, down-regulated ISGs; EVR, early virologic response; GWAS, genome-wide association studies; HCV, hepatitis C virus; IFN, interferon; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IL, interleukin; IL28B, interleukin 28B; ISDR, interferon sensitivity determining region; ISGs, interferon stimulated genes; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); NR, no response; Peg, pegylated; RBV, ribavirin; ROC, receiver operating characteristic; RTD, real-time detection; PCR, polymerase chain reaction; RTD-PCR, real-time detection-polymerase chain reaction; SNP, single nucleotide polymorphism; SVR, sustained viral response; TR, transient response; Up-ISGs, up-regulated ISGs.**

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ment. However, the clinical relevance of the expression of ISGs as predictive factors for the outcome of treatment has not yet been fully evaluated.

In parallel to gene expression analysis, genome-wide association studies (GWAS) have been used to identify loci associated with the response to treatment; genetic variation in interleukin 28B (IL28B) was found to predict hepatitis C treatment-induced viral clearance.[10–12]

In this study, with a relatively large cohort of CH-C patients treated with Peg-IFN and RBV, we validated the clinical relevance of the expression of hepatic ISGs as predictive factors for the outcome of treatment. In addition, we demonstrated that the expression of hepatic ISGs was closely related to genetic variation in IL28B.

Materials and Methods

Patients

We enrolled 168 patients with CH-C at the Graduate School of Medicine, Kanazawa University Hospital and its related hospitals, Japan (Table 1, Supplementary Table 1). The cohort included 92 men and 76 women, ranging from 21 to 73 years of age, who were registered prospectively in 2005 and 2007. All patients had HCV

Table 1. Comparison of Clinical Factors Between Patients With and Without NR

Clinical category	SVR+TR		NR		Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
No. of patients	n = 125		n = 43			—	
Age and sex							
Age, *y*	57	(30–72)	56	(30–73)	.927	—	
Sex (M vs F)	68 vs 57		24 vs 19		.872	—	
Liver factors							
F stage (F1-2 vs F3-4)	95 vs 30		20 vs 23		.001	4.18 (1.61–11.5)	.003
A grade (A0-1 vs A2-3)	68 vs 57		19 vs 24		.248	—	
ISGs (Mx, IFI44, IFIT1)							
(<3.5 vs ≥3.5)	103 vs 22		12 vs 31		<.001	16.2 (6.21–47.8)	<.001
Laboratory parameters							
HCV-RNA (*KIU/mL*)	2300	(126–5000)	1930	(140–5000)	.725	—	
BMI (kg/m^2)	23.2	(16.3–34.7)	23.4	(19.5–40.6)	.439	—	.107
AST (*IU/L*)	46	(18–258)	64	(21–283)	.017	—	
ALT (*IU/L*)	60	(16–376)	82	(18–345)	.052	—	
γ-GTP (*IU/L*)	36	(4–367)	75	(26–392)	<.001	—	
WBC ($/mm^3$)	4800	(2100–11,100)	4800	(2500–8200)	.551	—	
Hb (*g/dL*)	14	(9.3–16.6)	14.4	(11.2–17.2)	.099	—	
PLT ($\times 10^4/mm^3$)	15.7	(7–39.4)	15.2	(7.6–27.8)	.378	—	
TG (*mg/dL*)	98	(30–323)	116	(45–276)	.058	—	
T-Chol (*mg/dL*)	167	(90–237)	160	(81–214)	.680	—	
LDL-Chol (*mg/dL*)	82	(36–134)	73	(29–123)	.019	—	
HDL-Chol (*mg/dL*)	42	(20–71)	47	(18–82)	.098	—	
FBS (*mg/dL*)	94	(60–291)	96	(67–196)	.139	—	
Insulin (*μU/mL*)	6.6	(0.7–23.7)	6.8	(2–23.7)	.039	—	
HOMA-IR	1.2	(0.3–11.7)	1.2	(0.4–7.2)	.697	—	
Viral factors							
ISDR mutations ≤1 vs ≥2	80 vs 44		34 vs 9		.070	5.09 (1.69–17.8)	.003
Treatment factors							
Total dose administered							
Peg-IFN (*μg*)	3840	(960–7200)	3840	(1920–2880)	.916	—	
RBV (*g*)	202	(134–336)	202	(36–336)	.531	—	
Achieved administration rate							
Peg-IFN (%)							
≥80%	84		28		.975	—	
<80%	42		14				
RBV (%)							
≥80%	76		24		.745	—	
<80%	50		18				
Achievement of EVR	101/125 (81%)		0/43 (0%)		<.001	—	

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; EVR, early virologic response; γ-GTP, γ-glutamyl transpeptidase; ISDR, interferon sensitivity determining region; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); WBC, leukocytes; HOMA-IR, homeostasis model assessment of insulin resistance; Hb, hemoglobin; RBV, ribavirin; PLT, platelets; TG, triglycerides; TR, transient response; T-chol, total cholesterol; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; FBS, fasting blood sugar; CI, confidence interval.

genotype 1b and high viral loads (≥100K IU/mL) measured by quantitative Cobas Amplicor assays (Roche Diagnostics Co Ltd, Tokyo, Japan). All patients had undergone liver biopsy before combination therapy. Exclusion criteria for patients not eligible for Peg-IFN and RBV combination therapy were as follows: (1) pregnant women or women of childbearing potential, nursing mothers, or male patients whose partner might become pregnant; (2) patients with hepatocellular carcinoma; (3) patients with serious complications in the heart, kidneys, or lungs; (4) patients with autoimmune diseases, such as autoimmune hepatitis, and primary biliary cirrhosis; and (5) patients infected with the hepatitis B virus. Informed consent was obtained from all patients, and ethics approval for the study was obtained from the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science.

All patients were administered Peg-IFN-α 2b (Schering-Plough KK, Tokyo, Japan) and RBV combination therapy for 48 weeks. Peg-IFN was given in weekly doses and adjusted to body weight according to the manufacturer's instructions (45 kg or less, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; and 91 kg or more, 150 μg/dose). Similarly, RBV (Schering-Plough KK) was administered in daily doses adjusted to body weight according to the manufacturer's instructions (60 kg or less, 600 mg/day; 61–80 kg, 800 mg/day; and 81 kg or more, 1000 mg/day).

The final outcome of treatment was assessed 24 weeks after the cessation of combination therapy. We defined treatment outcomes according to the decrease in viremia as follows: sustained viral response (SVR), clearance of HCV viremia 24 weeks after the cessation of therapy; transient response (TR), no detectable HCV viremia at the cessation of therapy but relapsed during the follow-up period; and no response (NR), HCV viremia detected at the cessation of therapy. An early virologic response (EVR) (complete EVR) was defined as undetectable HCV-RNA in the serum by 12 weeks. HCV genotypes were determined according to the method of Okamoto et al. Serum HCV RNA was determined using qualitative and quantitative COBAS Amplicor assays (Roche Diagnostics Co, Ltd, Tokyo, Japan). The grading and staging of chronic hepatitis were histologically assessed according to the method of Desmet et al (Table 1).[13]

Preparation of Liver Tissue Samples

Liver biopsy samples were taken from all patients before treatment. The biopsy samples were divided into 2 parts: the first part was immersed in formalin for histologic assessment, and the second was immediately immersed in RNAlater (QIAGEN, Valencia, CA) for RNA isolation. Liver tissue RNA was isolated using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. Isolated RNA was stored at −70°C until use.

Affymetrix Genechip Analysis

The quality of the isolated RNA was estimated after electrophoresis using an Agilent 2001 Bioanalyzer (Agilent, Santa Clara, CA). Aliquots of total RNA (50 ng) isolated from the liver biopsy specimens were subjected to amplification using the WT-Ovation Pico RNA Amplification System (NuGen, San Carlos, CA) according to the manufacturer's instructions. Approximately 10 μg of complementary DNA (cDNA) was amplified from 50 ng of total RNA, and 5 μg of cDNA was used for fragmentation and biotin labeling using the FL-Ovation cDNA Biotin Module V2 (NuGen) according to the manufacturer's instructions. Biotin-labeled cDNA was suspended in 220 μL of hybridization cocktail (NuGen), and 200 μL was used for hybridization to the Affymetrix Human 133U Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA) containing 54,675 probes. After stringent washing, the microarray chips were stained with streptavidin-phycoerythrin, and probe hybridization was determined using a GeneChip Scanner 3000 (Affymetrix). Data files (CEL) were obtained using the GeneChip Operating Software 1.4 (Affymetrix).

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). The data were log transferred, normalized, centered, and applied to the average linkage hierarchical clustering with centered correlation.

For genechip analysis, we selected 37 representative ISGs. Hepatic gene expression profiling was obtained from 30 CH-C patients before and 1 week after the initiation of IFN and RBV combination therapy and the 100 most up-regulated genes were selected (submitted for publication). ISGs were suppressed in patients with a rapid viral response and up-regulated in patients with a slow viral response before treatment. Using the 100 treatment-induced genes, we evaluated hepatic gene expression in 30 patients before treatment. Hierarchical clustering analysis showed that a cluster of 37 ISGs was up-regulated in patients with a slow viral response.

Pathway analysis was performed using MetaCore (GeneGo, St. Joseph, MI). Functional ontology enrichment analysis was performed to compare the gene ontology process distribution of differentially expressed genes ($P < .01$).

Quantitative Real-time Detection-Polymerase Chain Reaction

We performed quantitative real-time detection (RTD)-polymerase chain reaction (PCR) (RTD-PCR) using TaqMan Universal Master Mix (PE Applied Biosystems, Carlsbad, CA). Primer pairs and probes for myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse) (Mx1), 2′-5′-oligoadenylate synthelase 3 (OAS3), interferon-induced protein 44 (IFI44),

interferon-induced protein 44-like (IFI44L), 2′-5′-oligoadenylate synthetase 2 (OAS2), ubiquitin specific peptidase 18 (USP18), radical S-adenosyl methionine domain containing 2 (RSAD2), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon induced with helicase C domain 1 (IFIH1), XIAP associated factor 1 (XAF1), cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial (CMPK2), epithelial stromal interaction 1 (EPSTI1), hect domain and RLD 6 (HERC6), poly (ADP-ribose) polymerase family, member 9 (PARP9), phospholipid scramblase 1 (PLSCR1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from the TaqMan assay reagents library. Primer pairs and probes for IL28B were designed as previously described.[12] The standard curve was obtained in every assay using the RNA obtained from a normal liver.[14,15] The expression values were normalized by GAPDH, and normalized values indicate the relative fold expression to a normal liver.

Amino Acid Substitutions of ISDR in the Nonstructural 5A Region

The nucleotide sequence of ISDR in the nonstructural 5A region was determined by direct sequencing of PCR amplified materials.[4] Mutant-type ISDR was defined as containing 2 or more aa substitutions.

Genetic Variation of IL28B Polymorphism

A single nucleotide polymorphism (SNP) of IL28B was evaluated in 91 patients whose hepatic gene expression profiling was obtained. We genotyped 32 patients using Affymetrix Genome-Wide Human SNP Array 6.0 as previously described.[12] The results for rs8105790, rs11881222, rs8099917, and rs7248668 were retrieved from a database to evaluate the association of these SNPs. rs12979860 was determined by direct sequencing, and rs8099917 was determined using TaqMan Pre-Designed SNP Genotyping Assays (PE Applied Biosystems) as recommended by the manufacturer.

Statistical Analysis

The Mann-Whitney U test was used to analyze continuous variables. Fisher exact test and χ^2 test were used for the analysis of categorical data. The overall plausibility of the treatment response groups was assessed using Fisher C statistic (Supplementary Table 2).[16,17] C is defined by $C = -2 \Sigma \ln(p_i)$, where p_i is the probability (P value) of each independent statement (clinical factors). C follows a χ^2 distribution with 2k degrees of freedom, k being the number of independent statements (clinical factors).[16] A nonsignificant C value means that the treatment response in the 2 groups was not statistically independent.

Multivariate analysis was performed using a stepwise logistic regression model. Each cut-off point for the continuous variables was decided by analysis of the receiver operating characteristic (ROC) curve. A P value of less than .05 was considered significant. Statistical analyses were performed using JMP7 for Windows (SAS Institute, Cary, NC).

Results

Response Rate and Clinical Characteristics

The clinical characteristics of the patients are shown in Table 1 and Supplementary Table 1. All of the patients were infected with HCV genotype 1b and had a high viral load (>100K IU/mL). No patients were coinfected with the hepatitis B virus (HBV). The intention-to-treat analysis showed that SVR, TR, and NR were observed in 70 (42%), 55 (33%), and 43 (25%) patients, respectively (Supplementary Table 1). Before comparing patients with 3 different responses, the overall plausibility of the treatment response groups was assessed using Fisher C statistic. Fisher C statistic utilizes the P values obtained by comparing pretreatment factors including age, gender, liver factors, laboratory parameters, and viral factors. Because the SVR and TR groups could not be defined as different, they were grouped together and compared with NR (Table 1, Supplementary Table 2).

Eleven patients with NR discontinued the therapy after 24 weeks because of an insufficient effect, namely, serum HCV-RNA was still detectable at this time. The remaining patients completed 48 weeks of Peg-IFN and RBV combination therapy. The administration rate of Peg-IFN with 80% or more was achieved in 67% of patients, and the administration rate of RBV with 80% or more was achieved in 60% of patients (Table 1).

Analysis of Hepatic Gene Expression

Prior to treatment, 91 of 168 patients (Supplementary Table 3) were randomly selected, and their hepatic gene expression was determined using Affymetrix genechip analysis.

Hierarchical clustering using 37 representative ISGs (see Materials and Methods) demonstrated 2 clear clusters of patients: one was a group composed of patients with up-regulated ISGs (Up-ISGs), and the other was a group consisting of patients with down-regulated ISGs (Down-ISGs) (Figure 1). In patients with Up-ISGs, 21 (49%) showed NR, whereas 8 (17%) patients with Down-ISGs showed NR ($P = .002$). In contrast, 14 (33%) patients with Up-ISGs showed SVR, whereas 27 (56%) patients with Down-ISGs showed SVR ($P = .03$). There were no significant differences in the frequency of advanced stages of liver fibrosis (F3-F4) between patients with Up-ISGs and patients with Down-ISGs (18 [42%] and 17 [35%], respectively, $P = .664$). These data indicated that the up-regulation of ISGs in the liver before treatment was strongly associated with resistance to IFN treatment.

Host and Viral Factors Associated With the Response to Combination Therapy

To evaluate the multiple host and viral factors associated with the response to Peg-IFN and RBV combination therapy in all patients, univariate and multivariate analyses were performed. To assess the expression of hepatic ISGs, 15 genes (Mx1, OAS3, IFI44, IFI44L, OAS2,

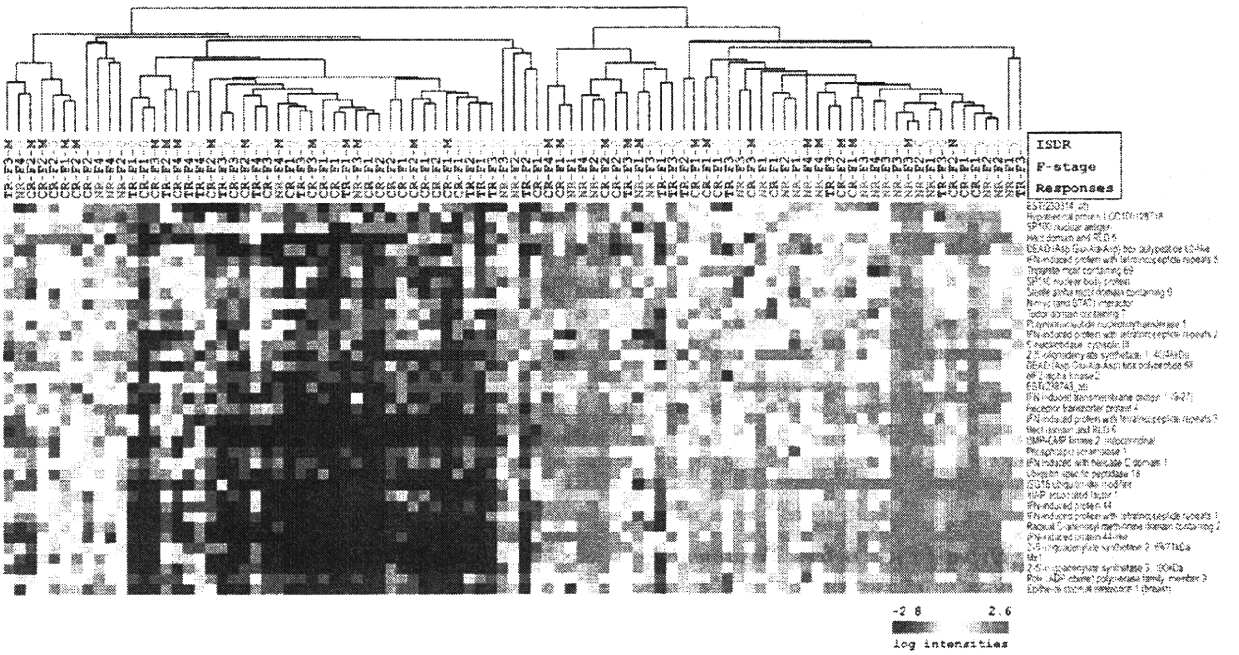

Figure 1. Hierarchical clustering analysis of 91 patients using 37 representative ISGs. Responses to therapy (SVR, TR, and NR), fibrosis stage (F1–F4), and status; ISDR mutations are also shown. ISDR mutation ≥2 = M, ≤1 = 0.

USP18, RSAD2, IFIT1, IFIH1, XAF1, CMPK2, EPSTI1, HERC6, PARP9, and PLSCR1) out of 37 representative ISGs were selected for their expression values of probe intensity, and their expression was confirmed in liver tissue obtained from 168 patients by RTD-PCR. Although there were significant correlations of their expression with each other, except RARP9 and PLSCR1 (Supplementary Table 4), the dynamic range of gene expression was high for 3 genes, namely, Mx1, IFI44, and IFIT1 (Supplementary Figure 1*A*). We averaged the expression values of Mx1, IFI44, and IFIT1 and used them for further study.

When we compared patients with SVR+TR and NR, the fibrosis stage of the liver ($P = .001$), expression of hepatic ISGs ($P < .001$), aspartate aminotransferase (AST) serum level ($P = .017$), γ-glutamyl transpeptidase (γ-GTP) ($P < .001$), low-density lipoprotein cholestrol (LDL-Chol) ($P = .019$), and insulin (μU/mL) ($P = .039$) were significantly different prior to treatment (Table 1). For treatment factors, the total dose and administration of IFN and RBV were not significantly different between these 2 groups. EVR was observed in 101 (81%) patients, and the proportion was significantly different ($P < .001$) between patients with SVR+TR and NR (Table 1).

Regression analysis of pretreatment factors showed a strong correlation among γ-GTP, alanine aminotransferase (ALT), and aspartate aminotransferase (AST); and homeostasis model assessment-insulin resistance (HOMA-IR), fasting blood sugar, and insulin; and total cholesterol (T-Chol), high-density lipoprotein cholestrol (HDL-Chol), and LDL-Chol (data not shown). We selected fibrosis stage, ISGs, HCV-RNA, ISDR mutation, and body mass index (BMI) as factors for multivariate analysis. Stepwise multivariate logistic regression analysis was performed using the selected factors. From the ROC curve, we set the cut-off value for the expression of ISGs as 3.5 (Supplementary Figure 1*B*). The results showed that expression of hepatic ISGs (<3.5), fibrosis stage (F1-F2), and ISDR mutation (≥2) were significant pretreatment factors contributing to SVR+TR (Table 1).

Clinical Parameters Associated With the Expression of Hepatic ISGs

Univariate and multivariate analyses revealed that the expression of hepatic ISGs was a strong predictor of the treatment outcome for SVR+TR patients. We next examined which clinical parameters were associated with the expression of hepatic ISGs (Table 2). Univariate analysis showed that the expression of ISGs was strongly correlated with the serum levels of γ-GTP ($P < .001$) and AST ($P < .001$) and weakly correlated with HCV-RNA, fasting blood sugar, insulin, HOMA-IR, triglyceride (TG), and LDL-Chol. Multivariate analysis showed that γ-GTP ($P < .001$), HCV-RNA ($P < .001$), and LDL-Chol ($P = .048$) were significantly associated with hepatic ISGs. Noticeably, the expression of ISGs was negatively correlated with HCV-RNA in SVR+TR patients ($P = .009$), whereas this correlation was not evident in NR patients ($P = .298$) (Table 2, Supplementary Figure 2). These results may indicate that endogenous ISGs suppress HCV in SVR+TR patients, whereas they are not active in NR patients.

Table 2. Clinical Factors Associated With Expression of Hepatic Interferon-Stimulated Genes

	Univariate analysis				Multivariate analysis			
Clinical factor	β	95% CI		*P* value	β	95% CI		*P* value
AST (*IU/L*)	0.274	0.13	0.42	<.001	—	—	—	
γ-GTP (*IU/L*)	0.326	0.18	0.47	<.001	0.288	0.14	0.43	<.001
HCV-RNA (*KIU/mL*)	−0.170	−3.19	−0.02	.025	−0.255	−0.40	−0.11	<.001
SVR+TR	−0.237	−0.32	−0.05	.009	—	—	—	
NR	−0.168	−0.57	0.18	.298	—	—	—	
FBS (*mg/dL*)	0.182	0.03	0.35	.021	—	—	—	
Insulin (*μU/mL*)	0.190	0.03	0.34	.016	—	—	—	
HOMA-IR	0.181	0.03	0.33	.017	—	—	—	.073
TG (*mg/dL*)	0.201	0.05	0.35	.011	—	—	—	.089
LDL-Chol (*mg/dL*)	−0.177	−0.33	−0.02	.025	−0.143	−0.28	0.00	.048

γ-GTP, γ-glutamyl transpeptidase; AST, aspartate aminotransferase; FBS, fasting blood sugar; TG, triglycerides; TR, transient response; NR, no response; SVR, sustained viral response; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-chol, low-density lipoprotein cholesterol; CI, confidence interval; β, β coefficient; CI, confidence interval.

Expression of Hepatic ISGs Before Treatment Is Associated With Genetic Variation of IL28B

Recently, a GWAS successfully identified the genomic locus associated with the treatment response to Peg-IFN and RVB combination therapy for CH-C. Genetic variation in IL28B predicts HCV treatment-induced viral clearance.[11,12] We determined the genetic variation in IL28B of 32 patients[12] (Table 3). The SNPs rs8105790, rs11881222, rs8099917, and rs7248668 had a significant association with treatment response (odds ratio: 24.7–27.1, $P = 1.84 \times 10^{-30}$–2.68×10^{-32}). These SNPs are located in block 2 of the IL28B haplotype and show significant linkage disequilibrium in the HapMap data.[12] Ge et al[11] reported a different SNP (rs12979860) that was located between rs11881222 and rs8099917. The nucleotide sequence of rs12979860 was determined by direct sequencing, and the results are shown in Table 3. There was a strong association of rs12979860 and the other 4 SNPs indicating that this SNP was located within the same haplotype block. We confirmed these findings in multiple samples from Japanese patients (data not shown).

We selected rs8099917 for further study and evaluated it using TaqMan Pre-Designed SNP Genotyping Assays. The G nucleotide of rs8099917 was associated with a poor response to treatment (minor allele), whereas the T was associated with a fair response to treatment (major allele).[12] Out of 91 patients (Supplementary Table 3), the proportion of major homozygotes (TT), heterozygotes (TG), and minor homozygotes (GG) were 66% (60/91), 30% (27/91), and 4% (4/91), respectively (Table 4); 86% (51/60) of the major genotype (TT) patients had SVR or TR, whereas 65% (20/31) with the minor genotypes (TG or GG) had NR ($P < .001$).

Interestingly, hepatic gene expression profiles revealed that patients with the minor genotype showed higher expression of hepatic ISGs, whereas patients with the major genotype showed lower expression of hepatic ISGs (Figures 2 and 3). To examine further the relationship of the genetic variation in IL28B and its expression levels, we evaluated the expression of IL28B in the liver by RTD-PCR (Figure 3). IL28B expression was approximately 10-fold less than the expression of ISGs. Although IL28B expression tended to be higher in some patients with the major genotype, there was no significant difference in IL28B expression in the liver between the major and minor genotypes (Figure 3*A*). Nevertheless, the expression of ISGs was clearly high in patients with the minor genotype ($P < .0001$) (Figure 3*B*). IL28 activates signal tranducers and activators of transcription 1 (STAT1) through downstream signaling from a heterodimeric class II cytokine receptor that consists of IL-10 receptor β (IL-10Rβ) and IL-28 receptor α (IL-28Rα).[18,19] Therefore, we examined the correlation between the expression of IL28B and ISGs. IL28B expression correlated with the expression of ISGs ($r = 0.44$, $P < .001$); however, the correlation was different according to the SNP genotype. We observed a steep-slope correlation for the minor genotype and a slow-slope correlation for the major genotype (Figure 3*C* and *D*). Interestingly, 4 minor homozygotic (GG) patients showed a steeper correlation than the heterozygotes (TG) (Figure 3*D*). Thus, the IL28B polymorphism might differentially regulate the expression of ISGs in the liver, leading to the different treatment outcomes.

We performed univariate and multivariate analyses to identify the clinical factors associated with the major and minor genotypes (Table 4). Univariate analysis showed that higher hepatic ISGs and lower body mass index were significantly associated with the minor genotype; however, multivariate analysis showed that only hepatic ISGs (≥3.5) were associated with the minor genotype ($P < .001$; OR, 18.1; 95% confidence interval: 3.95–113). We further compared the predictive capacity of multivariate models using the expression of hepatic ISGs (<3.5 vs ≥3.5) or the IL28B genotype (major vs minor) (Supplementary Table 6). The predictive performance and fitness of the multivariate model using the IL28B genotype was superior to that using the expression of hepatic ISGs. However, when these factors were included in the same model, the expression of hepatic ISGs was still useful for the predictive model independent of the IL28B genotype (Supplementary Table 6).

Table 3. Clinical Characteristics of 32 Patients Genotyped by GWAS and 5 SNPs in Strong Linkage Disequilibrium With IL28B,[11] Including rs12979860

Patient No.	Response	Age, y	Sex	F stage	ISGs	IL28B	RefSNP (chr pos) Minor allele	rs8105790 (44424341) C	rs11881222 (44426763) G	rs12979860 (44430627) T	rs8099917 (44435005) G	rs7248668 (44435661) A
1	SVR	42	M	1	4.20	83.8		TT	AA	CC	TT	GG
2	SVR	59	M	1	2.62	45.5		TT	AA	CC	TT	GG
3	SVR	41	F	1	1.54	1.3		TT	AA	CC	TT	GG
4	TR	57	M	1	3.18	21.7		TT	AA	CC	TT	GG
5	TR	68	F	1	1.43	20.3		TT	AA	CC	TT	GG
6	SVR	44	M	1	0.97	4.6		TT	AA	CC	TT	GG
7	SVR	61	M	2	2.15	6.1		TT	AA	CC	TT	GG
8	SVR	50	M	2	3.25	66.4		TT	AA	CC	TT	GG
9	SVR	49	M	2	1.25	ND		TT	AA	CC	TT	GG
10	TR	59	F	2	1.29	17.4		TT	AA	CC	TT	GG
11	SVR	48	F	2	1.00	90.2		TT	AA	CC	TT	GG
12	TR	65	F	2	2.86	36.4		TT	AA	CC	TT	GG
13	NR	34	M	3	0.82	17.8		TT	AA	CC	TT	GG
14	SVR	55	M	3	0.83	13.8		TT	AA	CC	TT	GG
15	TR	68	M	3	0.75	20.6		TT	AA	CC	TT	GG
16	SVR	64	M	3	0.94	15.7		TT	AA	CC	TT	GG
17	SVR	67	F	3	1.50	25.7		TT	AA	CC	TT	GG
18	SVR	48	M	4	1.69	7.9		TT	AA	CC	TT	GG
19	NR	66	F	1	4.57	16.5		TC	AG	CT	TG	GA
20	SVR	52	F	1	5.23	29.3		TC	AG	CT	TG	GA
21	NR	55	F	1	8.25	57.2		TC	AG	CT	TG	GA
22	SVR	49	F	1	5.36	ND		TC	AG	CT	TG	GA
23	TR	44	M	1	2.08	7.0		TC	AG	CT	TG	GA
24	NR	63	M	1	2.77	10.5		TC	AG	CT	TG	GA
25	NR	61	F	2	3.98	39.1		TC	AG	CT	TG	GA
26	NR	42	M	2	4.89	5.9		TC	AG	CT	TG	GA
27	SVR	49	M	3	3.31	6.9		TC	AG	CT	TG	GA
28	TR	71	F	3	5.53	27.3		TC	AG	CT	TG	GA
29	TR	63	M	3	3.40	33.5		TC	AG	CT	TG	GA
30	NR	70	F	3	4.78	8.1		TC	AG	CT	TG	GA
31	TR	62	F	3	3.53	14.0		TC	AG	CT	TG	GA
32	NR	56	M	4	7.37	30.8		CC	GG	TT	GG	AA

NOTE. The Pearson correlation of the r^2 estimates for adjacent pairs; rs8099917 vs rs8105790, rs8099917 vs rs11881222, rs8099917 vs rs12979860, and rs8099917 vs rs7248668 = 0.99, 0.99, 0.98, and 0.97, respectively.
IL28B, interleukin 28B; GWAS, genome-wide association studies; ISGs, interferon stimulated genes; SNP, single nucleotide polymorphism; SVR, sustained viral response; TR, transient response; NR, no response; M, male; F, female.

To examine further the different hepatic gene expression of patients with the major or minor genotypes, pathway analysis of differentially expressed genes between the 2 groups was performed. By comparing the expression of hepatic genes between patients with the major and minor genotypes, 1359 differentially expressed genes were identified ($P < .01$; 711 genes were up-regulated with the minor genotype, and 648 genes were up-regulated with the major genotype). Pathway analysis of these genes demonstrated that signaling pathways related to interferon action, apoptosis, and Wnt signaling were up-regulated in the liver of patients with the minor genotype, whereas B-cell-, dendritic cell-, and natural killer cell-related genes were up-regulated in the liver of patients with the major genotype (Supplementary Figure 3). These results suggest that IL28B may be involved in innate and adaptive immune responses and that different antiviral signaling pathways might be involved in the liver of patients with different SNPs.

Discussion

Multiple viral and host factors may be related to the treatment response to Peg-IFN and RBV combination therapy. For the viral factors, a higher number of aa substitutions in the ISDR of nonstructural 5A region was strongly associated with a favorable response to IFN-α monotherapy in patients with genotype-1 HCV.[4]

Besides viral factors, host factors such as age, gender, fibrotic stage of the liver, and the presence of steatosis and insulin resistance were associated with the treatment outcome.[20] Analysis of hepatic gene expression demonstrated that the up-regulation of ISGs in the liver before treatment may be related to a poor treatment response.[6-9] To reveal the underlying mechanism of treatment resistance, 2 reports compared gene expression profiling in the liver before and during therapy and showed that patients with up-regulated ISGs in the liver prior to treatment failed to further induce ISGs following the ad-

Table 4. Comparison of Clinical Factors Between Patients With Major (TT) and Minor (TG+GG) Alleles

Clinical category	TT		TG+GG		Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
No. of patients	n = 60		n = 31			—	
Treatment response							
SVR+TR vs NR	51 vs 9		11 vs 20		<.001	—	
Age and gender							
Age, *y*	56	(30–69)	56	(30–71)	.843	—	
Sex (M vs F)	39 vs 21		19 vs 12		.518	—	
Liver factors							
F stage (F1-2 vs F3-4)	36 vs 24		23 vs 17		.905	—	
A grade (A0-1 vs A2-3)	27 vs 33		20 vs 11		.075	—	
ISGs (Mx, IFI44, IFIT1)							
(<3.5 vs ≥3.5)	46 vs 14		5 vs 26		<.001	18.1 (3.95–113)	<.001
Laboratory parameters							
HCV-RNA (*KIU/mL*)	2055	(160–5000)	1970	(126–5000)	.602	—	
BMI (kg/m^2)	24.5	(16.3–40.5)	22.9	(19.1–26.6)	.006	—	.077
AST (*IU/L*)	59	(22–258)	54	(21–283)	.227	—	
ALT (*IU/L*)	75	(24–376)	60	(18–236)	.077	—	
γ-GTP (*IU/L*)	61	(4–392)	53	(20–229)	.517	—	.167
WBC ($/mm^3$)	4450	(2100–11,100)	4600	(2500–8200)	.947	—	
Hb (*g/dL*)	14.2	(11.4–16.7)	14.5	(11.2–17.2)	.606	—	
PLT ($\times 10^4/mm^3$)	15.4	(7–39.4)	16.2	(9.2–27.7)	.832	—	
TG (*mg/dL*)	98	(58–248)	131	(30–303)	.053	—	.055
T-Chol (*mg/dL*)	172	(115–222)	168	(129–237)	.910	—	
LDL-Chol (*mg/dL*)	84	(42–123)	69	(51–107)	.052	—	.055
HDL-Chol (*mg/dL*)	44	(18–72)	45	(29–77)	.218	—	
FBS (*mg/dL*)	95	(59–291)	96	(66–206)	.849	—	
Insulin (*μU/mL*)	7.5	(0.7–23.2)	9.2	(2–23.2)	.195	—	
HOMA-IR	1.3	(0.3–11.7)	1.2	(0.4–9.6)	.339	—	
Viral factors							
ISDR mutations (≤1 vs ≥2)	38 vs 22		23 vs 7		.194	—	.083
Treatment factors							
Total dose administrated							
Peg-IFN (*μg*)	3960	(1500–7200)	3840	(1920–5760)	.377	—	
RBV (*g*)	203	(26–336)	201	(106–268)	.777	—	
Achieved administration rate							
Peg-IFN (%)							
≥80%	41		17		.207	—	
<80%	19		14				
RBV (%)							
≥80%	34		19		.671	—	
<80%	26		12				
Achievement of EVR	40/60 (62%)		9/31 (29%)		<.001	—	

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; EVR, early virologic response; γ-GTP, γ-glutamyl transpeptidase; ISDR, interferon sensitivity determining region; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); WBC, leukocytes; HOMA-IR, homeostasis model assessment of insulin resistance; Hb, hemoglobin; RBV, ribavirin; PLT, platelets; TG, triglycerides; TR, transient response; T-chol, total cholesterol; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; FBS, fasting blood sugar; CI, confidence interval.

ministration of IFN and could not eliminate HCV.[6,7] We performed a similar analysis and observed that these findings were more evident in liver lobular cells than in infiltrating lymphocytes in the portal area (submitted for publication). Thus, both viral and host factors might be closely related to the treatment response to Peg-IFN and RBV combination therapy. However, the clinical relevance and relationships of these factors have not been fully evaluated. In this study, we validated the clinical significance of the expression of hepatic ISGs on treatment outcome using a relatively large cohort of patients and compared its significance with other viral and host factors. To compare the patients with SVR, TR, and NR, we assessed the overall plausibility of each group using Fisher C statistic,[16] and patients with SVR and TR were grouped together for further analysis.

We examined hepatic gene expression in 91 of 168 patients using the Affymetrix genechip. Expression profiling using 37 representative ISGs (see Materials and Methods), which were selected from gene expression profiling comparing pretreatment and under treatment liver, differentiated 2 groups of

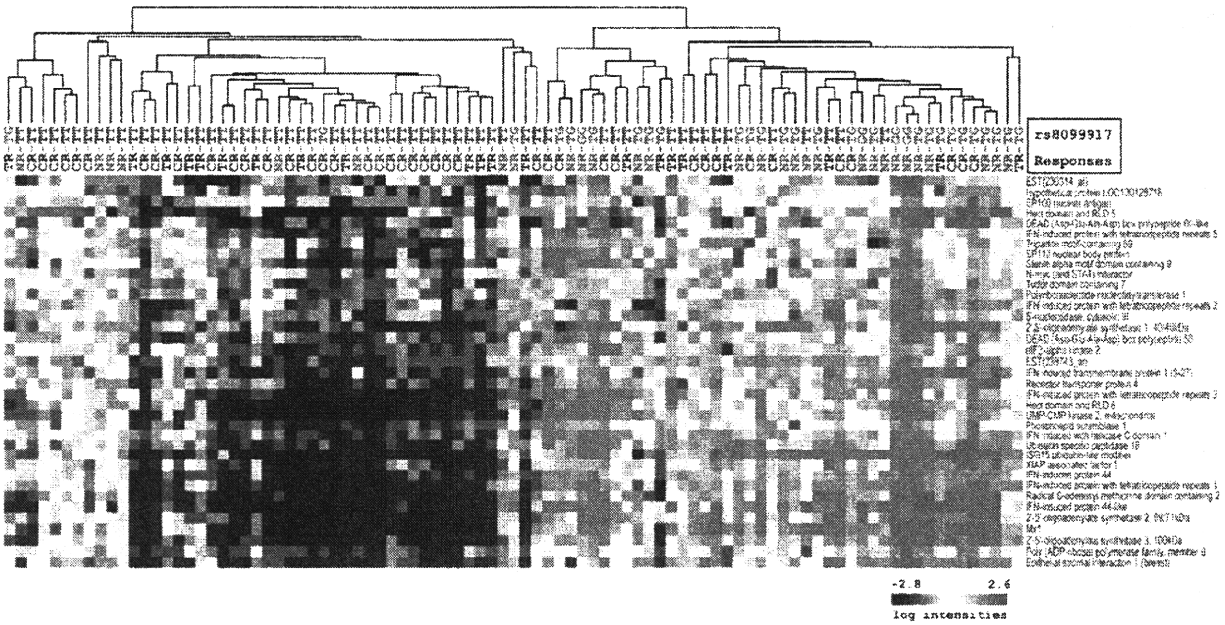

Figure 2. Hierarchical clustering analysis of 91 patients with the defined genotype of IL28B. Responses to therapy (SVR, TR, and NR) and IL28B genotype (TT, TG, or GG) are shown. The structure of the dendrogram and heat map is the same as in Figure 1.

patients: the Up-ISG and Down-ISG groups (Figure 1). The proportion of patients with NR to treatment was significantly higher in the Up-ISGs group.

Multivariate analysis showed that hepatic ISGs (<3.5), fibrosis stage (F1-F2), and ISDR mutations (≥2) significantly contributed to the outcome for the SVR+TR group (Table 1). Discriminate analysis using variables selected by multivariable analysis predicted the SVR+TR patients with 82% accuracy and NR patients with 79% accuracy. However, the accuracy decreased to 67% for SVR+TR patients and 53% for NR patients when the expression of hepatic ISGs was removed from the variables (data not shown). Interestingly, the expression of hepatic ISGs was strongly correlated with γ-GTP and weakly correlated with insulin resistance. A recent study describing the association between insulin resistance and poor treatment outcome might be partially explained by this observation.[20]

In this study, we utilized 3 ISGs (Mx1, IFI44, and IFIT1) out of 15 validated by RTD-PCR. The expression values of these ISGs were higher than those of other ISGs (Supplementary Figure 1*A*). We averaged these ISGs and set the cut-off value as 3.5 from the ROC curve (Supplementary Figure 1*B*). The sensitivity, specificity, and positive and negative predictive values on the likelihood of achieving SVR+TR using this cut-off value were 82% (103/125), 72% (31/43), 90% (103/115), and 58% (31/53), respectively. The results were compared with those observed for the 15 ISGs (Supplementary Table 5). These results showed that the 3.5 cut-off value for Mx1, IFI44, and IFIT1 would be valuable for clinical use.

Despite the importance of the expression of hepatic ISGs, viral factors may also allow us to predict the outcome of treatment. Multivariate analysis showed that ISDR mutations (≥2) independently contributed to the treatment outcome, although univariate analysis did not show significance (P = .07); therefore, ISDR might be uniquely and differentially involved in treatment resistance.

What causes the differences in the expression of hepatic ISGs? In parallel to the gene expression analysis, a GWAS was applied to identify genomic loci associated with treatment response, and a polymorphism in IL28B was found to predict hepatitis C treatment-induced viral clearance.[10–12] To examine the relationship between the genetic variation of IL28B and hepatic gene expression, we determined the IL28B polymorphism in 91 patents (Table 3). The patients with the minor genotype (TG or GG) had an increased expression of hepatic ISGs compared with the patients with major genotype (TT) (Figures 2 and 3). In European-Americans, the proportion of major homozygotes is 39% (CC at rs1297986), 49% for heterozygotes (TC), and 12% for minor homozygotes (TT).[11] Although the proportion of minor homozygotes was much less in this study (GG, 4%), as reported in a previous study in Japan,[12] more patients are required for proper evaluation. It is interesting that the expression of hepatic ISGs in minor homozygotes (GG) was higher than in heterozygotes (TG) in this study.

The results clearly showed that the differences in the expression of hepatic ISGs before treatment are associated with the IL28B polymorphism and results in different treatment outcomes. Although we could not detect significant differences in the expression levels of IL28B depending on the different SNP, some patients with the major genotype showed a higher expression of IL28B. Because IL28B expression was approximately 10-fold less than the expression of ISGs, the lower

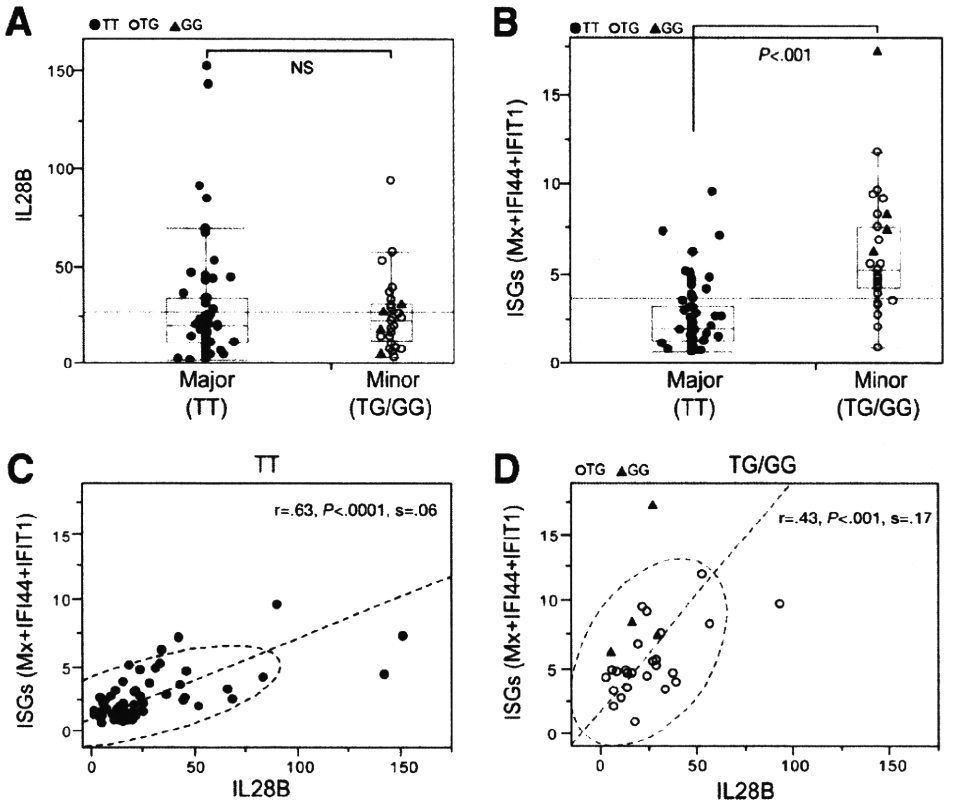

Figure 3. (*A*) IL28 expression in the liver of 91 patients with the major (TT) or minor (TG or GG) genotype (rs8099917). (*B*) Expression of ISGs in the liver of patients with the major (TT) or minor (TG or GG) genotype (rs8099917). (*C*) Relationship between IL28 and ISGs in the liver of patients with the major (TT) genotype (rs8099917). (*D*) Relationship between IL28 and ISGs in the liver of patients with the minor (TG or GG) genotype (rs8099917).

expression of IL28B may be a reason for the decreased ability to distinguish differences in its expression. Another possibility may be the specificity of the IL28B primers used in this study; because IL28B shares a 98.2% nucleotide sequence homology with IL28A, IL28B specific primers are not available.[21] When the expression of IL28B and hepatic ISGs were compared, a significant correlation was observed, and, interestingly, IL28B and ISGs derived from different SNPs were correlated in a different way (Figure 3*C* and *D*). It appeared that hepatic ISGs were more induced by the reduced amounts of IL28B in patients with the minor genotype. The mechanism behind these findings has yet to be determined; however, IL28B interacts with a heterodimeric class II cytokine receptor that consists of IL-10 receptor β (IL-10Rβ) and IL-28 receptor α (IL-28Rα).[18,19] It is possible that IL28B could mediate antiviral signaling through IL-10 signaling as well as STAT1 activation. The Th 2 dominant signaling of IL28B may modulate signaling pathways in livers with CH-C and contributes to the different expression of ISGs. Another possibility may be that the cell origin of hepatic ISGs is different. A recent study revealed cell-type specific ISG expression in macrophages and hepatocytes, which could be related to the IFN response.[22] As more of the B-cell-, dendritic cell-, and natural killer cell-related genes were up-regulated in the liver of patients with the major genotype, ISGs could be expressed by these cells, whereas they are expressed by hepatocytes in the liver of patients with the minor genotype. It is known that the induction of ISGs in lymphocytes is lower than that in hepatocytes. The precise mechanism should be investigated further as a different regulatory mechanism for the expression of ISGs may be present.

In conclusion, we presented the clinical relevance of the expression of hepatic ISGs for the treatment outcome of Peg-IFN and RBV combination therapy. The different expressions of hepatic ISGs before treatment might be due to polymorphisms in IL28B. Further studies are required to clarify the detailed pathways of IL28B and hepatic gene expression through molecular biologic and immunologic aspects.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.04.049.

Appendix 1. The Hokuriku Liver Study Group (HLSG) is Composed of the Following Members:

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Participating investigators are listed in Appendix 1.

Conflicts of interest

The authors disclose no conflicts.

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MAJOR ARTICLE

La Protein Required for Internal Ribosome Entry Site–Directed Translation Is a Potential Therapeutic Target for Hepatitis C Virus Replication

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Background. Translation of the hepatitis C virus (HCV) is mediated by an internal ribosome entry site (IRES). Here, we analyzed the functional relevance of La protein for replication of HCV using an infectious HCV clone, JFH-1.

Methods. A single-nucleotide mutation from A to U was introduced at the 338th nucleotide in the stem-loop domain IV structure of HCV IRES, which stabilized stem-loop IV and abolished translation and replication of JFH-1 almost completely.

Results. During JFH-1 replication, translation initiation factors required for HCV IRES activity, including La protein, polypyrimidine tract binding protein (PTB), PSMA7, and PCBP2, were significantly induced in Huh-7.5 cells. Interestingly, JFH-1 infection increased telomerase activity and induced the expression of human telomerase RNA (hTR) in Huh-7.5 cells. In 37 tissue specimens from patients with chronic hepatitis C, La protein significantly correlated with the representative essential telomerase components hTR, p23, and HSP90 ($P < .001$). Recombinant adenovirus that expressed short-hairpin RNA against La protein successfully suppressed the levels of La protein and core protein of JFH-1 to 30% of that in the control cells.

Conclusions. HCV infection might be strongly related to telomerase activity in the liver through La protein induction. Inhibition of La protein substantially repressed JFH-1 replication; therefore, La protein is a potential therapeutic target for HCV.

Hepatitis C virus (HCV) is a positive-strand, enveloped RNA virus that belongs to the genus *Hepacivirus* in the family *Flaviviridae*. A human liver infected with HCV develops chronic hepatitis, cirrhosis, and in some instances, hepatocellular carcinoma [1]. Although a combination of ribavirin and interferon has become a routine means of treating infected patients, the results are often unsatisfactory, especially in patients with a high viral load [2]. Identification of host factors that regulate HCV replication in infected patients could be helpful in the development of a novel antiviral treatment strategy. It has been reported that various host factors are associated with HCV infection; however, only a few proteins have been functionally shown with an infectious HCV clone to regulate HCV replication [3].

The translation of HCV is initiated by a highly structured RNA segment, the internal ribosome entry site (IRES), which occupies most of the 5′ nontranslated RNA [4]. Many canonical and noncanonical translation initiation factors, such as La protein [5], polypyrimidine tract binding protein (PTB) [6], and eukaryotic initiation factor 3 (eIF3), interact with HCV IRES and might regulate HCV translation. Previously, we reported that HCV IRES activity is highly dependent on these initiation factors, and it correlated with the expression of La protein [7, 8]. However, the functional relevance of these translation initiation factors on HCV

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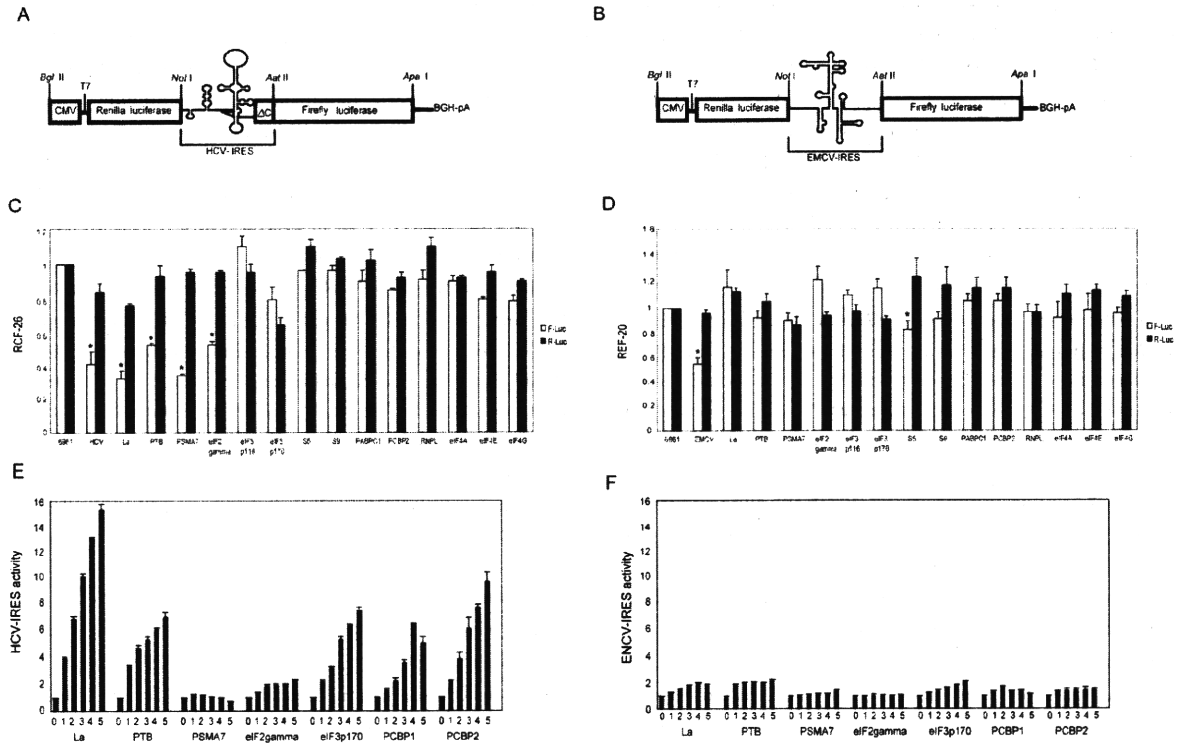

Figure 1. Organization of the transcriptional unit of plasmids pRL-HL (*A*) and pRL-EL (*B*). *C* and *D*, Suppression of 14 canonical and noncanonical initiation factors by specific antisense oligonucleotides. Changes in *Renilla* luciferase (RL) and firefly luciferase (FL) (hepatitis C virus–internal ribosome entry site [HCV-IRES]–directed translation) activities in RCF-26 (*C*). Changes in RL and FL (encephalomyocarditis virus [ENCV]–IRES–directed translation) activities in REF-20 (*D*). **P* < .05. *E* and *F*, In vitro translation of pRL-HL and pRL-EL in rabbit reticulocyte lysate. The plasmids pRL-HL or pRL-EL (0.05 μg) and increasing amounts of expression vectors (0–0.125 μg) of La protein, polypyrimidine tract binding protein (PTB), eIF3p 170, eIF2γ, PSMA7, PCBP1, and PCBP2 were co-translated in rabbit reticulocyte lysate. The fold increases in relative HCV IRES activity (*E*) and ENCV-IRES activity (*F*) are shown. **P* < .05. *Lane 0*, 0 μg; *lane 1*, 0.025 μg; *lane 2*, 0.05 μg; *lane 3*, 0.075 μg; *lane 4*, 0.1 μg; *lane 5*, 0.125 μg. **P* < .05.

replication had not been fully evaluated. In this study, we found that the expression of La protein is induced by HCV infection, and this induced La protein–activated telomerase activity in a human hepatoma cell line. The results indicate La protein is a potential therapeutic target for HCV infection.

EXPERIMENTAL PROCEDURES

Expression vector plasmids. The FLAG tag fusion La protein expression vector (pCMV-La-FLAG) was created by polymerase chain reaction (PCR), using the La protein expression vector (pCMV-La) as the template [8]. The forward primer 5′-AAT GAA ATC AGA AGA AA-3′ contains an *Xba* I site, and the reverse primer 5′-TGA TCT AGA TTA CTT ATC GTC GTC ATC CTT GTA ATC CTG GTC TCC AGC ACC ATT TTC TGT TTT CTG TTG -3′ contains *Xba* I and FLAG sites.

Cell lines. Human hepatocellular carcinoma 7 (Huh-7) cells and Huh-7.5 cells (provided by Professor C. M. Rice, Rockefeller University) were maintained in Dulbecco modified Eagle medium (DMEM; Gibco BRL), which contained 10% fetal bovine serum and 1% penicillin/streptomycin. The RCF-26 was a stably transformed cell line from Huh-7 cells that constitutively expressed dicistronic RNA transcripts containing sequences encoding 2 reporter proteins—*Renilla* luciferase and firefly luciferase—separated by a functional HCV IRES of genotype 1b (Figure 1*A*) [7]. The REF-20 was a stably transformed cell line from Huh-7 cells that constitutively expressed dicistronic RNA transcripts in which HCV IRES was replaced with encephalomyocarditis virus (EMCV) IRES (Figure 1*B*).

Antisense oligodeoxynucleotide. The antisense phosphorothioate oligodeoxynucleotides (oligos) designed for HCV IRES, La protein, PTB, eIF3, eIF2γ, S9, poly(A)-binding protein cytoplasmic 1 (PABPC1), PCBP2, RNPL, and control randomized oligo 6961 were described elsewhere [8]. Antisense oligos for PSMA7, S5, eIF4A, eIF4E, eIF4G, and EMCV IRES were synthesized. The nucleotide sequences of the antisense oligos were 5′-CTC ATG CCG GCG GGC GGC CG-3′ for PSMA7,

5′-GTC ATC CTG AGA ACA CAG CC-3′ for S5, 5′-GAC ATG ATC CTT AGA AAC TA-3′ for eIF4A, 5′-GCC ATC TTA GAT CGA TCT GA-3′ for eIF4E, 5′- GAC ATG ATC TCC TCT GTG AT-3′ for eIF4G, and 5′-TCC ATA TTA TCA TCG TGT TT-3′ for EMCV IRES. The antisense oligos (1.0 μmol/L) were transfected into RCF-26 (Figure 1*C*) or REF-20 (Figure 1*D*). After 24 h of transfection, *Renilla* luciferase (cap-dependent translation) and firefly luciferase (HCV or EMCV-directed translation) activities were measured with the Dual-Luciferase Reporter Assay System (Promega).

In vitro translation of pRL-HL and pRL-EL in rabbit reticulocyte lysate. In vitro translation of pRL-HL and pRL-EL was carried out in transcription and translation–coupled rabbit reticulocyte lysate systems (Promega). In 25 μL of the transcription and translation reaction mixture, 0.05 μg of pRL-HL or pRL-EL was cotranslated with an increasing amount of plasmid DNA (up to 0.125 μg) of La, PTB, PSMA7, eIF2-γ, eIF3p170, PCBP1, and PCBP2, which were cloned using the T7 promoter. A 3-μL aliquot was then used to measure *Renilla* luciferase and firefly luciferase activities using the Dual-Luciferase Reporter Assay System (Promega).

Site-directed mutagenesis. The plasmid pJFH-1 was used as the template for introduction of the site-directed mutation at nucleotide 338 in the 5′ nontranslated RNA. The site-directed mutagenesis reaction was performed using the Pfu Turbo DNA polymerase PCR system (Stratagene), according to the manufacturer's instructions.

Transfection of JFH-1 and JFH-1 338U into Huh-7.5 cells. Ten micrograms of synthetic RNA transcribed from pJFH-1 or pJFH-1 338U was used for electroporation. Cells were then pulsed at 260 V and 950 μF using the Gene Pulser II apparatus (Bio-Rad Laboratories).

Infection of Huh-7.5 cells with JFH-1. Seventy-two hours after transfection, the culture medium was collected, cleared by low-speed centrifugation at 2000 revolutions per minute at 760g for 10 min, and passed through a Millipore filter (pore size, 0.45 μm; Millipore Corporation). Part of the filtered culture medium was diluted 50-fold or 10-fold with DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin. Diluted culture medium (1 mL) was used for injection of cells into a well of a 6-well plate or a well containing cover slips and incubated for 4 h. At 3 days after infection, inoculated cells grown on cover slips were fixed and stained using anti-core antibody, as described below. The amounts of HCV RNA, La-RNA, and human telomerase RNA (hTR)-RNA in inoculated cells were determined by quantitative real-time detection (RTD)-PCR.

Western blot analysis and immunofluorescence staining. The expression levels of La protein and PTB in cells were evaluated by Western blotting using mouse anti-La antibody (SW5) and rabbit anti-PTB antibody, as described elsewhere [9]. The expression of HCV core protein, PSMA7, eIF2γ, PCBP2, and FLAG-tagged La protein was evaluated with mouse anti-core antibody (Affinity BioReagents), mouse anti-PSMA7 antibody (Antibodies Direct), rabbit anti-eIF2γ antibody (Abcam), mouse anti-huRNP E2 (23-G) antibody (Santa Cruz Biotechnology), and mouse anti-FLAG antibody (Sigma), respectively. For immunofluorescence staining, anti–core monoclonal antibodies and Alexa Fluor 488 goat anti–mouse immunoglobulin G antibody (Invitrogen) were used.

Quantitative RTD-PCR. The primer pairs and probes for La protein, PTB, eIF3 p170, GAPDH, and HCV were obtained as described elsewhere [8]. The primer pairs and probes for PSMA7, eIF2γ, PCBP2, hTR, p23, Hps90, and β-actin were obtained from the TaqMan assay reagents library. One microgram of isolated RNA was reverse-transcribed to complementary DNA using SuperScript II RT (Invitrogen) according to the manufacturer's instructions, and the resulting complementary DNA was amplified with appropriate TaqMan assay reagents [10].

Telomerase activity assay. The plasmids pCMV-La-FLAG and pCR3.1 were transfected into Huh-7 cells using Fugene 6 transfection reagent (Roche Applied Science). Forty-eight hours after transfection, the amounts of hTR-RNA in the transfected cells were determined by RTD-PCR. The expression of the FLAG-tag fusion La protein was evaluated by Western blot analysis. Telomerase activity was measured with a PCR-based telomerase repeat amplification protocol (TRAP) assay, performed with the TRAPEZE kit (Invitrogen) according to the manufacturer's instructions. Each reaction product was amplified in the presence of a 36-base pair internal telomerase assay standard. The PCR products were fractionated by electrophoresis on a 10% polyacrylamide gel and then visualized by staining with SYBR Green (Molecular Probes).

Construction of recombinant adenovirus expressing short-hairpin RNA for La protein. The short-hairpin RNA expression plasmid (pSh-La), which expresses short-hairpin RNA for La protein (seq: 5′-CCG GCC AAG GCA GAA CTC ATG GAA ACT CGA GTT TCC ATG AGT TCT GCC TTG GTT TG-3′), was purchased from Sigma. The pSh-La was digested with the enzymes *Hin*d III and *Bam*HI, and the excised fragment, including the short-hairpin RNA, was transferred to the adenoviral expression plasmid. The adenoviral expression plasmid and bovine growth hormone plasmid were cotransfected into 293A cells using the CellPhect Transfection kit (GE Healthcare) to produce crude adenoviral stocks. These stocks were purified using the Adeno-X Virus Purification kit (Clontech Laboratories) and stored at −80°C. The titers of the adenoviral stocks were adjusted to 4.0×10^9 PFU/mL.

Twelve hours after JFH-1 RNA transfection, the cells were washed 3 times with phosphate-buffered saline, and then Ad-shLa or Ad-Null was added at a multiplicity of infection of 10.

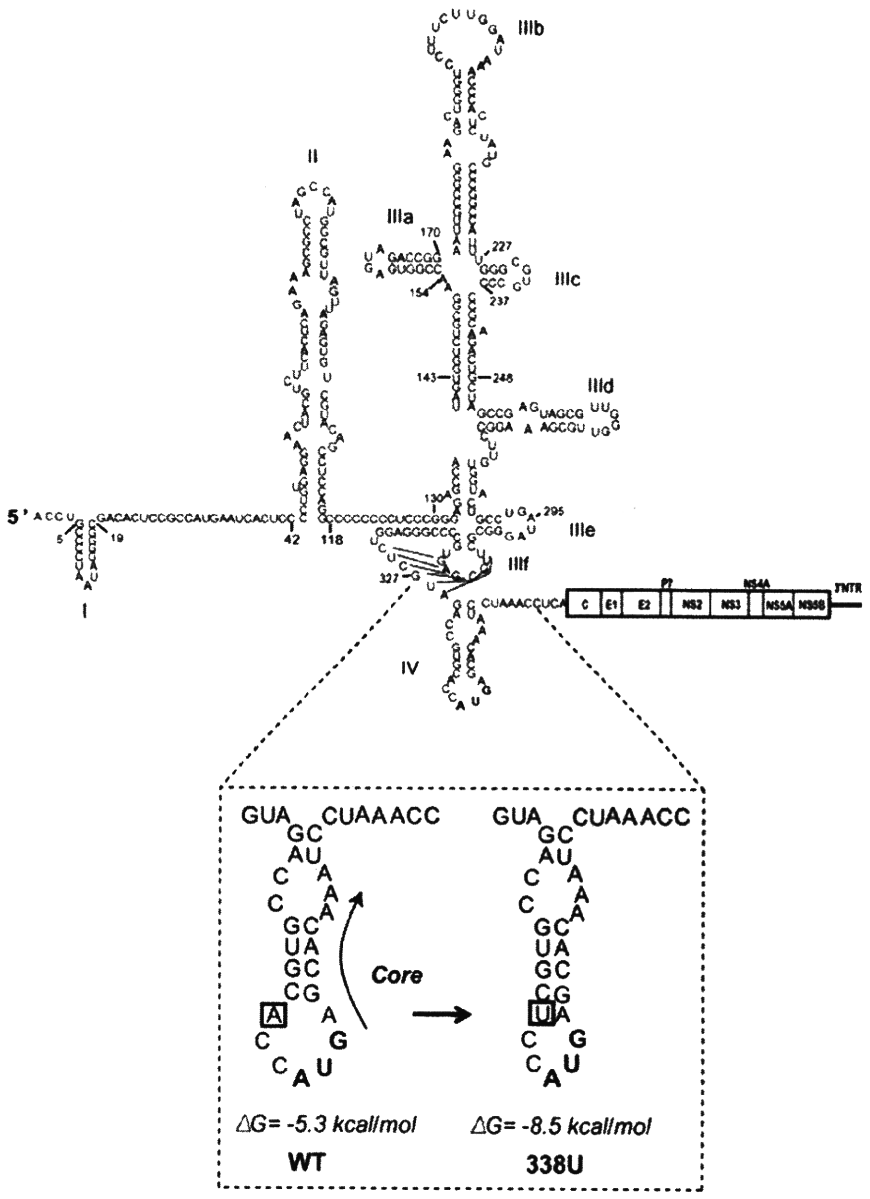

Figure 2. Organization of the full-length JFH-1 and the mutation at nucleotide 338 of stem loop IV.

One hour after injection, the cells were washed 3 times with phosphate-buffered saline, and complete culture medium was added.

Statistical analysis. Results were expressed as mean values ± standard deviation. Significance was tested by 1-way analysis of variance with Bonferroni methods, and differences were considered statistically significant at $P < .05$.

RESULTS

Dependence of HCV IRES activity on translation initiation factors. To confirm that HCV IRES activity was highly dependent on translation initiation factors, antisense oligonucleotides designed for 14 translation initiation factors were transfected into RCF-26 and REF-20 cells, and HCV or EMCV IRES

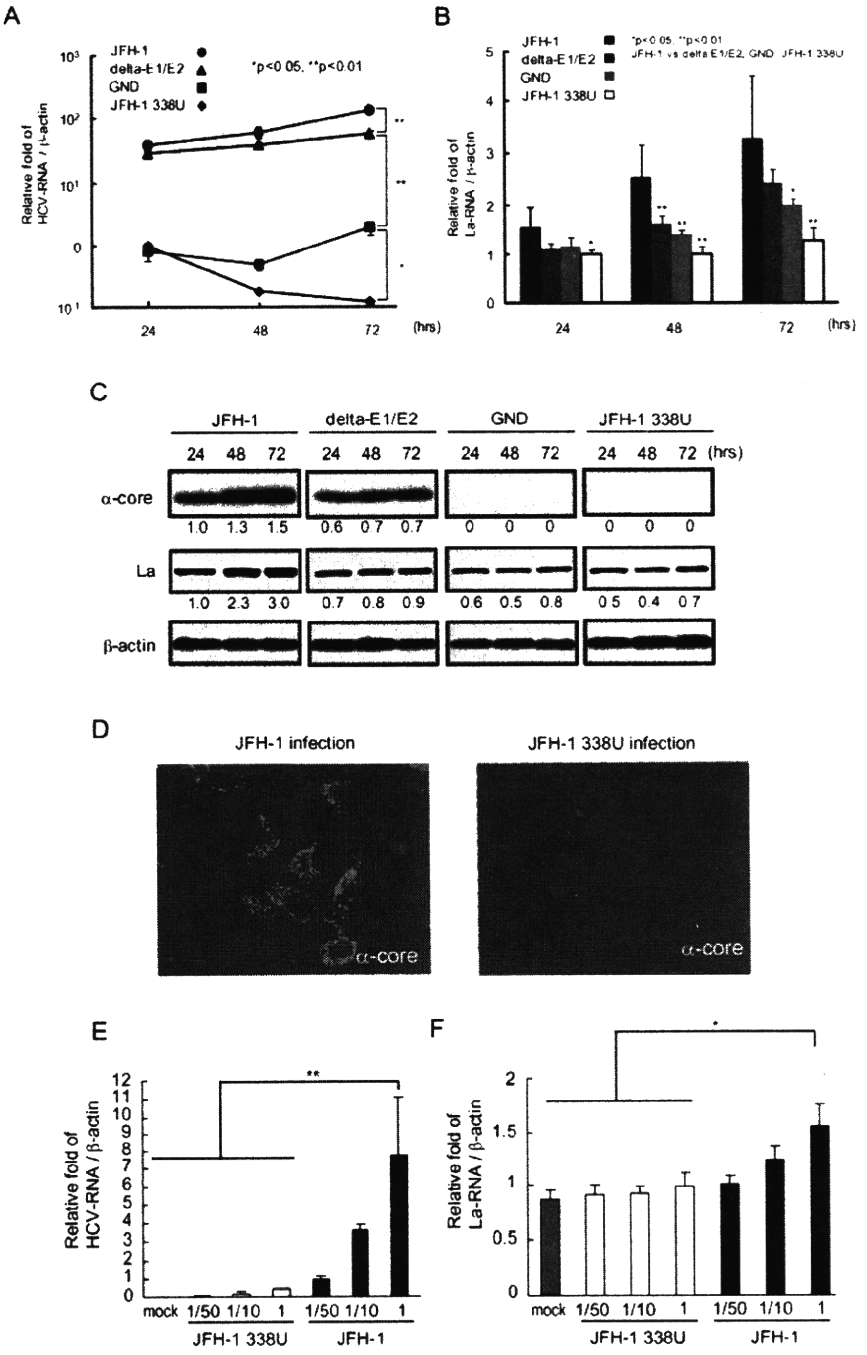

Figure 3. *A*, Hepatitis C virus (HCV) RNA replication determined by real-time detection–polymerase chain reaction (RTD-PCR) in JFH-1, JFH-1/delta E1-E2, JFH-1/GND, and JFH-1 338U transfected cells. $^{*}P < .05$. $^{**}P < .01$. *B*, La RNA expression determined by RTD-PCR in JFH-1, JFH-1/delta E1-E2, JFH-1/GND, and JFH-1 338U transfected cells. $^{*}P < .05$. $^{**}P < .01$. *C*, Western blots for detection of HCV core protein and La protein in JFH-1, JFH-1/delta E1-E2, JFH-1/GND and JFH-1 338U transfected cells. *D*, Immunofluorescence staining of core protein in Huh-7.5 cells infected with JFH-1 or JFH-1 338U. *E* and *F*, HCV RNA and La RNA determined by RTD-PCR in Huh-7.5 cells infected with serial dilution of JFH-1 or JFH-1 338U. $^{*}P < .05$, $^{**}P < .01$.

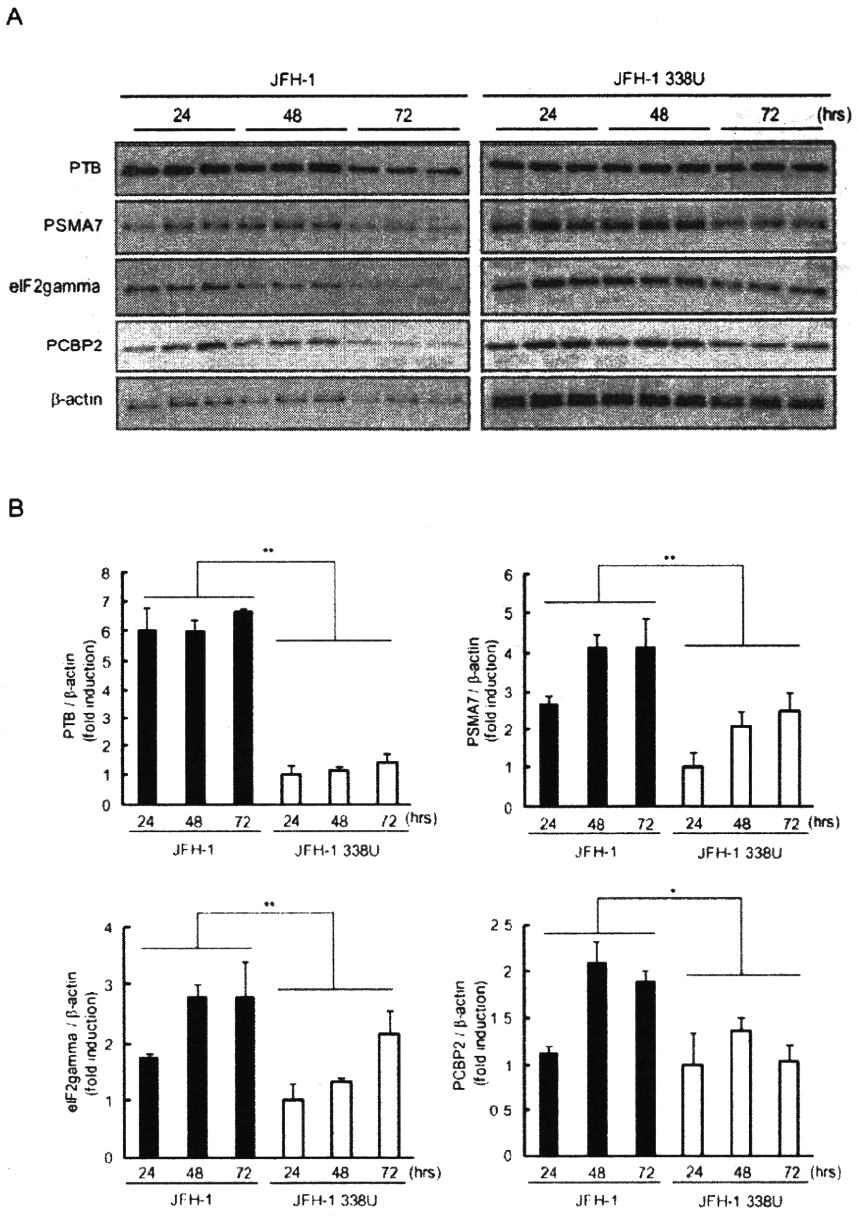

Figure 4. *A*, Protein expression of PTB, PSMA7, eIF2γ, and PCBP2 determined with Western blotting in Huh-7.5 cells after transfection with JFH-1 RNA and JFH-1 338U RNA. *B*, Quantitative densitometric analysis of protein expression. $^{*}P < .05$, $^{**}P < .01$.

activity was evaluated. The activities of *Renilla* luciferase and firefly luciferase expressed in these cells reflect cap-dependent and HCV or EMCV IRES directed translation, respectively (Figure 1*B* and 1*C*). The suppression of La protein, PTB, PSMA7, and eIF2γ by the antisense oligonucleotides in RCF-26 significantly repressed firefly luciferase activities, whereas *Renilla* luciferase activities were mostly maintained (Figure 1*C*). In contrast, these translation initiation factors did not affect EMCV IRES activity in REF-20 cells (Figure 1*D*).

These findings were also evaluated in rabbit reticulocyte lysates (RRL). With increasing amounts of expression vectors or La protein, PTB and eIF3 p170, the HCV IRES activity increased significantly (7-fold to16-fold) (Figure 1*E*). Although the depletion of PCBP2 did not affect HCV IRES activity in the RCF-

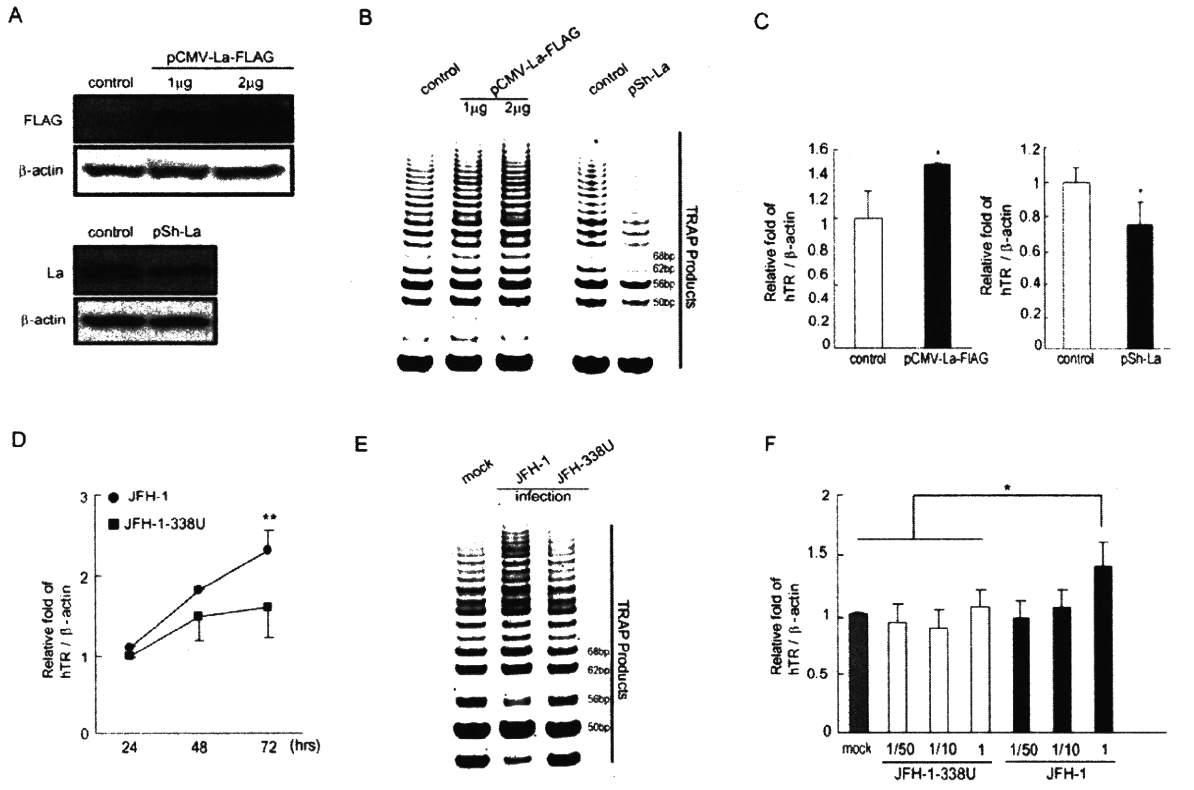

Figure 5. *A*, Western blot analysis of pCMV-La-FLAG or pSh-La transfected Huh-7 cells. *B*, The telomerase repeat amplification protocol (TRAP) assay in pCMV-La-FLAG or pSh-La transfected Huh-7 cells. *C*, Human telomerase RNA (hTR) expression in pCMV-La-FLAG or pSh-La transfected Huh-7 cells by real-time detection–polymerase chain reaction (RTD-PCR). $*P < .05$. *D*, The hTR expression in JFH-1 or JFH-1 338U RNA-transfected Huh-7.5 cells by RTD-PCR. $**P < .01$. *E*, The TRAP assay in JFH-1 or JFH-1 338U infected Huh-7 cells. *F*, Effect of JFH-1 or JFH-338U infection on hTR expression in Huh-7.5 by RTD-PCR. $*P < .05$.

26 cells, it stimulated HCV IRES activity up to 10-fold in the RRL (Figure 1*E*). On the other hand, EMCV IRES activity increased modestly by up to 2-fold (Figure 1*F*). These findings confirmed previous findings that HCV IRES activity is highly dependent on cellular factors.

Construction of translation incompetent full-length infectious HCV clone. JFH-1 is a genotype 2a-derived full length infectious HCV clone [11]. To evaluate the essential role played by IRES activity in HCV replication, we constructed a translation incompetent JFH-1 by introducing a single-nucleotide mutation from adenine to uracil at the position of nucleotide 338 (the third nucleotide upstream of the initiation codon of the core protein) in the 5′ nontranslated RNA (JFH-1 338U) (Figure 2). This mutation decreased the free energy (ΔG = 5.3 to −8.5 kcal/mol) and stabilized the folding structure of stem-loop domain IV that includes the initiation codon of the core protein. This mutation impairs ribosomal access to the AUG codon for translation initiation of viral proteins, as reported elsewhere [12].

Transfection of JFH-1 RNA into Huh-7.5 cells resulted in a substantial increase in viral RNA and core protein, as determined with RTD-PCR (Figure 3*A*), indirect immunofluorescence staining (data not shown), and Western blot analysis (Figure 3*C*). In contrast, translation-incompetent JFH-1 338U resulted in no evidence of viral replication or protein translation (as shown in Figure 3*A* and 3*C*). This indicated the functional importance of stem-loop IV not only for translation initiation but also for viral replication. Therefore, we used JFH-1 338U as an appropriate negative control in additional experiments.

Expression of La protein induced by JFH-1 in Huh-7.5 cells. Previously, we reported that expression of La protein was induced in the livers of patients with chronic hepatitis C, and the expression of La protein showed significant correlation with HCV RNA in tissue specimens from these patients [8]. In this study, we explored whether HCV might directly induce expression of La protein. The synthetic RNAs of JFH-1, JFH-1/delta E1-E2, JFH-1/GND [11], and JFH-1 338U were transfected into Huh-7.5 cells, and the expression of La RNA was

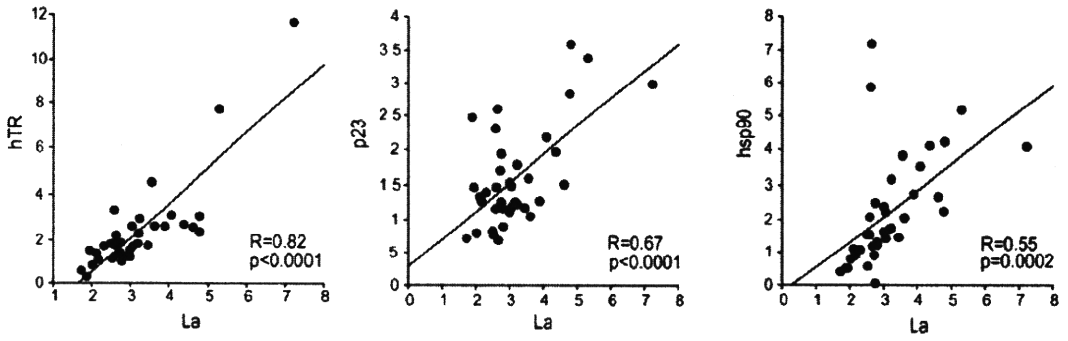

Figure 6. Correlation between expression of La protein and activity of human telomerase RNA (hTR), p23, and Hsp90 in liver biopsy specimens obtained from 37 patients with chronic hepatitis C.

evaluated after transfection by RTD-PCR and Western blot analysis. JFH-1 RNA peaked at 72 h after transfection (Figure 3*A*), as did the level of HCV core protein (Figure 3*C*). The JFH-1/delta E1-E2 RNA peak was significantly hampered compared with JFH-1 owing to the defective feature of infection. JFH-1 338U RNA and JFH-1/GND RNA was almost negligible at 72 h after transfection (Figure 3*A*) and a significant decline in JFH-1 338U RNA was noted. Under these conditions, La RNA was mostly induced in Huh-7.5 cells transfected with JFH-1, compared with JFH-1/delta E1-E2, JFH-1/GND, and JFH-1 338U (Figure 3*B*).

Similarly, expression of La protein was significantly increased after JFH-1 replication in Huh-7.5 cells, whereas only a slight increase was noticed in Huh-7.5 cells transfected with JFH-1/GND or JFH-1 338U (Figure 3*C*). The results indicated that JFH-1 replication induced La protein in Huh-7.5 cells.

To examine these findings further, the culture medium of the Huh-7.5 cells that included infectious HCV particles was collected and used to infect fresh Huh-7.5 cells at dilutions of 1:1, 1:10, and 1:50. HCV infection in the Huh-7.5 cells was confirmed by the expression of core protein (Figure 3*D*) and the presence of HCV RNA (Figure 3*E*). HCV infection was dependent on the amount of inoculated virus, as shown in Figure 3*E*. In contrast, there was no evidence of infection when using culture medium from Huh-7.5 cells transfected with JFH-1 338U RNA (Figure 3*D*, 3*E*). La RNA was significantly increased by the infection of virus derived from JFH-1 but not that derived from JFH-1 338U (Figure 3*F*). These results suggest that HCV infection itself could induce La protein in Huh-7.5 cells.

As for other initiation factors such as PTB, PSMA7, eIF2γ, and PCBP2, which were shown to be essential factors for HCV IRES activity (Figure 1), we also evaluated their gene expression according to the replication of JFH-1 (Figure 4). Western blotting of each initiation factor after JFH-1 RNA transfection showed significantly increased PTB, PSMA7, eIF2γ, and PCBP2. The increase was significantly greater in JFH-1 RNA transfected cells than in JFH-1 338U RNA transfected cells. Thus, HCV induces these initiation factors, and in turn, they served for HCV replication. Importantly, these relationships might be true in the tissue lesions of chronic hepatitis C. There were also significant correlations between the expression of these initiation factors and HCV RNA in the tissue specimens from patients with chronic hepatitis C, although the correlation between PTB, eIF3 p170, and HCV RNA was less than La protein, PSMA7, eIF2γ, PCBP2, and HCV RNA (data not shown) [8].

Activation of telomerase activity by La protein through the increase of human telomerase RNA. We next investigated the functional relevance of induced La protein in hepatocytes. Human telomerase plays an important role in cellular senescence and carcinogenesis. Human telomerase reverse transcriptase and hTR, as an RNA template, are core components of telomerase activity. In addition, other telomerase components, such as Hsp90 and p23 [13], have been reported to be essential for telomerase activity. There is a report that La protein is one of the telomerase components interacting with hTR [14]; however, the functional relevance of La protein for telomerase activity has not yet been validated.

The FLAG-tagged La protein expression vector pCMV-La-FLAG or short-hairpin RNA for La protein expression vector pSh-La was transfected into Huh-7 cells, and transduction was confirmed by Western blotting using anti-FLAG antibody or anti-La protein antibody (Figure 5*A*). Telomerase activity detected by the TRAP assay in cells overexpressing La protein was significantly higher than that found in control cells. In contrast, telomerase activity was repressed in La protein–repressed Huh-7 cells (Figure 5*B*). To reveal the mechanism underlying the up-regulation of telomerase activity by La protein, we measured the changes in the expression of human telomerase reverse transcriptase and hTR by RTD-PCR. Although no significant changes were observed in the expression of human telomerase

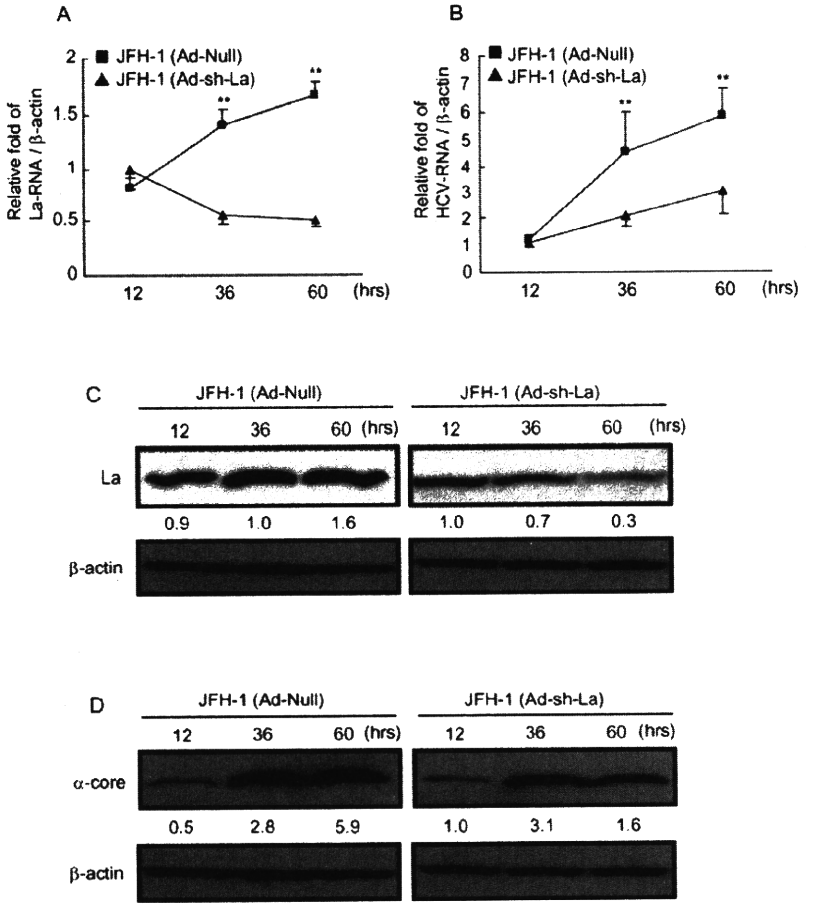

Figure 7. Suppression of La protein expression and its effect on of hepatitis C Virus (HCV) replication. *A*, Effect of Ad-shLa on La protein expression in JFH-1-transfected cells. $^{**}P < .01$. *B*, Effect of Ad-shLa on HCV replication. $^{**}P < .01$. *C*, Western blotting of La protein in JFH-1-transfected Huh-7.5 cells after infection with Ad-Null or Ad-shLa. *D*, Western blotting of HCV core protein in JFH-1-transfected Huh-7.5 cells after infection with Ad-Null or Ad-shLa.

reverse transcriptase, the expression of hTR was modestly but significantly increased by the overexpression of La protein and decreased by the repression of La protein, respectively (Figure 5*C*).

This finding was confirmed in Huh-7.5 cells transfected with JFH-1 RNA, which showed significantly higher expression of hTR than those transfected with translation-replication incompetent JFH-1 338U (Figure 5*D*). Moreover, JFH-1 infection similarly activated telomerase activity (Figure 5*E*) and induced hTR (Figure 5*F*) in Huh-7.5 cells, whereas JFH-1 338U infection did not activate telomerase activity or induce hTR. Therefore, the data strongly suggest that HCV infection could activate telomerase activity by increasing La protein and hTR.

When the relationship between La protein and telomerase components was evaluated in tissue biopsy specimens from patients with chronic hepatitis C, the expression of La protein strongly correlated with hTR. Moreover, it correlated significantly with the representative telomerase components p23 and HSP90 (Figure 6).

Repression of replication of JFH-1 in Huh-7.5 cells by recombinant adenovirus expressing short-hairpin RNA against La protein. Expression of La protein is induced by HCV infection, and it activates telomerase activity in Huh-7.5 cells. Therefore, it could be important to suppress La protein not only for the inhibition of HCV, but also for reducing the oncogenic potential of hepatocytes infected with HCV.

We constructed recombinant adenovirus expressing short-hairpin RNA against La protein (Ad-shLa). JFH-1 RNA was transfected into Huh-7.5 cells and 12 h after transfection, cells were exposed with Ad-shLa or control adenovirus (Ad-Null) for 1 h. At 12, 36, and 60 h after injection, changes in the levels of HCV RNA and La protein were evaluated by RTD-PCR and