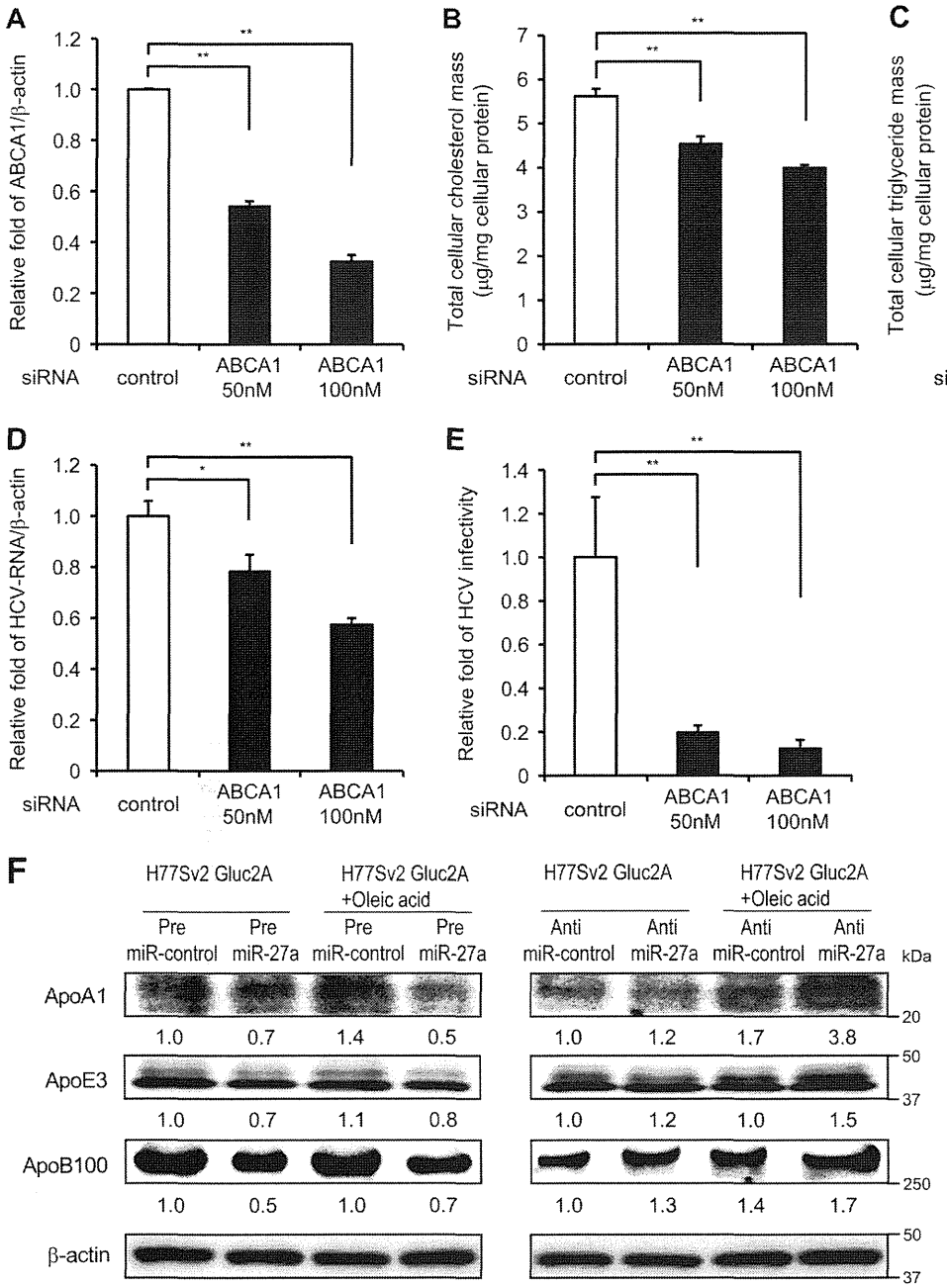

FIG 8 Suppression of ABCA1 inhibits HCV replication and infection. Huh-7.5 cells were transfected with H77Sv2 Gluc2A RNA and siRNA to ABCA1 or control siRNA. ABCA1 expression was quantified at 72 h posttransfection by RTD-PCR ($n = 6$). (A) Knockdown efficiency of ABCA1 in Huh-7.5 cells by siRNA. (B) TG concentration in cells ($n = 6$). (C) TCHO concentrations in cells ($n = 6$). (D) HCV RNA assay by RTD-PCR ($n = 6$). (E) HCV infectivity. Huh-7.5 cells were infected with HCVcc derived from ABCA1 knockdown Huh-7.5 cells. HCV RNA was quantified at 72 h postinfection by RTD-PCR ($n = 6$). Experiments were performed in duplicate and repeated three times. Values are means ± standard errors. *, $P < 0.01$; **, $P < 0.005$. (F) Regulation of ApoA1, ApoE2, and ApoB100 by miR-27a. Experiments were performed under the same conditions as Fig. 6B and C and repeated three times.

series of mutations were introduced into the putative miR-27a binding sites (MT-1, MT-2, and MT-1,2). The Luc activity of the WT was significantly repressed by pre-miR-27a and increased by anti-miR-27a. However, there was a smaller change in Luc activity caused by pre- and anti-miR-27a in the single mutants (MT-1 and MT-2) and no change in Luc activity in the double mutant (MT-1,2) (Fig. 7D and E). These results show that miR-27a targets ABCA1 to decrease the lipid content of cells.

The functional relevance of ABCA1 in lipid metabolism and HCV replication in Huh-7.5 cells was examined by inhibiting ABCA1 with an siRNA (Fig. 8). siRNA to ABCA1 repressed the expression of ABCA1 in a dose-dependent manner (Fig. 8A). Under this condition, the cellular TG and TCHO levels decreased significantly (Fig. 8B and C) and HCV RNA levels also decreased to 57% of the control. More strikingly, HCV infectivity decreased to 12% of the control (Fig. 8D and E).

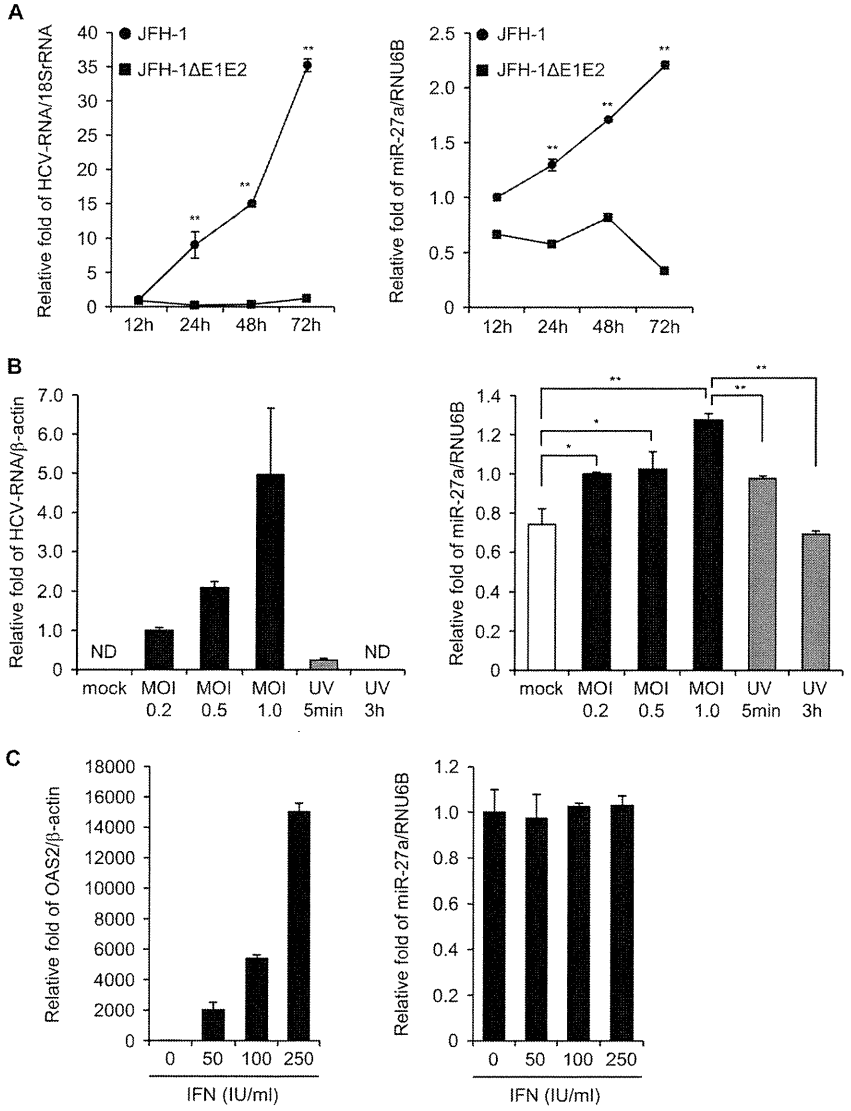

FIG 9 miR-27a is upregulated by HCV infection. (A) Kinetics of HCV replication and induction of miR-27a. Huh-7.5 cells were transfected with JFH-1 RNA or infection-incompetent JFH-1ΔE1E2 RNA (20). At 12, 24, 48, and 72 h posttransfection, HCV RNA (left) and miR-27a (right) levels were quantified by RTD-PCR ($n = 6$). (B) Induction of miR-27a and UV-irradiated HCV particles. Huh-7.5 cells were infected with infectious HCV (multiplicity of infection [MOI] of 0.2, 0.5, or 1) or UV-inactivated HCV. At 72 h postinfection, HCV RNA (left) and miR-27a (right) were quantified by RTD-PCR ($n = 6$). *, $P < 0.01$; **, $P < 0.005$; ND, not detected. (C) Induction of miR-27a and IFN-α treatment. Huh-7.5 cells were treated with different doses of IFN-α. At 24 h posttreatment, OAS2 (left) and miR-27a (right) were quantified by RTD-PCR ($n = 6$). All experiments were performed in duplicate and repeated three times. Values are means ± standard errors.

Several reports have demonstrated the importance of apolipoproteins, including the major components of VLDL and LDL apoE3 (36) and apoB100 (11), in the production of infectious HCV particles. More recently, the functional relevance of ApoA1 in HCV replication and particle production has been reported (37). Here the expression of apoA1, apoB100, and apoE3 was repressed by pre-miR-27a and increased by anti-miR-27a, suggesting that miR-27a regulates the expression of apolipoproteins to reduce the production of infectious HCV particles (Fig. 8F).

Regulation of miR-27a expression through C/EBPα. miR-27a forms a gene cluster with miR-23a and miR-24-2, and both of these miRNAs are regulated by the same promoter (38). However, no detailed analysis of the regulation of this promoter has been carried out. Because the expression of miR-27a was upregulated more in CH-C liver than CH-B liver, it could be speculated that HCV infection induces the expression of miR-27a. To examine this, we evaluated the expression of miR-27a during HCV infection (Fig. 9). The expression of miR-27a increased, correlating with the increase in JFH-1 RNA, while infection-incompetent JFH-1ΔE1E2 did not induce miR-27a expression (Fig. 9A). In addition, UV-irradiated HCV particles did not induce miR-27a expression (Fig. 9B). However, IFN-α treatment did not induce the expression of miR-27a (Fig. 9C). Thus, HCV infection was essential for induction of miR-27a expression.

We identified a C/EBPα binding site (−614 to −606), a key regulator of adipocyte differentiation, in the promoter region of miR-27a. Interestingly, H77Sv2 Gluc2A and tunicamycin

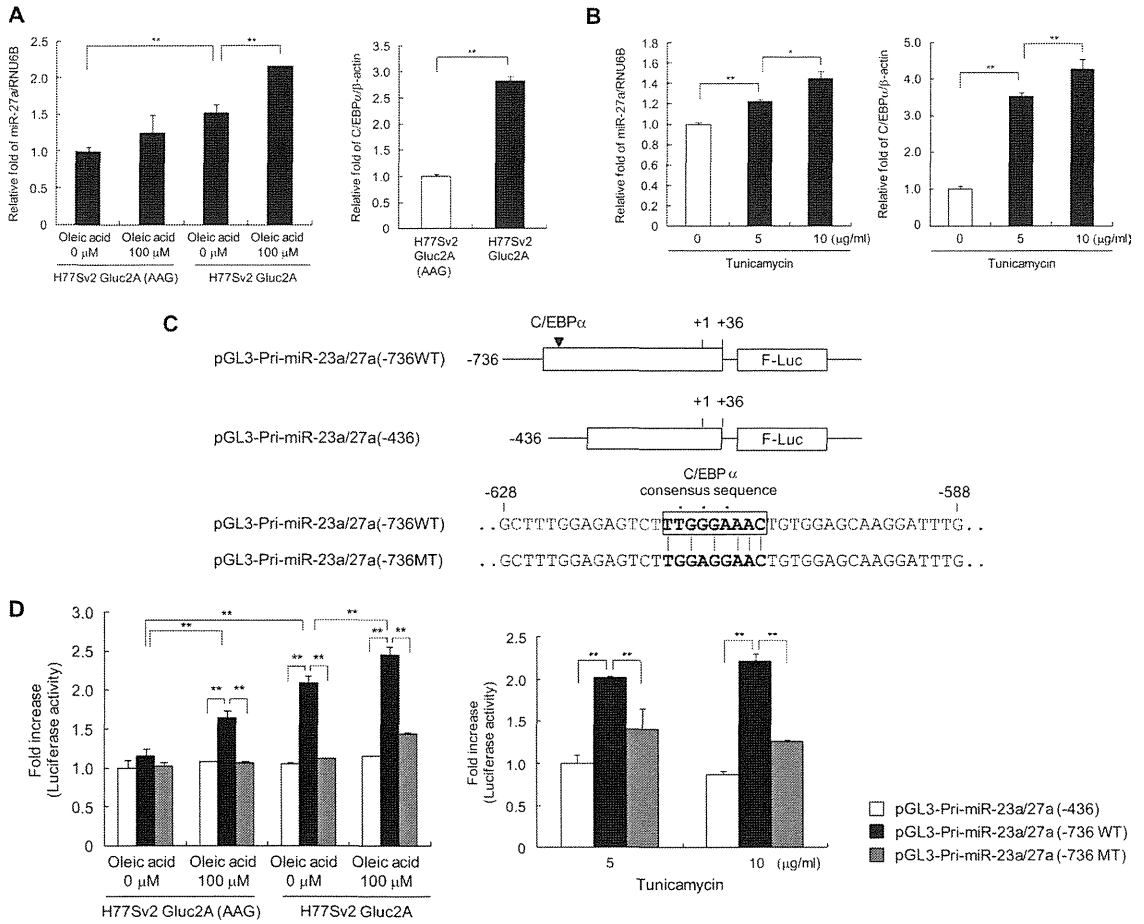

FIG 10 miR-27a is regulated by the adipocyte differentiation factor C/EBPα. (A) Induction of miR-27a and C/EBPα expression by oleic acid and HCV replication. Huh-7.5 cells were transfected with H77Sv2 Gluc2A RNA or H77Sv2 Gluc2A (AAG) RNA. At 24 h posttransfection, oleic acid (100 μM) was added to the culture medium. At 72 h after oleic acid treatment, miR-27a (left) and C/EBPα (right) levels were quantified by RTD-PCR ($n = 6$). (B) Induction of miR-27a and C/EBPα expression by tunicamycin. Huh-7.5 cells were treated with different doses of tunicamycin. At 24 h after tunicamycin treatment, miR-27a (left) and C/EBPα (right) levels were quantified by RTD-PCR ($n = 6$). (C) miR-27a promoter luciferase constructs. pGL3-Pri-miR-23a/27a(−736WT) includes −700 to +36 bp relative to the transcription initiation site of pri-miR-23a~27a~24-2. pGL3-Pri-miR-23a/27a (−436) includes −400 to +36 bp relative to the transcription initiation site of pri-miR-23a~27a~24-2, which lacks the consensus C/EBPα binding site. pGL3-Pri-miR-23a/27a(−736MT) has mutations at the −736WT C/EBPα binding site. (D) miR-27a promoter activity in Huh-7.5 cells following HCV infection and oleic acid (left) or tunicamycin (right) treatment. Reporter constructs lacking the C/EBPα binding site did not respond to any of these conditions ($n = 6$). All experiments were performed in duplicate and repeated three times. Values are means ± standard errors. *, $P < 0.01$; **, $P < 0.005$.

significantly induced the expression of miR-27a and C/EBPα (Fig. 10A and B). To analyze the induction of miR-27a through C/EBPα, we constructed a Luc reporter construct that included the upstream promoter region (−736) of miR-27a [pGL3-Pri-miR-23a/27a(−736WT)] together with a short promoter construct (−436) lacking the C/EBPα binding site [pGL3-Pri-miR-23a/27a(−436)]. In addition, three nucleotide mutations were introduced into the C/EBPα consensus binding site to construct pGL3-Pri-miR-23a/27a(−736MT) (Fig. 10C). The activity of pGL3-Pri-miR-23a/27a(−736WT), but not that of pGL3-Pri-miR-23a/27a(−736MT) or pGL3-Pri-miR-23a/27a(−436), which both lack a C/EBPα binding site, was induced by HCV replication, lipid overload, and tunicamycin treatment (Fig. 10D). These results indicate that the regulation of miR-27a by HCV replication, lipid overload, and ER stress is mediated through C/EBPα.

Pre-miR-27a enhances IFN signaling through the reduction of lipid storage. Finally, we assessed whether miR-27a influences IFN signaling. IFN-α treatment stimulated IFN signaling in a dose-dependent manner by increasing p-STAT1 expression in Huh-7.5 cells (Fig. 11A). Oleic acid impaired this induction of p-STAT1, while pre-miR-27a restored the expression of p-STAT1 and anti-miR-27a impaired this induction by oleic acid. These findings were observed in both HCV-replicating and non-HCV-replicating cells (Fig. 11A).

HCV replication deduced from Gluc activity is shown in Fig. 11B. IFN sensitivity could be estimated by the relative fold changes in Gluc activity from the baseline activity (in the absence of IFN). The results demonstrated that oleic acid reduced IFN sensitivity, while pre-miR-27a increased IFN sensitivity under either condition with or without oleic acid (Fig. 11B).

These findings were further studied with clinical samples. The expression of miR-27a was evaluated in liver biopsy specimens obtained from 41 patients who received pegylated IFN (Peg-IFN) and ribavirin (RBV) combination therapy (Fig. 12A). Interestingly, the expression of miR-27a was significantly higher

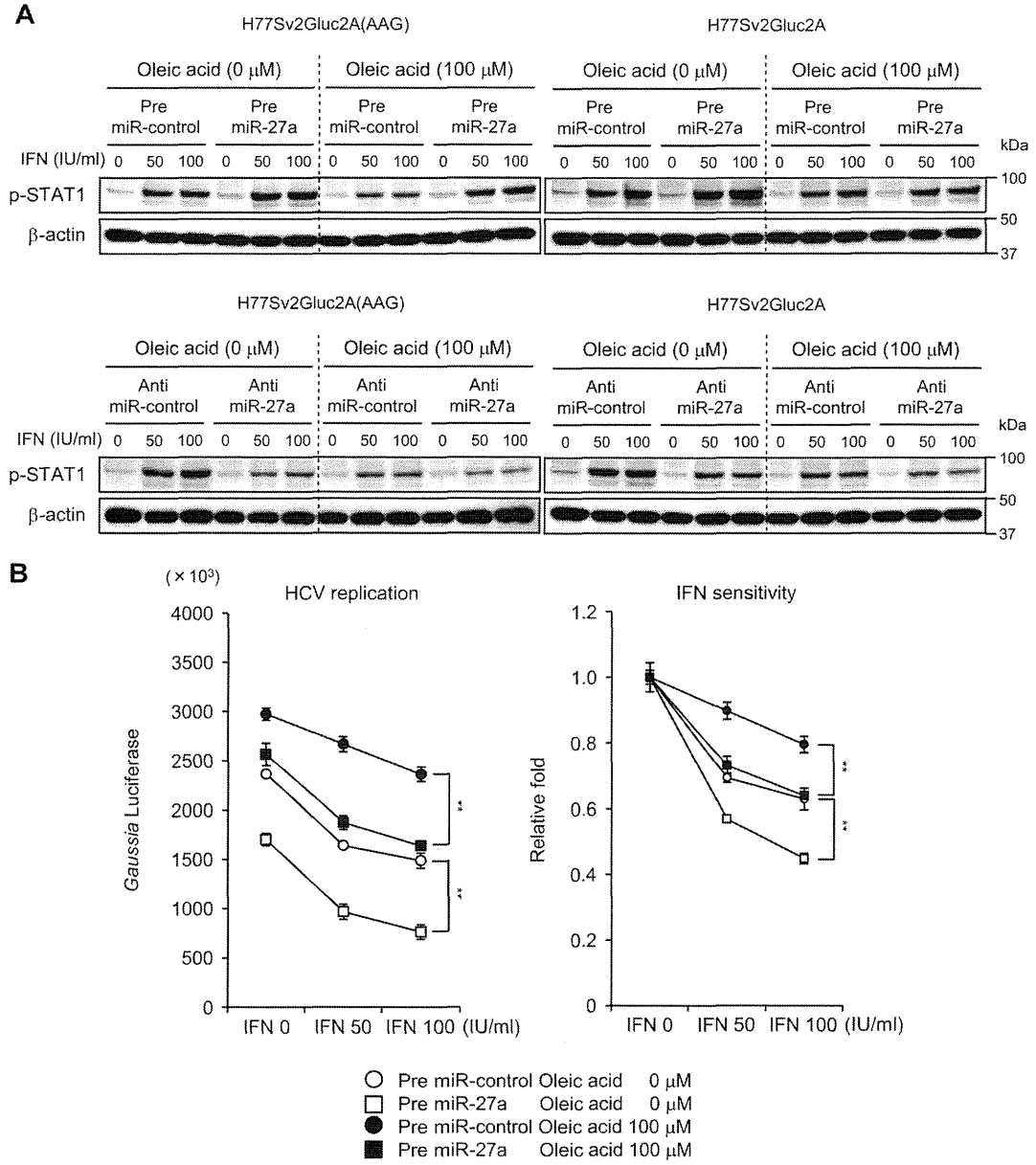

FIG 11 miR-27a restores IFN signaling impaired by lipid overload. (A) Induction of p-STAT1 expression by miR-27a. Huh-7.5 cells were transfected with H77Sv2 Gluc2A RNA or H77Sv2 Gluc2A (AAG) RNA and pre- or anti-miR-control or pre- or anti-miR-27a. At 24 h posttransfection, oleic acid (100 μM) was added to the culture medium. At 48 h after oleic acid treatment, the cells were treated with different doses of IFN-α. At 24 h after IFN treatment, p-STAT1 expression levels were determined by Western blotting. Experiments were repeated three times. (B) Absolute values of Gluc activity (left) and *n*-fold changes in Gluc activity (right) indicate IFN sensitivity ($n = 6$). Experiments were performed in duplicate and repeated three times. Values are means ± standard errors. *, $P < 0.01$; **, $P < 0.005$.

in patients with severe steatosis (grade 3 or 4) than in those with mild steatosis (grade 1 or 2) (Fig. 12B). Importantly, patients with a favorable response to treatment (sustained virological response or transient response) expressed higher miR-27a levels than patients with a poor response (nonresponse) (Fig. 12C). Although there was no significant difference in miR-27a expression according to the interleukin-28B (IL-28B) genotype (Fig. 12D and E), 17 patients had a treatment-resistant IL-28 genotype (TG at rs8099917) (39–41) and 6 of these with a favorable response to treatment expressed significantly higher miR-27a levels than the 11 with a poor response (Fig. 12E). These data suggest that miR-27a enhances IFN signaling and increases the response to IFN treatment.

DISCUSSION

Previously, we examined miRNA expression in HCC and noncancerous background liver tissue infected with HBV and HCV and showed the presence of infection-specific miRNAs that were differentially expressed according to HBV or HCV infection, but not according to the presence of HCC (2). In this study, we pursued the functional analysis of these miRNAs. Among 19 infection-specific miRNAs, we first focused on 6 that were upregulated by

A

No.	Age	Sex	Histological stage			Treatment response			IL28B SNP	
		M:F	F1	F2	F3	SVR	TR	NR	TT	TG
41	54.9±11.2	22:19	15	15	11	17	9	15	24	17

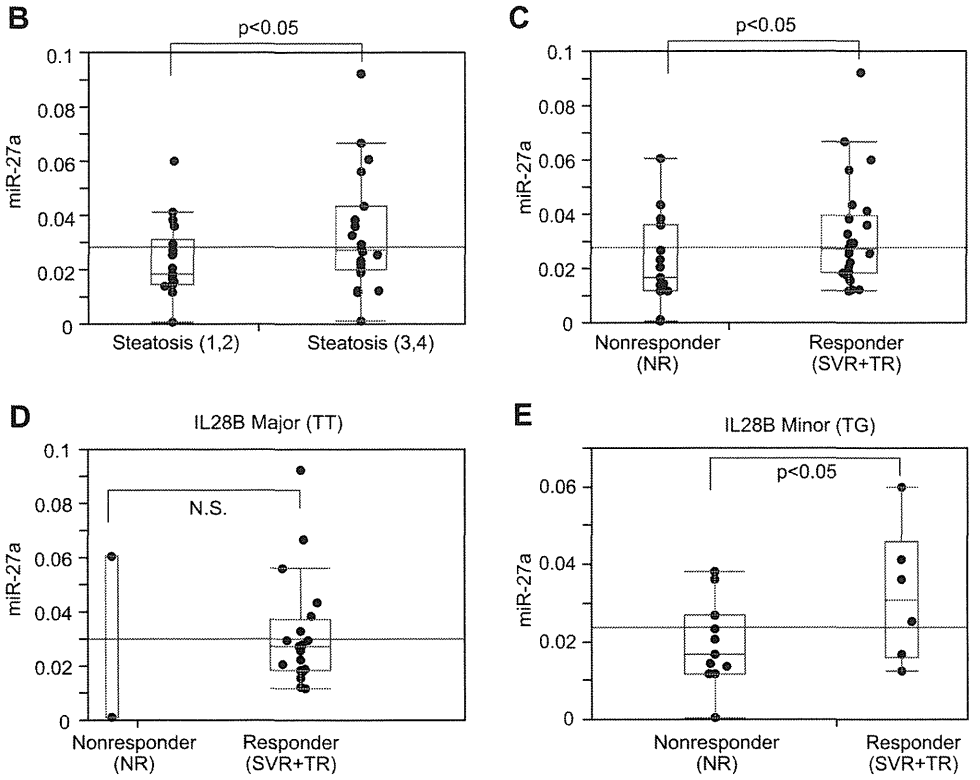

FIG 12 Expression of miR-27a in clinical samples. (A) Clinical characteristics of 41 patients who received Peg-IFN and RBV combination therapy. M:F, male/female ratio; SVR, sustained virological response; TR, transient response; NR, nonresponse; SNP, single nucleotide polymorphism. (B) Significant upregulation of miR-27a expression in the livers of patients with severe steatosis. Steatosis grades 1 and 2, $n = 19$; steatosis grades 3 and 4, $n = 22$. (C) Significant upregulation of miR-27a expression in the livers of patients with a favorable response to treatment (SVR or TR). Nonresponders; $n = 15$; responders, $n = 26$. (D) No significant difference in miR-27a expression between nonresponders and responders of the IL-28B major genotype (treatment-sensitive genotype) was observed. Nonresponders, $n = 3$; responders, $n = 21$. N.S., not significant. (E) Significant upregulation of liver miR-27a was observed in responders of the IL-28B minor genotype (treatment-resistant genotype). Nonresponders, $n = 11$; responders, $n = 6$.

HCV infection, as they were expected to have a positive role in HCV replication. However, inhibition experiments with a series of specific anti-miRNAs showed an unexpected increased in HCV replication. Closer examination clarified that miR-27a had a negative effect on HCV replication. Interestingly, profiling of gene expression in Huh-7.5 cells in which miR-27a was inhibited or overexpressed showed that miR-27a could target lipid metabolism signaling pathways. In support of these findings, the lipid content (TG and TCHO) of Huh-7.5 cells was significantly increased by anti-miR-27a and repressed by pre-miR-27a (Fig. 2 and 3). More importantly, miR-27a was involved in HCV particle formation, as demonstrated by iodixanol gradient centrifugation (Fig. 4). Anti-miR-27a reduced the buoyant density of HCV particles and increased HCV replication and infectivity, while pre-miR-27a decreased HCV replication and dramatically repressed HCV infectivity. In the buoyant-density experiment, the infectious HCV peaks were identical to the RNA peak and the lower infectious virus peak was not observed. We cannot explain this discrepancy from other studies; however, the method used to purify the virus particles could be one reason.

miR-27a regulated many lipid metabolism-related transcription factors, such as RXRα, PPARα, PPARγ, FASN, SREBP1, and SREBP2 (Fig. 5 and 6). We also confirmed that miR-27a targets RXRα in human Huh-7.5 cells, which is concordant with a previous study showing that miR-27a targets RXRα in rat hepatic stellate cells (32). Moreover, we newly demonstrated that the gene for the lipid transporter ABCA1 is a target of miR-27a. ABCA1 mediates the efflux of TCHO and phospholipids to the lipid-poor apolipoproteins ApoA1 and ApoE, which then form nascent HDLs (34, 35). It also mediates the transport of lipids between the Golgi apparatus and the cell membrane. Recently, the knockdown of ABCA1 in rat hepatoma cells increased TG secretion to the culture medium and decreased the cellular levels of FFA (29), while liver-specific ABCA1 knockout mice fed a high-fat diet showed increased plasma TG concentrations and decreased TG and TCHO contents in the liver (42). Thus, ABCA1 regulates the lipid content

of hepatocytes, as well as HDL synthesis. In this study, we confirmed that the repression of ABCA1 decreased cellular TG and TCHO levels in Huh-7.5 cells and, importantly, decreased HCV replication and strikingly repressed HCV infection (Fig. 8).

LXR/RXRα was previously shown to activate the ABCA1 promoter (34), but we clearly demonstrated here that miR-27a directly targets ABCA1. Pre-miR-27a repressed the Luc activity of a reporter construct fused with the ABCA1 3′ UTR, while anti-miR-27a increased it. We also found that miR-27a regulates the expression of ABCA1 in a 3′ UTR sequence-specific manner, as a series of mutations introduced into putative miR-27a binding sites abrogated its regulation (Fig. 7). In addition to these findings, we showed that miR-27a repressed the expression of the apolipoproteins ApoA1, ApoB100, and ApoE3, which were recently shown to play important roles in the production and formation of infectious HCV particles (Fig. 8) (11, 36, 37). Thus, miR-27a may regulate lipid metabolism by reducing lipid synthesis and increasing lipid secretion from cells.

As the expression of miR-27a was upregulated more in CH-C liver than in CH-B liver, it is speculated that miR-27a expression is induced by HCV infection. Indeed, we clearly demonstrated that miR-27a expression was induced by HCV infection, lipid overload, and tunicamycin-induced ER stress (Fig. 9). Furthermore, the adipocyte differentiation-related transcription factor C/EBPα was involved in this regulation. A central role for C/EBPα in the development of adipose tissue has been suggested, as it was found to be sufficient to trigger the differentiation of preadipocytes into mature adipocytes (43). Thus, HCV infection might trigger lipogenesis in hepatocytes by inducing C/EBPα, as shown in this study. Conversely, the induction of C/EBPα expression by miR-27a had a negative effect on lipogenesis and HCV replication. Therefore, miR-27a might play a negative feedback role in HCV infection-induced lipid storage in hepatocytes. Moreover, HCV replication might be hampered by HCV-induced miR-27a, which would partially explain the low HCV titer in CH-C liver.

Besides the anti-HCV effect of miR-27a observed in this study, an antivirus effect against murine cytomegalovirus (MCMV) infection was observed previously (44, 45). MCMV replication was initiated by miR-27a degradation from a viral transcript, while miR-27a had a negative effect on MCMV replication. It was also reported that miR-27a was the target of *Herpesvirus saimiri* U-rich RNAs and was downregulated in transformed T lymphocytes (46). Therefore, the functional relevance of miR-27a in transformed T cells should be explored in a future study. In this study, miR-27a was upregulated by HCV infection, which is in sharp contrast to MCMV and *H. saimiri* infection. Therefore, the differences in antiviral action and host cell interactions also need to be explored further.

Our assessment of miR-27a expression in patients receiving Peg-IFN and RBV combination therapy showed that those with high miR-27a levels had a more favorable treatment response (Fig. 12). Moreover, miR-27a significantly enhanced IFN signaling (Fig. 11), suggesting that it might have therapeutic benefits in combination with IFN therapy, especially in patients with the IFN-resistant IL-28B genotype, who show a more severe steatosis than those with the IFN-sensitive IL-28B genotype (39–41). Further studies should be performed to confirm these findings with more clinical samples.

Although miR-27a has been shown to be upregulated in cancers of the breast, kidney, ovary, and gastric region, its downregulation has been reported in colorectal cancer, malignant melanoma, oral squamous cell carcinoma, and acute promyelocytic leukemia (47). However, its importance in HCC remains controversial, with one report observing its upregulation compared with the level in normal liver tissue (48), while another showed lower miR-27a expression in HCC than in paired nontumor tissues (49). Moreover, our previous findings on HBV-related and HCV-related HCC showed no miR-27a upregulation compared with the level in the paired background liver (1.14-fold, $P = 0.49$).

In summary, we have revealed the important role of miR-27a in HCV replication for the first time. These findings will be applicable in the improvement of the therapeutic effects of anti-HCV therapy, especially in patients showing treatment resistance and severe hepatic steatosis.

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We have no potential competing interests to declare.

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Clinical Cancer Research

Imaging, Diagnosis, Prognosis

Association of *Interleukin-28B* Genotype and Hepatocellular Carcinoma Recurrence in Patients with Chronic Hepatitis C

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Abstract

Purpose: Several single-nucleotide polymorphisms (SNP) in the interleukin-28B (*IL-28B*) locus have recently been shown to be associated with antiviral treatment efficacy for chronic hepatitis C (CHC). However, such an association with hepatocellular carcinoma (HCC) is unkno3 we investigated the association between the *IL-28B* genotype and the biology and clinical outcome of patients with HCC receiving curative treatment.

Experimental Design: Genotyping of 183 patients with HCC with CHC who were treated with hepatic resection or radiofrequency ablation (RFA) was carried out, and the results were analyzed to determine the association between the *IL-28B* genotype (rs8099917) and clinical outcome. Gene expression profiles of 20 patients with HCC and another series of 91 patients with CHC were analyzed using microarray analysis and gene set enrichment analysis. Histologic and immunohistochemical analyses were also conducted.

Results: The TT, TG, and GG proportions of the rs8099917 genotype were 67.8% (124 of 183), 30.6% (56 of 183), and 1.6% (3 of 183), respectively. Multivariate Cox proportional hazard analysis showed that the *IL-28B* TT genotype was significantly associated with HCC recurrence ($P = 0.007$; HR, 2.674; 95% confidence interval, 1.16–2.63). Microarray analysis showed high expression levels of IFN-stimulated genes in background liver samples and immune-related genes in tumor tissues of the *IL-28B* TG/GG genotype. Histologic findings showed that more lymphocytes infiltrated into tumor tissues in the TG/GG genotype.

Conclusions: The *IL-28B* genotype is associated with HCC recurrence, gene expression, and histologic findings in patients with CHC. *Clin Cancer Res; 19(7); 1827–37. ©2013 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide and the third most common cause of cancer mortality (1). HCC usually develops in patients suffering from chronic hepatitis B or chronic hepatitis C (CHC). Although hepatic resection has been considered the most efficient therapy for HCC, it is only suitable for 20% to 35% of patients because of poor hepatic reserve (2). Radiofrequency ablation (RFA) has therefore been introduced as a minimally invasive therapy for such cirrhotic patients and is widely applicable with little effect on hepatic reserve. Moreover, randomized (3, 4) and nonrandomized (5, 6) controlled studies revealed no statistical difference in patient survival between resection and RFA.

Despite these curative treatments of HCC, its recurrence remains common. Several studies have identified potential risk factors for HCC recurrence, including the presence of cirrhosis, high α-fetoprotein (AFP) levels, large tumor foci, and tumor multiplicity (7, 8).

The interleukin-28B (*IL-28B*) gene, also known as IFN-λ3, is a newly described member of the family of IFN-related cytokines (9) and shares the same biologic properties as type I IFNs (10). Recently, several single-nucleotide polymorphisms (SNP) in the *IL-28B* locus have been associated with the effectiveness of pegylated-IFN and ribavirin combination therapy for CHC (11, 12). We previously confirmed this relationship and revealed that the *IL-28B* genotype is associated with the expression of hepatic IFN-stimulated genes (ISG) in patients with CHC (13). Others have also described an association between the *IL-28B* genotype and the outcome of CHC therapy, biochemical factors, and histologic findings (14, 15); however, the relationship between the *IL-28B* genotype and the biology and clinical course of HCC remains unknown. In this study,

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Translational Relevance

Several single-nucleotide polymorphisms (SNP) in the interleukin-28B (*IL-28B*) locus have recently been shown to be associated with antiviral treatment efficacy in chronic hepatitis C (CHC). In this study, we investigated the association between the *IL-28B* genotype (rs8099917) and the biology and clinical outcome of patients with hepatocellular carcinoma (HCC) receiving curative treatment. Patients with the *IL-28B* TT genotype had a significantly higher incidence of HCC recurrence than patients with the TG/GG genotype. Gene expression profile and histologic analysis showed that the immune response and chronic hepatitis inflammation were more severe in patients with the TT genotype. Conversely, the expression of IFN-stimulated genes was upregulated and the immune response to tumors was more intense in those with the TG/GG genotype. These finding suggest that such molecular mechanisms may affect HCC recurrence.

therefore, we investigated the association between the *IL-28B* genotype and clinical outcome after initial curative treatment of HCC and clarified the molecular features in relation to the *IL-28B* genotype.

Materials and Methods

Patients

A total of 852 patients were admitted to the Department of Gastroenterology, Kanazawa University Hospital, Kanazawa, Japan between January 2000 and March 2012 for the treatment of developed HCC. The major background liver disease was hepatitis C virus (HCV; $n = 502$), followed by hepatitis B virus ($n = 148$). Treatment of HCC included surgical resection in 175 patients and RFA in 390 patients. The choice of treatment procedure was determined according to the extent of the tumor and the hepatic functional reserve as assessed by Child's classification that forms the Japanese HCC Guidelines (16, 17). In some cases indicated for surgical resection, we conducted RFA on patients who refused surgical resection, and we consequently excluded these patients on the basis of Japanese HCC guidelines.

Study inclusion criteria were: (i) Child–Pugh class A or B; (ii) the presence of up to 3 tumors, each 3 cm or less; (iii) HCV infection (positive for HCV RNA, patients with sustained viral response were excluded); (iv) radical treatment by either surgical resection or RFA; and (v) availability of blood samples for genetic analyses (Supplementary Fig. S1). Consequently, 183 patients were studied and their baseline characteristics are reported in Table 1. Informed consent was obtained from all patients before therapy. The experimental protocol was approved by the Human Genome, Gene Analysis Research Ethics Committee of Kanazawa University (Approval No. 260), and the study was conducted in accordance with the Declaration of Helsinki.

Diagnosis of HCC

HCC diagnosis was based predominantly on image analysis. Patients underwent dynamic computed tomography (CT) and/or dynamic MRI and abdominal angiography with CT imaging in the arterial and portal flow phase. HCC was diagnosed if a liver nodule showed hyperattenuation in the arterial phase and washout in the portal or delayed phase or showed typical hypervascular staining on digital subtraction angiography (18).

Table 1. Clinical features of 183 patients with HCC at entry by *IL-28B* genotype

Variables	*IL-28B* TT genotype ($n = 124$)	*IL-28B* TG/GG genotype ($n = 59$)	*P*
Sex (male:female)	76:48	32:27	0.422
Age, y (≤70:>70)	64:60	32:27	0.754
Platelet count ($\times 10^4/mm^3$; ≤10:>10)	68:56	28:31	0.429
ALT, IU/L (≤40:>40)	44:80	25:34	0.416
γ-GTP, IU/L (≤50:>50)	46:78	21:38	0.871
Albumin, g/dL (≤3.5:>3.5)	41:83	12:47	0.084
Protrombin activity, % (≤70:>70)	28:96	9:50	0.325
Total bilirubin, mg/dL (≤2:>2)	7:117	1:58	0.440
Child–Pugh class (A:B)	77:29	43:10	0.352
Therapy (resection: RFA)	19:105	10:49	0.830
Period of therapy (2000-05:2006-12)	41:83	21:38	0.741
History of IFN therapy (yes:no)	56:68	26:33	0.999
Tumor number (solitary: 2–3)	80:44	42:17	0.406
Tumor size, mm (≤20:>20)	83:41	36:23	0.508
AFP, ng/mL (≤20:>20)	60:64	37:22	0.082
DCP, AU/L (≤40:>40)	75:49	39:20	0.516

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Method of treatment

Hepatic resection was carried out under intraoperative ultrasonographic monitoring and guidance. Anatomic resection was conducted in 9 patients and nonanatomic resection was conducted in 20 patients. Curative resection was defined as removal of all recognizable tumors with a clear margin (19). RFA was conducted using either the radiofrequency interstitial tumor ablation system (RITA; RITA Medical Systems Inc.) or the cool-tip system (Tyco Healthcare Group LP). All procedures were conducted according to the manufacturer's protocol. In the case of RFA, dynamic CT was conducted 1 to 3 days after therapy and the ablated area was evaluated. Complete ablation was defined as no enhancement in the ablated area on the dynamic CT. When complete ablation was not achieved, additional ablation was considered.

Follow-up

All patients were followed up by ultrasound and contrast enhancement 3-phase CT or MRI every 3 months. Local tumor progression was defined as the reappearance of tumor progression adjacent to the treated site and distant recurrence as the emergence of one or several tumor(s) not adjacent to the treated site. Patients with confirmed recurrence received further treatment such as resection, RFA, transarterial chemoembolization, and chemotherapy depending on the condition. Time to recurrence (TTR) was defined as the period from the date of therapy until the detection of tumor recurrence, death, or the last follow-up assessment. For TTR analysis, the data were censored for patients without signs of recurrence.

Genetic variation of the *IL-28B* polymorphism

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. An *IL-28B* SNP (rs8099917) was determined using TaqMan Pre-Designed SNP Genotyping Assays as described previously (12). A custom assay was created by Applied Biosystems for rs12979860. We determined *IL-28B* genetic variations in all patients included in this study.

Affymetrix genechip analysis

Resected cancer and noncancerous liver tissue specimens were immediately frozen in liquid nitrogen and kept at −80°C until required for RNA preparation. 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 required. The quality of isolated RNA was estimated after electrophoresis using an Agilent 2001 Bioanalyzer. Microarray analysis using an Affymetrix Human 133 Plus 2.0 microarray chip was conducted as described previously (13). The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at National Center for Biotechnology Information (NCBI, Bethesda, MD; accession number GSE41804).

Gene set enrichment analysis

Affymetrix GeneChip array data were normalized, preprocessed, and analyzed using R (20) and Bioconductor (21) software. Raw CEL file data were normalized using the MAS 5.0 algorithm as implemented in the *affy* package. Normalized data were $\log_2$ transformed and assessed using gene set enrichment analysis (GSEA), which is a bioinformatics method to assess whether genes with known biological/molecular function are concomitantly upregulated or downregulated in a certain gene expression dataset (22). GSEA was conducted using a parametric analysis of gene set enrichment (PAGE; ref. 23). The Gene Ontology gene set collection C5 of the Molecular Signatures Database (22) was downloaded from the Broad Institute and loaded into the R environment.

We also investigated the gene set differentially expressed HCC-infiltrating mononuclear inflammatory cells studied previously (24). *Z* scores and *P* values of all gene sets were calculated using the *PGSEA* package and an estimate was made as to whether certain gene sets, and therefore functional gene categories, were differentially regulated in HCC tissue from patients with the *IL-28B* TT genotype and the *IL-28B* TG/GG genotype.

Hierarchical clustering

Hierarchical clustering was conducted with Cluster software using Pearson's correlation distance metric and average linkage followed by visualization in Treeview software.

Histologic liver analysis

Noncancerous liver tissue that had been surgically resected from patients with HCC and liver specimens obtained by needle biopsy from the background liver of patients with HCC were fixed in 10% buffered formalin and embedded in paraffin. Each paraffin-embedded specimen was sliced into 3 to 4 μm sections and stained with hematoxylin and eosin. Each specimen was semiquantitatively analyzed by assigning a score according to each of the following features: (i) severity of inflammatory cell infiltration (0 for none, 1 for minimal, 2 for mild, 3 for moderate, and 4 for severe) in the periportal, intralobular, and portal areas; (ii) the severity of the F stage of fibrosis (0 for F0, 1 for F1, 2 for F2, 3 for F3, and 4 for F4; ref. 25); the degree of lymphoid aggregates in the portal area (0 for none, 1 for mild, 2 for scattered, 3 for clustered, 4 for lymph follicle without germinal center, and 5 for lymph follicle with germinal center); the severity of portal sclerotic change, perivenular fibrosis, and pericellular fibrosis (on a scale of 0–4 with 0 for none to 4 for severe); the severity of damage to the bile duct (on a scale of 0–4 with 0 for none to 4 for disappearance); the existence of bridging necrosis (0 for none and 1 for existence); the severity of irregular regeneration of hepatocytes as described previously (on a scale of 0–4 with 0 for none to 4 for severe; ref. 26); the grade of steatosis (on a scale of 0–4 with 0 for none to 4 for severe). The irregular regeneration score was based on the findings of a map-like distribution, anisocytosis, and pleomorphism

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of the hepatocytes, bulging of the regenerated hepatocytes and proliferation of atypical hepatocytes and oncocytes.

Immunohistochemistry

Paraffin-embedded specimens were sliced into 3 to 4 μm sections, deparaffinized, and subjected to heat-induced epitope retrieval at 98°C for 40 minutes. After blocking endogenous peroxidase activity using 3% hydrogen peroxide, the slide was incubated with appropriately diluted primary antibodies. Antihuman CD4, antihuman CD8 and antihuman CD14 mouse monoclonal antibodies were used to evaluate the immunoreactivity of HCC using a DAKO EnVision+TM kit, as described in the manufacturer's instructions.

We semiquantitatively analyzed tumor tissues by assigning a score to the severity of CD4-positive and CD8-positive lymphocyte infiltration in the tumor tissue (0 for none, 1 for mild, 2 for moderate, and 3 for severe).

Statistical analysis

Fisher exact probability test was used to compare categorical variables and the Mann–Whitney *U* test was used to compare continuous variables; a *P* value of less than 0.05 was considered statistically significant. The TTR survival curve was analyzed using the Kaplan–Meier curve and compared by the log-rank test. Univariate Cox regression analysis was conducted to identify TTR predictors out of clinical and biologic parameters [sex, age, *IL-28B* genotype, therapy, platelet count, alanine aminotransferase (ALT), γ-GTP, albumin, prothrombin activity, bilirubin, Child–Pugh class, history of IFN therapy, AFP, and des-γ-carboxy prothrombin (DCP)] and tumor factors (size and number).

Multivariate analysis was conducted using the Cox regression model with backward elimination (27). The significance level for removing a factor from the model was set to 0.05. A bootstrap technique was applied to confirm the choice of variables (27). One thousand bootstrap samples were generated using resampling with replacement and Cox regression analysis with backward elimination was applied to each sample. The percentage of samples (from the total of 1,000) for which each variable was included in the model was calculated. In multivariate analysis, we evaluated two models that contained either Child–Pugh class or its components to avoid multicollinearity. Data analysis was conducted with R software. We used functions from the Regression Modeling Strategies library for validation with the bootstrap technique (28).

Results

Patient characteristics and *IL-28B* genotype frequency

We genotyped 183 patients with HCC for the *IL-28B* rs8099917 TT, TG, and GG genotypes and observed respective proportions of 67.8% (124 of 183), 30.6% (56 of 183), and 1.6% (3 of 183), which is a similar distribution to that observed in several Japanese studies of patients with CHC (13, 14, 29, 30). Although the prevalence of the TG/GG genotype was higher than that of the general population (12%–16%; refs. 12, 31, 32), there was no significant difference between our result and that of HCV-infected patients in a previous study. There was also no significant difference in clinical variables between the TT and TG/GG genotypes (Table 1).

We next genotyped 160 of 183 cases for rs12979860 and our findings were largely in concordance with those of rs8099917, with the exception of 1 case (0.6%). The haplotype of the case showed that rs8099917 was TT and rs12979860 was CT suggesting that these 2 loci are in a haplotype block with a high level of linkage disequilibrium, as previously reported (13, 30). Genotype distribution analysis showed that rs8099917 was in Hardy–Weinberg equilibrium, so we selected it for further investigation.

During the median follow-up period of 2.5 years (range, 0.3–7.2 years), 118 of 183 patients developed HCC recurrence. Local tumor progression was seen in 13 patients treated by RFA and in only 1 patient treated by resection. The local tumor progression rate and distant recurrence rate were 2.6% and 21.2% in the first year and 8.3% and 54.2% within 2.5 years, respectively. These results are comparable with previous reports by others (33, 34). The type of recurrence was also comparable between *IL-28B* genotype groups.

Associations between the *IL-28B* genotype and HCC clinical outcome

HCC TTR was also analyzed using multivariate Cox regression analysis using 15 clinical parameters and the *IL-28B* genotype. With a significance level of 0.05 for removing a variable in a Cox regression with backward elimination, the *IL-28B* genotype was selected as the final model (Table 2). To confirm this decision, a bootstrapping technique was applied. The percentages of inclusion among the 1,000 samples created by the bootstrapping technique for variables are shown in Table 2. The percentage of inclusion for the *IL-28B* genotype was 80.4%. Frequencies of another variable were lower than 40%. The bootstrap procedure result confirmed the variables chosen for the final model.

In univariate Cox regression analyses, the *IL-28B* genotype was associated with HCC recurrence (Table 2). The TTR survival curve was analyzed using the Kaplan–Meier curve and log-rank test (Fig. 1), and patients with the *IL-28B* TT genotype showed a significantly shorter median TTR (1.61 years) than those with the *IL-28B* TG/GG genotype (2.58 years; $P = 0.007$).

Histologic analysis of noncancerous liver tissues of *IL-28B* TT and TG/GG genotypes

To clarify the molecular mechanism influencing HCC recurrence, we histologically analyzed 141 noncancerous liver tissues according to previously published criteria (Table 3; ref. 26). The mean score of the degree of inflammatory cell infiltration in the periportal area was significantly higher in TT genotype patients (2.804) than TG/GG genotype patients (2.513; $P = 0.025$); the degree of inflammatory cell infiltration in the intralobular area was also

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Table 2. Cox regression analysis and relative frequency of variables inclusion with $P < 0.05$ (in 1,000 bootstrap samples)

Variables	Univariate		Multivariate		Frequency (%)
	HR (95% CI)	*P*	HR (95%CI)	*P*	
IL-28B allele: major vs. minor	2.674 (1.161–2.627)	0.007	2.674 (1.161–2.627)	0.007	80.4
Tumor size, mm: >20 vs. ≤20	1.303 (0.881–1.880)	0.193			39.8
AFP, ng/mL: >20 vs. ≤20	1.674 (0.948–1.968)	0.094			33.2
γ-GTP, IU/L: >50 vs. ≤50	1.188 (0.865–1.804)	0.235			32.8
Therapy: RFA vs. resection	1.218 (0.826–2.266)	0.223			31.6
DCP, AU/L: >40 vs. ≤40	1.524 (0.920–1.945)	0.127			27.4
ALT, IU/L: >40 vs. ≤40	0.277 (0.721–1.544)	0.782			23.6
Child–Pugh class: A vs. B	0.025 (0.653–1.515)	0.980			19.2
Period of therapy: 2000-05 vs. 2006-12	0.886 (0.818–1.701)	0.375			15.8
History of IFN therapy: yes vs. no	0.570 (0.771–1.605)	0.569			15.8
Sex: male vs. female	0.108 (0.697–1.496)	0.914			14.6
Tumor number: solitary vs. 2-3	0.263 (0.845–1.857)	0.263			13.4
Platelet count ($\times 10^4/mm^3$): >10 vs. ≤10	0.118 (0.680–1.407)	0.906			12.6
Age: per 1 y	0.621 (0.986–1.028)	0.534			8.4

higher in the TT genotype (2.522) than the TG/GG genotype (2.308), although this did not reach statistical significance ($P = 0.08$). Furthermore, the mean score of the degree of hepatocyte anisocytosis was significantly higher in the TT genotype (1.891) than the TG/GG genotype (1.385; $P = 0.024$). Anisocytosis is characterized by viability of cell size with focal dysplastic change and indicates irregular regeneration of hepatocytes. The irregular regeneration score was higher in the TT genotype (2.207) than the TG/GG genotype (1.795), albeit not significantly ($P = 0.105$).

IL-28B TT and TG/GG genotype gene expression profiles in the noncancerous liver

We next compared the gene expression profile of HCC tissues and noncancerous liver tissues of both the *IL-28B* TT and *IL-28B* TG/GG genotype. Ten patients with HCC were selected from each *IL-28B* genotype and their gene expression was determined using Affymetrix genechip analysis (Supplementary Table S1). We recently reported that expression of hepatic ISGs is downregulated in individuals with the *IL-28B* TT genotype, whereas the expression of other immune response-related genes was shown to be upregulated (13). Therefore, to validate our expression data, we compared the expression of ISGs and other immune response-related genes in the present study with that of the previous study. We analyzed the expression data of 20 patients from the current study in addition to another series of 91 patients with CHC from our previous study.

One-way hierarchical clustering using 28 representative ISGs showed that patients with the *IL-28B* TG/GG genotype

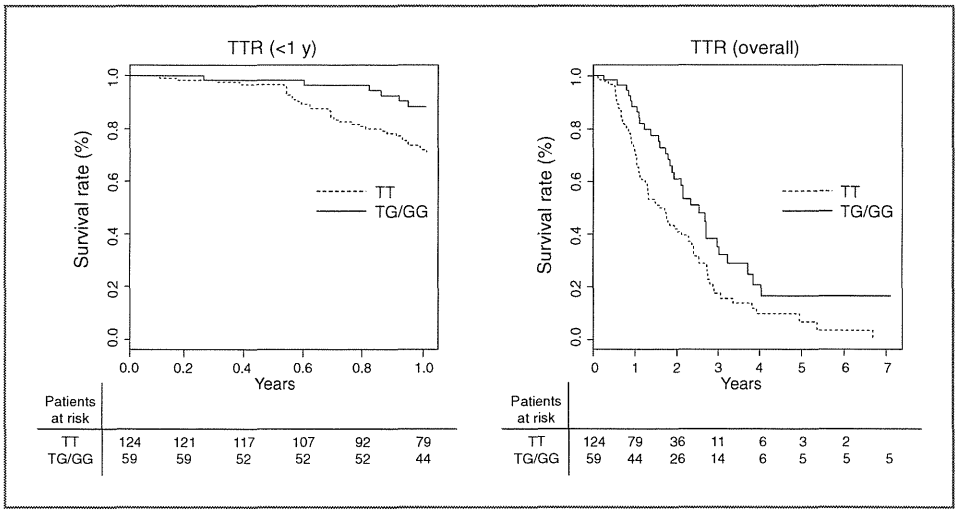

Figure 1. Kaplan–Meier curves of early and overall TTR in relation to *IL-28B* genotype. The patients with the *IL-28B* TT genotype showed a significantly shorter median TTR (1.61 years) than those with the *IL-28B* TG/GG genotype (2.58 years; $P = 0.007$).

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Table 3. Comparison of liver histology between *IL-28B* major and minor genotypes

Variable	*IL-28B* TT genotype ($n = 92$)	*IL-28* TG/GG genotype ($n = 39$)	*P* value
Score of inflammatory cell infiltration			
Periportal	2.804	2.513	0.032
Intralobular	2.522	2.308	0.082
Portal	2.946	2.846	0.322
Fibrosis	3.587	3.436	0.428
Portal lymphoid reaction	4.098	3.949	0.363
Damage of bile duct	0.380	0.256	0.216
Portal sclerotic change	0.076	0.077	0.990
Perivenular fibrosis	1.133	1.000	0.447
Pericellular fibrosis	1.163	0.821	0.045
Bridging fibrosis	0.957	0.641	0.106
Irregular regeneration	2.207	1.795	0.105
Anisocytosis	1.891	1.385	0.024
Bulging	0.326	0.436	0.485
Map-like distribution	1.370	1.333	0.881
Oncocytes	1.326	1.051	0.227
Nodularity	1.185	1.231	0.849
Atypical hepatocytes	0.467	0.692	0.304
Steatosis	1.707	1.692	0.951

NOTE: Data shown as mean.

had higher expression of hepatic ISGs, whereas patients with the TT genotype showed lower expression of hepatic ISGs in CHC tissues and noncancerous background liver tissue, confirming our previous data (Fig. 2A and Supplementary Table S2). Expression of hepatic ISGs in HCC tissues was lower than in background liver tissues, with no relationship to the *IL-28B* genotype. Hierarchical clustering of 51 representative immune response-related genes from the Gene Ontology gene set of the Molecular Signatures Database indicated that their expression was upregulated in TT genotype compared with TG/GG genotype tissues, with the exception of HCC tissues (Fig. 2B and Supplementary Table S2). Upregulation of immune response-related genes suggests that hepatic inflammation is more severe in TT genotype patients, which is consistent with our histologic findings and recent studies that reported an association between high serum ALT levels and the *IL-28B* TT genotype (14, 29).

Gene expression profile of HCC tissues from *IL-28B* TT and TG/GG genotypes

We applied PAGE to identify gene sets differentially regulated between the different *IL-28B* genotypes from the whole gene expression profiles derived from HCC tissues. Analysis of groups of genes involved in a specific function enables significant differences to represent a biologically meaningful result (23). Many gene sets associated with the immune system (e.g., the immune system process, T-cell activation, regulation of T-cell activation, and T-cell proliferation) showed a significant increase in their expression in patients with HCC with the *IL-28B* TG/GG genotype (Supplementary Table S3). This PAGE profile was consistent with the hierarchical clustering of 51 immune response-related genes (Fig. 2B) and suggests that the immune response to tumors might be more intensive in *IL-28B* TG/GG genotype HCC than *IL-28B* TT genotype HCC.

Lymphocyte infiltration into HCC tissues with the *IL-28B* TG/GG genotype

To verify our PAGE profile, we histologically compared HCC tissue of 20 cases of the *IL-28B* TT genotype and 12 cases of the TG/GG genotype using immunohistochemical staining with antibodies against helper T cells (CD4) and cytotoxic T cells (CD8). The mean score of the degree of $CD8^+$ lymphocyte infiltration in the tumor tissue was significantly higher in the TG/GG genotype (1.75) than the TT genotype (1.175; $P = 0.047$; Supplementary Table S4). A representative case is shown in Fig. 3. There was no morphologic alteration associated with the *IL-28B* genotype. Immunohistochemical analysis showed intratumoral infiltration of $CD4^+$ and $CD8^+$ lymphoid cells and slight infiltration of monocytes/macrophages in HCC of the *IL-28B* TG/GG genotype, compared with little infiltration of lymphocytes or monocytes/macrophages in HCC of the *IL-28B* TT genotype.

Furthermore, the gene set differentially expressed in HCC-infiltrating mononuclear inflammatory cells from our previous study (24) was upregulated in HCC of the *IL-28B* TG/GG genotype (Z score, -9.879; $P < 0.001$). One-way hierarchical clustering was carried out of 122 genes involved in the gene set differentially expressed in HCC-infiltrating

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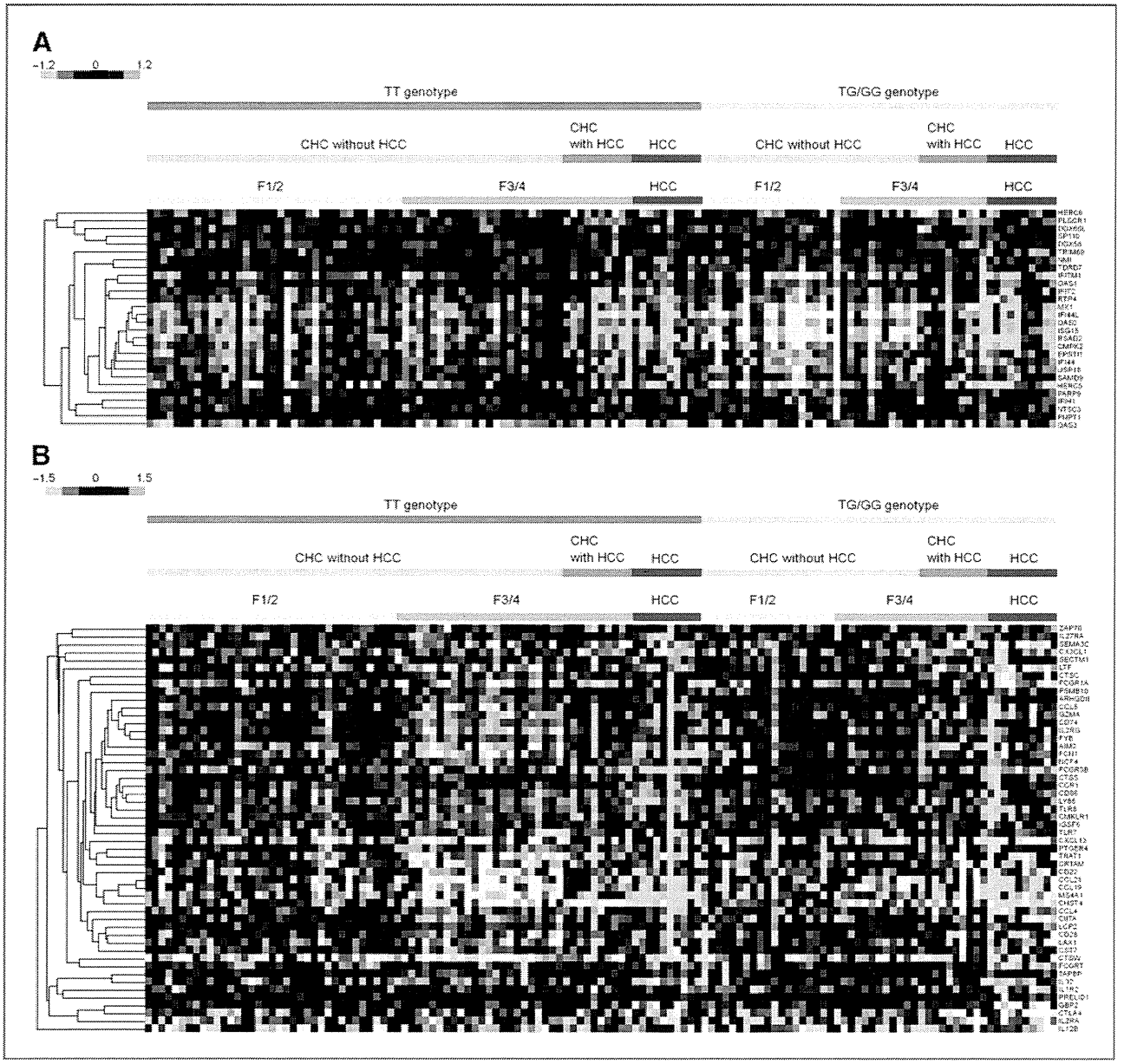

Figure 2. A, one-way hierarchical clustering of 28 representative ISGs of 111 patients with the *IL-28B* genotype. B, one-way hierarchical clustering analysis of 51 representative immune response-related genes of 111 patients with the *IL-28B* genotype.

mononuclear inflammatory cells. Most of the 122 genes were expressed at high levels in many HCC tissues of *IL-28B* TG/GG genotype patients (Supplementary Fig. S2).

Discussion

IL-28B is a recently identified, novel IFN-λ family member that shares the same biologic properties as type I IFNs (9). Recent reports have shown a significant association between *IL-28B* allelic variants and treatment outcome in CHC (11, 12). *IL-28B* genotyping is therefore considered to be a suitable pretreatment predictor of treatment response for individual patients, and also an indicator of biochemical and histologic findings in patients infected with HCV (14). In this study, we determined that the *IL-28B* genotype is associated with HCC recurrence in patients with CHC as patients with the *IL-28B* TT genotype showed a significantly higher incidence of recurrence than those with the *IL-28B* TG/GG genotype after curative therapy. To our knowledge, this is the first study to reveal an association between the *IL-28B* genotype and HCC recurrence and molecular features in patients with CHC.

To date, there are several contradicting results about the association of the *IL-28B* genotype and progression of liver disease including the development of HCC. Fabris and colleagues and Eurich and colleagues reported that

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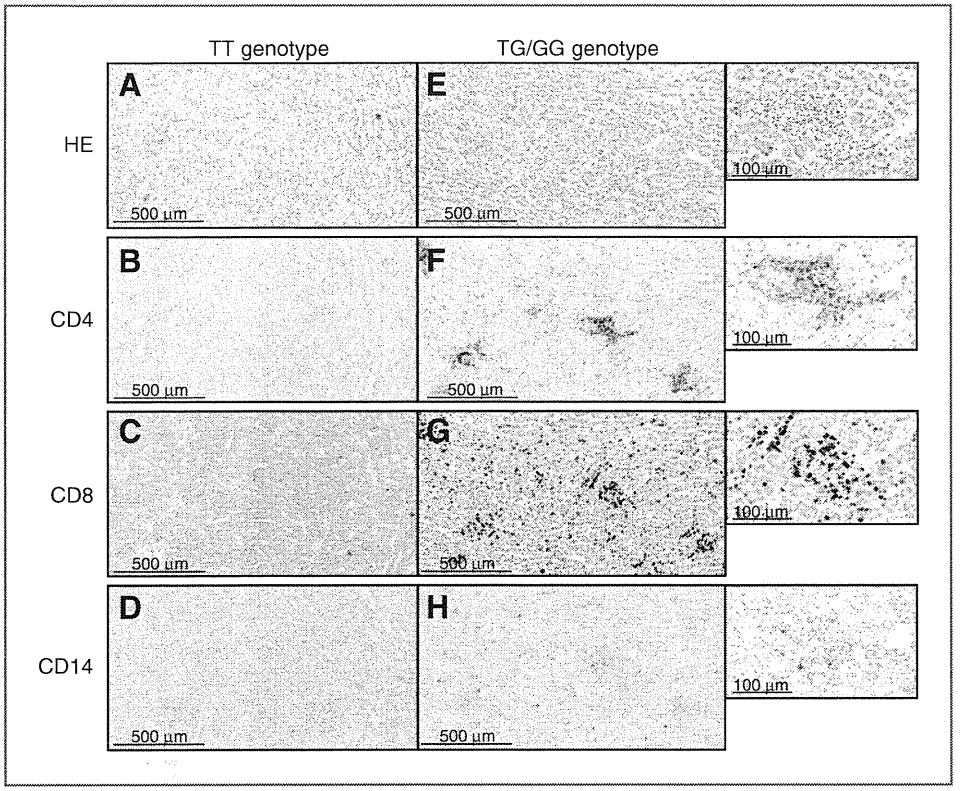

Figure 3. Expression of CD4, CD8, and CD14 in tumor-infiltrating mononuclear cells in HCC tissues. Immunohistochemical analysis of noncancerous liver tissues of *IL-28B* TT (A–D) and TG/GG genotypes (E–H). Samples were analyzed by hematoxylin and eosin staining (A and E), CD4 staining (B and F), CD8 staining (C and G), and CD14 staining (D and H).

patients with a T allele in rs12979860 (G allele in rs8099917) were at a high risk of progressing to liver cirrhosis and HCC (35, 36), however, these reports have not yet been confirmed by others: A large-scale European genome-wide association study (GWAS) recently identified a weak protective role for the rs12979860 T allele in the progression of fibrosis during HCV infection (37), whereas a Japanese GWAS identifying a susceptibility locus for HCV-induced HCC found no association of rs12979860 and rs8099917 SNPs with HCC (38). In support of these findings, Joshita and colleagues reported no association between the *IL-28B* genotype and the incidence of primary HCC (39). These results show a good concordance with those of the present study, which revealed that the *IL-28B* genotype was not associated with HCC incidence before treatment (Table 1). Furthermore, closer histologic assessment showed a high score of periportal inflammation and pericellular fibrosis in the rs8099917 TT genotype (CC in rs12979860). This suggests that our patient selection process was not biased, and that our results are in agreement with the Japanese study and are comparable with the European study.

To date, the reasons for contradicting results about the association of the *IL-28B* genotype and progression of liver disease have not been clear, however, clinical bias such as patient number, history of treatment, virus genotype, and titer and racial differences may affect the results. It should be noted that significant differences in genotype frequencies with respect to ethnic/racial groups have previously been reported for *IL-28B* SNPs (11). To overcome these limitations, a future cross-sectional prospective study should be conducted.

Several risk factors for HCC recurrence have previously been reported, including the presence of cirrhosis, high AFP levels, and multiplicity of tumors 7, 8). However, multivariate analysis and the bootstrap procedure of the present study revealed that the *IL-28B* genotype was independent indicators for recurrence, suggesting that it is stronger predictors of HCC recurrence than other factors.

The expression of hepatic ISGs was higher in *IL-28B* TG/GG genotype patients than *IL-28B* TT genotype patients with CHC in this study. This confirms our previous findings in a different cohort and those of another research group (13, 40). Several ISGs have been reported to have antiproliferative and proapoptotic functions (41, 42), and IFN-α (type I IFN) has also been found to inhibit metastasis and human HCC recurrence after curative resection mediated by angiogenesis (43). Indeed, *IL-28B* rs8099917 is associated with early HCC recurrence (<1 year), possibly because of the intrahepatic metastasis of HCC in this study (Fig. 1 and Supplementary Table S5). These reports and our findings suggest that high expression of hepatic ISGs might cause the low HCC recurrence in the *IL-28B* TG/GG genotype, although the mechanism of this association remains unknown.

Microarray, histologic, and immunohistochemical analysis in the present study showed that the immune response was more severe in chronic hepatitis and noncancerous tissue of *IL-28B* TT genotype compared with TG/GG genotype patients. Serum ALT levels were also higher in the

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IL-28B TT genotype, albeit not significantly. These results support previous findings that showed higher serum ALT levels and more severe liver inflammation in TT genotype compared with TG/GG genotype patients with HCC (14, 29). Irregular regeneration of hepatocytes develops as a result of repeated cycles of necrosis and regeneration of hepatocytes and was previously reported to be an important predictive factor for the development of HCC (26). We histologically showed that the degree of hepatocyte anisocytosis was more severe in noncancerous livers of TT genotype than TG/GG genotype patients, perhaps because of *IL-28B* genotype-dependent hepatic inflammation. This might also affect the late recurrence of HCC (>1 year) as a result of the multicentric occurrence of HCC in background liver disease. In the late recurrence group, *IL-28B* TT genotype patients showed a shorter TTR than *IL-28B* TG/GG genotype patients although this did not reach statistical significance ($P = 0.086$; Supplementary Fig. S3; Supplementary Table S6).

Previous studies showed that the gene expression profile of noncancerous liver tissue was associated with late recurrence HCC and the multicentric occurrence of HCC (44). However, the gene set expression of these studies did not differ between the *IL-28B* TT and TG/GG genotypes in the present study. Although the reason for this discrepancy is unclear, the *IL-28B* genotype may affect early recurrence more than late recurrence, and the limited number of patients and the short follow-up period may affect statistical comparisons. Therefore, further investigations with a large series of patients are necessary to clarify whether *IL-28B* genotype-dependent inflammation influences HCC recurrence.

On the other hand, the gene expression profile and histologic analyses showed that more lymphocytes infiltrate into the tumor tissue of the *IL-28B* TG/GG genotype than the TT genotype. Chew and colleagues previously showed that 14 intratumoral immune gene signatures were able to identify molecular cues driving the tumor infiltration of lymphocytes and predict the survival of patients with HCC, particularly during the early stages of disease (45). We can confirm that the expression of some of these 14 genes was higher in TG/GG genotype than TT genotype patients (Supplementary Fig. S4), supporting the association of the *IL-28B* genotype, HCC recurrence, and histologic findings. The presence of lymphocyte infiltration in HCC was also reported as a negative predictor of HCC recurrence after liver transplantation (46), and this phenomenon may contribute to a lower incidence of HCC recurrence in the TG/GG genotype.

It may seem contradictory that the immune response in noncancerous liver was more severe in TT genotype than TG/GG genotype patients despite the fact that the expression of immune genes was higher in tumor tissue and more lymphocytes infiltrated the tumor in the TG/GG genotype compared with the TT genotype. Although we are unable to explain this contradiction, it is conceivable that the host immune reaction has a differential role between tumor and nontumor tissue. Moreover, HCV-specific T-cell immune responses, which are essential for disease control, are attenuated in patients with CHC, and T-cell exhaustion has recently been implicated in the deficient control of chronic viral infections. On the other hand, little is known on self- and tumor-specific T-cell responses in patients with HCC. While several reports have shown the existence of exhausted T cells in a tumor environment, impaired T-cell responses to tumors are unlikely to be simply explained by T-cell exhaustion (47).

Anergy or other functional statuses such as suppressive immunity by tumor cells should be considered in tumor immunity. Therefore, differences in immunity to viral antigens and self- and tumor-antigens could explain our findings, although further work should be carried out to confirm these conclusions. We have preliminarily confirmed that the ratio of regulatory T cells is higher in the peripheral blood of *IL-28B* TT genotype HCC patients than *IL-28B* TG/GG genotype patients, although there is no significant difference between non-HCC *IL-28B* TT genotype and *IL-28B* TG/GG genotype patients (data not shown). Although the cause of this phenomenon is unclear, our gene expression profile of noncancerous liver and tumor tissues suggests paradoxical roles for the immune response in CHC and HCC depending on *IL-28B* genotype; it will be necessary to clarify these mechanisms in future investigations.

Recently, a sustained virologic response (SVR) to CHC antiviral treatment was shown to be associated with a lower risk of HCC recurrence (48). Although we did not include patients with SVR in the current study, we nevertheless observed that they showed a longer recurrence-free survival than patients infected with HCV, independent of *IL-28B* genotype (data not shown). This result together with the association between the *IL-28B* genotype and response to antiviral treatment promotes recommendations for aggressive CHC antiviral treatment, especially in cases with the *IL-28B* TT genotype.

RFA is a recently developed technique and its efficacy has been reported equal to that of surgical resection, especially in early-stage HCC (3–6). In the European Association for the Study of the Liver–European Organisation for Research and Treatment of Cancer (EASL-EORTC) guidelines, RFA is considered the standard care for patients with Barcelona Clinic Liver Cancer stage 0-A tumors not suitable for surgery and whether or not RFA can be considered a competitive alternative to resection is uncertain (49). In our study, the local tumor progression rate was not statistically different between RFA and resection cases. However, further studies with an appropriate sample population are necessary to clarify the comparison of RFA and resection. The present study has some limitations. It was a retrospective cohort and a single-center study, so it was difficult to completely eliminate bias. Further prospective studies of a larger series of patients should be conducted to validate our results. As a consequence of the small sample size and even smaller number of patients undergoing surgical resection, we could not show an association between *IL-28B* genotype and HCC

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recurrence in the surgical resection group (data not shown). However, we did find no significant difference in TTR between RFA and surgical resection, confirming previous findings.

In conclusion, we found that the *IL-28B* rs8099917 TT genotype is associated with shorter TTR in patients with HCC with CHC. Microarray analysis showed a high expression of ISGs in background liver and high expression of immune system-related genes in tumor tissues of the *IL-28B* TG/GG genotype. Histologic findings also showed that more lymphocytes infiltrated into tumor tissues in the TG/GG genotype. The *IL-28B* genotype therefore is a candidate useful genetic marker to predict HCC recurrence as well as the response to pegylated-IFN and ribavirin combination therapy for CHC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Honda, Y. Sakai, M. Moriyama, S. Kaneko
Development of methodology: M. Moriyama
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Hodo, A. Tanaka, K. Arai, T. Yamashita, T. Yamashita, A. Sakai, Y. Nakanuma, M. Moriyama, S. Kaneko
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Hodo, M. Honda, M. Sasaki, M. Moriyama
Writing, review, and/or revision of the manuscript: Y. Hodo, M. Honda, M. Moriyama
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Hodo, A. Tanaka, Y. Nomura, K. Arai, E. Mizukoshi, M. Sasaki, M. Moriyama, S. Kaneko
Study supervision: Y. Sakai, E. Mizukoshi, M. Moriyama

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全自動生物化学発光免疫測定装置「BLEIA®-1200」専用試薬「BLEIA®‘栄研’HCV抗体」の性能評価

国立病院機構 長崎医療センター・臨床研究センター（肝臓内科）
八橋　弘・中村　実可・釘山　有希・佐々木　龍
戸次　鎮宗・橋元　悟・裵　成寛・大谷　正史
佐伯　哲・長岡　進矢・小森　敦正・阿比留正剛

はじめに

わが国には約150～200万人に及ぶC型肝炎ウイルス（hepatitis C virus：以下HCVと略す）のキャリアが存在すると推定されている。これらのキャリアは長期間炎症が持続し，肝硬変，肝癌へ移行する割合が非常に高いことから，HCV感染者のスクリーニングと早期治療が重要視されている。

1989年にChiron社がHCVを発見し，非構造領域NS3，NS4のC100-3を用いた第一世代のHCV抗体試薬が開発されたが，検出率はHCV感染の約70％であった。その後，core，NS3およびNS4を用いた第二世代，さらにNS5抗原を加えた第三世代の試薬が測定系に用いられるようになり，感度および特異度はほぼ100％となった[1]。

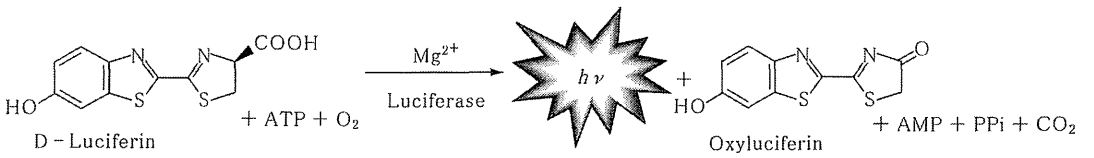
平成24年度までのわが国におけるC型肝炎検診は，肝炎ウイルス検診等実施要領（健発0329第25号）にあるように，まずHCV抗体の検出（定性）およびHCV抗体検査（半定量）[2]が行われ，HCV抗体陽性と判定された検体を抗体価の違いによって高力価，中力価および低力価の3群に分類し，さらにHCV抗原検査[3)4)]，HCV核酸増幅検査[5]を経て，「現在，C型肝炎ウイルスに感染している可能性が極めて高い」または「現在，C型肝炎ウイルスに感染していない可能性が極めて高い」と判定している。平成25年度からの肝炎ウイルス検診等実施要領[6]は一部手順が改正され，適切に力価分けが可能なHCV抗体検査と核酸増幅検査の組み合わせとなり，「現在，C型肝炎ウイルスに感染している可能性が高い」と判定された方には医療機関の受診を強く勧めることになる。

今回，新たにHCV抗体の検出（定性）およびHCV抗体検査（半定量）を目的として開発された，生物発光酵素免疫測定法（Bioluminescent enzyme immunoassay：BLEIA法）を測定原理とする第三世代の試薬「BLEIA®‘栄研’HCV抗体」（栄研化学株式会社）について，基本性能の評価，患者検体による臨床性能評価および肝炎ウイルス検診等実施要領による分類等を行ったので，その結果を報告する。

Evaluation of *BLEIA® EIKEN HCV ANTIBODY* dedicated for automated bioluminescent enzyme immunoassay analyzer BLEIA®-1200

Hiroshi Yatsuhashi et al　*National Hospital Organization Nagasaki Medical Center*

Key words：C型肝炎，HCV抗体検査，生物発光，酵素免疫測定法，BLEIA法



ATP：Adenosine－5'－triphosphate　　PPi：Pyrophosphate
AMP：Adenosine－5'－monophosphate　　hν：光のエネルギー

図1　ホタルのルシフェリン-ルシフェラーゼ発光原理

I．対　象

対象は，国立病院機構 長崎医療センター（以下，国立長崎医療センター）にてインフォームドコンセントが得られた，C型肝炎と診断した100例および非C型肝炎と診断した80例の肝疾患の検体を用いた。HCVのGenotypeの内訳は1aが1例，1bが80例，2aが10例および2bが9例であった。C型肝炎の病期内訳は慢性肝炎が82例，肝硬変が14例および肝癌が4例であり，非C型肝炎の内訳は慢性肝炎が34例，肝硬変が16例，自己免疫性肝炎が7例，原発性胆汁性肝硬変が20例および脂肪肝が3例であった。いずれも治療開始前の検体である。

セロコンバージョンパネル等を用いたHCV抗体の検出性能の確認には5種のパネル（PHV901，PHV904，PHV905，PHV906，PHV907（SeraCare Life Sciences社））およびLow Titer Performance Panel（PHV105（SeraCare Life Sciences社）），Mixed Titer Performance Panel（PHV205（SeraCare Life Sciences社））を用いた。PHV906，PHV907，PHV105は，医薬品・医療用具等安全性情報175号の「C型肝炎ウイルス抗体の検出を目的とする体外診断用医薬品の適正使用について」記載のセロコンバージョンパネルである。

平成23年度肝炎ウイルス検診等実施要領による分類については，栄研化学株式会社提供の企業健康診断検体5,000例を使用した。なお，本健康診断検体はインフォームドコンセントを得たものであり，かつ連結不可能匿名化されたものである。

II．方　法

1．BLEIA法

BLEIA法は生物発光の一種であるホタルルシフェラーゼ発光を原理としている[7]。ホタルルシフェラーゼ発光は，アデノシン三リン酸（ATP），マグネシウムイオン，溶存酸素の存在下で酵素であるホタルルシフェラーゼにより基質であるルシフェリンがオキシルシフェリンに変換され，光を放出する（**図1**）。BLEIA法ではKajiyama，Tatsumiらによって開発された耐熱性ビオチン化ルシフェラーゼ[8)~10)]を標識した抗ヒト抗体（標識IgG抗体）と，リコンビナントHCV抗原固相化磁性粒子を用いた免疫サンドイッチ法によって，抗HCV抗体価を測定している。検体を希釈した後，磁性粒子を加え抗HCV抗体を捕捉する。洗浄後，標識IgG抗体を加えて反応させ，再洗浄後に基質を加えて発光強度を測定する（**図2**）。判定は1カットオフインデックス（COI）以上を陽性とし，力価別においてはHCV抗体陽性検体のうち，1以上40COI未満を低力価，40以上400COI未満を中力価，400COI以上を高力価と判定する。

BLEIA® '栄研' HCV抗体（BLEIA法）の検討として，1. 同時再現性・日差再現性，2. 希釈直線性，3. C型・非C型肝炎患者検体での検討，4. 他法との相関および乖離検体例の分析，5. セロコンバージョンパネル等を用いたHCV抗体の検出性能の確認，6. 肝炎ウイルス検診等実施要領による分類の確認を行った。

2．CLEIA法

CLEIA法の測定は，ルミスポット® '栄研'