Table I Patients' Characteristics

Number of patients	57
Sex (male/female)	34/23
Age, y, mean ± SD (range)	47.4 ± 14.7 (19-67)
Type of liver transplant, n	
Deceased donor	14
Living donor	43
Primary diagnosis	
Hepatitis C virus cirrhosis	22
Hepatitis B virus cirrhosis	8
Primary biliary cirrhosis	8
Primary sclerosing cholangitis	3
Alcohol cirrhosis	3
Wilson disease	2
Biliary atresia	3
Budd-Chiari syndrome	1
Citrullinemia	1
Cryptogenic	2
Fulminant hepatic failure	4
Months posttransplantation, mean ± SD (range)	41.8 ± 26.5 (0-92.4)
New transplantation, n (%)	3 (5.3)
Within 12 months posttransplantation, n (%)	9 (15.8)
12 to 36 months posttransplantation, n (%)	20 (35.1)
Over 36 months posttransplantation, n (%)	25 (43.9)

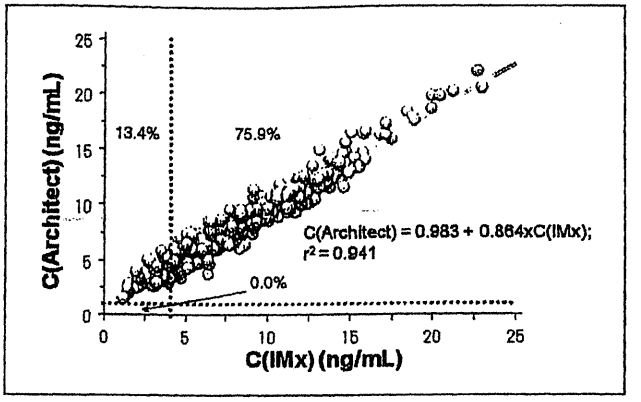

Figure 1. Correlation of tacrolimus (FK) trough concentrations measured by the microparticle enzyme immunoassay (MEIA) and chemiluminescent assay (CMIA) methods. The association between the 2 methods was excellent. C(ARCHITECT) = 0.983 + 0.864 × C(IMx); r^2 = 0.941. C(ARCHITECT) is the FK trough concentration measured by the CMIA method; C(IMx) is the FK trough concentration measured by the MEIA method. Of all samples, 13.4% were below the limit of quantification (4.1 ng/mL) in the MEIA method, whereas all samples were within the limit of qualification in the CMIA method.

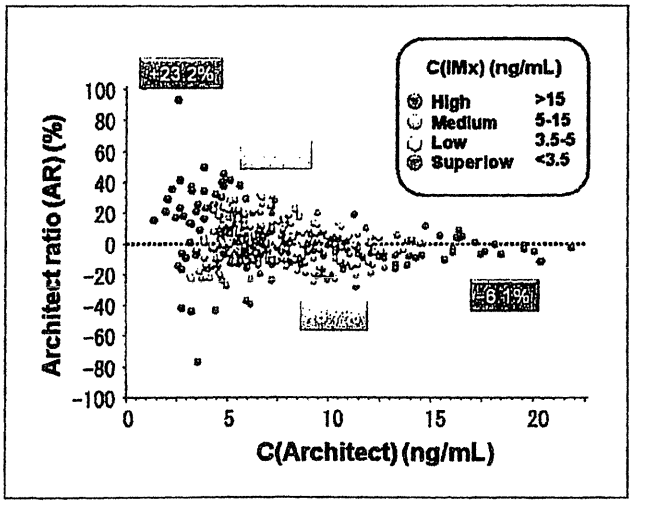

Figure 2. Correlation of tacrolimus trough concentration measured by the microparticle enzyme immunoassay (MEIA) and chemiluminescent assay (CMIA) methods using the ARCHITECT ratio (AR). The mean AR was 23.2% in samples with tacrolimus (FK) level less than 3.5 ng/mL and 6.8% in samples with FK level between 3.5 and 5 ng/mL, as measured by the MEIA. AR was defined as [C(ARCHITECT) – C(IMx)]/C(ARCHITECT).

those with hematocrit more than 45% accounted for 7.5% (n = 30).

The association between the 2 methods showed excellent correlation (Figure 1). C(ARCHITECT) = 0.983 + 0.864 × C(IMx); r^2 = 0.941. However, the discrepancy in the FK level between the 2 methods in terms of AR was large in samples with low FK level, especially less than 5 ng/mL. AR was 23.2% in samples with FK level less than 3.5 ng/mL (MEIA) and 6.8% in samples with FK level between 3.5 and 5 ng/mL (MEIA) (Figure 2). In contrast, there was excellent correlation between the 2 methods, with AR –3.7% (FK level between 5 and 15 ng/mL) and –6.1% (FK level over 15 ng/mL; Figure 2).

Analysis of the effect of hematocrit on FK level showed that the CMIA-MEIA discrepancy was larger in samples with low hematocrit (<25%), AR = –12.8%, as well as in samples with high hematocrit (>45%), AR = 11.4% (Figure 3).

In 14 of the 57 patients, the dose of FK was reduced due to renal dysfunction in 2 patients and stable graft function with more than 2 years after liver transplantation in 12 patients. The follow-up period in these 14 patients was 4.4 ± 1.8 years (range, 2.1-6.2 years). None of the patients in this study showed signs of rejection.

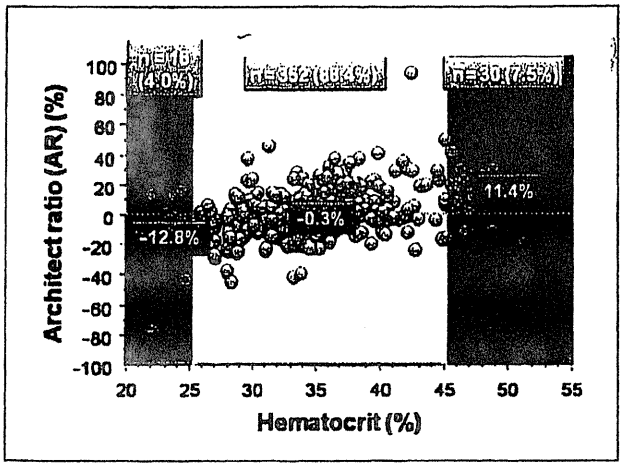

Figure 3. Correlation of ARCHITECT ratio (AR) with hematocrit (n = 398). The discrepancy in tacrolimus (FK) level measured by the chemiluminescent assay (CMIA) and microparticle enzyme immunoassay (MEIA) methods was larger in samples with low hematocrit (<25%, AR = –12.8%) and also in samples with high hematocrit (>45%, AR = 11.4%). AR was defined as [C(ARCHITECT) – C(IMx)]/C(ARCHITECT).

DISCUSSION

Therapeutic drug monitoring (TDM) is necessary when using tacrolimus due to the narrow therapeutic range and to minimize the side effects. The ideal TDM is estimated by calculating the area under the curve using several FK levels such as C0, C2, and C6. The efficacy C0 (trough) monitoring makes a suitable substitute to the AUC as it is simple and easy to measure and requires a single measure. Therefore, C0 monitoring is widely used and is an accepted measure in most transplant programs. Other methods of monitoring tacrolimus include detection of calcineurin inhibition and mixed lymphocyte reaction (MLR), which are more complex and difficult to apply in daily clinical practice. Other candidate methods for TDM include the cylex Immuknow assay, cytokines (eg, interleukin [IL]-5, IL-2, interferon [IFN]-γ, IFN-α, IL-2 receptor), neopterin, serum amyloid A, and lymphotoxin,[7,8] although their clinical application has not been established yet due to inadequate evidence of suitability for TDM.

Accurate detection of tacrolimus level in liver transplant recipients is important; however, the limitation of detecting low concentrations of tacrolimus has hampered efforts to lower the target FK level in stable liver transplant recipients. The LOQ of currently available MEIA in our hospital is 4.1 ng/mL, but we frequently encounter liver transplant recipients with normal liver function who show no sign of rejection and in whom the FK trough level is about 3.5 ng/mL. The LOQ in the CMIA method is as low as ~1 ng/mL. In our study, we maintained the FK trough level at less than 3.5 ng/mL in 13.4% liver transplant recipients.

Comparison of these 2 methods showed that the FK level varies in 2 manners. One factor relates to the hematocrit level. In the MEIA method, it has been known that there could be a substantial error in patients with low or high hematocrit.[9,10] In contrast, there is minimal error in relation to the hematocrit level in the CMIA method (Figure 3). Thus, it is likely that the discrepancy in the results of the 2 methods is due to the shortfall in the MEIA method. The more important second factor is the FK level itself. The correlation between the 2 methods was excellent at the high FK level (ie, >5 ng/mL), whereas the discrepancy between the values obtained by the 2 methods increased in samples of patients with low FK level (ie, <5 ng/mL). The FK level was underestimated, and the discrepancy ratio was more than 23% by the MEIA method at FK levels <3.5 ng/mL but was 6.8% at FK levels between 3.5 and 5 ng/mL (Figure 2). Thus, the MEIA method has a substantial error when FK level is <5 ng/mL, whereas the CMIA method is relatively accurate even in the range of 1 to 5 ng/mL.

Accurate measurement of serum FK level is necessary for TDM of FK, especially during long-term and stable periods after liver transplantation. Maintaining low FK level in stable liver transplant recipients could result in a better preservation of kidney function and less side effects such as infection, hypertension, diabetes, and hyperlipidemia. It would be useful to use once-a-day FK tablet, where lower FK trough level might be effective in avoiding rejection.

Several other methods are available for measurement of FK level in addition to CMIA and MEIA, such as the enzyme-multiplied immunoassay technique (EMIT), high-performance liquid chromatography with ultraviolet detection (HPLC-UV), and most recently mass spectrometry. Although the relationship between CMIA method and methods other than MEIA has not yet been investigated using clinical samples, to date, the CMIA method is the most accurate and clinically useful for real-time TDM of tacrolimus in liver transplant recipients.

CONCLUSIONS

We conclude based on the results of the present study that the CMIA method is superior to the

MEIA method for measurement of serum FK level, especially low FK levels (<5 ng/mL). Using this method, we can investigate the effects of low FK level in terms of reducing the chance of various short- and long-term side effects in liver transplant recipients.

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ORIGINAL ARTICLE

Guanylate-binding protein 2 mRNA in peripheral blood leukocytes of liver transplant recipients as a marker for acute cellular rejection

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Keywords
acute cellular rejection, guanylate-binding protein 2 (GBP2), interferon regulatory factor 1 (IRF1), liver transplantation, peripheral blood leukocytes.

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Summary

Previously, we reported guanylate-binding protein 2 (GBP2) and interferon regulatory factor 1 (IRF1) elevated in the rat peripheral blood during acute cellular rejection (ACR), which are identified from transcriptome analysis of liver graft, as leukocyte-related gene in liver. In this study, we investigated whether these two genes could differentially diagnose ACR from other types of liver dysfunction (LD) clinically. The mRNAs from leukocytes of 19 patients with ACR and 27 with LD, as well as from liver biopsies of 12 patients with ACR and 12 with LD, were analysed by real-time PCR for GBP2 and IRF1 expression. Sensitivity and specificity were calculated using receiver operator characteristic (ROC) curves. Guanylate-binding protein 2 (GBP2) and interferon regulatory factor 1 (IRF1) gene expression levels in ACR samples were higher than that in controls, and GBP2 expression in blood was higher than that in LD (26.4 ± 3.1 and 15.6 ± 1.9, $P = 0.0203$). Multivariate analysis showed that the ratio GBP2/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was independent of ACR-related factors (OR = 0.911, $P = 0.035$). GBP2 expression levels in ACR were also higher than that in liver transplantation patients with hepatitis C or no LD. Using a cut-off value of 20, the sensitivity and specificity of GBP2/GAPDH based on ROC curve analysis were 63% and 85% respectively. GBP2 in the patients with LD may be useful for diagnosis of ACR.

Introduction

Liver transplantation with efficient immunosuppressive therapies is an established treatment for end-stage liver disease. However, acute cellular rejection (ACR) still occurs in 50–70% of transplanted patients [1–4], and can potentially lead to severe liver dysfunction (LD) and failure. The underlying genetic and molecular mechanisms of ACR remain poorly understood and liver biopsy remains the only accurate diagnostic method for ACR. However, such biopsy is invasive and moderate to severe complications needing transfusion or interventional therapies occur in up to 5% of cases [5].

We hypothesized that because ACR is a response to the transplanted tissue, and immunosuppressants work by affecting the recipient's leukocytes, changes in peripheral blood could reflect the intragraft gene expression. Using the rat allo- and iso-liver transplantation models with transcriptome analysis, we identified previously two leukocyte-associated genes, the guanylate-binding protein 2 (GBP2) and interferon regulatory factor 1 (IRF1), which reflected the state of ACR [6]. Both genes were upregulated in liver grafts and peripheral leukocytes in ACR [6]. These genes were ACR-specific and not related to other liver dysfunctions (LDs), such as bile duct ligation [6].

The present study was designed on the premise that GBP2 and IRF1 expression levels are higher in peripheral blood leukocytes of patients with ACR compared with patients with LD. Accordingly, we measured GBP2 and IRF1 mRNA expression levels in human peripheral blood

leukocytes in patients with ACR and LD, and assigned appropriate cut-off values for the diagnosis of ACR. Routine biochemical analysis was used to assess LD, and after liver biopsy, the cases were categorized as either LD or ACR with LD. Acute cellular rejection was then differentially diagnosed from the overall pool of patients with LD. Multivariate analysis was applied for comparison with LD before liver transplantation, and finally, a threshold was assigned for the diagnosis.

Materials and methods

Patients and specimens

Peripheral blood leukocytes and liver biopsy specimens were obtained from patients who received liver transplantation. From 1999 to 2007, we performed 86 liver transplantations in 84 recipients in our institution. This study used samples from patients with LD, defined as either total bilirubin >2.0 mg/dl, aspartate aminotransferase (AST) >40 U/l or alanine aminotransferase (ALT) >40 U/l. Subsequently, patients with LD were divided into those with ACR and other types of LD following liver biopsy examination (all paired blood and liver specimens were examined pathologically on the same day by two pathologists), according to the following criteria: diagnosis by either or both the pathologists as ACR with LD; or no pathologic diagnosis of ACR (LD cases). Samples obtained within 2 weeks after ACR treatment or from patients with severe complications (rejection, infection, or recurrence of primary disease) were excluded.

Guanylate-binding protein 2 and interferon regulatory factor 1 mRNA levels were evaluated using peripheral blood leukocytes from 46 patients (ACR 19, LD 27) and from 20 control blood samples obtained from donors during the last 4 years. To compare the effects of LD and liver transplantation status, we also included eight liver samples from patients with hepatitis C who received liver transplantation but did not develop LD. Furthermore, we also used eight protocol biopsy samples after liver transplantation as controls. Only fresh biopsy samples (24 in total; ACR/LD 12, LD 12) were used for this analysis.

The Institutional Review Board of Osaka University approved the study protocol and all patients provided written informed consent.

Purification of peripheral blood leukocytes

Immediately prior to liver biopsy, 8 ml of peripheral blood was collected from each patient in a Vacutainer® CPT™ cell preparation tube containing sodium citrate (Becton Dickinson, Franklin Lakes, NJ). The blood samples were centrifuged immediately at 17 000 × *g* for 20 min and the separated leukocytes were placed into a 15-ml centrifugation tube, mixed with 10 ml of phosphate-buffered saline (PBS), and then centrifuged at 800 × *g* for 10 min. After washing with 1 ml PBS, the cells were resuspended with 1 ml TRIzol Reagent (Molecular Research Center, Cincinnati, OH, USA) and stored at −80 °C until RNA isolation.

Liver biopsy and pathologic examination

Parts of the liver biopsy samples were immediately immersed in RNAlater (Qiagen, Valencia, CA, USA), before freezing in liquid nitrogen and storing at −80 °C. Hematoxylin and eosin (H&E)-stained sections of the samples were examined by two independent experienced pathologists blinded to the clinical information. Specimens diagnosed as ACR were graded according to the Banff classification [7–9]: mild (RAI: 4–5) or moderate (RAI: 6–7) ACR. After biopsy evaluation, the patients were followed to confirm that the pathologic diagnosis matched the clinical course.

Isolation of RNA

Frozen liver biopsy samples were disrupted in TRIzol reagent using Tissue Lyzer (Qiagen, Haan, Germany). Total RNA was purified from the tissue samples by TRIzol reagent according to the protocol provided by the manufacturer. Isolated RNA was quantified and assessed for purity by UV spectrophotometry. The quality of RNA was confirmed using an Agilent 2100 Bioanalyzer and RNA 6000 LabChip kit (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNAs with intact 18S and 28S RNA were used for subsequent experiments.

Quantitative RT-PCR

Total RNA (1 μg) was subjected to reverse transcription to generate complementary DNA (cDNA) using the Reverse Transcription System (Promega, Madison, WI, USA). The expression levels of GBP2 and IRF-1 were quantified using a real-time thermal cycler, LightCycler®, and detection system (Roche Diagnostics, Mannheim, Germany). LightCycler-DNA master SYBR green I (Boehringer, Mannheim) was used to detect the amplification products. Briefly, a 20-μl reaction volume containing 2 μl of cDNA and 0.2 μmol/l of each primer was applied to a glass capillary. In this assay, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The following primers were used: human GBP2 (forward; 5′-GGATATATTTGGCCCTTTAGAAGAA-3′, reverse; 5′-CTTTTTCCTTTTCTGAGAGTGACTG-3′), human IRF-1 (forward; 5′-AGCTCAGCTGTGCGAGTGTA-3′, reverse; 5′-TAGCTGCTGTGGTCATCAGG-3′), and human GAPDH (forward; 5′-CAACTACATGGTTTACATGTTC-3′,

reverse; and, 5′-GCCAGTGGACTCCACGAC-3′). These primers were designed using web-based software PRIMER3 (version 0.9, Whitehead Research Institute http://primer3.sourceforge.net/). The PCR for each gene was performed with cycling conditions of 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for15 s, annealing at 62 °C for10 s, and extension at 72 °C for18 s. Quantitative mRNA analysis was performed using LightCycler® analysis software (Roche Diagnostics) as recommended by the manufacturer. The relative gene expression levels were expressed as quantified gene expression divided by quantified GAPDH levels.

Statistical analysis

Data are expressed as mean ± standard error (SEM). Differences were tested by Student's *t*-test or chi-squared test and considered statistically significant at $P < 0.05$. Cut-off values for diagnosis were ascertained using the receiver operator characteristic (ROC) curve, and the sensitivity and specificity were calculated for each cut-off value. Multivariate analysis was performed by multiple logistic regression. All statistical analysis was performed using StatView version 5.0 (SAS Institute, Cary, NC, USA).

Results

Clinical course and serial changes in GBP2 and IRF1 mRNA levels

Figure 1 shows serial changes in GBP2 and IRF1 mRNA expression levels in a representative patient diagnosed by liver biopsy and treated for ACR. The patient (58-year-old male) underwent transplantation using a left-lobe liver graft with middle hepatic vein necessitated because of cryptogenic liver cirrhosis. Six days after the surgery, a liver biopsy was performed to assess liver damage. Acute cellular rejection was diagnosed and the patient was placed on steroid pulse therapy (MPSL, Fig. 1). Immediately before the start of this treatment, GBP2 and IRF1 mRNA levels in the peripheral blood leukocytes showed a transient increase. However, the levels returned to pre-transplantation control levels during continued treatment.

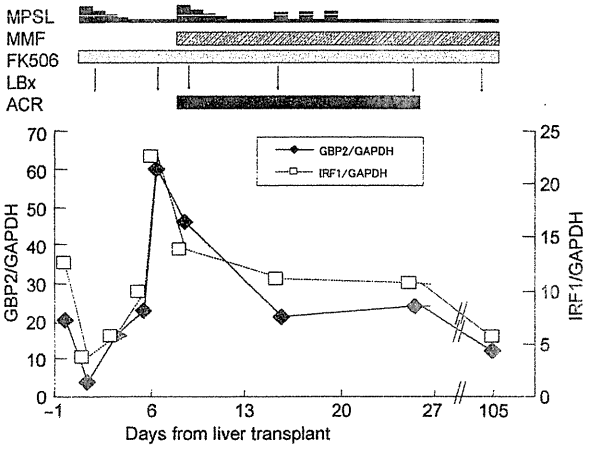

Figure 1 Serial changes in GBP2 and IRF1 mRNA expression levels during acute cellular rejection (ACR) and in response to treatment in a representative patient. The patient underwent living-relative liver transplantation of left liver grafts as a result of cryptogenic liver cirrhosis. MPSL, methylprednisolone; MMF, mycophenolate mofetil; LBx, liver biopsies.

Patients' characteristics

Table 1 summarizes the differences between patients of the ACR/LD and LD groups at liver biopsy. Assessment of type of liver damage (ACR and LD) using peripheral blood leukocyte mRNA levels required equivalent background liver damage. There were no differences in the primary disease for liver transplantation, including hepatitis C. Liver biopsy date after liver transplantation in the ACR/LD group was closer to the date of surgery than in LD patients, but the difference was not statistically significant. Biochemical analysis indicated that AST and ALT levels were >40 U/l and total bilirubin >2.0 mg/dl in both groups. However, the levels of ALT, alkaline phosphatase (ALP), and gamma glutamyl transpeptidase (γGTP) were significantly higher in the ACR/LD group than in LD patients.

Table 1. Patients' characteristics.

	ACR/LD	LD	*P*-value
n	19	27	
Age	51.7 ± 1.7	52.7 ± 1.6	0.685
Gender			
M	12	16	0.790
F	7	11	
Primary disease			
HCV	7	13	0.394
PBC/PSC/AIH	4	7	
Cryptogenic	6	3	
Others*	2	4	
Days after transplantation	104 ± 55	219 ± 70	0.193
AST	146 ± 61	67 ± 11	0.194
ALT	118 ± 16	69 ± 9	0.018
ALP	578 ± 142	220 ± 32	0.019
γGTP	272 ± 56	126 ± 23	0.019
Total bilirubin	12.9 ± 2.1	11.2 ± 2.0	0.550
PT-INR	1.30 ± 0.07	1.36 ± 0.05	0.470

Data are mean ± SD.
ACR, acute cellular rejection; LD, liver dysfunction other than ACR.
*HBV, citrullinemia.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γGTP, gamma glutamyl transpeptidase.

GBP2 and IRF1 mRNA levels in ACR and LD

Biochemical analysis showed no liver damage in ACR and LD patients. Donor peripheral blood was then used as a control and GBP2 and IRF1 mRNA levels were compared with those of ACR/LD and LD patients. The expression levels of GBP2 and IRF1 genes were higher in both ACR and LD patients as compared with the control group (Fig. 2). GBP2 mRNA level, but not that of IRF1 mRNA, was significantly higher in the ACR/LD group than in LD group.

In contrast, liver biopsy analysis showed higher levels of both GBP2 and IRF1 mRNAs in ACR/LD than LD, although not statistically significant (Fig. 3). We had compared previously the same two genes in the rat allo-transplantation model (ACR) and iso-model (no ACR) [6]. We therefore used protocol liver biopsy samples assessed by routine biochemical analysis at 1, 2, and 5 years after liver transplantation. Both GBP2 and IRF1 mRNA levels were higher in the ACR/LD group than in protocol liver biopsy samples.

The GBP2 and IRF1 genes are mainly expressed in leukocytes [6], and leukocyte infiltration is a feature of ACR [7]; therefore, their mRNA levels in peripheral blood should correlate with those in the liver. Accordingly, we compared the mRNA expression levels in paired samples of five patients with ACR/LD and five with LD (Fig. 4), both samples were obtained on the same day in each patient. There were significant correlations in the expression levels of both GBP2 and IRF1 between peripheral blood and liver ($P < 0.05$).

GBP2 mRNA levels in the diagnosis of ACR

The above results showed higher GBP2 mRNA levels in peripheral blood of ACR/LD patients, and also high levels of ALT, ALP, and γGTP in ACR/LD patients as compared with LD. In the next step, multiple logistic regression analysis was conducted to assess whether GBP2 mRNA levels in peripheral blood is an independent diagnostic factor for ACR. The ratio of GBP2/GAPDH mRNA and ALP level were identified as independent factors, with an odds ratio for GBP2/GAPDH of 0.911 (Table 2).

Guanylate-binding protein 2 mRNA levels in peripheral blood of ACR patients were also compared with those of patients with hepatitis C (before transplantation) and in normal liver after transplantation (blood samples were obtained at the protocol liver biopsy as described above). GBP2 mRNA levels in the peripheral blood of ACR/LD patients were significantly higher than those of patients with severe liver damage and normal liver (both control and transplanted patients; Table 3).

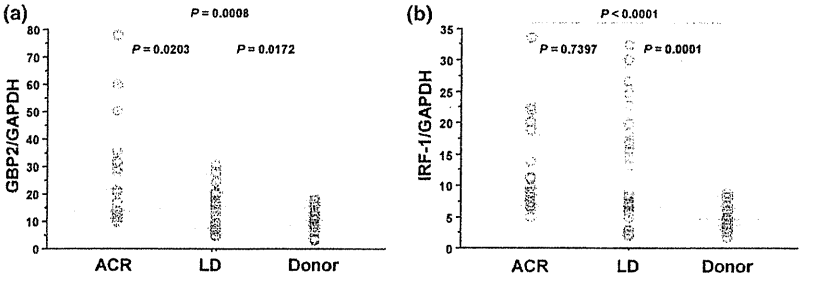

Figure 2 GBP2 (a) and IRF1 (b) mRNA expression levels in peripheral blood leukocytes from 19 ACR and 27 LD patients. Twenty donor blood leukocytes were used as controls. The pathologic diagnosis was made for each case as described in Materials and Methods. In the box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. ACR, acute cellular rejection; LD, other liver dysfunction.

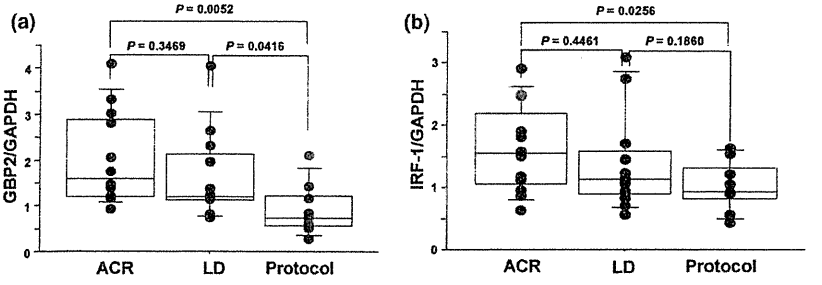

Figure 3 GBP2 (a) and IRF1 (b) mRNA expression levels in liver biopsies from 12 patients with ACR, 12 with LD, and protocol liver biopsy (no-dysfunction transplanted liver) specimens as controls. ACR, acute cellular rejection; LD, other liver dysfunction.

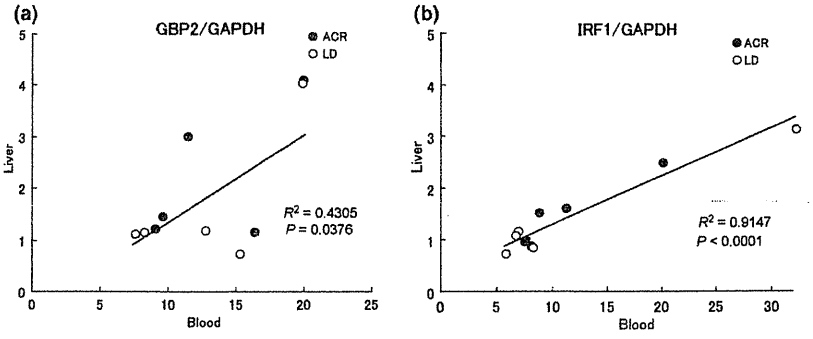

Figure 4 Correlation between GBP2 (a) and IRF1 (b) mRNA expression levels in peripheral blood leukocytes with those in liver tissues. We compared GBP2 and IRF1 expression levels of paired peripheral blood and liver biopsy samples from five ACR and five LD patients. Closed circles, ACR, acute cellular rejection; open circles, LD, other liver dysfunction.

Table 2. Results of multiple logistic regression analysis for the diagnosis of acute cellular rejection/liver dysfunction (ACR/LD).

	P	Odds ratio	95% confidence interval
GBP2/GAPDH	0.035	0.911	0.856–0.970
ALT	0.175	0.990	0.975–1.005
ALP	0.043	0.997	0.993–1.000
γGTP	0.891	1.000	0.994–1.005

GBP2, guanylate-binding protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γGTP, gamma glutamyl transpeptidase.

The differential diagnosis of ACR from hepatitis C recurrence is one of the most difficult issues to be resolved in respect of the damaged transplanted liver. To resolve this issue, we compared GBP2 mRNA levels in the HCV (+) recipients. Among these patients, GBP2 mRNA in ACR only were higher than those in LD (14.6 ± 2.1 vs. 11.2 ± 4.1), although the differences were not statistically significant ($P = 0.2160$).

Differential diagnosis of ACR from liver dysfunctions

To determine the cut-off value for the GBP2/GAPDH ratio for the diagnosis of ACR, a ROC curve was used to calculate the sensitivity and specificity (Fig. 5). The cut-off values ranged from 9 to 30. Using a GBP2/GAPDH cut-off value of 20, the sensitivity was 63% and specificity was 85% (Table 4).

Table 3. Guanylate-binding protein 2 (GBP2) mRNA expression levels in patients with acute cellular rejection (ACR), liver dysfunction other than ACR, donors, preoperative patients with hepatitis C, and in the protocol biopsy after liver transplantation.

	n	GBP2/GAPDH	*P*-value vs. ACR
ACR/LD	19	26.4 ± 3.1	–
LD	27	15.6 ± 2.5	0.0203
Donor	20	10.3 ± 1.0	0.0008
HCV(+) preLTx	8	16.9 ± 1.6	0.0362
Protocol	8	15.3 ± 2.6	0.0268

ACR, acute cellular rejection; LD, liver dysfunction other than ACR; HCV (+) pre LTx, preoperative patients with hepatitis C; GBP2, guanylate-binding protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

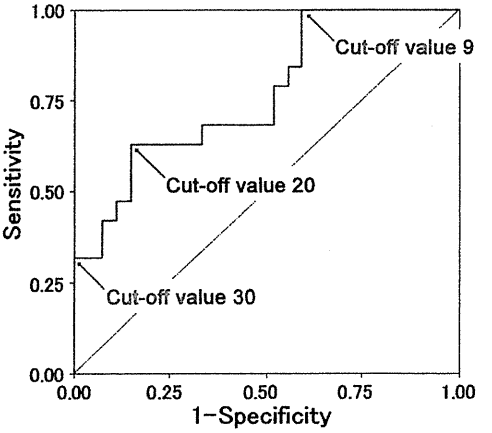

Figure 5 Receiver operator characteristic (ROC) curve for diagnosis of acute cellular rejection (ACR) using GBP2 mRNA expression levels. The GBP2/GAPDH cut-off values are indicated on the figure. Sensitivity and specificity at each cut-off values are detailed in Table 4.

Table 4. Sensitivity and specificity for acute cellular rejection (ACR) diagnosis using guanylate-binding protein 2 (GBP2) mRNA expression levels in peripheral blood leukocytes.

	GBP2/GAPDH cut-off value		
	9	20	30
Sensitivity (%)	100	63.2	31.6
Specificity (%)	40.7	85.2	100
Positive predictive value (%)	54.3	75.0	100
Negative predictive value (%)	100	76.7	67.5
Efficacy (%)	65.2	76.1	71.7

GBP2, guanylate-binding protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

Acute cellular rejection following transplantation is a systemic response against the grafted liver. Based on this hypothesis, we identified previously two leukocyte-related peripheral blood biomarkers (GBP2 and IRF1) from transcriptome analysis of rat liver [6]. In the present study, these same genes were investigated by mRNA expression analysis in human peripheral blood during ACR. Indeed, liver biopsy for the differential diagnosis of LD carries safety issues, and several clinicians have called for direct peripheral blood biomarkers [10–14]. However, such investigations into peripheral blood biomarker for ACR diagnosis have not so far been carried out.

The reported peripheral blood markers for ACR are related to T-cell related immune responses including regulatory T-cell levels [10–13] and apoptosis [14]. The systemic state in ACR is still unclear, although it might also involve regulatory T cells. Furthermore, IRF-1 is known to suppress regulatory T-cell function under suppression of FOXP3 [15]. On the other hand, both of our peripheral blood markers for ACR are associated with interferon (IFN)-related mechanisms [16–18]. Briefly, IRF1, which is a transcriptional factor regulated by the IFN-STAT signaling pathway [19,20], regulates GBP2 expression levels [16,17]. This system in turn elicits antiviral activity [21], macrophage activation [22], and fibroblast proliferation [23]. These results indicated that the Th1 cytokine, IFN-gamma, would stimulate IRF1, with subsequent increase in GBP2, and thus downregulate regulatory T-cell activity via suppression of FOXP3. Indeed, immunosuppressive drugs decrease the ratio of Th1/Th2 cytokines and increase regulatory T cells [24]. As ACR does not adequately respond to immunosuppressive drug, our speculation seems reasonable. In support of the hypothesis on the systemic status during ACR, one report described another peripheral blood marker for ACR [10]. AIF-1 is a promising peripheral blood marker for ACR, which increased in parallel with changes in IFN-gamma and other Th1 markers in rats [10].

Interferon regulatory factor 1 (IRF1) and guanylate-binding protein 2 (GBP2) are good peripheral blood markers for ACR detection, because unlike FOXP3 they are upregulated during ACR. Unfortunately, there was no significant difference in IRF1 expression between ACR and LD samples, as would be expected from a reported marker of ACR-related pathway. Perhaps, GBP2 would be enhanced as a downstream factor of IRF1, and might therefore be more sensitive for ACR detection, although the relationship between GBP2 and ACR remains unclear in this and previous reports [23]. In addition, GBP2 levels increased rapidly prior to detection of pathologic changes, supporting the notion that GBP2 is an enhanced factor in ACR. It is quite difficult to identify peripheral blood markers by direct 'omics' analysis, because such molecules, e.g. FOXP3, are often present at low concentrations. Thus, although GBP2 expression levels were high in ACR, the population of GBP2-related cells might be too small to show detectable changes in peripheral blood [25].

Another current topic of diagnosis in damaged liver is the challenge for the distinction of ACR from hepatitis C recurrence in HCV (+) patients. In this study, GBP2 mRNA levels in ACR were higher than those in hepatitis C recurrence; however, there were no statistically significant differences because of limited number of HCV (+) patients. To ensure the differential diagnosis of ACR from hepatitis C recurrence using GBP, further investigation would be necessary.

In summary, this study showed the potential clinical usefulness of GBP2 as a new peripheral blood marker for ACR. The GBP2 gene-related pathway and the previously reported ACR-related genes are downstream of IFN-gamma signaling. It is therefore probable that IFN-gamma-related pathways play a key role in ACR [26,27]. Prospective clinical analyses will be necessary to achieve precise diagnosis.

Authorship

HN, YD, MM 'designed research/study'. SK, HN 'performed research/study'. HN 'contributed important reagents'. HE, YT, MT 'collected data'. SK, SM, HN, TA 'analysed data'. SK 'wrote the paper'.

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Effects of Preceding Interferon Therapy on Outcome After Surgery for Hepatitis C Virus-Related Hepatocellular Carcinoma

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Background and Objectives: Interferon (IFN) can eradicate hepatitis C virus (HCV)-RNA from serum and hepatic tissue, and suppress the development of hepatocellular carcinoma (HCC). Despite such effectiveness, HCC develops even in HCV patients successfully treated with IFN therapy.

Methods: HCV-related HCC patients who underwent curative hepatectomy for HCC were divided into three groups according to preceding IFN for HCV infection therapy and the therapeutic effect: responders group (n = 23), non-responders group (n = 46), and no-IFN group (n = 215). Postoperative outcome was retrospectively examined in the three groups.

Results: AST and ALT were significantly lower in responders group than non-responders group ($P < 0.001$, $P = 0.001$) and no-IFN group ($P = 0.001$, $P = 0.002$). Platelet count was significantly higher in responders group than other groups ($P = 0.008$, $P = 0.001$). The percentage of cirrhotic patients in responders group was significantly lower than other groups ($P = 0.017$, $P = 0.014$). Multivariate analysis identified preceding IFN therapy to be associated with disease-free survival at marginal significance ($P = 0.086$), and as a significant independent factor for overall survival ($P = 0.042$).

Conclusions: Preceding IFN therapy for HCV infection improves postoperative outcome in HCV-related HCC patients treated successfully with IFN.

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KEY WORDS: **hepatocellular carcinoma (HCC); interferon (IFN); hepatitis C virus (HCV); hepatic resection**

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Approximately 80% of Japanese HCC patients have a history of chronic infection with hepatitis C virus (HCV), which is a known cause of HCC [1,2]. Recent advances in imaging modalities and treatment have brought some improvement to the prognosis of patients with HCV-related HCC, but the outcome remains unsatisfactory. Even after curative hepatic resection for HCV-related HCC, the rate of tumor recurrence within 1 year is 20–40%, rising to about 80% by 5 years [3,4]. This high recurrence rate and the progression of the underlying hepatic damage due to HCV-related chronic hepatitis (CH) or cirrhosis result in unfavorable postoperative outcome in patients with HCV-related HCC.

Interferon (IFN) is the only agent known to be effective against HCV infection [5–10]. It can eradicate HCV-RNA from peripheral blood and hepatic tissue, prevent deterioration of liver dysfunction in patients with HCV infection, and suppress the development of HCC. HCV-infected patients treated with IFN, especially those who develop sustained virological response (SVR), defined as negative HCV-RNA polymerase chain reaction at 6 months after the end of treatment, enjoy the benefits of such treatment [11,12]. However, despite such effectiveness of IFN therapy, there have been recently some reports of development of HCC even in HCV patients who had gained SVR following IFN therapy [13,14]. With regard to the HCC development in patients treated successfully with IFN, HCV-related HCC patients can be divided into three groups according to the clinical background of preceding IFN therapy; successfully treated, unsuccessfully treated, or not treated with IFN. However, to date, there have been few studies on the correlation between clinical background of previous IFN therapy for HCV infection and surgical outcome of HCV-related HCC [15,16].

In the present retrospective study, we reviewed HCV-related HCC patients who had undergone surgery in our hospital. We analyzed the factors that affected postoperative outcome including history of previous IFN therapy and the effect of such therapy.

MATERIALS AND METHODS

The present study included 284 patients with HCC who had undergone curative hepatic resection at the Department of Surgery, Osaka University Hospital between January 1990 and December 2008. Patients with HCC grade Vp3, Vp4, Vv2, and Vv3, defined according to the classification system of the Liver Cancer Study Group of Japan, were excluded from this study [17]. Curative resection was defined as complete removal of all macroscopically evident tumors. Patients who had undergone surgery for recurrent HCC were also excluded from this

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study. Among the 284 patients, 215 patients were not treated with IFN (no IFN group). The remaining 69 patients received IFN therapy for HCV infection. In the latter group, HCC had not been detected at the IFN therapy, and was detected after the IFN therapy. The IFN therapy was performed not for HCC, but for HCV-related hepatitis. The administration of IFN therapy was determined based on the informed consent between each physician and patient. The response to IFN therapy was assessed retrospectively based on changes in HCV-RNA. Based on the response, patients were divided into the responders group and non-responders group; 23 patients whose HCV-RNA disappeared after IFN therapy were categorized as the responders group, and 46 patients whose HCV-RNA did not disappear after IFN therapy in non-responders group. Figure 1 summarizes the classification of the enrolled patients. The type, dosage, and duration of IFN administration before surgery varied, though all patients received IFN-α.

Hospital records were retrospectively reviewed for clinical factors including previous history of IFN therapy, tumor- and surgery-related factors. The surgical procedure was selected based on the extent of the tumor and residual liver function. The HCC staging was performed according to the classification system of the Liver Cancer Study Group of Japan [17]. The histological grade of differentiation of HCC was determined according to the Edmondson–Steiner classification, and was based on the areas of the tumor with the highest grade [18]. Non-cancerous lesion of the liver was divided histopathologically into chronic hepatitis CH and liver cirrhosis (LC).

Patients were followed up after hepatic resection at regular intervals of 3–4 months with physical examination, tumor markers including alpha-fetoprotein (AFP), and protein induced by vitamin K absence or antagonists-II (PIVKA-II), liver biochemical tests, abdominal ultrasonography, and abdominal computed tomography (CT) to check for intrahepatic recurrence, and chest radiography and bone scintigraphy for extra-hepatic recurrence. The median duration of clinical follow-up after the initial hepatectomy was 51.2 months.

Data were expressed as mean ± standard deviation. Differences between groups were assessed by the chi-square test, Fisher's exact test, or the Mann–Whitney U test. Survival rates were calculated according to the Kaplan and Meier method and compared using the log-rank test. Statistical analysis was performed using StatView (version 5.0, SAS Institute Inc., Cary, NC). A P-value <0.05 was considered statistically significant. The study protocol was approved by the Human Ethics Review Committee of Osaka University Hospital and a signed consent form was obtained from each patient.

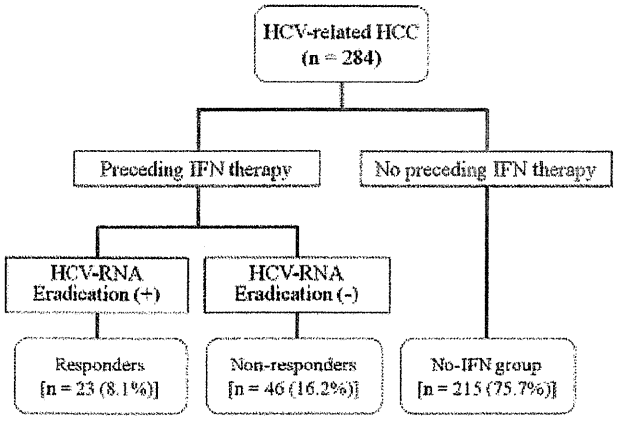

Fig. 1. Distribution of patients enrolled in this study according to the clinical background of preceding IFN therapy. HCV, hepatic C virus; HCC, hepatocellular carcinoma; IFN, interferon.

RESULTS

The study group comprised 222 (78.2%) men and 62 (21.8%) women, with a mean age of 65 (range, 39–79). Table I summarizes the clinicopathological characteristics of the responders group, the non-responders group, and the no-IFN group. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly lower in the responders group than the non-responders group ($P < 0.001$, $P = 0.001$) and no-IFN group ($P = 0.001$, $P = 0.002$). There were no significant differences in the levels of AST and ALT between the non-responders group and the no-IFN group. Platelet count was significantly higher in the responders than that in the non-responders ($P = 0.008$) and that in the no-IFN group ($P = 0.001$). In the responders group, histopathological status of the non-cancerous liver tissue obtained at surgery was CH in 16 patients (69.6%) and LC in seven patients (30.4%). The percentage of patients of the responders group with LC was significantly lower than that of the non-responders group (28/46, 60.9%; $P = 0.017$) and that of the no-IFN group (123/215, 57.2%; $P = 0.014$). Liver function assessed by Child–Pugh classification was not different among the three groups. Other clinical factors listed in Table I were also not different among the three groups, including tumor- and surgical-related factors. Adjuvant therapy of IFN was administered in a small number of patients (n = 14, 4.9%), and the frequency of such patients was not different among the three groups.

For all the 284 patients, the 1-, 3-, and 5-year disease-free survival (DFS) rates were 70.8%, 36.7%, and 22.8%, respectively. There was no significant difference in DFS between the IFN group (the responders group and the non-responders group) and the no-IFN group ($P = 0.396$). However, the DFS of the responders group (1 year: 89.2%, 3 years: 59.4%, 5 years: 59.4%) was significantly better than that of the no-IFN group (1 year: 70.8%, 3 years: 35.7%, 5 years: 21.6%; $P = 0.039$), and tended to be better than that of the non-responders group (1 -year: 60.4%, 3 years: 32.3%, 5 years: 16.9%; $P = 0.051$; Fig. 2). However, there was no significant difference in DFS between the non-responders group and the no-IFN group ($P = 0.673$). The 1-, 3-, and 5-year overall survival rates for all patients were 94.5%, 80.4%, and 66.9%, respectively. The overall survival rates of the IFN group (responders group and non-responders group) tended to be higher than those of the no-IFN group ($P = 0.093$). The 1-, 3-, and 5-year overall survival rates for the responders group were 100%, 100%, and 100%, respectively, and were significantly higher than the non-responders group (1-year: 94.4%, 3 years: 78.6%, 5 years: 55.4%; $P = 0.026$) and the no-IFN group (1 year: 94.0%, 3 years: 79.0%, 5 years: 66.1%; $P = 0.009$; Fig. 3). There was no significant difference in overall survival between the non-responders group and the no-IFN group ($P = 0.904$).

Univariate analysis was performed between DFS and various clinicopathological factors (Table II). Microscopic vascular invasion (negative vs. positive), tumor stage (I, II vs. III, IV), number of nodules (single vs. multiple), the diameter of largest tumor nodules (<5 cm vs. ≥5 cm), AFP level (<5 ng/m vs. ≥5 ng/m), and preceding IFN therapy (responders vs. non-responders, no-IFN) were significant factors ($P < 0.001$, $P = 0.006$, $P = 0.008$, $P = 0.021$, $P = 0.017$, $P = 0.037$). Multivariate analysis for DFS using the above six factors identified the number of nodules and microscopic vascular invasion as significant independent factors ($P = 0.014$, $P = 0.041$; Table III). In the same analysis, preceding IFN therapy showed a borderline significance with DFS ($P = 0.086$). The diameter of the largest tumor nodules and AFP level also tended to be associated with DFS ($P = 0.090$, $P = 0.098$).

Univariate analysis for overall survival using various clinicopathological factors demonstrated that microscopic vascular invasion (negative vs. positive), preceding IFN therapy (responders vs. non-responders, no-IFN), number of nodules (single vs. multiple), diameter of largest nodules (<5 cm vs. ≥5 cm), and AFP level (<5 ng/m vs. ≥5 ng/m) were significant factors ($P = 0.004$, $P = 0.009$, $P = 0.015$,

TABLE I. Clinicopathological Characteristics of Patients With HCV-Related HCC

	IFN group			*P*-value		
	Responders (n = 23)	Non-responders (n = 46)	No-IFN (n = 215)	Responders versus non- responders	Responders versus no-IFN	Non- responders versus no-IFN
Clinical factors						
Gender (male/female)	19/4	33/13	170/45	0.323	0.793	0.278
Age (years)	66 ± 7	64 ± 7	65 ± 7	0.355	0.653	0.424
Alcohol abuse (+/−)	14/9	27/19	132/83	0.862	0.961	0.733
HCV serotype (1/2/unknown)	19/4/0	35/5/6	166/29/20	>0.999	0.795	0.969
HBs Ag (+/−)	1/22	1/45	7/208	>0.999	0.562	>0.999
AST (IU/L)	28 ± 13	49 ± 27	46 ± 21	<0.001	0.001	0.184
ALT (IU/L)	26 ± 16	52 ± 36	47 ± 29	0.001	0.002	0.233
Platelet count ($\times 10^4/\mu l$)	16.4 ± 3.3	13.0 ± 5.3	13.2 ± 4.5	0.008	0.001	0.867
Albumin (g/dl)	4.0 ± 0.4	3.8 ± 0.6	3.8 ± 0.5	0.142	0.175	0.975
Total bilirubin (mg/dl)	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.3	0.114	0.104	0.362
Prothrombin time (%)	77 ± 10	77 ± 8	76 ± 9	0.719	0.888	0.442
Hepaplastin test (%)	81 ± 13	78 ± 12	77 ± 12	0.244	0.217	0.834
Child-Pugh (A/B)	22/1	37/9	187/28	0.148	0.326	0.265
Non-cancerous lesion (CH/LC)	16/7	18/28	92/123	0.017	0.014	0.648
Tumor-related factors						
AFP (ng/ml)	1,791 ± 6,654	545 ± 1,444	851 ± 4,004	0.226	0.332	0.610
PIVKA-II (mAU/ml)	1,773 ± 5,433	1,418 ± 4,733	2,006 ± 4,879	0.786	0.837	0.459
Preoperative TAE (+/−)	10/13	23/23	119/96	0.609	0.278	0.509
Postoperative IFN (+/−)	1/22	1/45	12/203	>0.999	>0.999	0.476
Number of nodules (single/multiple)	17/6	33/13	152/63	0.849	0.747	0.888
Tumor diameter (cm)	3.5 ± 1.9	3.2 ± 2.0	3.6 ± 2.6	0.287	0.725	0.171
Vascular invasion (+/−)	2/21	2/44	18/197	0.596	>0.999	0.543
Stage (I/II/III/IV)	5/12/4/2	12/24/7/3	45/109/49/12	0.967	0.890	0.671
Edmondson-Steiner grade (I, II/III, IV/unknown)	13/10/0	26/16/4	117/89/9	0.672	0.980	0.541
Surgery-related factors						
Hr (0/S/1/2)	13/3/3/4	29/5/7/5	116/41/37/21	0.869	0.615	0.543
Volume of resection (g)	152 ± 118	137 ± 151	165 ± 162	0.393	0.682	0.186
Blood loss (ml)	1,022 ± 1,583	996 ± 702	1,167 ± 1,217	0.770	0.571	0.197
Operation time (min)	253 ± 128	236 ± 99	236 ± 99	0.337	0.940	0.174
Transfusion (+/−)	4/19	7/39	52/163	>0.999	0.465	0.187

Data are expressed as mean ± standard deviation.
IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

$P = 0.034$, $P = 0.045$; Table II). Multivariate analysis for overall survival using the above five factors identified number of nodules, microscopic vascular invasion, and preceding IFN therapy as significant independent factors ($P = 0.025$, $P = 0.037$, $P = 0.042$; Table III).

HCC recurred postoperatively in nine (39.1%) patients of the responders group, 29 (63.0%) of the non-responders group, and in 157 (73.0%) of the no-IFN group. Table IV summarizes the clinical characteristics of patients with recurrent HCC at diagnosis of the recurrence. AST and ALT levels in the responders group were

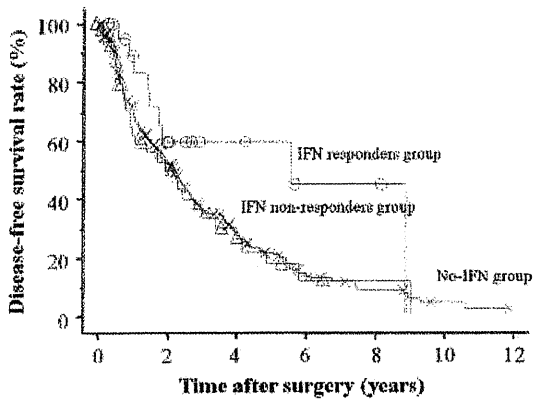

Fig. 2. Disease-free survival after initial surgery for HCC in the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 23), open triangles: non-responders (n = 46), crosses: no-IFN (n = 215). IFN: interferon.

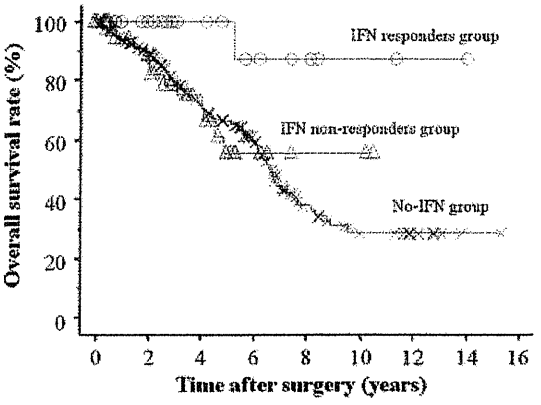

Fig. 3. Overall survival calculated from the initial surgery for HCC for the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 23), open triangles: non-responders (n = 46), crosses: no-IFN (n = 215). IFN: interferon.

TABLE II. Univariate Analysis of Disease-Free Survival and Overall Survival of Patients With HCV-Related HCC

	Number of patients	Disease-free survival	Overall survival
Clinical factors			
Gender (male/female)	222/62	0.732	0.789
Age, years (<66/≥67)	143/141	0.682	0.842
Alcohol abuse (+/−)	172/112	0.955	0.572
HCV genotype (1/2/unknown)	220/40/25	0.612	0.427
AST (IU/L) (<40/≥40)	126/158	0.496	0.547
ALT (IU/L) (<40/≥40)	122/162	0.216	0.301
Total bilirubin (mg/dl) (<1.0/≥1.0)	252/32	0.890	0.587
Albumin (g/dl) (<3.5/≥3.5)	114/170	0.174	0.171
Prothrombin time (%) (<70/≥70)	77/207	0.693	0.875
Hepaplastin test (%) (<70/≥70)	75/209	0.427	0.398
Platelet count ($\times 10^4/\mu l$) (<10/≥10)	83/201	0.176	0.123
Child-Pugh (A/B)	246/38	0.866	0.594
Non-cancerous lesion (CH/LC)	126/158	0.247	0.177
Tumor-related factors			
AFP (ng/ml) (<5/≥5)	54/230	0.021	0.045
PIVKA-II (mAU/ml) (<400/≥400)	190/83	0.130	0.142
Preceding IFN (responders/non-responders, no-IFN)	23/261	0.037	0.009
Preoperative TAE (+/−)	152/132	0.863	0.562
Postoperative IFN (+/−)	14/270	0.222	0.253
Number of nodules (single/multiple)	202/82	0.008	0.015
Tumor diameter (cm) (<5/≥5)	232/52	0.017	0.034
Vascular invasion (+/−)	22/262	<0.001	0.004
Stage (I, II/III, IV)	207/77	0.006	0.197
Edmondson-Steiner grade (I, II/III, IV)	156/115	0.328	0.265
Surgery-related factors			
Hr (0/S, 1, 2)	158/126	0.313	0.893
Intraoperative blood loss (L) (<1/≥1)	151/133	0.289	0.270
Operation time (min) (<240/≥240)	141/143	0.221	0.493
Transfusion (+/−)	63/221	0.756	0.180

IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

significantly lower than those in the non-responders group ($P = 0.047$, $P = 0.045$) and those in the no-IFN group ($P = 0.028$ and $P = 0.034$). There were no significant differences in AST and ALT levels between the non-responders and no-IFN groups. Platelet count was significantly higher in the responders group than that in the no-IFN group ($P = 0.029$) and tended to be higher than that in the non-responders group ($P = 0.079$). Figure 4A shows the distribution of interval between initial hepatectomy and recurrence. In most patients, HCC recurred within 2 years in the three groups, and the distribution of the interval was not different among the three groups. In all groups, the

TABLE III. Multivariate Analysis of Disease-Free Survival and Overall Survival of Patients With HCV-Related HCC

	OR	95% CI	*P*-value
Disease-free survival			
AFP (ng/ml) (<5/≥5)	1.427	0.937–2.174	0.098
Preceding IFN (responders/non-responders, no-IFN)	1.809	0.919–3.561	0.086
Number of nodules (single/multiple)	1.707	1.022–2.850	0.041
Tumor diameter (cm) (<5/≥5)	1.391	0.951–2.037	0.090
Vascular invasion (−/+)	2.331	1.186–4.587	0.014
Stage (I, II/III, IV)	1.287	0.715–2.315	0.401
Overall survival			
AFP (ng/ml) (<5/≥5)	1.689	0.847–3.367	0.137
Preceding IFN (responders/non-responders, no-IFN)	7.750	1.076–55.798	0.042
Number of nodules (single/multiple)	1.622	1.062–2.476	0.025
Tumor diameter (cm) (<5/≥5)	1.381	0.842–2.268	0.200
Vascular invasion (−/+)	2.247	1.049–4.808	0.037

IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies. OR, odds ratio, 95% CI, 95% confidence interval.

TABLE IV. Clinicopathological Characteristics of Patients With Recurrent HCC in the Responders Group, the Non-Responders Group, and the No-IFN Group

	IFN group			*P*-value		
	Responders (n = 9)	Non-responders (n = 29)	No-IFN (n = 157)	Responders versus non-responders	Responders versus no-IFN	Non-responders versus no-IFN
Clinical factors						
Gender (male/female)	9/0	22/7	126/31	0.164	0.212	0.590
Age (years)	67 ± 7	66 ± 6	67 ± 7	0.641	0.971	0.378
AST (IU/L)	30 ± 25	50 ± 28	55 ± 28	0.047	0.028	0.786
ALT (IU/L)	32 ± 26	53 ± 33	54 ± 34	0.045	0.034	0.902
Platelet count ($\times 10^4/\mu l$)	14.8 ± 3.3	12.2 ± 3.7	11.8 ± 3.5	0.079	0.029	0.720
Albumin (g/dl)	3.9 ± 0.3	3.7 ± 0.4	3.6 ± 0.4	0.122	0.085	0.782
Total bilirubin (mg/dl)	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.3	0.216	0.242	0.757
Prothrombin time (%)	76 ± 8	75 ± 12	75 ± 11	0.894	0.942	0.918
Hepaplastin test (%)	75 ± 11	74 ± 11	73 ± 13	0.872	0.817	0.907
Child-Pugh (A/B)	8/1	25/4	130/27	>0.999	>0.999	0.791
Tumor-related factor						
AFP (ng/ml)	51 ± 112	60 ± 98	81 ± 305	0.983	0.848	0.757
PIVKA-II (mAU/ml)	90 ± 83	258 ± 712	200 ± 696	0.491	0.640	0.744
Latency to recurrence (years)	2.6 ± 2.8	2.0 ± 2.0	2.2 ± 2.1	0.497	0.561	0.707
Recurrence site (intrahepatic/extrahepatic)	8/1	29/0	150/7			
Intrahepatic recurrence (single/multiple)	6/2	11/18	57/93			

Data are expressed as mean ± standard deviation.
IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

majority of first recurrence sites were residual liver [responders group: 89% (8/9), non-responders group: 100% (29/29), no-IFN group: 94% (150/157)] (Fig. 4B). In the responders group, among eight patients with intrahepatic recurrence, solitary recurrence was seen in six patients (75.0%). On the other hand, the percentage of solitary intrahepatic recurrence was 37.9% (11/29) in the non-responders group and 38.0% (57/150) in the no-IFN group. In the responders group, surgery, percutaneous therapy, and transarterial chemoembolization (TACE) was selected in three, four, and two patients for treatment of recurrence, respectively (Fig. 4C). The proportion of patients in whom surgery or percutaneous therapy was selected for treatment in the responders group (7/9, 77.8%) was significantly higher than that of the non-responders group (7/29, 24.1%, $P = 0.006$) and the no-IFN group (28/157, 17.8%, $P < 0.001$).

Figure 5 shows the overall survival from diagnosis of the first HCC recurrence in the three groups. The overall survival rate of the

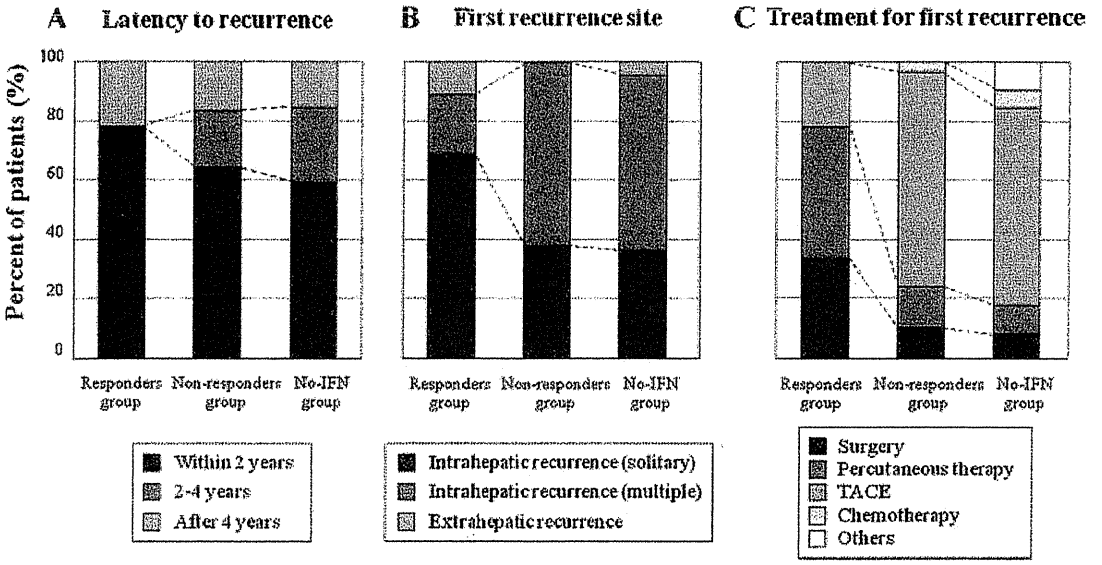

Fig. 4. A: Distribution of the latency from the initial hepatectomy to HCC recurrence for the responders, the non-responders, and the no-IFN group. B: Distribution of the first recurrence site in patients with HCC recurrence of the responders, the non-responders, and the no-IFN group. C: Distribution of selected treatment for first HCC recurrence in the responders, the non-responders, and the no-IFN group. IFN: interferon, TACE: transcatheter arterial chemoembolization.

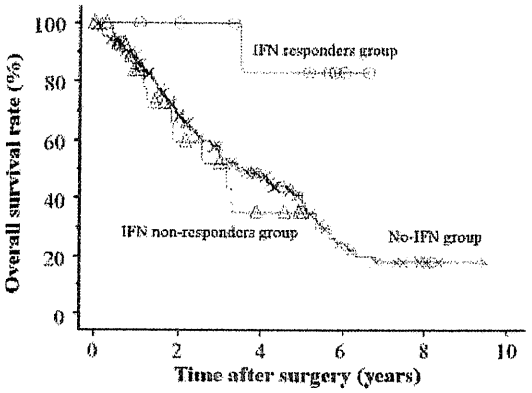

Fig. 5. Overall survival rates calculated from the diagnosis of first HCC recurrence in the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 9), open triangles: non-responders (n = 29), crosses: no-IFN group (n = 157). IFN: interferon.

responders group was significantly higher than that of the non-responders group ($P = 0.012$) and that of no-IFN group ($P = 0.011$).

DISCUSSION

The present study demonstrated that a significantly better DFS from the initial hepatectomy in the responders group than the other two groups. This result was similar to that reported previously by Uenishi et al. [16]. Based on the pattern of the DFS curve of the responders group in this study, the recurrence rate appeared to decrease mainly in 2 years later. We have reported that DFS curves for postoperative HCC patients in the early (within 2 years) and late (4 years after surgery) represented both intrahepatic metastasis and multicentric carcinogenesis, respectively [19]. Based on this viewpoint, the decrease in recurrence in the responders group was probably mainly due to the suppression of new multicentric carcinogenesis. A number of investigators have reported that suppression of increased liver inflammation, as assessed by AST and ALT, contributes to inhibition of hepatocarcinogenesis and postoperative intrahepatic recurrence after HCC surgery, which is more likely to originate from multicentric carcinogenesis [20,21]. IFN has been reported also to be effective in eradication of HCV-RNA from the serum and hepatic tissue and prevention of deterioration of liver dysfunction in patients with HCV infection [5–8,10]. It is possible that the suppression of new multicentric carcinogenesis seen in the IFN responders group of this study was due to these effects of IFN therapy. This speculation is supported by the findings of the present study that the levels of aminotransferases and platelet count in the responders group were significantly lower and higher, respectively, than those of the other groups, at the initial hepatectomy and first recurrence, and that the frequency of LC in the responders group was significantly lower than that of the other groups.

On the other hand, IFN has been reported to have anti-tumor effects [22–24]. These anti-tumor effects of IFN had been actually verified also in IFN-alpha/5-fluorouracil combination therapy for advanced HCC in a series of studies by our group [25–32]. Additionally, in a previous report by Uenishi et al. [16], only one patient developed postoperative recurrence about 5 years after the initial surgery among 11 patients of the responders group, and the recurrence pattern of the responders group was also suggestive of the inhibitory effect of IFN on metastasis originating from the primary HCC. Taken together, also in the present study, the decrease of recurrence might be potentially derived from the suppression of intrahepatic metastasis by IFN.

In the present study, overall survival from the initial hepatectomy in the responders group was also significantly better than those of the other two groups. This improvement of overall survival was caused by the aforementioned decrease of HCC recurrence rate in the responders group. In addition, in the responder group, the percentage of patients who underwent selective surgery or percutaneous therapy for the treatment of recurrent HCC was higher than other groups. In general, the treatment for the postoperative HCC recurrence is frequently restricted for the residual liver function, which is one of the reasons for the unfavorable postoperative outcome [3,4]. Considering such restriction of the treatment, the improved liver function by IFN therapy was also speculated to contribute to the better overall survival. Finally, it could be argued that IFN therapy was the main reason for the improvement in both DFS and overall survival rates in the responders group.

To date, several studies examined the impact of IFN therapy after curative loco-regional treatment for HCC [33–37]. For example, in a randomized controlled trial, Ikeda et al. [33] reported that IFN therapy suppressed tumor recurrence after surgery or ethanol injection for HCV-related HCC. Kubo et al. [36] also reported that postoperative IFN therapy significantly decreased recurrence after resection of HCV-related HCC in a randomized controlled trial. That several randomized controlled trials indicated improved posttreamtemt outcome in patients with HCV-related HCC who received postoperative IFN therapy, adds support to our conclusion of the effectiveness of preceding IFN therapy.

Since the present study is retrospective in nature, few details of IFN therapy are unavailable. For example, the duration of HCV-RNA clearance was not clear in several patients treated with IFN. Therefore, in this study, we could not divide patients of the responders group into those with SVR or not. In order to examine more strictly the effectiveness of preceding IFN therapy for surgical outcome, is prospectively designed study is necessary.

CONCLUSIONS

The present study demonstrated the effectiveness of IFN therapy for HCV infection administered before HCC resection as assessed by evaluating the disease-free and overall survival. IFN therapy for HCV might be essential not only for the treatment of HCV infection but also for improvement of prognosis of patients who are susceptible to the development of HCC.

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Association of Gene Expression Involving Innate Immunity and Genetic Variation in Interleukin 28B With Antiviral Response

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Innate immunity plays an important role in host antiviral response to hepatitis C viral (HCV) infection. Recently, single nucleotide polymorphisms (SNPs) of *IL28B* and host response to peginterferon α (PEG-IFNα) and ribavirin (RBV) were shown to be strongly associated. We aimed to determine the gene expression involving innate immunity in *IL28B* genotypes and elucidate its relation to response to antiviral treatment. We genotyped *IL28B* SNPs (rs8099917 and rs12979860) in 88 chronic hepatitis C patients treated with PEG-IFNα-2b/RBV and quantified expressions of viral sensors (*RIG-I, MDA5*, and *LGP2*), adaptor molecule (*IPS-1*), related ubiquitin E3-ligase (*RNF125*), modulators (*ISG15* and *USP18*), and *IL28* (*IFNλ*). Both *IL28B* SNPs were 100% identical; 54 patients possessed rs8099917 TT/rs12979860 CC (*IL28B* major patients) and 34 possessed rs8099917 TG/rs12979860 CT (*IL28B* minor patients). Hepatic expressions of viral sensors and modulators in *IL28B* minor patients were significantly up-regulated compared with that in *IL28B* major patients (≈3.3-fold, $P < 0.001$). However, expression of *IPS-1* was significantly lower in *IL28B* minor patients (1.2-fold, $P = 0.028$). Expressions of viral sensors and modulators were significantly higher in nonvirological responders (NVR) than that in others despite stratification by *IL28B* genotype (≈2.6-fold, $P < 0.001$). Multivariate and ROC analyses indicated that higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio were independent factors for NVR. IPS-1 down-regulation in *IL28B* minor patients was confirmed by western blotting, and the extent of IPS-1 protein cleavage was associated with the variable treatment response. *Conclusion:* Gene expression involving innate immunity is strongly associated with *IL28B* genotype and response to PEG-IFNα/RBV. Both *IL28B* minor allele and higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio are independent factors for NVR. (HEPATOLOGY 2012;55:20-29)

Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma in many patients.[1] Pegylated interferon α (PEG-IFNα) and ribavirin (RBV) combination therapy has been used to treat chronic hepatitis C (CH-C) to alter the natural course of this disease. However, 20% patients are nonvirological responders (NVR) whose HCV-RNA does not become negative during the 48 weeks of PEG-IFNα/RBV combination therapy.[2] In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near interleukin 28B

Abbreviations: CH-C, chronic hepatitis C; γ-GTP, γ-glutamyl transpeptidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HMBS, hydroxymethylbilane synthase; IL28, interleukin 28; IPS-1, IFNβ promoter stimulator 1; ISG15, interferon-stimulated gene 15; MDA5, melanoma differentiation associated gene 5; NVR, nonvirological responders; PEG-IFNα, pegylated interferonα; SNP, single nucleotide polymorphism; RIG-I, retinoic acid-inducible gene I; RBV, ribavirin; RNF125, ring-finger protein 125; ROC, receiver operator characteristic; SVR, sustained viral responder; TVR, transient virological responder; USP18, ubiquitin-specific protease 18; VR, virological responder.

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(*IL28B*) that encodes for type III IFNλ3 were shown to be strongly associated with a virological response to PEG-IFNα/RBV combination therapy.[3-5] In particular, the rs8099917 TG and GG genotypes were shown to be strongly associated with a null virological response to PEG-IFNα/RBV.[3] However, mechanisms involving resistance to PEG-IFNα/RBV have not been completely elucidated.

The innate immune system has an essential role in host antiviral defense against HCV infection.[6] The retinoic acid-inducible gene I (RIG-I), a cytoplasmic RNA helicase, and related melanoma differentiation associated gene 5 (MDA5) play essential roles in initiating the host antiviral response by detecting intracellular viral RNA.[7,8] The IFNβ promoter stimulator 1 (IPS-1)—also called the caspase-recruiting domain adaptor inducing IFNβ, mitochondrial antiviral signaling protein, or virus-induced signaling adaptor—is an adaptor molecule. IPS-1 connects RIG-I sensing to downstream signaling, resulting in *IFNβ* gene activation.[9-12] RIG-I sensing of incoming viral RNA has been shown to be modified by LGP2,[8,13] a helicase related to RIG-I and MDA5 lacking caspase-recruiting domain. The ubiquitin ligase ring-finger protein 125 (RNF125) has been shown to conjugate ubiquitin to RIG-I, MDA5, and IPS-1 and this suppresses the functions of these proteins.[14] Further, these molecules are ISGylated by the IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein,[15] and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18) to regulate the RIG-I/IPS-1 system.[16,17] Moreover, the NS3/4A protease of HCV specifically cleaves IPS-1 as part of its immune-evasion strategy.[9,18] Therefore, the RIG-I/IPS-1 system and its regulatory systems have essential roles in the innate antiviral response.

Recently, we demonstrated that baseline intrahepatic gene expression levels of the RIG-I/IPS-1 system were prognostic biomarkers of the final virological outcome in CH-C patients who were treated with PEG-IFNα/RBV combination therapy.[19] We found that up-regulation of *RIG-I* and *ISG15* and a higher expression ratio of *RIG-I/IPS-1* could predict NVR for subsequent treatment with PEG-IFNα/RBV combination therapy.[19] However, association of gene expression involving innate immunity and genetic variation of *IL28B* has not yet been elucidated. Hence, the aim of this study was to determine gene expression involving the innate immune system in different genetic variations of *IL28B* and elucidate the relation of gene expression to final virological outcome of PEG-IFNα/RBV combination therapy in CH-C patients.

Patients and Methods

Patients. Among histologically proven CH-C patients admitted at the Musashino Red Cross Hospital, 88 patients with HCV genotype 1b and a high viral load (>5 log IU/mL by TaqMan HCV assay; Roche Molecular Diagnostics, Tokyo, Japan) were included in the present study (Table 1). Patients with decompensated liver cirrhosis, autoimmune hepatitis, or alcoholic liver injury were excluded. No patient had tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody or had received immunomodulatory therapy before enrollment. Forty-two patients had been enrolled in a previous study that determined hepatic gene expression involving innate immunity.[19] Written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Treatment Protocol. The patients were administered subcutaneous injections of PEG-IFNα-2b (PegIntron, MSD, Whitehouse Station, NJ) at a dose of 1.5 $\mu g\ kg^{-1}\ week^{-1}$ for 48 weeks. RBV (Rebetol, MSD) was administered concomitantly over this treatment period, administered orally twice daily at 600 mg/day for patients who weighed less than 60 kg and 800 mg/day for patients who weighed between 60-80 kg. The dose of PEG-IFNα-2b was reduced to 0.75 $\mu g\ kg^{-1}\ week^{-1}$ when either neutrophil count was less than $750/mm^3$ or platelet count was less than $80 \times 10^3/mm^3$. The dose of RBV was reduced to 600 mg/day when the hemoglobin concentration decreased to 10 g/dL. More than 80% adherence was achieved in all patients.

Measurement of Hepatic Gene Expression. Liver biopsy was performed immediately before initiating

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Potential conflict of interest: Nothing to report.

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

Table 1. Patient Characteristics and *IL28B* Genotype

	IL28B Major*	*IL28B* Minor†	*P*-value‡
Patients, n	54	34	
Age (SD), year	58.8 (10.0)	59.1 (10.3)	0.918§
Sex, n (%)			0.051‖
Male	13 (24.1)	15 (44.1)	
Female	41 (75.9)	19 (55.9)	
BMI (SD), kg/m^2	22.7 (3.5)	23.5 (3.6)	0.193§
ALT (SD), IU/L	61.3 (50.7)	62.4 (44.7)	0.962§
γ-GTP (SD), IU/L	36.7 (25.9)	57.3 (52.4)	0.010§
LDL-cholesterol (SD), mg/dL	103.3 (29.8)	91.8 (26.9)	0.067§
Hemoglobin (SD), g/dL	14.1 (1.4)	14.4 (1.3)	0.186§
Platelet count (SD), ×10^{3}3/μL	161 (6.4)	163 (4.4)	0.489§
Fibrosis stage, n (%)			0.532‖
F1, 2	38 (70.4)	26 (76.5)	
F3, 4	16 (29.6)	8 (23.5)	
Viral load (SD), ×10^{63} IU/mL	1.7 (1.4)	1.9 (2.0)	0.788§
%HCV core 70 & 91 a.a. double mutation¶	8.9	43.5	0.001‖
%ISDR wild**	43.5	51.7	0.486‖
Viral response, n (%)			<0.001‖
SVR	17 (31.5)	13 (38.2)	
TVR	26 (48.1)	3 (8.8)	
NVR	11 (20.4)	18 (52.9)	

Unless otherwise indicated, data are given as mean (SD).

*rs8099917 TT and rs12979860 CC.

†rs8099917 TG and rs12979860 CT.

BMI, body mass index; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; SVR, sustained virological response; TVR, transient virological response; NVR, nonvirological response.

‡Comparison between *IL28B* major and minor genotypes.

§Mann-Whitney *U* test.

‖Chi-square test.

¶HCV core mutation was determined in 68 patients.

**ISDR was determined in 75 patients.

the therapy. After extraction of total RNA from liver biopsy specimens, the messenger RNA (mRNA) expression of the positive and negative cytoplasmic viral sensor (*RIG-I, MDA5*, and *LGP2*), the adaptor molecule (*IPS-1*), the related ubiquitin E3-ligase (*RNF125*), the modulators of these molecules (*ISG15* and *USP18*), and *IFNλ* (*IL28A/B*) was quantified by real-time quantitative polymerase chain reaction (PCR) using target gene-specific primers. In brief, total RNA was extracted by the acid-guanidinium-phenol-chloroform method using Isogen reagent (Nippon Gene, Toyama, Japan) from the liver biopsy specimen, which was 0.2-0.4 cm in length and 13G in diameter. Complementary DNA (cDNA) was transcribed from 2 μg of total RNA template in a 140-μL reaction mixture using the SYBR RT-PCR Kit (Takara Bio, Otsu, Japan) with random hexamer. Real-time quantitative PCR was performed using Smart Cycler version II (Takara Bio) with the SYBR RT-PCR Kit (Takara Bio) according to the manufacturer's instructions. Assays were performed in duplicate and the expression levels of target genes were normalized to the expressions of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene and hydroxymethylbilane synthase (*HMBS*), an enzyme that is stable in the liver, as quantified using real-time quantitative PCR as internal controls. For accurate normalization, a set of two housekeeping genes was used in the present study. Sequences of the primer sets were as follows: *RIG-I*, 5′-AAAGCATGCA TGGTGTTCCAGA-3′, 5′-TCATTCGTGCATGCTC ACTGATAA-3′; *MDA5*, 5′-ACATAACAGCAACATG GGCAGTG-3′, 5′-TTTGGTAAGGCCTGAGCTGG AG-3′; *LGP2*, 5′-ACAGCCTTGCAAACAGTACAAC CTC-3′, 5′-GTCCCAAATTTCCGGCTCAAC-3′; *IPS-1*, 5′-GGTGCCATCCAAAGTGCCTACTA-3′, 5′-CAGC ACGCCAGGCTTACTCA-3′; *RNF125*, 5′-AGGGCA CATATTCGGACTTGTCA-3′, 5′-CGGGTATTAAAC GGCAAAGTGG-3′; *ISG15*, 5′-AGCGAACTCATCT TTGCCAGTACA-3′, 5′-CAGCTCTGACACCGACA TGGA-3′; *USP18*, 5′-TGGTTCTGCTTCAATGACT CCAATA-3′, 5′-TTTGGGCATTTCCATTAGCACT C-3′; *IFNλ*: 5′-CAGCTGCAGGTGAGGGA-3′, 5′-G GTGGCCTCCAGAACCTT-3′; *GAPDH*, 5′-GCACC GTCAAGGCTGAGAAC-3′, 5′-ATGGTGGTGAAGA CGCCAGT-3′; *HMBS*, 5′-AAGCGGAGCCATGTCT GGTAAC-3′, 5′-GTACCCACGCGAATCACTCTCA-3′.

Genotyping for* IL28B *(rs8099917 and rs12979860) Polymorphism. Genetic polymorphism in a tagged SNP located near the *IL28B* gene (rs8099917 and rs12979860) was determined by direct sequencing of PCR-amplified DNA. In brief, after extraction from whole blood samples, genomic DNA was amplified by PCR. Sequences of the primer sets were: rs8099917, 5′-ATCCTCCTCTCATCCCTCA TC-3′, 5′-GGTATCAACCCCACCTCAAAT-3′; rs129 79860, 5′-GGACGAGAGGGCGTTAGAG-3′, 5′-AG GGACCGCTACGTAAGTCAC-3′.

Both strands of the PCR products were sequenced by the dye terminator method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Chiba, Japan); nucleotide sequences were determined by a capillary DNA sequencer ABI3730xl (Applied Biosystems). Homozygosity (rs8099917 GG and rs12979860 TT) or heterozygosity (rs8099917 TG and rs12979860 CT) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (rs8099917 TT and rs12979860 CC) was defined as having the *IL28B* major allele.

Western Blotting. Western blotting was performed using samples from 14 patients (six from *IL28B* major patients and eight from *IL28B* minor patients) as described.[19] In brief, liver biopsy specimens of