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Variants in *IL28B* in Liver Recipients and Donors Correlate With Response to Peg-Interferon and Ribavirin Therapy for Recurrent Hepatitis C

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BACKGROUND & AIMS: Patients with hepatitis C virus (HCV)–related liver disease frequently undergo orthotopic liver transplantation, but recurrent hepatitis C is still a major cause of morbidity. Patients are treated with peg-interferon and ribavirin (PEG-IFN/RBV), which has substantial side effects and is costly. We investigated genetic factors of host, liver donor, and virus that might predict sensitivity of patients with recurrent hepatitis C to PEG-IFN/RBV. **METHODS:** Liver samples were analyzed from 67 HCV-infected recipients and 41 liver donors. Liver recipient and donor DNA samples were screened for single nucleotide polymorphisms near the *IL28B* genes (rs12980275 and rs8099917) that affect sensitivity to PEG-IFN/RBV. HCV RNA was isolated from patients and analyzed for mutations in the core, the IFN sensitivity-determining region, and IFN/RBV resistance-determining regions in nonstructural protein 5A. **RESULTS:** In liver recipients and donors, the *IL28B* single nucleotide polymorphism rs8099917 was significantly associated with a sustained viral response (SVR; $P = 0.003$ and $P = .025$, respectively). Intrahepatic expression of *IL28* messenger RNA was significantly lower in recipients and donors that carried the minor alleles (T/G or T/T) in rs8099917 ($P = .010$ and .009, respectively). Genetic analyses of *IL28B* in patients and donors and of the core and nonstructural protein 5A regions encoded by HCV RNA predicted an SVR with 83% sensitivity and 82% specificity; this was more effective than analysis of any single genetic feature. **CONCLUSIONS: In patients with recurrent HCV infection after orthotopic liver transplantation, combination analyses of single nucleotide polymorphisms of *IL28B* in recipient and donor tissues and mutations in HCV RNA allow prediction of SVR to PEG-IFN/RBV therapy.**

Keywords: ISDR; IRRDR; Genetic Analysis; Genetic Variations.

Hepatitis C virus (HCV) infection affects 170 million people worldwide and can lead to decompensated cirrhosis and hepatocellular carcinoma.[1,2] As a result, HCV-related liver disease is the leading indication for orthotopic liver transplantation (OLT) worldwide.[3,4] However, several reports have shown that post-OLT patient and graft survival are significantly negatively affected by HCV recurrence after OLT.[5,6] This can be mitigated by achievement of a sustained virological response (SVR) with pegylated interferon and ribavirin (PEG-IFN/RBV) therapy.[7] However, many patients cannot tolerate curative doses or do not respond to therapy with PEG-IFN/RBV.[6,8] Because of the substantial cost of therapy, both financial and with regard to side effects, it would be ideal to be able to predict which patients would benefit from PEG-IFN/RBV therapy for recurrent HCV.[9,10]

Many reports have demonstrated that HCV-RNA mutations, including those of amino acid residues 70 and 91 in the Core region,[11] and those in the interferon sensitivity determining region (ISDR)[12] and variable region 3 domain[13] in the nonstructural protein 5A (NS5A), were significantly associated with IFN sensitivity in patients infected with genotype 1 HCV. We previously reported that these genetic mutations have a significant impact on patients' responsiveness to PEG-IFN/RBV therapy for recurrent hepatitis C after OLT.[14] However, in addition to viral factors, host factors can also be used to predict IFN sensitivity. For example, Asahina et al demonstrated that the pretreatment induction level of IFN-stimulated genes (ISGs) was significantly associated with SVR to PEG-IFN/RBV therapy.[15] In addition, it was recently reported that single nucleotide polymorphisms near the IL28B gene on chromosome 19q13, rs12980275, or rs8099917 are significantly associated with the sensitivity of IFN/RBV combination therapy for chronic hepatitis

Abbreviations used in this paper: DW, double-wild; ETR, end of treatment response; HCV, hepatitis C virus; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; ISG, interferon-stimulated gene; mRNA, messenger RNA; NR, nonresponse; NS5A, nonstructural protein 5A; OLT, orthotopic liver transplantation; PCR, polymerase chain reaction; PEG-IFN, pegylated interferon; RBV, ribavirin; SVR, sustained viral response.

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C.[16-21] In the present study, we examined the impact of genetic variations of IL28B in recipients and donors, and of genetic variations in HCV-RNA on the responsiveness to IFN/RBV therapy for recurrent hepatitis C after OLT.

Patients and Methods

Patients

Recipients enrolled in this study underwent OLT for HCV-related liver disease, had normal ejection fraction, lung capacity, and renal function (creatinine clearance >70 mL/min), were treated with PEG-IFN/RBV combination therapy after OLT, were negative for hepatitis B virus and human immunodeficiency virus, and positive for HCV-RNA. At Kyushu University Hospital, 112 liver transplantations were performed between April 1999 and March 2009 on HCV-infected patients. PEG-IFN/RBV therapy was administered to 78 of these recipients. Eleven recipients were excluded from this analysis because of ongoing therapy with PEG-IFN/RBV therapy (n = 5) or because their therapy was discontinued secondary to side effects (n = 6). Therefore, 67 recipients were retrospectively analyzed. A total of 41 of their donors were available for analysis. All OLTs were performed after obtaining informed consent from recipients and donors, and the current study was approved by the Kyushu University ethics committee.

Surgical Technique and Immunosuppression

The surgical procedure for the recipients has been described previously.[22] The choice of resected segments for donation was dictated by the need to obtain a graft volume >35% of the recipient's standard liver volume. Simultaneous splenectomies for 41 recipients (61%) were performed to prevent pancytopenia due to antiviral therapy. The induction and maintenance of immunosuppression was achieved using a calcineurin inhibitor, mycophenolate mofetil, and steroids in most cases. Cyclosporine and tacrolimus were used for 35 and 32 recipients, respectively. The response rate to antiviral therapy was comparable (data not shown). Steroids were administered intraoperatively (methylprednisolone, 1000 mg) and were tapered off by 6 months after transplantation. In 8 cases, steroid-free immunosuppression was performed using basiliximab (Simulect; Novartis Pharma, Tokyo, Japan).

Antiviral Treatment Regimen and Assessment of the Therapeutic Effects

The primary doses of PEG-IFNα-2b (Pegintron; Schering-Plough Inc, Kenilworth, NJ) and RBV (Rebetol; Schering-Plough Inc) were 0.5 μg/kg per week and 200 mg/day and were increased to 1.5 μg/kg per week and 800 mg/day in a stepwise manner according to individual tolerance within the first 12 weeks. The proportion of patients receiving >70% of the full treatment dose (1.0 μg/kg/week) during 80% of the treatment period was 79% (53 of 67). Hematopoietic growth factors including granulocyte colony-stimulating factor and erythropoietin were not used. Antiviral therapy was discontinued in cases with severe depression, renal dysfunction, or autoimmune hepatitis. The viral titers were assessed in all patients using a polymerase chain reaction (PCR)–based quantitative assay (Amplicor Monitor or PCR Cobas TaqMan system; Roche Diagnostics, Mannheim, Germany). SVR was defined as an undetectable level of HCV RNA at 6 months after completion of treatment, while a nonresponse (NR) was defined as a detectable level of HCV RNA at the end of treatment. End of treatment response (ETR) was defined as an undetectable HCV-RNA at the end of treatment.

Analysis of Genetic Variations of IL28B and HCV-RNA

DNA from recipients and donors was extracted from exenterated liver tissue at OLT and biopsied liver tissue after OLT. PCR and direct sequencing were performed using TaKaRa Ex-Taq polymerase (Takara Bio Inc, Tokyo, Japan) and a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc, Tokyo, Japan), respectively. The PCR primers for single nucleotide polymorphisms near the IL28B gene were used as previously described.[18] For the genetic analysis of HCV-RNA in the Core and NS5A regions, reverse transcription using Superscript 3 First-Strand Synthesis SuperMix (Invitrogen, Tokyo, Japan) and nested PCR were performed as described previously.[14] The numbering of amino acids was performed according to the polyprotein of HCV genotype 1b prototype HCV-J (GenBank accession no. D90208). HCV-J was used as the consensus sequences for Core and ISDR. To evaluate the association between the genetic variations in IL28B and HCV-RNA mutations, the patients were divided into 2 groups based on the presence or absence of mutations at amino acid residues 70 and 91 in the Core (a double-wild [DW] group and non-DW group) and on the numbers of mutations in the ISDR (ISDR ≥ 2 and ISDR < 2) and interferon/ribavirin resistance-determining region (IRRDR) (IRRDR ≥ 6 and IRRDR < 6) regions.

Real-Time PCR of IL28 Messenger RNA

Total RNA was extracted from resected liver tissue derived from the recipient, donated liver tissue derived from the donor, and biopsied liver tissue after OLT using reagents for RNA extraction including ISOGEN and Ethachinmate (Nippon Gene, Tokyo, Japan). The synthesis of first-strand complementary DNA and quantitative reverse transcription PCR were performed using TaqMan EZ RT-PCR Core Reagents (Applied Biosystems) according to manufacturer's protocol. A standard curve was prepared by serial 10-fold dilutions of complementary DNA from Huh7 cell lines stimulated by vesicular stomatitis virus infection. Primers and a TaqMan-probe were designed for the IL28 genes as previously reported.[18]

Table 1. Comparison of the Data Among Patients Showing ETR, SVR, and NR

	ETR (n = 47)	NR (n = 20)	SVR (n = 23)	*P* value
Pretransplantation factor				
Recipient's age (*y*), mean ± SD	55.8 ± 7.3	56.9 ± 7.6	56.0 ± 6.8	NS
Recipient's sex (*male/female*), n	28/19	13/7	15/8	NS
Donor's age (*y*), mean ± SD	34.8 ± 9.6	31.8 ± 10.4	32.1 ± 8.8	NS
Donor's sex (*male/female*), n	30/17	16/4	16/7	NS
HCV genotype (*1/2*), n	40/7	19/1	21/2	NS
MELD score, mean ± SD	12.4 ± 4.7	10.6 ± 4.3	11.7 ± 4.7	NS
Pretransplantation viral load (*logIU/mL*), mean ± SD	5.7 ± 0.8	5.8 ± 0.9	5.7 ± 0.7	NS
History of IFN therapy (*yes/no*), n	14/27	7/11	5/14	NS
Intraoperative factor, mean ± SD				
Intraoperative bleeding (*mL*)	5449 ± 4265	7018 ± 5571	5701 ± 4378	NS
Operation time (*min*)	808 ± 155	877 ± 191	803 ± 174	NS
GV/SLV (%)	40.4 ± 6.9	41.7 ± 8.1	39.4 ± 5.8	NS
Post-transplantation factor				
Acute cellular rejection (*yes/no*), n	7/30	2/13	2/18	NS
Bile duct complication (*yes/no*), n	10/37	3/17	4/19	NS
CMV infection (*yes/no*), n	5/32	2/13	3/17	NS
Steroid pulse therapy (*yes/no*), n	4/37	1/16	1/17	NS
Time to antiviral therapy from transplantation (*y*), mean ± SD	1.2 ± 1.3	1.0 ± 1.1	1.3 ± 1.2	NS
Pretreatment viral load (*logIU/mL*), mean ± SD	6.4 ± 0.7	6.5 ± 0.6	6.1 ± 0.8	NS
Pathological activity score, mean ± SD	1.27 ± 0.62	1.32 ± 0.58	1.36 ± 0.58	NS
Pathological fibrosis score, mean ± SD	0.93 ± 1.07	0.74 ± 0.99	1.17 ± 1.16	NS
Pretreatment ALT level (*IU/L*), mean ± SD	53.0 ± 47.0	73.8 ± 48.7	63.7 ± 48.7	NS
Pretreatment WBC level (*per μL*), mean ± SD	4107 ± 1292	4441 ± 2414	3953 ± 1127	NS
Pretreatment Hb level (*mg/dL*), mean ± SD	11.7 ± 1.5	10.9 ± 1.4	11.8 ± 1.5	NS
Pretreatment Plt level (*per μL*), mean ± SD	18.3 ± 9.9	21.7 ± 12.4	18.7 ± 10.0	NS

ALT, alanine aminotransferase; CMV, cytomegalovirus; ETR, end of treatment response; GV, graft volume; Hb, hemoglobin; HCV, hepatitis C virus; IFN, interferon; MELD, model for end-stage liver disease; NR, null response; Plt, platelet; SLV, standard liver volume; SVR, sustained viral response; WBC, white blood cell.

Statistical Analysis

Data are expressed as means ± standard deviation. The statistical analyses were performed using Student *t* test and Fisher's exact probability test. SPSS software (version 15.0, SPSS, Inc, Chicago, IL) was used for all analyses. A difference of $P < .05$ was considered to be significant.

Results

Characteristics of the Patients

The age of the recipients was 56.1 ± 7.4 years; 41 patients were male. The age of the donors was 33.9 ± 9.9 years; 36 patients were male. The viral titers before OLT and PEG-IFN/RBV therapy were 5.7 ± 0.9 log IU/mL and 6.4 ± 0.7 log IU/mL, respectively. Sixty-eight percent of recipients with HCV genotype 1 (40 of 59) and 88% of recipients with genotype 2 (7 of 8) exhibited an ETR. Forty percent of recipients (23 of 57) attained an SVR in the current study. The patient characteristics are presented in Table 1. There were no significant differences in pretransplantation factors among SVR, ETR, and NR patients.

Correlation Between Genetic Variations in IL28B and IFN Sensitivity After OLT

Genetic variations in rs8099917 and rs12970275 of IL28B were evaluated for all recipients and donors, and the match rate of the haplotype between rs8099917 and rs12980275 was 94% (101 of 108; Supplementary Table 1). Of the 67 recipients and 41 donors enrolled in this study, 19 (28%) recipients and 11 (27%) donors had the minor allele (T/G or T/T) in rs8099917. We first examined the correlation of IL28B genetic variation in recipients with the responsiveness to IFN therapy after OLT. With regard to the recipient genotype, the SVR rate was significantly higher in the recipients carrying the major homozygous allele than in those with the minor heterozygous or homozygous allele (54% vs 11%; $P = .003$, Figure 1*A*). Interestingly, the SVR rate was also significantly higher in the recipients transplanted with the liver grafts from donors carrying the major homozygous allele (44% vs 9%; $P = .025$, Figure 1*B*). Combined analyses revealed that the SVR rate was significantly increased when both donors and recipients were major-allele homozygotes (56%; $P = .005$), whereas it was lower in the recipients who carried the major homozygous allele but received a minor heterozygote or homozygote allele transplant, or those carrying the minor allele who received a transplant from a major-allele homozygous donor (10%). SVR was not seen in heterozygote or homozygote minor allele recipients transplanted with liver tissue from heterozygote or homozygote minor allele donors (Figure 1C). The achievement of ETR was also significantly associated with single nucleotide polymorphisms

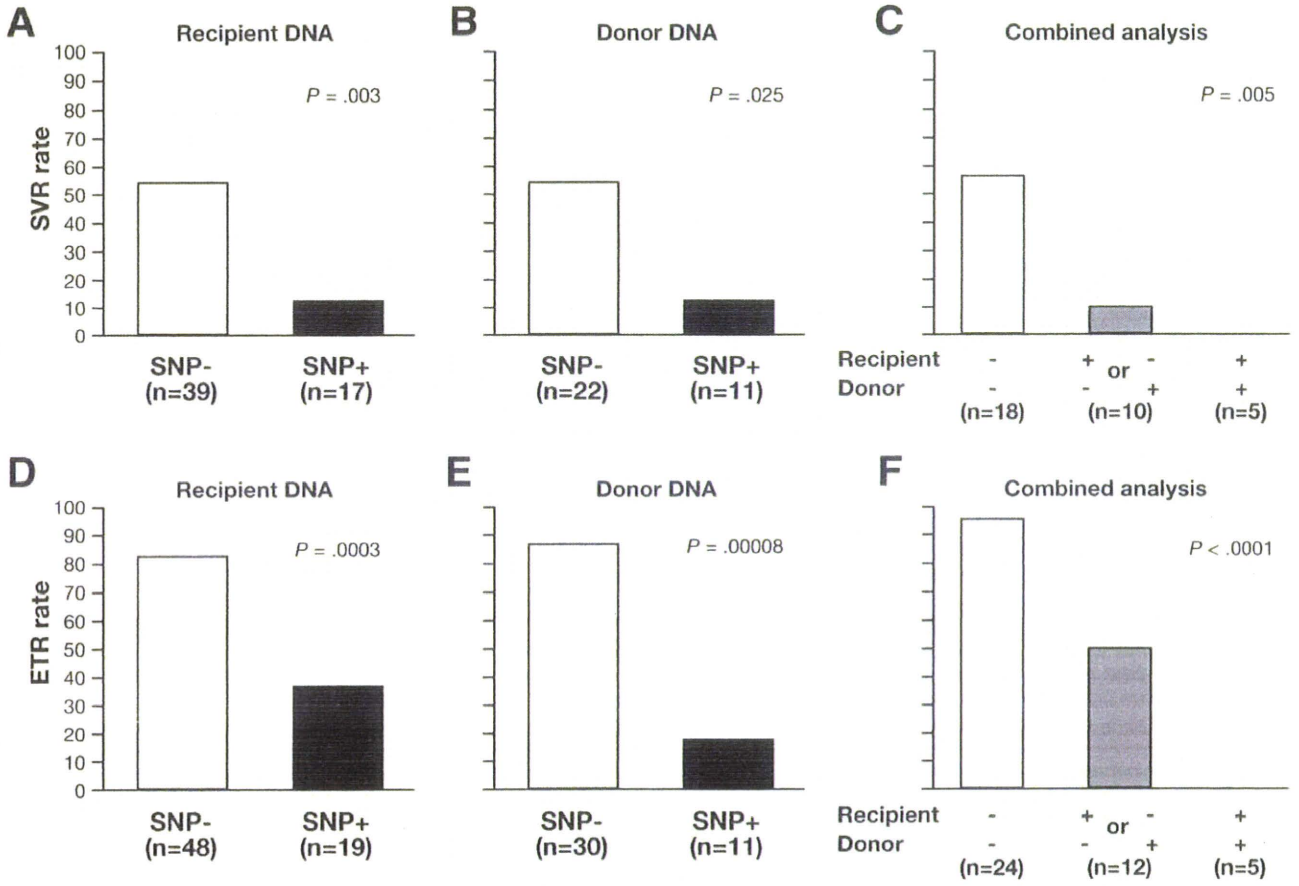

Figure 1. Association of the IL28B genetic variation in rs8099917 with the sensitivity to interferon/rivavirin combination therapy for recurrent hepatitis C virus infection after orthotopic liver transplantation. The sustained viral response (*SVR*) and end of treatment response (*ETR*) ratios in the recipients (*A*, *C*) and donors (*B*, *D*) carrying the major homozygous allele (*white bar*) or the minor allele (*black bar*) are shown. Combined analyses of the factors are shown in (*E*) and (*F*). The SVR and ETR ratios are shown for the recipients carrying the major-allele homozygote who received a transplant from donors carrying the same allele (*white bar*), the major homozygous allele in either the recipients or the donors (*gray bar*), and the recipients carrying the minor-allele homozygote who received a transplant from donors carrying the same minor allele (*black bar*). Statistical analysis was performed using Fisher's exact probability test. SNP, single nucleotide polymorphism.

in the IL28B gene of both the recipients and the donors (Figure 1*D–F*). These results indicate that IL28B genetic variations of not only the recipients but also the donors are significantly associated with the response to PEG-IFN/RBV therapy for recurrent hepatitis C after OLT.

IL28 Messenger RNA Expression and IL28B Genetic Variation

To analyze the correlation between IL28B genetic variation and IL28 messenger (mRNA) expression, the expression level of IL28 mRNA was compared between major allele homozygous and minor allele–positive recipients and/or donors. The amounts of IL28 mRNA in the livers were comparable with those in peripheral blood mononuclear cells (P = NS; Supplementary Figure 1). The levels of IL28 mRNA in the resected livers were significantly higher in the patients carrying the major allele homozygote than those carrying the minor allele heterozygote or homozygote (8.88% ± 6.09% vs 4.45% ± 3.31%; P = .010, Figure 2). In addition, the expression of IL28 mRNA in the liver grafts was also higher in the donors carrying the major allele homozygote than those carrying the minor allele heterozygote or homozygote (6.82% ± 6.51% vs 2.61% ± 1.75%; P = .009, Figure 2). In the transplanted liver before antiviral therapy, a high level of IL28 mRNA expression was observed only in the recipients who were homozygous for the major allele and who were transplanted with a liver from a recipient with the same allele, whereas other combinations exhibited a lower level of IL28 mRNA expression in the transplanted livers (Figure 2). These results suggest that IL28B genetic variation in both recipients and donors is closely associated with the expression of IL28 mRNA in the transplanted livers and might be involved in determining IFN sensitivity after OLT. To confirm the potential role of IL28 mRNA expression in IFN sensitivity, the expression of IL28 mRNA in the transplanted liver was significantly higher in the SVR patients than that in the NR patients (8.03% ± 6.22% vs 2.34% ± 1.82%; P = .046; Figure 3).

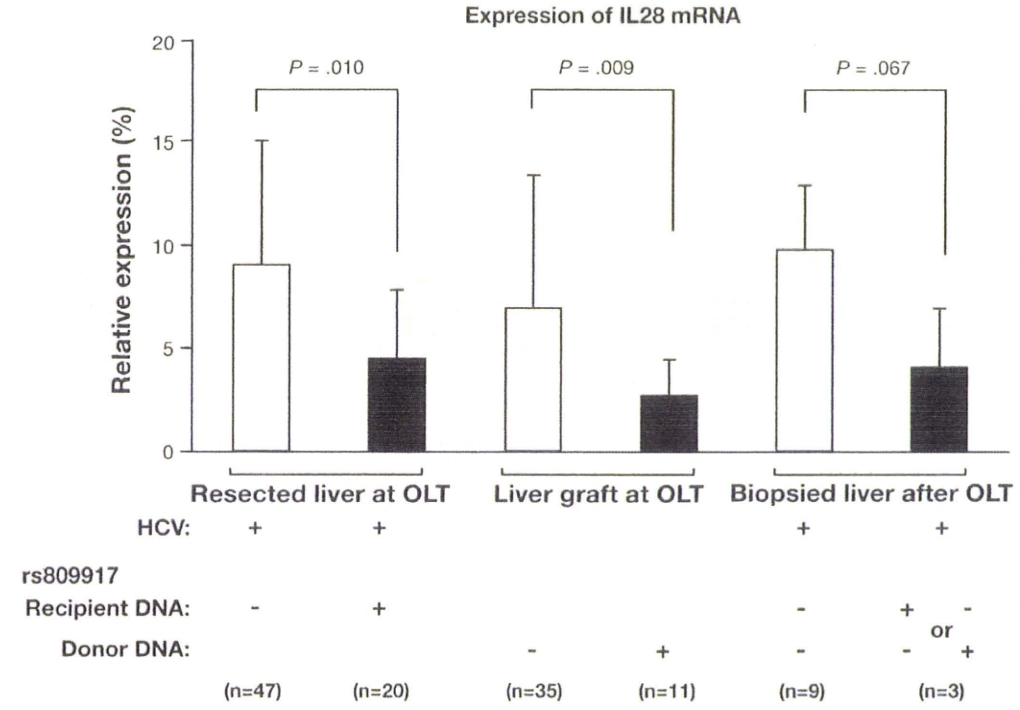

Figure 2. The expression of IL28 messenger RNA (*mRNA*) in the resected livers (n = 67), the liver grafts (n = 46), and the transplanted livers (n = 12). *White* and *black bars* indicate the major allele homozygote and minor allele heterozygote or homozygote, respectively. In the transplanted liver, the *white bar* indicates the major allele homozygote in both recipients and donors. The *black bar* indicates cases carrying the major homozygous allele (either the recipients or the donors). The statistical analysis was performed using Student's *t* test. HCV, hepatitis C virus; OLT, orthotopic liver transplantation.

CLINICAL-LIVER, PANCREAS, AND BILIARY TRACT

Association Between Genetic Variations in IL28B and Mutations in HCV-RNA

Recently, we demonstrated the significant impact of HCV-RNA mutations in the Core and NS5A regions on IFN sensitivity after OLT.[14] Therefore, we next examined the association between genetic variations of IL28B in resected liver tissue and mutations in HCV-RNA on the IFN sensitivity after OLT. The mutation rates in amino acid residues 70 and 91 in the Core region were 48% (25 of 52) and 35% (18 of 52), respectively, and in the non-DW, carrying mutation in either positions, ratio was 44% (23 of 52). The number of mutations in the ISDR and IRRDR of NS5A were 1.56 ± 1.83 (range, 0−7) and 5.00 ± 2.74 (range, 1−13), respectively. The minor allele positive ratio at rs8099917 in the non-DW group was significantly higher than that in the DW group (41% vs 13%; P = .025; Figure 4*A*). On the other hand, minor allele−positive ratios in the ISDR < 2 and IRRDR < 6 groups were comparable with those in the ISDR ≥ 2 and IRRDR ≥ 6 groups (31% vs 25% and 33% vs 19%, respectively; P = NS, Figure 4*B* and *C*). These results suggest that viral mutations in the Core but not in the NS5A region are associated with IL28B genetic variation.

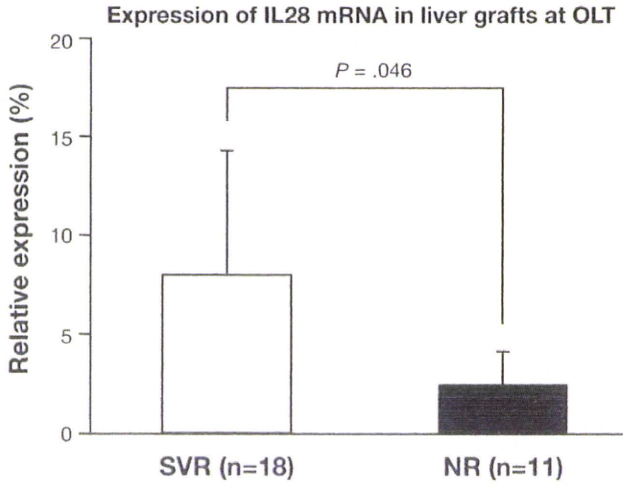

Figure 3. Expression of IL28 messenger RNA in the transplanted livers in recipients with sustained viral response (*SVR*) (*white*, n = 18) and nonresponse (*NR*) (*black*, n = 11). The statistical analysis was performed using Student's *t* test. OLT, orthotopic liver transplantation.

Combined Genetic Analysis of the IL28B Gene and HCV-RNA in the Prediction of IFN Sensitivity After OLT

Although the sensitivity and specificity of using IL28B genetic variations to predict the achievement of SVR were 62% to 87% in the chronic hepatitis C patients, the specificity of the genetic variation for predicting SVR was lower in recipients after OLT (Table 2).[16,18,19] Therefore, we assessed the impact of HCV-RNA mutations on

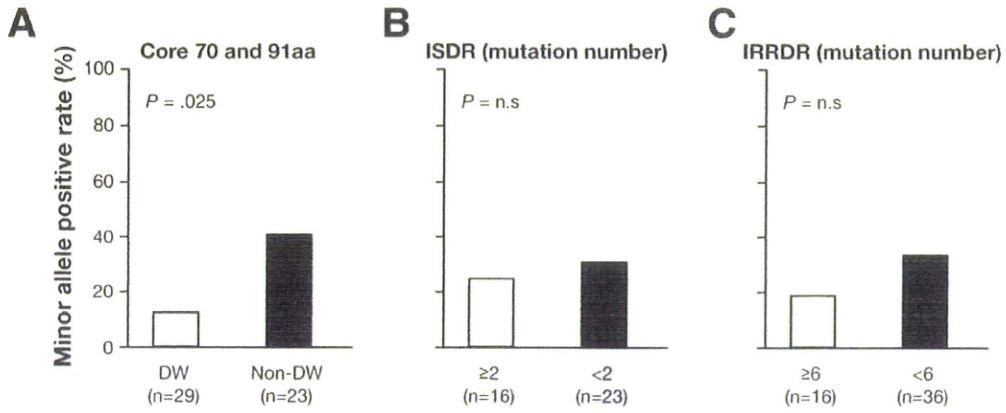

Figure 4. Association of genetic variations of IL28B in rs8099917 and mutations of hepatitis C virus (HCV)-RNA in the resected HCV-infected liver. (*A*) Comparison of the minor allele-positive rate in rs8099917 between a double-wild (*DW*) group with no mutations in amino acids 70 and 91 in the Core region (*white bar*) and a non-DW bearing 1 or more such mutations (*black bar*). (*B*) Comparison between the number of mutations of the interferon sensitivity-determining region (*ISDR*) ≥2 group (*white bar*) and <2 group (*black bar*). (*C*) Comparison between the number of mutations in the interferon-ribavirin resistance-determining region (*IRRDR*) ≥6 group (*white bar*) and <6 group (*black bar*).

the achievement of SVR in the recipients carrying the major allele homozygote and in those receiving transplantation from donors carrying the major allele homozygote. In our previous report, IFN sensitivity was scored according to the viral factors that participate in the IFN sensitivity.[14] Briefly, the patients were divided into 4 groups based on the number of positive factors, including the DW in the Core region, ISDR ≥ 2 and IRRDR ≥ 6 in the NS5A region, and this positive number was used as a prediction score. In the recipients carrying the major allele homozygote, the percentage of patients achieving SVR based on a prediction score of 0 (n = 8) was 13%, and those achieving SVR based on a score of 1 (n = 14), 2 (n = 7), or 3 (n = 4) were 43%, 86%, and 100%, respectively (P = .006; Figure 5*A*). In the donors carrying the major allele homozygote, the percentage of those achieving SVR based on a score of 0 (n = 9) was 11%, and those achieving SVR based on a score of 1 (n = 6), 2 (n = 4), or 3 (n = 2) were 33%, 75%, and 100%, respectively (P = .037; Figure 5*B*). These results suggest that combined genetic analysis of IL28B in both recipients and donors and of the Core and NS5A regions in HCV-RNA has the potential to predict SVR to PEG-IFN/RBV therapy after OLT.

Table 2. Comparison of Sensitivity and Specificity for SVR Between the Current Analysis (After Liver Transplantation) and Previous Analysis (Before Liver Transplantation)

	Sensitivity (%)	Specificity (%)
Current analysis (after Liver Transplantation)		
rs8099917 (Recipient DNA)	91	45
rs8099917 (Donor DNA)	91	45
Core aa70, 91 (Double Wild)	57	74
ISDR mutation number ≥2	64	71
IRRDR mutation number ≥6	75	78
Combined analysis (rs8099917 and HCV-RNA)	83	82
Previous analysis (before Liver Transplantation)		
rs8099917 (Tanaka et al 2009)	81	87
rs12979860 (McCarthy et al 2010)	63	76
rs12979860 (Montes-Cano et al 2010)	67	62

HCV, hepatitis C virus; IRRDR; interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; SVR, sustained viral response.

Discussion

In this study, we demonstrated that genetic variations in IL28B of both recipients and donors were significantly associated with IFN sensitivity, including SVR and ETR after OLT. These genetic variations were significantly associated with IL28 mRNA expression in both the resected liver derived from the recipients and in the donated liver. In addition, the current study revealed that HCV-RNA mutations in the Core but not in the NS5A region were significantly associated with IL28B genetic variations. Furthermore, the combined genetic analysis of IL28B and HCV-RNA was useful to predict the response to PEG-IFN/RBV therapy in patients with recurrent HCV infection after OLT.

The predictive factors for IFN sensitivity have been investigated extensively and several viral and host factors have been identified. Among the viral factors identified, the viral genotype is the most important and well-established predictive factor determining IFN sensitivity.[23] The SVR rate in patients with genotype 1 has been reported to be low (40%−50%), while that in patients with genotypes 2 and 3 has been reported to be high (70%−80%).[24] In addition, many reports have shown that mutations in the Core and NS5A regions are useful for predicting the response to IFN therapy.[11–13,24–26]

Several host factors have also been reported to be associated with the efficacy of IFN-centered antiviral therapy. The increased expression of ISGs at baseline

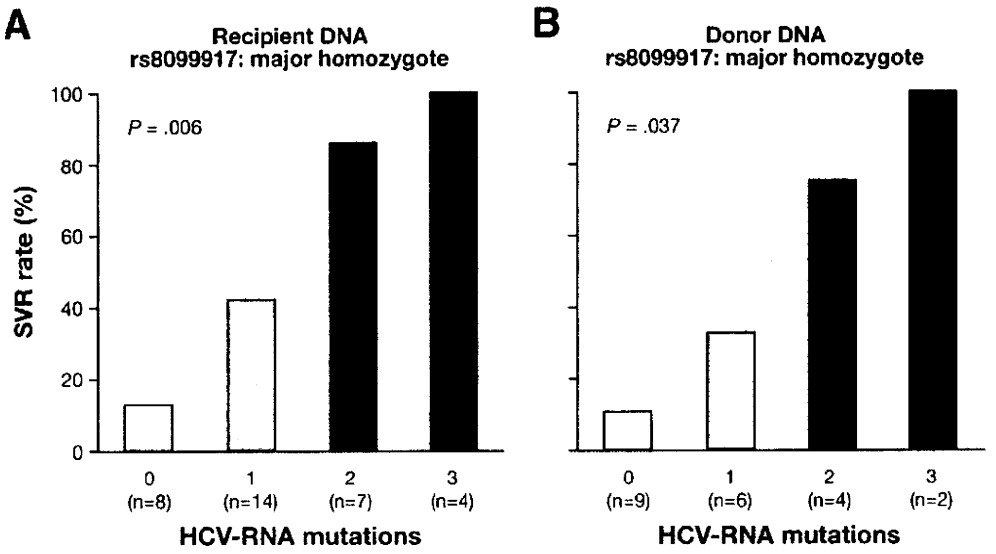

Figure 5. Combined genetic analyses of hepatitis C virus (HCV)-RNA mutations in the Core and nonstructural protein 5A regions for the prediction of sustained viral response (*SVR*) in the recipients carrying the major allele homozygote who received liver transplants from donors carrying the same allele. The interferon sensitivity score was calculated as the total number of cases positive for the following viral factors: double-wild (*DW*) at aa70 and 91, interferon sensitivity-determining region (*ISDR*) ≥2, and interferon/ribavirin resistance-determining region (*IRRDR*) ≥6. The achievement percentage of SVR among the 4 groups is indicated.

in patients predicts that they will be NR to IFN therapy,[15,27,28] and a significant association between the presence of genetic variations in IL28B and the response to PEG-IFN/RBV therapy has been reported in genome-wide association studies.[16-21] In these studies, the expression level of IL28 mRNA in the PBMCs of patients carrying the minor heterozygous or homozygous allele was significantly lower.[18,19] Other studies have shown that IL28B transduces signals through the receptor complexes in a manner different from other type I IFNs, but uses the common Janus activating kinase-signal transducer and activation of transcription pathway to induce ISGs.[29,30] The discrepancies between the impaired transcription of IL28B due to genetic variations and the increased expression of ISGs suggest the participation of other factors in determining the efficacy of IFN therapy.

Although IFN-centered antiviral therapy is significantly associated with post-transplantation graft prognosis in patients infected with HCV,[7] the efficacy of the IFN therapy after OLT is unsatisfactory[8] and the treatment is frequently accompanied by severe side effects.[9] Therefore, in addition to the development of an optimal therapeutic regimen for HCV infection after OLT, establishment of a reliable marker or set of markers to predict the sensitivity to IFN therapy is needed. We have previously reported that viral RNA mutations in the Core and NS5A regions are significantly associated with IFN sensitivity after OLT.[14] In addition, the current study revealed that IL28B genetic variation in both recipients and donors is also associated with IFN sensitivity after OLT. Although the sensitivity and specificity of genetic variations of IL28B for predicting the achievement of SVR have been reported to be high in chronic hepatitis C patients,[16,18,19] the current analysis revealed that the specificity was lower in cases of recurrent hepatitis C after OLT than in chronic hepatitis C patients. By using a combination of genetic analyses, the efficacy of the post-transplantation PEG-IFN/RBV therapy might be predicted before OLT. Large-scale prospective analyses of the association between IFN sensitivity after OLT and genetic variations in both IL28B and HCV will be needed in future studies. In addition, the molecular mechanism underlying the association between IFN sensitivity and genetic variation of IL28B and HCV should be clarified. Furthermore, it might be feasible to predict the IFN sensitivity based on the genetic analyses of viral and host factors, thereby allowing for the individualization of antiviral therapy, including dose-escalated IFN therapy,[31,32] simultaneous splenectomy for pancytopenia,[32,33] and the use of new antivirals such as proteases inhibitors.[34] Further clinical investigation is needed to improve the post-transplantation antiviral therapy for recurrent HCV after OLT.

Previous reports have demonstrated an association between the clinical tolerance of the graft and tissue chimerism, including hepatocytes.[35-37] In the present study, to determine the correlation between the tissue chimerism in the transplanted liver and the impact of IL28B genetic variation in both the recipients and donors, short tandem repeat analysis and evaluation of minor allele frequency in the transplanted liver were performed (Supplementary Figure 2). Although liver tissue chimerism after OLT was demonstrated using short tandem repeat analysis and cloning in this study, we could not deter-

mine the cell types present and whether the cells were made up of hepatocytes, endothelial cells, or infiltrating lymphocytes due to the limited sample volumes. However, the coexistence of cells derived from donors and recipients in the local environment supports the notion that genetic variation of IL28B in both donors and recipients participates in the determination of IFN sensitivity after OLT.

In conclusion, the combination of genetic analysis of IL28B in both the recipient and donor, rather than either alone, together with HCV-RNA may be a reliable predictor of IFN efficacy in patients with recurrent hepatitis C after OLT. In addition, this analysis may also make it possible to select the optimal donors exhibiting high sensitivity to the IFN therapy after OLT.

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.2010.07.058.

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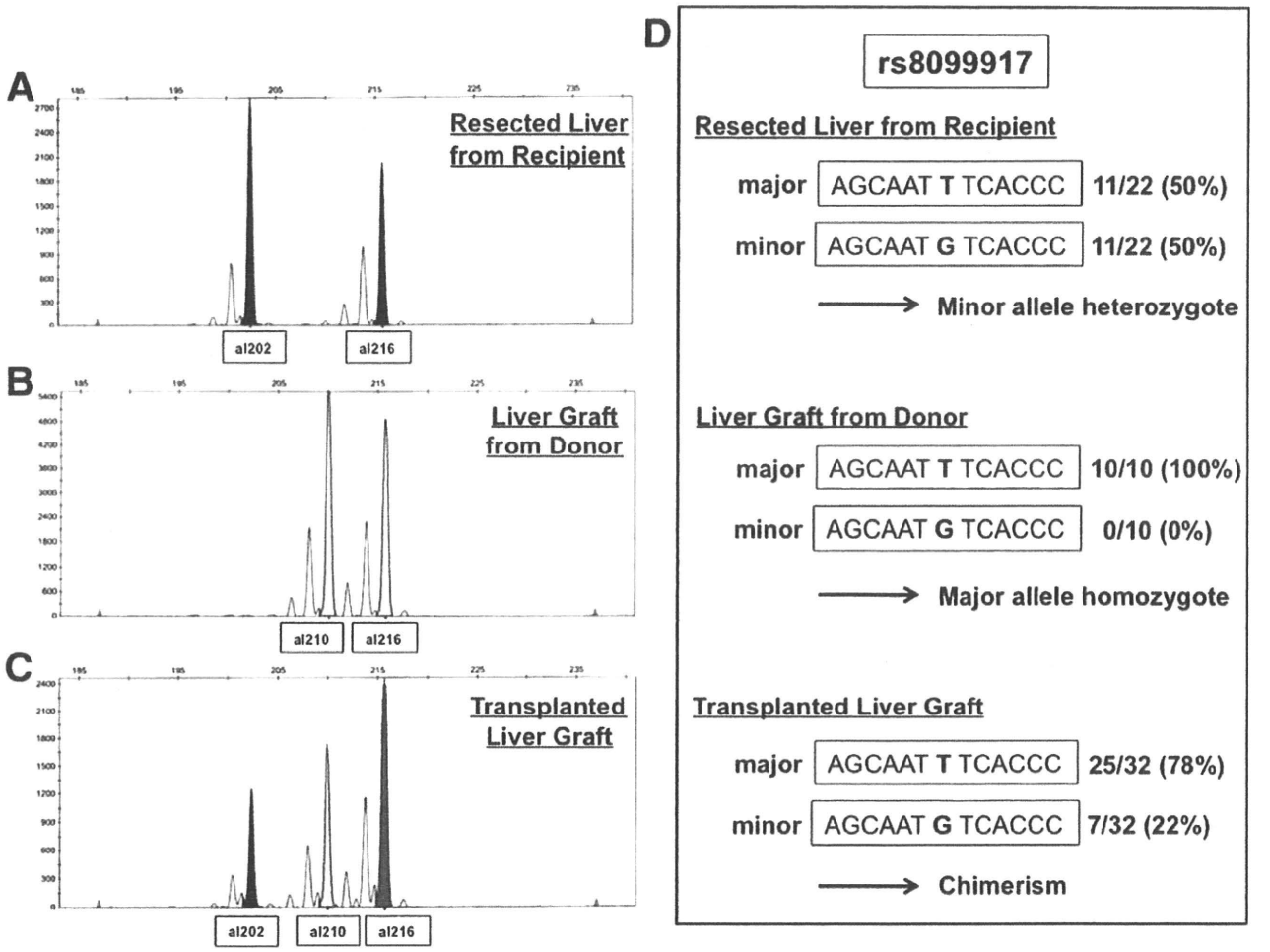
Conflicts of interest

The authors disclose no conflicts.

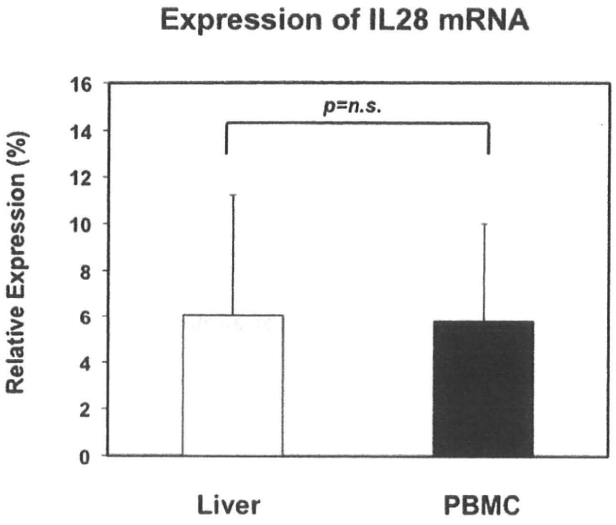
Supplementary Table 1. The Match Rate of Genetic Variation Between rs8099917 and rs12980275

	rs8099917		
	SNP(+), n (%)	SNP(−), n (%)	n (%)
rs12980275			
SNP(+)	26 (24)	3 (2.8)	29 (27)
SNP(−)	4 (3.7)	74 (69)	78 (73)
	30 (28)	77 (72)	108

Concordance rate: 93.5%.



Supplementary Figure 2. To determine the tissue chimerism following orthotopic liver transplantation (OLT), genotyping by short tandem repeat analysis (microsatellite analysis) of DNA samples extracted from the resected liver derived from the recipient, donated liver derived from the donor, and biopsied liver after OLT were performed in 15 cases. Five loci in 5 chromosomes were examined for each sample (D2S123, D5S107, D10S197, D11S904, and D13S175). Polymerase chain reaction products for the loci were analyzed with an Applied Biosystems 3130 genetic analyzer using Genemapper software (Applied Biosystems). To analyze the minor allele-positive rate in the transplanted liver after OLT in the case of the combination of minor allele heterozygotes in the recipient and the major allele homozygote in donor, cloning and sequencing of rs8099917 was performed. Extraction of DNA was performed on the resected liver derived from the recipient, donated liver derived from the donor, and biopsied liver after OLT. The products were cloned by the pT7Blue T-Vector (Takara Bio Inc). Short tandem repeat analyses (microsatellite analyses) at the D2S204 locus of the resected livers from recipients (*A*), the liver grafts from donors (*B*), and the transplanted livers (*C*). The *black* and *gray peaks* correspond to the alleles of recipients and donors, respectively. (*D*) A case of OLT for a recipient who carried the minor allele heterozygote who received the transplant from a donor who had the major allele homozygote.



Supplementary Figure 1. Expression of IL28B messenger RNA (*mRNA*) in livers (*white*) and the peripheral blood mononuclear cells (*PBMC*) (*black*). The statistical analysis was performed using Student's *t* test.

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Peripheral B Cells May Serve as a Reservoir for Persistent Hepatitis C Virus Infection

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Key Words
Hepatitis C virus · B cells · Retinoic acid-inducible gene-I · Interferon promoter-stimulator-1 · Interferon regulatory factor-3 · Interferon β

Abstract
A recent study by our group indicated that peripheral B cells in chronic hepatitis C (CHC) patients are infected with hepatitis C virus (HCV). This raised the logical question of how HCV circumvents the antiviral immune responses of B cells. Because type I interferon (IFN) plays a critical role in the innate antiviral immune response, IFNβ expression levels in peripheral B cells from CHC patients were analyzed, and these levels were found to be comparable to those in normal B cells, which suggested that HCV infection failed to trigger antiviral immune responses in B cells. Sensing mechanisms for invading viruses in host immune cells involve Toll-like receptor-mediated and retinoic acid-inducible gene-I (RIG-I)-mediated pathways. Both pathways culminate in IFN regulatory factor-3 (IRF-3) translocation into the nucleus for IFNβ gene transcription. Although the expression levels of RIG-I and its adaptor molecule, IFN promoter-stimulator-1, were substantially enhanced in CHC B cells, dimerization and subsequent nuclear translocation of IRF-3 were not detectable. TANK-binding kinase-1 (TBK1) and IκB kinase ε (IKKε) are essential for IRF-3 phosphorylation. Constitutive expression of both kinases was markedly enhanced in CHC B cells. However, reduced expression of heat shock protein of 90 kDa, a TBK1 stabilizer, and enhanced expression of SIKE, an IKKε suppressor, were observed in CHC B cells, which might suppress the kinase activity of TBK1/IKKε for IRF-3 phosphorylation. In addition, the expression of vesicle-associated membrane protein-associated protein-C, a putative inhibitor of HCV replication, was negligible in B cells. These results strongly suggest that HCV utilizes B cells as a reservoir for persistent infection.

Introduction

Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus that belongs to the *Flaviviridae* family [1]. It is responsible for public health problems worldwide and affects nearly 200 million people [2]. The liver is regarded as the primary target of HCV infection; however, HCV infection is also associated with B cell lymphoproliferative disorders such as mixed cryoglobulinemia and B cell non-Hodgkin lymphoma [3, 4]. In fact, epidemiological evidence suggests a close link between chronic HCV infection and B cell non-Hodgkin lymphoma [5,

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6]. A pathogenic role for HCV in B cell disorders has been further demonstrated by reports showing clinical resolution of the above-mentioned B cell disorders after successful anti-HCV treatment with interferons (IFNs) [3, 7]. Based on this circumstantial evidence, a possible role for B cells in HCV pathogenesis has been postulated, although this has not been conclusively demonstrated.

A body of evidence suggests that HCV RNA replication occurs in a variety of extrahepatic cells, including peripheral dendritic cells, monocytes and macrophages [8–10]. It has also been suggested that HCV preferentially infects B cells that express CD81, a putative HCV receptor molecule [11–14]. A recent study by our group verified that peripheral CD19+ B cells in chronic hepatitis C (CHC) patients were infected with HCV, which suggested a new viral reservoir during the course of natural HCV infection in humans [15]. Thus, we assume that HCV has an escape strategy for persistent infection of B cells.

Foy et al. [16] found that nonstructural (NS) HCV proteins could inhibit the activation of early signaling pathways, such as Toll-like receptor 3 (TLR3)- and retinoic acid-inducible gene-I (RIG-I)-mediated pathways, which lead to IFNβ production. These results indicated that HCV NS3/4A serine protease blocked IFN regulatory factor-3 (IRF-3) activation upon HCV infection in the human hepatoma cell line HuH-7. Subsequent studies have shown that NS3/4A blocks IFN promoter-stimulator-1 (IPS-1)-mediated signaling pathways by cleaving the IPS-1 molecule and impeding downstream IRF-3 activation [17]. Thus, HCV apparently has a strategy to evade host innate immunity. However, recent studies by Dansako et al. [18, 19] found that the effects of HCV NS3/4A protease on IFN production depended on the cell lines used, because a non-neoplastic human hepatocyte cell line, PH5CH8 [20], retained both TLR3- and RIG-I-mediated pathways, in contrast to HuH-7 cells, which lack the former pathway [21]. However, no studies have examined the effects of HCV infection on IFN responses of nonhepatic cell lines.

In this study, we aimed to understand the mechanisms by which HCV evades innate immune responses in CHC B cells. We found that the antiviral immune response, represented by IFNβ induction, was severely impaired in B cells of CHC patients. Our results strongly suggest that the IRF-3 activation cascade is impeded in B cells upon HCV infection. Thus, IFNβ gene transcription is not augmented, which may result in failed IFNβ-inducible antiviral responses in CHC B cells. Furthermore, the expression of vesicle-associated membrane protein-associated protein-C (VAP-C), a putative inhibitor of HCV replication, was negligible in B cells. These results support the notion that HCV can successfully reside in B cells, resulting in persistent infection. This is the first study describing analysis of the suppressive effects of HCV infection on antiviral innate immunity in peripheral B cells. Thus, this study offers new insights into the role of B cells in the pathogenesis of HCV.

Methods

Patients and Samples

A total of 24 CHC patients were enrolled in this study, with the following characteristics: 14 males and 10 females; mean age 62.4 ± 7.4 years; mean serum ALT levels 67.5 ± 36.0 IU/l; mean serum AST levels 66.7 ± 34.3 IU/l; 21 patients with HCV genotype 1b and 3 with HCV genotype 2a, and mean HCV RNA 1,752 ± 1,188 KIU/ml. All cases were confirmed to be negative for other viral infections, including hepatitis B virus and human immunodeficiency virus. The study protocols were approved by the Review Board of the National Institute of Infectious Diseases. All donors gave written informed consent. Controls were healthy blood donors at the Tokyo Red Cross Blood Center (Tokyo, Japan) who were confirmed to be negative for HCV, hepatitis B virus and human immunodeficiency virus.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia Biotech, Quebec, Que., Canada) density gradient centrifugation. CD19+ B lymphocytes were isolated from PBMCs by negative selection (B Cell Isolation Kit II, human; Miltenyi, Auburn, Calif., USA). The purity of isolated B cells was generally >95%, as assessed by flow cytometry.

Semiquantitative Real-Time PCR

Total RNA was extracted from lymphoid cells using Isogen (Nippon Gene Co. Ltd., Tokyo, Japan). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif., USA) with oligo(dT)12–18 primer (Invitrogen). PCR amplification was performed using SYBR Premix Ex Taq™ II (Takara Shuzo, Kyoto, Japan) with gene-specific primers (Bex Co. Ltd., Tokyo, Japan) available in the public database RTPrimerDB [22] under the codes 3542 for IFNβ and 3539 for GAPDH, and the Universal Probe Library Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp; Roche Applied Science) as follows: IPS-1 (No. 19, 04686926001), TIR domain-containing adaptor inducing IFN (TRIF; No. 37, 04687957001), suppressor of IκB kinase ε (IKKε) (SIKE; No. 56, 04688538001), heat-shock protein of 90 kDa (Hsp90; No. 25, 04686993001) and DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked (DDX3X; No. 69, 04688686001). The primer sequences for RIG-I were 5′-GTG CAA AGC CTT GGC ATG T-3′ (forward) and 5′-TGG CTT GGG ATG TGG TCT ACT C-3′ (reverse) [23], and for TLR3 they were 5′-GTT ACG AAG AGG CTG GAA TGG T-3′ (forward) and 5′-GCC AGG AAT GGA GAG GTC TAG A-3′ (reverse) [24].

Real-time PCR was carried out for 45 cycles at 94°C for 1 min and at 60°C for 25 s (two-step PCR) using a Light Cycler (Roche Diagnostics, Basel, Switzerland). Amplification of predicted fragments was confirmed by melt curve analysis and gel electropho-

resis. Standard curves were established with 10-fold serial dilutions of amplified products. Measured amounts of each mRNA were normalized to GAPDH mRNA. mRNA expression levels in normal B cells were arbitrarily defined as 1.0.

Immunoblot Analysis

To extract whole-cell proteins, cell pellets were suspended in modified RIPA buffer [150 mM NaCl, 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, 1.0% NP-40, 0.5% sodium deoxycholic acid and 0.1% SDS] containing Halt protease inhibitor cocktail (Pierce, Rockford, Ill., USA) and Halt phosphatase inhibitor cocktail (Pierce; 2×10^7 cells/ml). After 20 min of incubation on ice, cell extracts were centrifuged at 12,000 *g* for 10 min at 4°C, transferred to other tubes and stored at –80°C. Nuclear and cytoplasmic proteins were separated using a Nuclear Extraction Kit (Active Motif, Carlsbad, Calif., USA) according to the manufacturer's protocol. Protein concentration was determined using the BCA™ Protein Assay Kit – Reducing Agent Compatible (Pierce). Samples (whole-cell extract, 1 g; fractionated extract, 2×10^5 cells) were loaded onto 7.5 or 12.5% SDS acrylamide gels (Real Gel Plate; Bio Craft, Tokyo, Japan), followed by transfer to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature using Block Ace™ (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan). They were then sequentially probed with primary and secondary antibodies at 4°C overnight and for 1 h at room temperature, respectively.

For primary antibodies, we used anti-IFN rabbit polyclonal antibody (ab9662, 1/1,000 dilution; Abcam Inc., Cambridge, Mass., USA), anti-ACTB (β-actin) rabbit polyclonal antibody (4967, 1/1,000 dilution; Cell Signaling Technology, Danvers, Mass., USA), anti-TLR3 rabbit polyclonal antibody (ab62566, 1/1,000 dilution; Abcam), anti-TRIF rabbit polyclonal antibody (4596, 1/1,000 dilution; Cell Signaling Technology), anti-RIG-I rabbit polyclonal antibody (29010, 1/100 dilution; Immuno-Biological Laboratories Co. Ltd., Gunma, Japan), anti-IPS-1 rabbit polyclonal antibody (AT107, 1/2,000 dilution; Alexis Biochemicals, Farmingdale, N.Y., USA), anti-IRF-3 rabbit polyclonal antibody (18781, 1/100 dilution; Immuno-Biological Laboratories), anti-GAPDH mouse monoclonal antibody [5G4(6C5), 1/9,000 dilution; HyTest Ltd., Turku, Finland], anti-PARP-1 mouse monoclonal antibody (AM30, 1/500 dilution; Calbiochem, San Diego, Calif., USA), anti-TANK-binding kinase-1 (TBK1) rabbit polyclonal antibody (3504, 1/1,000 dilution; Cell Signaling Technology) and anti-IKKε rabbit polyclonal antibody (ab7891, 1/500 dilution; Abcam). Anti-VAP-C rabbit polyclonal antibody (2.66 g/ml) was produced by a group of the authors (H.K., K. Moriishi and Y.M.).

The secondary antibodies used were horseradish peroxidase-coupled donkey anti-rabbit Ig (NA934, 1/10,000 dilution; GE Healthcare Ltd.; UK, Buckinghamshire, UK) and horseradish peroxidase-coupled sheep anti-mouse Ig (NA931, 1/10,000 dilution; GE Healthcare UK). Protein bands were detected using ECL Plus™ Western Blotting Detection Reagents (GE Healthcare UK) and a LAS-3000 Image Analyzer (Fuji Film, Tokyo, Japan). Densitometric analysis was performed within a linear range using Image Gauge (Fuji Film). The density of each band (the amount of protein) was normalized against that of the corresponding β-actin.

Native PAGE for IRF-3 Dimer Detection

Native PAGE was performed using 7.5% SDS acrylamide gels (Real Gel Plate; Bio Craft). Gels were prerun with 25 mM Tris-Cl (pH 8.4) and 192 mM glycine with and without 0.2% deoxycholate in the cathode and anode chambers, respectively, for 30 min at 40 mA. Samples were extracted in lysis buffer (3×10^7 cells/ml; 50 mM Tris-Cl, pH 8.0, 1% NP40, 150 mM NaCl) containing Halt protease inhibitor cocktail and Halt phosphatase inhibitor cocktail, mixed with equal volumes of Tris-glycine native sample buffer (2×; Invitrogen), applied to the gel and electrophoresed for 60 min at 25 mA.

Immunoblotting was performed as described above. As a positive control for IRF-3 dimerization, HeLa cells were added with 100 g/ml polyriboinosinic-polyribocytidylic acid (poly I:C; kindly provided by Toray Co. Ltd., Tokyo, Japan). Three hours after incubation, cells were harvested and cell lysates were prepared as described above.

Poly I:C Transfection

CD19+ B lymphocytes isolated from PBMCs were cultured in RPMI-1640 medium containing 10% FCS, 2 mM L-glutamine, 1 mM HEPES, 0.05 mM β-mercaptoethanol, penicillin and streptomycin in a flat-bottom 96-well plate for 3 h (2.5×10^6 cells/well). To activate the RIG-I-mediated pathway, cells were transfected with 10 μg/ml poly I:C using Poly(I:C)/LyoVec (Invivogen, San Diego, Calif., USA). After 18 h of culture, transfected or non-transfected cells were dissolved in Isogen (Nippon Gene) for semi-quantitative real-time PCR assay. Three independent triplicate transfection experiments were performed in order to verify the reproducibility of the results.

Statistics

Unpaired (two-tailed) Student's t tests were applied at the 95% confidence level ($p < 0.05$) using Prism (version 4; GraphPad Software Inc., San Diego, Calif., USA) in all cases.

Results

Impaired IFN Responses in Peripheral B Cells of CHC Patients

We recently demonstrated that HCV infected and may have replicated in peripheral B cells of CHC patients [15]. This implied that HCV may have evaded the immune response by B cells, resulting in persistent infection. Because the induction of type I IFNs, including IFNβ, is crucial for host defense against invading viruses, we first examined constitutive IFNβ expression levels in peripheral B cells of CHC patients. As shown in figure 1a, IFNβ mRNA expression levels were not augmented in CHC B cells compared with normal B cells. The results of Western blotting analysis (fig. 1b) indicated that constitutive IFNβ protein expression levels were not enhanced in B cells of CHC patients, which supported the finding of unaltered IFNβ mRNA expression.

We then stimulated normal and CHC B cells using poly I:C transfection, which triggers RIG-I- and melanoma differentiation-associated gene-5-mediated IFNβ path-

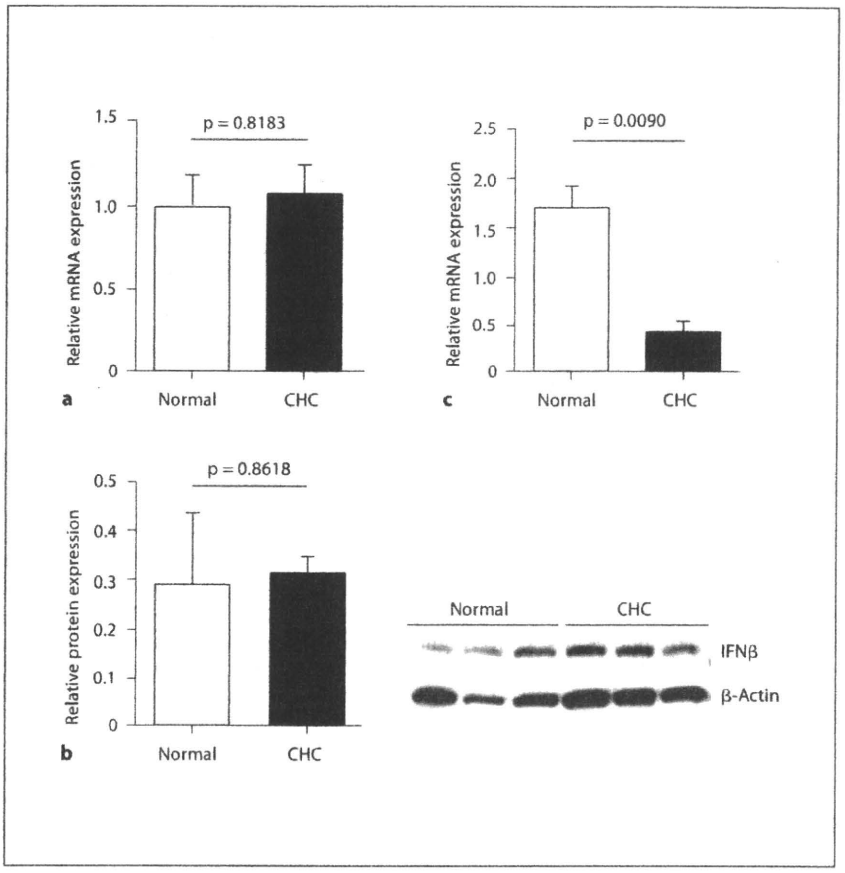

Fig. 1. IFNβ expression in CHC B cells. Fractionation of CD19+ B cells from PBMCs was performed as described in Methods. **a** IFNβ mRNA expression levels in CD19+ B cells isolated from normal individuals (n = 4) and CHC patients (n = 7) were measured in duplicate by quantitative real-time RT-PCR and normalized against those of the housekeeping gene GAPDH. mRNA expression levels in normal B cells were arbitrarily defined as 1.0. **b** Whole-cell extracts prepared from CD19+ B cells isolated from normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IFNβ and anti-ACTB antibodies. Relative IFNβ protein expression levels normalized against β-actin expression are shown. **c** CD19+ B cells isolated from normal individuals (n = 3) or CHC patients (n = 3) were transfected with poly I:C (10 g/ml). Eighteen hours after transfection, cells were harvested and total RNA was isolated. IFNβ mRNA expression levels were measured in duplicate using quantitative real-time RT-PCR and normalized against those of the housekeeping gene GAPDH. mRNA expression levels in untransfected normal or CHC B cells were arbitrarily defined as 1.0. Representative results from at least 2 independent experiments with similar results are shown.

ways. As shown in figure 1c, IFNβ mRNA expression levels in CHC B cells were much lower than those in normal B cells, suggesting that CHC B cells are defective with regard to IFNβ production upon stimulation with the intracellular delivery of poly I:C. In addition, the expression levels of IFN-stimulated genes, such as ISG-15 and ISG-56, in CHC B cells were also much lower than those in normal B cells upon poly I:C stimulation (data not shown).

Taken together, these results indicate that chronic HCV infection fails to induce an IFNβ response in CHC B cells. Subsequent experiments were designed to elucidate the underlying mechanism(s) by which HCV interrupted the IFN responses in CHC B cells.

Expression Levels of HCV Sensor Molecules in Peripheral B Cells of CHC Patients

We next examined the gene expression levels in peripheral B cells of two major viral sensors, TLR3 and RIG-I, as well as their corresponding adaptor molecules, TRIF and IPS-1, which are indispensable for initiating innate immune responses [25]. As shown in figure 2, TLR3, TRIF, RIG-I and IPS-1 expression levels were significantly enhanced in peripheral B cells of CHC patients. Expression of another cytoplasmic sensor molecule, melanoma differentiation-associated gene-5, was also enhanced (data not shown). These results demonstrate that the expression levels of cytoplasmic virus sensors as well as their adaptors are constitutively augmented in CHC B cells.

Expression, Dimerization and Nuclear Translocation of IRF-3 in CHC B Cells

The IRF-3 activation cascade, including phosphorylation, dimerization and nuclear translocation, is essential for IFNβ gene transcription [26]. We found that constitutive IRF-3 expression levels in CHC B cells were significantly lower than those in normal B cells ($p = 0.0018$) as assessed by Western blotting (fig. 3a). Furthermore, IRF-

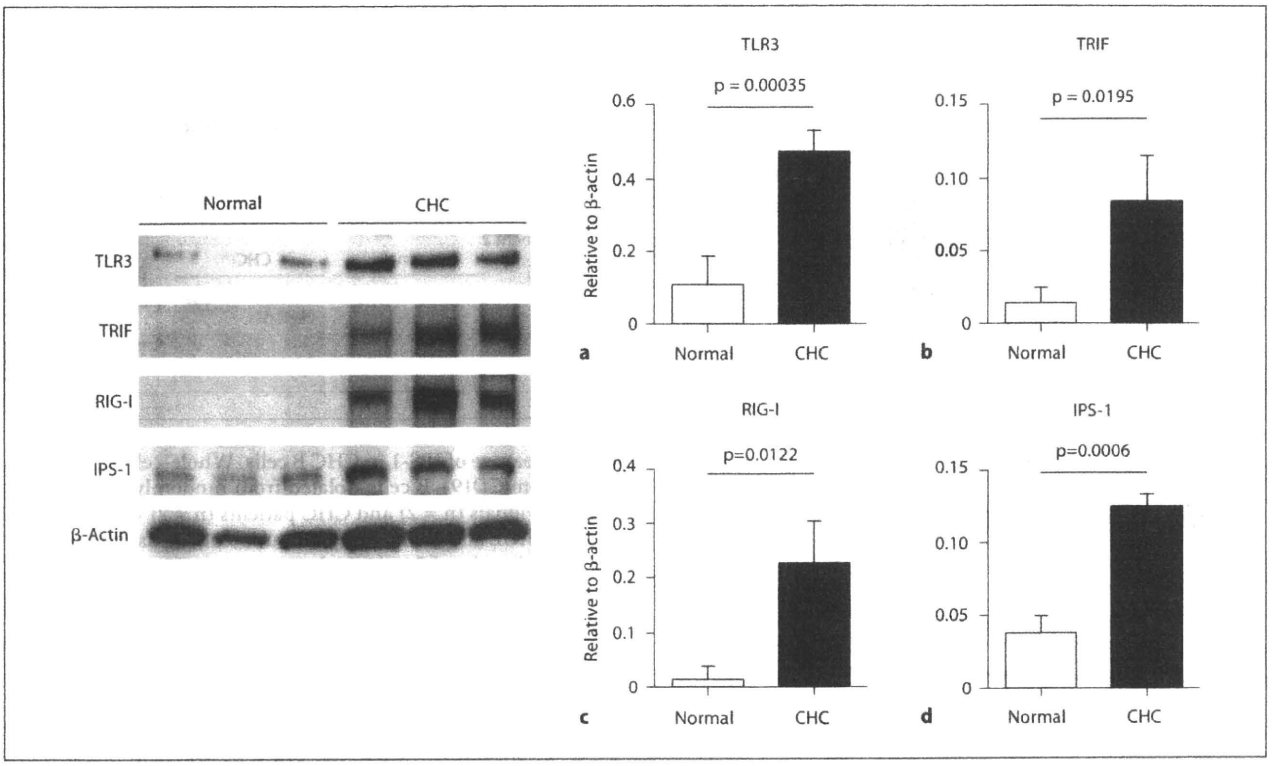

Fig. 2. Expression levels of HCV sensor and adaptor molecules in CHC B cells. Whole-cell extracts prepared from CD19+ B cells isolated from normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-TLR3, anti-TRIF, anti-RIG-I, anti-IPS-1 and control anti-ACTB antibodies. Relative protein expression levels normalized against β-actin expression are shown.

3 dimerization assessed by native PAGE was not observed in CHC B cells (fig. 3b). Consequently, IRF-3 nuclear translocation did not occur in CHC B cells (fig. 3c). Thus, these results indicate that the IRF-3 activation cascade does not proceed in CHC B cells, which may explain the lack of IFNβ responses to HCV infection.

Cleavage of IPS-1 in CHC B Cells

Several lines of evidence have indicated an essential role for the adaptor molecule IPS-1, also called Cardif, MAVS or VISA, which acts downstream of RIG-I in the IRF-3 signaling pathway [27]. Recent studies have confirmed that HCV can cleave IPS-1 via its NS3/4A protease activity [28]. As a result of this proteolytic cleavage, IPS-1 is dislodged from the mitochondria and becomes an inactive cytosolic fragment. This causes the failure of downstream signaling for IRF-3 activation. Therefore, we examined IPS-1 cleavage in CHC B cells by native PAGE. As shown in figure 4, IPS-1 cleavage was incomplete, and a substantial amount of uncleaved (intact) IPS-1 was detected. Thus, we concluded that impaired IRF-3 activation cannot be solely explained by IPS-1 cleavage in CHC B cells. These results suggest either that the NS3/4A protease is not expressed in CHC B cells or that IPS-1 in B cells is resistant to this protease. Because NS3 molecules were detected in CHC B cells by Western blotting [15], the latter seems to be more likely.

Stabilization or Inhibition of TBK1/IKKε Kinase Activity in CHC B Cells

Two protein kinases, TBK1 and IKKε, which are both located downstream of IPS-1, are essential for IFNβ production via IRF-3 phosphorylation [29]. Interestingly, the constitutive expression levels of both kinases were markedly enhanced in CHC B cells compared with those in normal B cells (fig. 5), although the downstream IRF-3 activation cascade was severely impaired, as shown in figure 3.

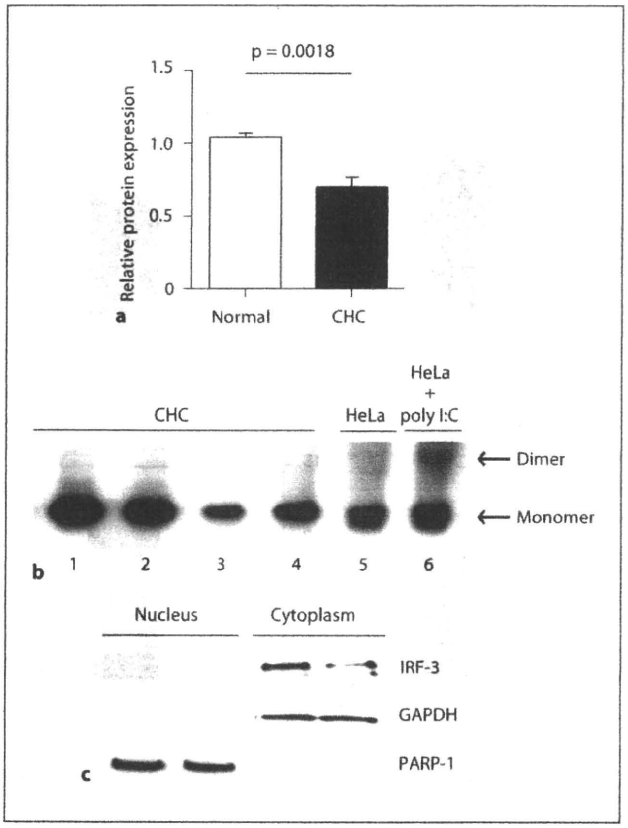

Fig. 3. Expression, dimerization and nuclear translocation of IRF-3 in CHC B cells. **a** Whole-cell extracts prepared from CD19+ B cells isolated from randomly selected normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IRF-3 and anti-ACTB antibodies. Relative protein expression levels of IRF-3 normalized against β-actin expression are shown. **b** Dimerization of IRF-3 in CD19+ B cells isolated from CHC patients (n = 4) were analyzed by native PAGE as described in Methods. As a positive control to detect IRF-3 dimer formation, cell extracts from HeLa cells stimulated with poly I:C were applied (lane 6). **c** Nuclear and cytoplasmic proteins were isolated from CD19+ B cells of CHC patients (n = 2) as described in Methods. Samples were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IRF-3, anti-GAPDH (cytoplasmic marker protein) and anti-PARP-1 (nuclear marker protein) antibodies.

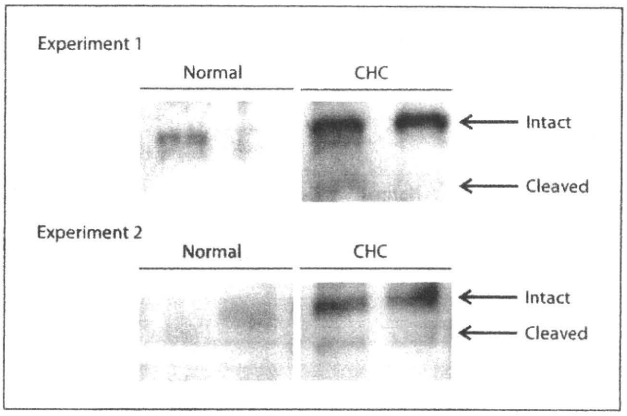

Fig. 4. Cleavage of IPS-1 in CHC B cells. Whole-cell extracts prepared from CD19+ B cells isolated from randomly selected normal individuals (n = 2) and CHC patients (n = 2) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IPS-1 antibody. Results of 2 independent experiments are shown.

Huang et al. [30] identified a protein called SIKE that interacts with IKKε and TBK1 and has an inhibitory effect on the IRF-3 activation pathway. Yang et al. [31] demonstrated that Hsp90 was important for stabilizing TBK1 and promoting IRF-3 phosphorylation by TBK1 in response to viral infection. The gene expression levels of these two molecules in B cells were analyzed by real-time PCR, as shown in figure 6a. SIKE expression levels were significantly enhanced in CHC B cells ($p = 0.0059$), while those of Hsp90 were significantly reduced ($p = 0.001$). These results strongly suggest that the kinase activities of TBK1 and IKKε are downregulated in CHC B cells, which may be responsible for the failure in IRF-3 activation and subsequent IFNβ transcription.

The DEAD box helicase DDX3X [32] is a critical component of TBK1-dependent type I IFN induction [33, 34]. As shown in figure 6a, DDX3X expression levels were significantly reduced in CHC B cells ($p = 0.0043$). This could be just a concomitant observation; however, this result is of interest assuming that HCV has an additional mechanism by which it interferes with IRF-3 activation.

VAP-C Expression in B Cells

Human VAP subtype A (VAP-A) and subtype B (VAP-B) are essential host factors for HCV replication because they bind to both NS5A and NS5B [35]. VAP-C is a splicing variant of VAP-B that lacks two thirds of the C terminus [36, 37]; therefore, it cannot interact with VAP-A, VAP-B or NS5A. A physiological role of VAP-C was recently demonstrated by Kukihara et al. [38], who found that VAP-C inhibited the association between VAP-A/B and NS5B, thereby reducing HCV replication efficiency. Interestingly, VAP-C expression in hepatocytes was found to be negligible, which may be advantageous for

HCV replication in the liver [38]. These results prompted us to examine VAP-C expression in B cells. As shown in figure 6b, VAP-C was expressed in CD19– cells (i.e. non-B cells), but not in CD19+ B cells. Together with the defect in antiviral immune responses of CHC B cells described above, this observation further supports the notion that HCV utilizes B cells as a reservoir for persistent infection.

Discussion

HCV infection of hepatocytes has long been an implicit assumption. However, this does not necessarily imply that hepatocytes are the exclusive target for HCV infection. HCV may seek other cellular compartments as reservoirs in the event that the liver becomes unsuitable for replication, possibly due to cellular destruction caused by the host immune response and/or the development of conditions such as cirrhosis and hepatocellular carcinoma. Our previous study suggested the possibility that HCV persistently infects peripheral B cells [15]. Consequently, we were extremely interested in how HCV is able to escape host innate immunity and persistently remain in peripheral B cells. The results of this study may provide plausible answers to these questions.

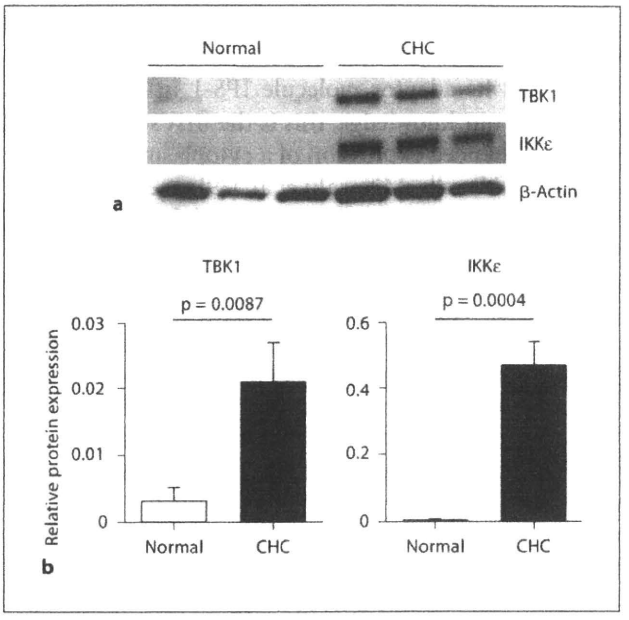

Fig. 5. Expression of TBK1 and IKKε in CD19+ B cells. **a** Whole-cell extracts prepared from CD19+ B cells isolated from normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-TBK1, anti-IKKε and anti-ACTB antibodies. **b** Relative protein expression levels of each protein normalized against β-actin expression are shown.

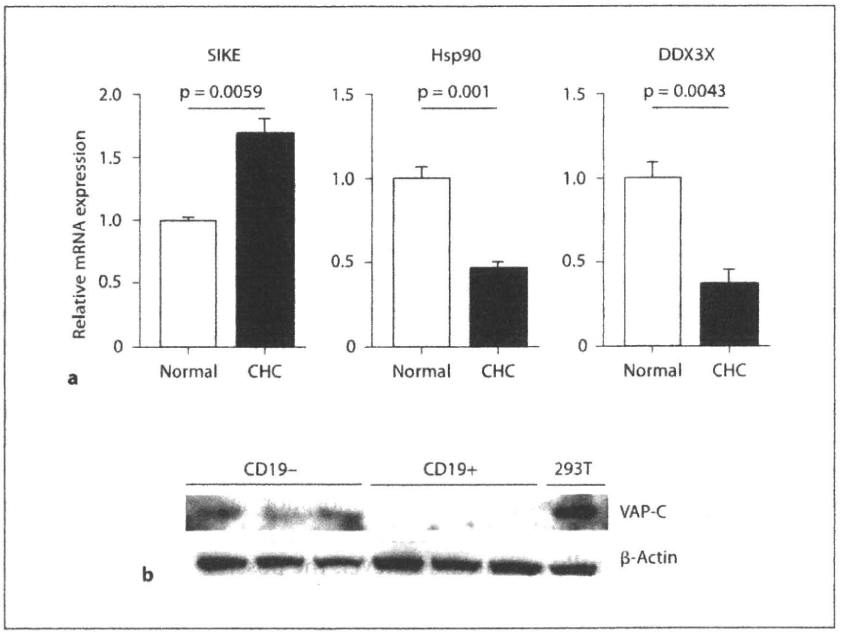

Fig. 6. Expression of SIKE, Hsp90, DDX3X and VAP-C in CD19+ B cells. **a** mRNA expression levels of SIKE, Hsp90 and DDX3X in CD19+ B cells isolated from normal individuals (n = 3–7) and CHC patients (n = 3–5) were measured in duplicate by quantitative real-time RT-PCR and normalized against those of the housekeeping gene GAPDH. mRNA expression levels in normal B cells were arbitrarily defined as 1.0. **b** Whole-cell extracts prepared from CD19– and CD19+ B cells isolated from normal individuals (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-VAP-C antibody. Whole-cell extracts from 293T cells were used as a positive control.