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Early Decrease in α-Fetoprotein, but Not Des-γ-Carboxy Prothrombin, Predicts Sorafenib Efficacy in Patients with Advanced Hepatocellular Carcinoma

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Key Words

Antitumor response · Chemotherapy · Des-γ-carboxy prothrombin · α-Fetoprotein · Hepatocellular carcinoma · Sorafenib · Tumor markers

Abstract

Objectives: The aim of this study was to investigate the relationships between early changes in the tumor markers α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP), and antitumor response in the early period following administration of sorafenib in patients with advanced hepatocellular carcinoma (HCC). ***Methods:*** Forty-eight advanced HCC patients were evaluated. AFP and DCP were measured at baseline, and after 2 and 4 weeks, and the antitumor responses were evaluated according to the RECIST criteria 4 weeks after starting sorafenib therapy. The ratios of each tumor marker were compared by stratifying the patients into the partial response (PR) + stable disease (SD) group or the progressive disease (PD) group. ***Results:*** Both 2 and 4 weeks after starting sorafenib therapy, the AFP ratio in the PR + SD group (n = 32) was significantly lower than in the PD group (n = 16; p = 0.002, p = 0.002). DCP was elevated in both the PR + SD group and the PD group 2 weeks and 4 weeks after starting sorafenib therapy. ***Conclusions:*** Evaluation of AFP ratios 2 and 4 weeks after starting sorafenib therapy may be useful for predicting antitumor response. On the other hand, early elevation of DCP does not necessarily suggest treatment failure by sorafenib, as DCP elevation can occur despite therapeutic efficacy.

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Introduction

Sorafenib is a molecularly targeted multikinase inhibitor that suppresses both signal transduction of tumor growth and angiogenesis by inhibiting Raf kinase, and VEGF and PDGF receptor kinase [1]. The SHARP Study and the Asia-Pacific Study [2, 3], two large-scale, phase III, clinical studies, demonstrated that sorafenib significantly prolongs time to progression (TTP) and improves overall survival (OS) in patients with advanced hepatocellular carcinoma (HCC), and confirmed its efficacy in improving prognosis in these patients for the first time as a systemic chemotherapeutic agent. Accordingly,

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sorafenib has been recognized as the only standard systemic chemotherapeutic agent for patients with advanced HCC for whom resection and local therapy are not indicated [4–6].

α-Fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are well-known and widely used serological tumor markers in the screening and diagnosis of HCC [7–11]. These tumor markers are also useful as indicators of the therapeutic effect by evaluating serial changes in these values before and after tumor resection and local ablation therapy. Although numerous studies have reported the relationships between the changes in tumor markers during treatment and antitumor response [12–19], there have been no comprehensive reports evaluating the relationship between prognosis and serial changes in AFP and DCP during treatment with sorafenib. Even in the SHARP Study and the Asia-Pacific Study, this relationship was not evaluated, despite the lack of systemic chemotherapeutic agents other than sorafenib that improve prognosis in advanced HCC.

Accordingly, we investigated cumulative TTP and OS stratified by antitumor effects based on image analysis, and assessed the relationship between antitumor effects and changes in AFP and DCP in the early period of sorafenib administration in patients with advanced HCC.

Patients and Methods

Patient Eligibility

Between July 2009 and December 2010, a total of 52 patients with advanced HCC were consecutively started on sorafenib (Nexavar®; Bayer Health Care Pharmaceuticals, West Haven, Conn., USA) therapy at the Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital. Inclusion criteria for this study were as follows: HCC was diagnosed either by needle biopsy or by the combination of typical radiological findings on dynamic multidetector row computed tomography (MDCT) and elevated AFP serum levels, according to the American Association for the Study of Liver Diseases [20]; patients were classified as having advanced HCC if they were not eligible for or had disease progression after surgical or locoregional therapies; Eastern Cooperative Oncology Group performance status score of 0–1; Child-Pugh liver function class A or B (≤7); adequate hepatic function (albumin level >2.5 g/dl, total bilirubin level <3.0 mg/dl, and alanine and aspartate aminotransferase levels <5 times the upper limit of normal); dynamic MDCT was obtained at baseline and after 4 weeks of sorafenib treatment in order to assess the therapeutic effects.

Of 52 patients, 48 patients meeting the inclusion criteria were enrolled. HCC stage was diagnosed according to the criteria of the Liver Cancer Study Group of Japan [21]. This study was approved by the Ethics Committee of the Musashino Red Cross Hospital and was performed in compliance with the Helsinki Declaration.

Sorafenib Therapy

The starting dosage of sorafenib was 800 mg/day p.o. However, out of concern regarding the possibility of having to discontinue sorafenib treatment at an early stage due to adverse events, the initial dosage was set at 400 mg/day for patients aged ≥80 years, and those with a body weight ≤40 kg or a history of treatment for varices or ascites. Sorafenib therapy was continued until the occurrence of potentially fatal adverse events.

Image-Based Evaluation of Antitumor Effects

Dynamic MDCT images were taken at baseline and after 4 weeks of sorafenib treatment. Tumor responses were defined as the time point response [(in accordance with the Response Evaluation Criteria In Solid Tumors (RECIST; version 1.1)] [22] 4 weeks after sorafenib administration where the confirmation of response was not required. Patients in whom the effect was rated as partial response (PR) or stable disease (SD) were pooled in the PR + SD group, while patients showing progressive disease (PD) comprised the PD group. MDCT images were obtained every 2–6 weeks after the first MDCT image, which was obtained 4 weeks after the start of sorafenib administration.

Measurement and Evaluation of Serum AFP and DCP

The HCC tumor markers analyzed were serum AFP and DCP at baseline, and 2 and 4 weeks after starting sorafenib administration. Because DCP levels are influenced by vitamin K and warfarin, patients ingesting these agents were excluded from DCP analysis. For each patient, the baseline concentration of each tumor marker was assigned a value of 1, and the ratios for each tumor marker 2 and 4 weeks after the start of administration were calculated.

Statistics

Statistical analyses were performed using Stat View J software (version 5; SAS Institute, Cary, N.C., USA). TTP and OS after the start of sorafenib administration were analyzed by the Kaplan-Meier method, while comparisons between the two patient groups were performed by log-rank test. Tumor marker levels were analyzed by Wilcoxon signed-rank test, and comparisons of the ratios for the tumor markers between the two patient groups were performed by the Mann-Whitney U test. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient Baseline Characteristics

Table 1 shows baseline characteristics of the 48 HCC patients enrolled in this study. The study cohort consisted of 38 males and 10 females, with a mean age of 69.9 ± 10.0 years. Six patients had never been treated for HCC, while the remaining 42 patients had previously undergone therapy. None of these previous treatments had involved molecularly targeted therapy. The starting dosage of sorafenib in this study was 800 mg/day in 26 patients and 400 mg/day in 22 patients. Criteria for starting sorafenib at 400 mg/day were as follows: (a) age ≥80 years

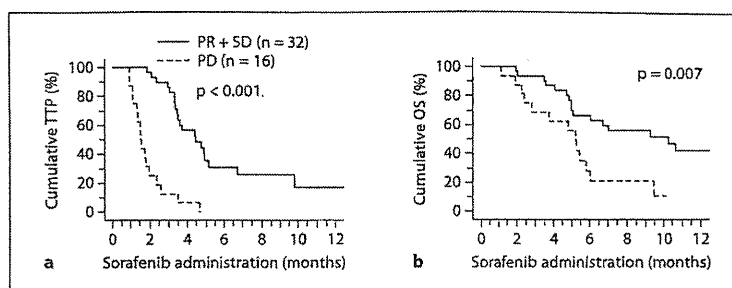

Fig. 1. Comparison of cumulative TTP (**a**) and OS (**b**) in the PR + SD and PD groups according to RECIST.

(n = 8); (b) body weight ≤40 kg (n = 2), and (c) history of treatment for varices or ascites (n = 12). The median baseline AFP level was 572 ng/ml (range, 2.3–148,000), and the median baseline DCP level was 424 mAU/ml (range, 15–305,000). The mean observation period was 7.2 ± 4.5 months.

Antitumor Responses 4 Weeks after the Start of Sorafenib Therapy

According to RECIST, 4 weeks after the start of sorafenib therapy, there were no complete responses, 2 PR, 30 SD, and 16 PD. The response rate was 4.2%, and the disease control rate was 66.7%.

Cumulative TTP and OS in the PR + SD and PD Groups

Cumulative TTP in the two groups according to RECIST is shown in figure 1a. The median observation period was 3.2 months. The median TTP was significantly longer in the PR + SD group than in the PD group (4.4 vs. 1.5 months; hazard ratio, 0.14; 95% CI, 0.06–0.29; $p < 0.001$).

Cumulative OS in the two groups according to RECIST is shown in figure 1b. The median observation period was 5.7 months. The median OS was significantly longer in the PR + SD group than in the PD group (10.3 vs. 5.2 months; hazard ratio, 0.36; 95% CI, 0.17–0.78; $p = 0.007$).

Comparison of Actual and Relative Levels of AFP at Baseline, and 2 and 4 Weeks after the Start of Sorafenib Therapy (Stratified by Antitumor Response)

AFP was not measured in 9 and 1 patients 2 and 4 weeks after starting sorafenib administration, respectively. Accordingly, AFP was analyzed in 39 and 47 patients 2 and 4 weeks after starting sorafenib administration, respectively.

Table 1. Baseline characteristics of the 48 HCC patients enrolled in this study

Mean age, years	69.9 ± 10.0
Male/female	38/10
HBV/HCV/NBNC	6/30/12
ECOG PS (0/1)	29/19
Child-Pugh score (5/6/7)	24/21/3
HCC stage (III/IVA/IVB)	11/18/19
Initial therapy/therapy for recurrence	6/42
Sorafenib starting dosage (800/400 mg)	26/22
Median serum AFP level, ng/ml	572
Range	2.3–148,000
Median serum DCP level, mAU/ml	424
Range	15–305,000
Mean observation period, months	7.2 ± 4.5

Numbers of patients are shown unless indicated otherwise. HBV/HCV = Hepatitis B/C virus; NBNC = non-HBV, non-HCV; ECOG = Eastern Cooperative Oncology Group; PS = performance status.

Data comparing actual AFP levels at baseline, and 2 and 4 weeks after starting sorafenib administration, both for the total patients and when stratified by antitumor response according to RECIST, are shown in table 2. Among the total number of patients, AFP showed no statistically significant differences between baseline and 2-week treatment levels, but in the PD group, AFP levels after 2 weeks of treatment were significantly elevated versus baseline levels ($p = 0.013$). Similarly, in the total number of patients, AFP showed no statistically significant differences between baseline and 4-week treatment levels, but in the PD group, AFP was significantly higher after 4 weeks of treatment compared with baseline levels ($p = 0.002$). In the PR + SD group, the median actual AFP level 4 weeks after starting sorafenib administration was higher than that at 2 weeks; however, there were no sig-

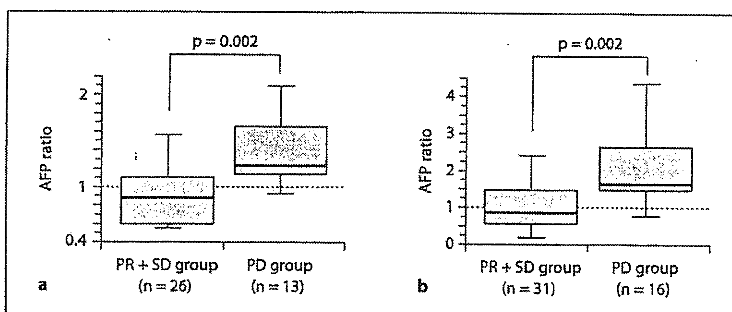

Fig. 2. AFP ratios 2 (**a**) and 4 weeks (**b**) after the start of sorafenib treatment in the PR + SD and PD groups according to RECIST.

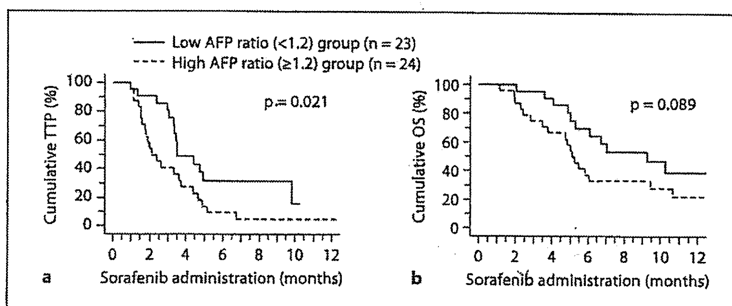

Fig. 3. Comparison of cumulative TTP (**a**) and OS (**b**) in the groups with low (<1.2) and high AFP ratio (≥1.2) 4 weeks after starting sorafenib therapy.

Table 2. Comparison of actual AFP levels (ng/ml) at baseline, and 2 and 4 weeks after the start of sorafenib therapy (stratified by antitumor response)

Groups	Baseline	After 2 weeks	p value	After 4 weeks	p value
Total	572 (2.3–148,000)	481 (2.2–163,300)	0.155	676 (1.1–281,700)	0.077
PR + SD	245.5 (2.3–148,000)	198 (2.2–163,300)	0.657	311 (1.1–281,700)	0.518
PD	2,321 (8.6–62,400)	3,303 (6.4–52,840)	0.013	6,258.5 (6.4–237,000)	0.002

nificant differences between AFP levels after 2 and 4 weeks (p = 0.423). On the other hand, in the PD group, the median actual AFP level 4 weeks after starting sorafenib administration was significantly higher than that after 2 weeks (p = 0.003).

Figure 2 compares the AFP ratios stratified by antitumor effects according to RECIST after 2 and 4 weeks of sorafenib treatment. AFP ratios 2 and 4 weeks after the start of sorafenib administration were 0.88 (range, 0.28–1.79) and 0.88 (range, 0.07–3.17) in the PR + SD group, and 1.24 (range, 0.74–2.12) and 1.63 (range, 0.64–7.35) in the PD group. At both time points, the ratio in the PR + SD group was significantly lower than in the PD group (p = 0.002, p = 0.002).

Cumulative TTP and OS in the Groups with Low and High AFP Ratio 4 Weeks after the Start of Sorafenib Therapy

The median AFP ratio 4 weeks after the start of sorafenib therapy was 1.2 (0.1–7.4).

Cumulative TTP (according to RECIST) in the groups with low (<1.2) and high AFP ratio (≥1.2) 4 weeks after the start of sorafenib therapy is shown in figure 3a. The median TTP was significantly longer in the low AFP (n = 23) ratio group than in the high AFP ratio group (n = 24; 3.5 vs. 2.1 months; hazard ratio, 0.46; 95% CI, 0.23–0.91; p = 0.021).

Cumulative OS in the low (n = 23) and high AFP ratio groups (n = 24) 4 weeks after the start of sorafenib therapy

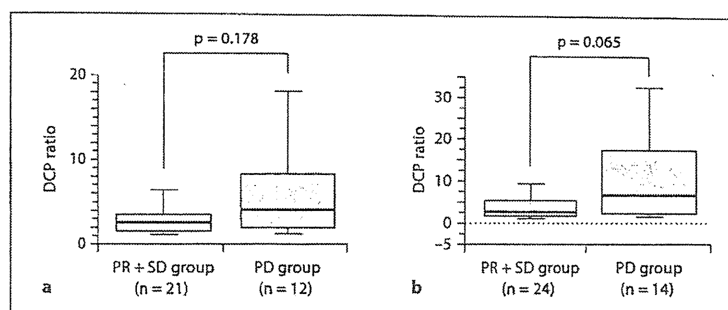

Fig. 4. DCP ratios 2 (**a**) and 4 weeks (**b**) after the start of sorafenib treatment in the PR + SD and PD groups according to RECIST.

Table 3. Comparison of actual DCP levels (mAU/ml) at baseline, and 2 and 4 weeks after the start of sorafenib therapy (stratified by antitumor response)

Patients	Baseline	After 2 weeks	p value	After 4 weeks	p value
Total	424.5 (15–305,000)	741 (26–798,000)	<0.001	2,025 (78–1,020,000)	<0.001
PR + SD	425.5 (15–216,000)	741 (30–323,000)	<0.001	1,715 (81–524,000)	<0.001
PD	575.5 (19–305,000)	6,186.5 (26–798,000)	0.002	20,550 (78–1,020,000)	0.001

is shown in figure 3b. The median OS tended to be higher in the low than in the high AFP ratio group (9.3 vs. 5.1 months; hazard ratio, 0.53; 95% CI, 0.25–1.12; p = 0.089).

Comparison of Actual and Relative Levels of DCP at Baseline, and 2 and 4 Weeks after the Start of Sorafenib Therapy (Stratified by Antitumor Response)

In the analysis of DCP, 7 patients who were taking vitamin K and 1 patient who was on warfarin were excluded. In addition, DCP was not determined in 7 and 2 patients 2 and 4 weeks after starting sorafenib administration, respectively. Accordingly, DCP was analyzed in 33 patients 2 weeks and in 38 patients 4 weeks after starting sorafenib administration.

Data comparing actual DCP levels at baseline, and 2 and 4 weeks after starting sorafenib administration, both for the total number of patients and patients stratified by antitumor response according to RECIST, are shown in table 3. Actual levels of DCP after 2 weeks of treatment were significantly higher than baseline levels in the total number of patients, the PR + SD group and the PD group. After 2 weeks of treatment, DCP was elevated in 97.0% (32/33) of the patients. Similarly, actual levels of DCP after 4 weeks of treatment were also significantly elevated from baseline levels in all patient groups; the total number of patients, the PR + SD group, and the PD group. After 4 weeks of treatment, DCP was elevated in 92.1% (35/38) of the patients.

Figure 4 compares the DCP ratios between the PR + SD and PD groups according to RECIST after 2 and 4 weeks of sorafenib therapy. The DCP ratios 2 and 4 weeks after the start of sorafenib administration were 2.57 (range, 0.87–10.02) and 2.72 (range, 0.30–13.46) in the PR + SD group, and 4.02 (range, 1.12–35.03) and 6.73 (range, 1.25–45.08) in the PD group. There were no significant differences between the PR + SD and PD groups at either time point (p = 0.178, p = 0.065).

Cumulative TTP and OS in the Low and High DCP Ratio Groups 4 Weeks after the Start of Sorafenib Therapy

The median DCP ratio 4 weeks after the start of sorafenib therapy was 4.0 (0.3–45.1).

Cumulative TTP (according to RECIST) in the low (<4) and the high DCP ratio (≥4) groups 4 weeks after the start of sorafenib therapy is shown in figure 5a. There were no significant differences in the median DCP ratio between the low (n = 19) and the high DCP ratio group (n = 19; 3.5 vs. 2.4 months; hazard ratio, 1.06; 95% CI, 0.51–2.19; p = 0.874).

Cumulative OS in the low (n = 19) and high DCP ratio groups (n = 19) 4 weeks after the start of sorafenib thera-

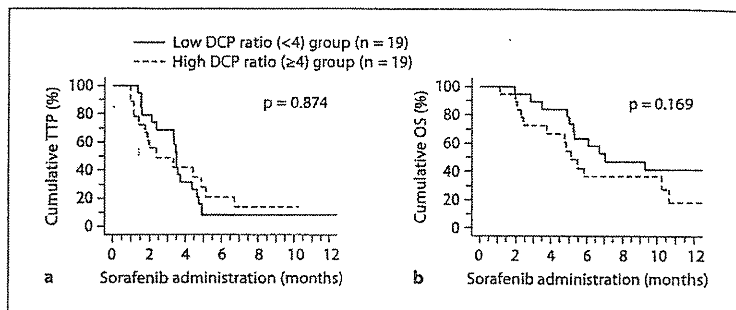

Fig. 5. Comparison of cumulative TTP (**a**) and OS (**b**) in the groups with low (<4) and high DCP ratio (≥4) 4 weeks after starting sorafenib therapy.

py is shown in figure 5b. There were no significant differences in median DCP ratio between the low and high DCP ratio groups (7.2 vs. 5.1 months; hazard ratio, 0.57; 95% CI, 0.25–2.55; p = 0.169).

Discussion

In the present study, we investigated the relationships between the changes in tumor markers, AFP and DCP, and antitumor responses in the early period following administration of sorafenib to patients with advanced HCC, and found that the relationship for AFP was different from that for DCP. With regard to AFP, both 2 and 4 weeks after starting sorafenib therapy, the ratio in the PR + SD group was significantly lower than in the PD group. On the other hand, DCP was elevated in both the PD group and the PR + SD group, and there were no statistically significant differences between the two groups either 2 or 4 weeks after starting sorafenib therapy. These results suggest that the time course changes for AFP in the early period after starting sorafenib administration are useful for predicting antitumor response assessed by image analysis.

Several studies have reported that the primary effect of sorafenib is inhibition of tumor growth rather than tumor shrinkage [2, 3, 23, 24]. In the SHARP Study, it was reported that the response rate in the sorafenib group based on RECIST criteria was only 2.3%, but both the cumulative TTP and cumulative survival duration were prolonged [2]. This can be considered an example of the limitations of using RECIST criteria, which focus on changes in the size of the entire tumor for evaluation of the therapeutic efficacy of molecularly targeted drugs. In the present study, cumulative TTP and OS were significantly better in the PR + SD group than in the PD group. In view of these findings, the primary clinical benefit of sorafenib is disease stabilization. Accordingly, it is important to evaluate treatment response in patients treated with sorafenib. In the present study, we analyzed the tumor marker response according to radiological response using the RECIST criteria. On the other hand, modified RECIST criteria were recently proposed as a method to assess arterial involvement [25]. Further investigation using these modified RECIST criteria is thus necessary.

In order to evaluate tumor responses, the formal recommendation of the panel of experts in HCC-Design Clinical Trials was to conduct imaging surveillance every 6–8 weeks using CT or MRI [4]. In our hospital, dynamic MDCT was obtained after 4 weeks of sorafenib treatment in order to assess early therapeutic effects. We found that antitumor responses 4 weeks after sorafenib administration correlated with both TTP and OS. Therefore, the present results indicate that it may be beneficial to evaluate the time point response 4 weeks after sorafenib administration in patients receiving sorafenib.

In the present study, AFP was significantly elevated in the PD group both 2 and 4 weeks after the start of administration compared with baseline. There has only been one report on AFP response after sorafenib therapy [26]. Shao et al. [26] reported the AFP responder group as patients whose AFP levels decreased to less than 0.8-fold of baseline levels within 1 month following sorafenib administration, while the non-responder group did not show this decrease. Consistent with our results, both the cumulative survival and TTP rates were significantly better in the AFP responder group than in the non-responder group. Hence, in the case of sorafenib therapy, changes in AFP levels may be correlated with the antitumor effects evaluated by image analysis, similarly to the course following other therapies for HCC, such as hepatic resection, radiofrequency ablation therapy, and transarterial chemoembolization. A comparison of the actual AFP levels 2 and 4

weeks after starting sorafenib administration in the PD group revealed that the median value after 4 weeks was significantly higher than that after 2 weeks. Even in the PR + SD group, the median value after 4 weeks was higher than that after 2 weeks. There were no significant differences between AFP levels after 2 and 4 weeks; thus, one of the reasons for this phenomenon was unevenness of AFP levels owing to the small sample size in this study.

With regard to DCP, there have been numerous reports that the time course change in DCP following treatment for HCC reflects therapeutic efficacy [17–19]. However, in the present study, we found that both the actual and relative levels of DCP were elevated in >90% of the patients, not only in the PD group but also in the PR + SD group, both 2 and 4 weeks after starting sorafenib therapy. To our knowledge, there have been no comprehensive clinical reports regarding the time course changes in DCP following sorafenib treatment. In a case report by Nakazawa et al. [27], DCP levels were markedly increased following treatment, even in patients who achieved a complete response on the basis of image analysis. From basic research, Murata et al. [28] reported that culturing a liver cancer cell line (HepG2) under hypoxic conditions resulted in increased DCP production by the cells. One possible mechanism for the increased DCP levels following sorafenib administration is that sorafenib-mediated inhibition of angiogenesis places tumor cells under hypoxic conditions, subsequently leading to increased DCP production. Thus, the increase in DCP levels following sorafenib administration may reflect HCC cell ischemia. Based on our results, increases in DCP soon after the start of sorafenib administration, regardless of antitumor effect, are not useful for assessing the antitumor responses, as DCP may increase in response to the ischemia caused by sorafenib.

Assessment by image analysis is the gold standard for evaluating antitumor responses of anticancer drugs [4, 22, 23]. However, such image analysis can be difficult in patients with multiple HCC lesions, vascular invasion, extrahepatic metastases, or ischemic tumors. In particular, patients in whom therapy using sorafenib is indicated are often in advanced stages of disease. There are limitations in using only radiological criteria to evaluate sorafenib treatment.

Our results suggest that the determination of early changes in AFP is useful for evaluating both antitumor response and prognostic efficacy of sorafenib, as assessed by TTP and OS, in patients with advanced HCC. In patients with advanced HCC treated with sorafenib, it is important to evaluate therapeutic efficacy as early as possible, as appropriate and early evaluation of sorafenib therapy can avoid unnecessary adverse events and allow second-line therapy when sorafenib therapy is not effective. In addition, determination of early changes in AFP is useful for evaluating the efficacy of new molecularly targeted agents currently under development. At present, there is no effective second-line treatment and we could not confirm whether continuing sorafenib administration would prolong the survival of patients with elevated AFP. Therefore, we cannot conclude that sorafenib therapy should be stopped in the case of elevated AFP ratio after 2 or 4 weeks of treatment. However, when an effective second-line treatment becomes available, an elevated AFP ratio may be a good indicator for switching to second-line therapy.

On the other hand, with regard to early changes in DCP, caution is required when assessing the antitumor response of sorafenib, as DCP elevation can occur irrespective of therapeutic effects.

In conclusion, our results suggest that early evaluation of AFP after starting sorafenib therapy is useful for predicting antitumor response. In contrast, early elevation of DCP does not necessarily suggest treatment failure of sorafenib. Appropriate and early evaluation of efficacy of sorafenib by AFP determination can provide valuable information that may influence subsequent decisions regarding patient management, thus avoiding unnecessary adverse events and allowing the opportunity for second-line therapy.

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Disclosure Statement

The authors declare that they have no financial conflicts of interest.

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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 80×10^3/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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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), $\times 10^3 3/\mu L$	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), $\times 10^{63}$ 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′-AAAGCATGCATGGTGTTCCAGA-3′, 5′-TCATTCGTGCATGCTCACTGATAA-3′; *MDA5*, 5′-ACATAACAGCAACATGGGCAGTG-3′, 5′-TTTGGTAAGGCCTGAGCTGGAG-3′; *LGP2*, 5′-ACAGCCTTGCAAACAGTACAACCTC-3′, 5′-GTCCCAAATTTCCGGCTCAAC-3′; *IPS-1*, 5′-GGTGCCATCCAAAGTGCCTACTA-3′, 5′-CAGCACGCCAGGCTTACTCA-3′; *RNF125*, 5′-AGGGCACATATTCGGACTTGTCA-3′, 5′-CGGGTATTAAACGGCAAAGTGG-3′; *ISG15*, 5′-AGCGAACTCATCTTTGCCAGTACA-3′, 5′-CAGCTCTGACACCGACATGGA-3′; *USP18*, 5′-TGGTTCTGCTTCAATGACTCCAATA-3′, 5′-TTTGGGCATTTCCATTAGCACTC-3′; *IFNλ*: 5′-CAGCTGCAGGTGAGGGA-3′, 5′-GGTGGCCTCCAGAACCTT-3′; *GAPDH*, 5′-GCACCGTCAAGGCTGAGAAC-3′, 5′-ATGGTGGTGAAGACGCCAGT-3′; *HMBS*, 5′-AAGCGGAGCCATGTCTGGTAAC-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′-ATCCTCCTCTCATCCCTCATC-3′, 5′-GGTATCAACCCCACCTCAAAT-3′; rs12979860, 5′-GGACGAGAGGGCGTTAGAG-3′, 5′-AGGGACCGCTACGTAAGTCAC-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

approximately 10 mg were homogenized in 100 μL of Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Next, 30 μg of protein was separated by NuPAGE 4%-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and blotted on polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-RIG-I (Cell Signaling Technology, Danvers, MA) or anti-IPS-1 (Enzo Life Science, Farmingdale, NY), followed by anti-β-actin (Sigma Aldrich, St. Louis, MO). After immunoblotting with horseradish peroxidase-conjugated secondary antibody, signals were detected by chemiluminescence (BM Chemiluminescence Blotting Substrate, Roche Applied Science, Mannheim, Germany). Optical densitometry was performed using ImageJ software (NIH, Bethesda, MD). Naive Huh7 cells were used for a positive control for full-length IPS-1, and cells transfected with HCV-1b subgenomic replicon[20] were used for a positive control for cleaved IPS-1.

Definitions of Response to Therapy. A patient negative for serum HCV-RNA during the first 6 months after completing PEG-IFNα-2b/RBV combination therapy was defined as a sustained viral responder (SVR), and a patient for whom HCV-RNA became negative at the end of therapy and reappeared after completion of therapy was defined as a transient virological responder (TVR). A patient for whom HCV-RNA became negative at the end of therapy (SVR + TVR) was defined as a virological responder (VR). A patient whose HCV-RNA did not become negative during the course of therapy was defined as an NVR. HCV-RNA was determined by TaqMan HCV assay (Roche Molecular Diagnostics).

Statistical Analysis. Categorical data were compared using the chi-square test and Fisher's exact test. Distributions of continuous variables were analyzed by the Mann-Whitney U test for two groups. All tests of significance were two-tailed and $P < 0.05$ was considered statistically significant.

Results

Patient Characteristics and IL28B *Genotype.* Table 1 shows patient characteristics according to *IL28B* genotype. SNPs at rs8099917 and rs12979860 were 100% identical; 54 patients were identified as having the major alleles (rs8099917 TT/rs12979860 CC; *IL28B* major patients) and the remaining 34 had the minor alleles (rs8099917 TG/rs12979860 CT; *IL28B* minor patients). Patients having a minor homozygote (rs8099917 GG or rs12979860 TT) were not found in this study, which is consistent with a recent report of the rarity of a minor homozygote in Japanese patients.[3] *IL28B* minor patients were significantly associated with a higher γ-glutamyl transpeptidase (γ-GTP) level and higher frequency of mutations at amino acid positions 70 and 91 of the HCV core region (glutamine or histidine mutation at amino acid position 70; methionine mutation at amino acid position 91). NVR rate was significantly higher in *IL28B* minor patients than in *IL28B* major patients.

Gene Expression Involving Innate Immunity and IFNλ *in the Liver.* Hepatic expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*) were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). Similarly, expressions of *ISG15* and *USP18* were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). In contrast, the hepatic expression of the adaptor molecule (*IPS-1*) was significantly lower in *IL28B* minor patients than that in *IL28B* major patients (Fig. 1). Hepatic expression of *RNF125* was similar among *IL28B* genotypes (Fig. 1). *IFNλ (IL28A/B)* expression was higher in *IL28B* minor patients, but not statistically significant (Fig. 1). Because expression of *RIG-I* and *IPS-1* were negatively correlated, the expression ratio of *RIG-I/IPS-1* in *IL28B* minor patients was significantly higher than in *IL28B* major patients (Fig. 1).

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, we compared levels of gene expression involving innate immunity and *IFNλ* based on the final virological response (Fig. 2). Overall, hepatic expressions of cytoplasmic viral sensors and the ISG15/USP18 system in NVR patients were significantly higher than those in VR patients. In a similar but opposite manner, hepatic expressions of *IPS-1* and *RNF125* in NVR patients were significantly lower than that in VR patients, and the expression of *IFNδ* was higher in NVR patients, but the differences were not statistically significant. Expression ratio of *RIG-I/IPS-1* was significantly higher in NVR patients than that in VR patients.

Because hepatic expressions of the RIG-I/IPS-1 and ISG15/USP18 systems were significantly related both to *IL28B* minor and NVR patients, *RIG-I* and *ISG15* expression levels and the *RIG-I/IPS-1* ratio between VR and NVR patients were further stratified by *IL28B* genotype (Fig. 3). Even in the subgroup of *IL28B* minor patients, the expressions of *RIG-I* and *ISG15* were significantly higher in NVR patients than those in VR patients. Similar tendencies were observed in a subgroup of *IL28B* major patients, in whom the *RIG-I/IPS-1* expression ratio was significantly higher in

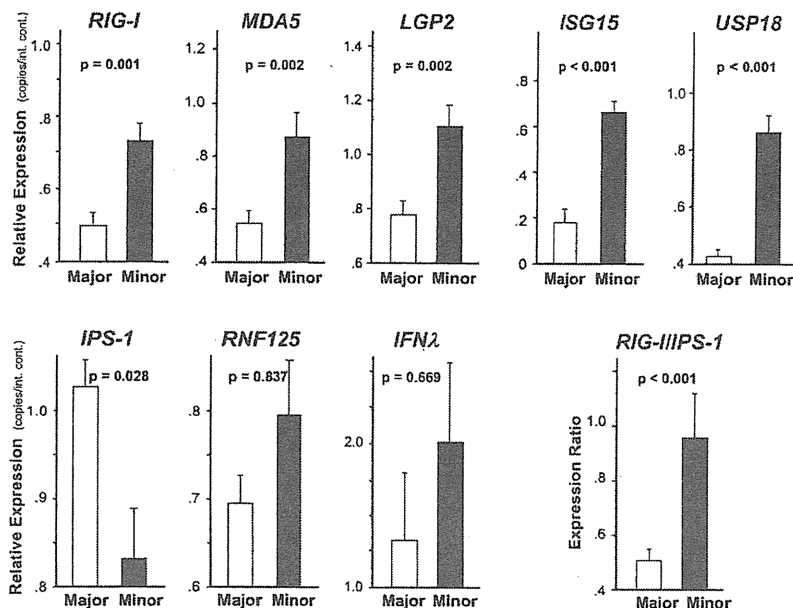

Fig. 1. Comparison of hepatic gene expression levels between *IL28B* major (rs8099917 TT/rs12979860 CC, n = 54) and *IL28B* minor patients (rs8099917 TG/rs12979860 CT, n = 34). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and expression ratio of the *RIG-I/IPS-1* are shown. Error bars indicate standard error. The *P*-values were determined by the Mann-Whitney *U* test.

NVR patients than in VR patients. However, in patients of the same virological response subgroup, *RIG-I* and *ISG15* expression levels and *RIG-I/IPS-1* ratio were higher in *IL28B* minor patients, and the difference in *ISG15* expression in subgroup of VR and NVR patients and that in *RIG-I/IPS-1* ratio in subgroup of VR patients was statistically significant between *IL28B* genotypes (Fig. 3).

Receiver Operator Characteristic (ROC) Analysis. To determine the usefulness of these gene quantifications and *IL28B* genotyping as predictors of NVR, an ROC analysis was conducted (Fig. 4A). The area under the ROC curve for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio was 0.712, 0.782, and 0.732, respectively, suggesting that quantification of these gene transcripts is useful for

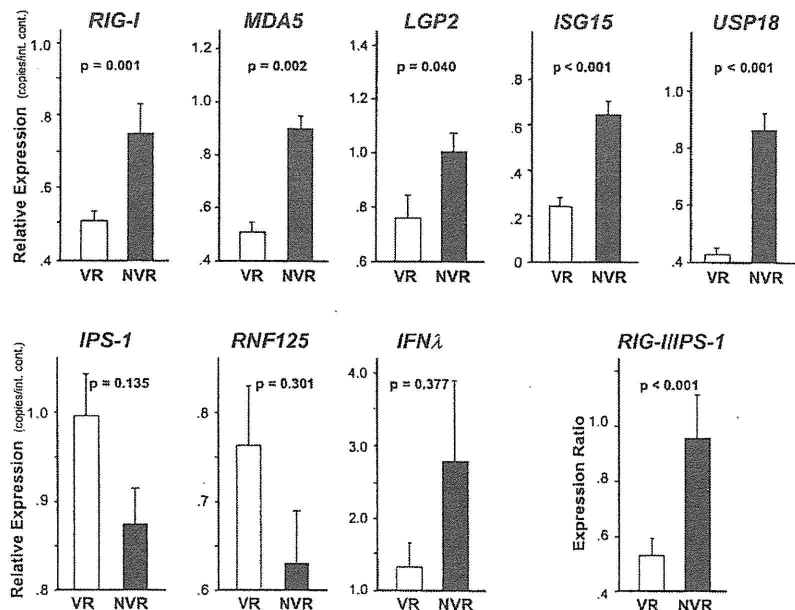

Fig. 2. Comparison of hepatic gene expression levels between virological responders (VR, n = 60) and nonvirological responders (NVR, n = 28). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The *P*-values were determined by the Mann-Whitney *U* test.

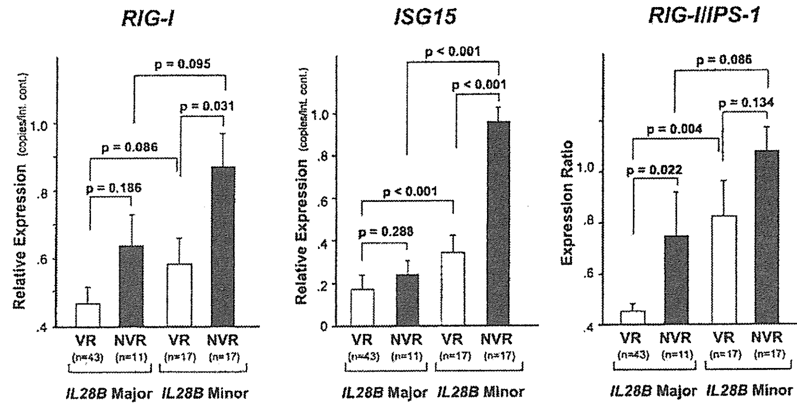

Fig. 3. Comparison of hepatic gene expression levels between virological responders (VR) and nonvirological responders (NVR) in subgroups of the *IL28B* genotype (*IL28B* Major, rs8099917 TT/rs12979860 CC; *IL28B* Minor, rs8099917 TG/rs12979860 CT). Expressions of *RIG-I* and *ISG15* as well as the *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The numbers of patients in each subgroup are shown in the bottom of the figure.

prediction of NVR (Table 2). The area under the ROC curve for *IL28B* genotype was 0.662, which was lower compared with that for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio.

When we stratified the patients by the cutoff value for *RIG-I* and *ISG15* expressions and *RIGI/IPS-1* ratio, no statistically significant difference was found in NVR rates among *IL28B* genotypes within the same subgroup (Fig. 4B).

Factors Associated with NVR. In univariate analysis, age, platelet counts, double mutation at amino acid positions 70 and 91 of the HCV core region, *IL28B* minor allele, and hepatic-expressions of *RIG-I*, *MDA5*, *LGP2*, *ISG15*, and *USP18*, and *RIG-I/IPS-1* ratio were significantly

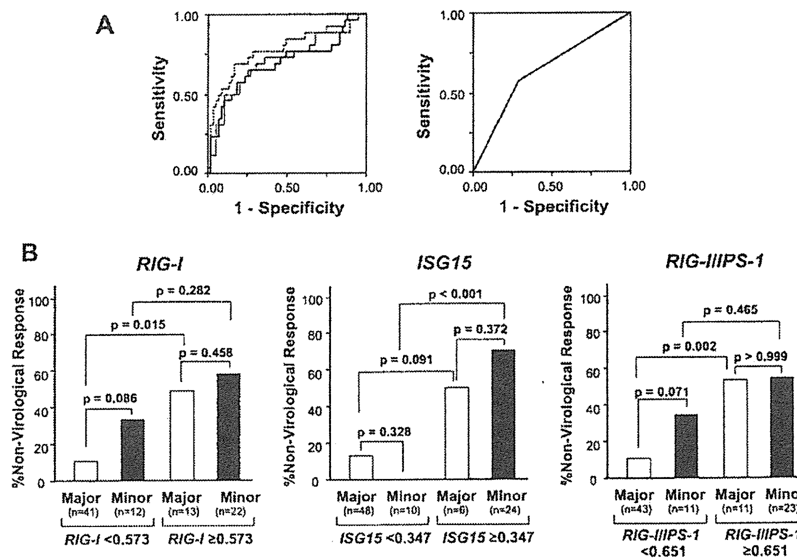

Fig. 4. (A) Receiver operator characteristics (ROC) curve for prediction of nonvirological response. ROC curves were generated to compare *RIG-I* (black line), *ISG15* (dotted line), and *RIG-I/IPS-1* ratio (gray line) (all in the left panel), and *IL28B* genotype (in the right panel). (B) Nonvirological response rate in *IL28B* major (rs8099917 TT/rs12979860 CC) and minor patients (rs8099917 TG/rs12979860 CT) in subgroups divided by the cutoff value of *RIG-I* and *ISG15* expression and the *RIG-I/ISG15* ratio determined by ROC analysis. Cutoff values of *RIG-I* and *ISG15* expression are expressed as expression copy number normalized to the expression of an internal control. The numbers of patients in each subgroup are shown in the bottom of the figure.

Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative as Well as Positive Predictive Values of Nonvirological Responses

Variables	AUC	95% CI	Cutoff	Sensitivity	Specificity	NPV	PPV
RIG-I (copies/int. control)	0.712	0.584-0.840	0.573	0.679	0.733	0.830	0.543
ISG15 (copies/int. control)	0.782	0.666-0.899	0.347	0.714	0.833	0.862	0.667
RIG-I/IPS-1 (copies/int. control)	0.732	0.611-0.852	0.651	0.679	0.750	0.833	0.559
IL28B genotype	0.662	0.537-0.787	TG*/CT†	0.607	0.717	0.796	0.500

AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.
*Genotype at rs8099917.
†Genotype at rs12979860.

associated with NVR (Table 3). Among these, multivariate analysis identified old age, HCV core double mutant, and higher hepatic expressions of *RIG-I* and *ISG15* as factors independently associated with NVR (Table 3).

IPS-1 and RIG-I Protein Expression in the Liver. Western blotting revealed that full-length and cleaved IPS-1 were variably present in all the samples from CH-C patients (Fig. 5A). Similar to mRNA

Table 3. Factors Associated with Nonvirological Response

Factors	Univariate Analysis		Multivariate Analysis*	
	Risk Ratio (95% CI)	*P*-value	Risk Ratio (95% CI)	*P*-value
Age (by every 10 year)	1.84 (1.10-3.14)	0.027	3.76 (1.19-11.7)	0.023
Sex				
Male	1			
Female	1.62 (0.59-4.42)	0.350		
BMI (by every 5 kg/m^2)	0.87 (0.46-1.65)	0.672		
Fibrosis stage				
F1/F2	1			
F3/F4	1.82 (0.69-4.85)	0.228		
Degree of steatosis				
<10%	1			
≥10%	1.46 (0.43-5.03)	0.544		
Albumin (by every 1 g/dL)	0.41 (0.11-1.56)	0.190		
AST (by every 40 IU/L)	0.89 (0.53-1.56)	0.681		
ALT (by every 40 IU/L)	0.85 (0.57-1.32)	0.481		
γ-GTP (by every 40 IU/L)	1.32 (0.82-2.07)	0.235		
Fasting blood sugar (by every 100 mg/dL)	1.35 (0.74-2.45)	0.340		
Hemoglobin (by every 1 g/dL)	0.93 (0.67-1.31)	0.683		
Platelet counts (by every 10^4/μL)	0.90 (0.82-0.99)	0.037	0.92 (0.78-1.08)	0.296
HCV load (by every 100 KIU/mL)	1.00 (1.00-1.00)	0.688		
Core 70 & 91 double mutation				
Wild	1		1	
Mutant	3.92 (1.14-13.5)	0.030	11.1 (1.40-88.7)	0.023
ISDR				
Nonwildtype	1			
Wildtype	1.38 (0.13-3.61)	0.513		
IL28B genotype				
Major allele†	1		1	
Minor allele‡	3.91 (1.52-10.0)	0.005	1.53 (0.20-11.9)	0.684
Hepatic gene expression (by every 0.1 copy/*int. control*)				
RIG-I	1.28 (1.10-1.50)	0.002	1.53 (1.07-2.22)	0.021
MDA5	1.53 (1.12-2.00)	0.001		
LGP2	1.34 (1.04-1.74)	0.026		
IPS-1	0.90 (0.78-1.04)	0.143		
RNF125	0.93 (0.83-1.04)	0.204		
ISG15	1.37 (1.16-1.62)	<0.001	1.28 (1.04-1.58)	0.021
USP18	1.67 (1.27-2.20)	<0.001		
IFNλ	1.02 (0.99-1.05)	0.170		
RIG-I/IPS-1 ratio (by every 0.1)	1.21 (1.07-1.36)	0.002		

Risk ratios for nonvirological response were calculated by the logistic regression analysis. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, IFN sensitivity determining region.
*Multivariate analysis was performed with factors significantly associated with nonvirological response by univariate analysis except for *MDA5*, *LGP2*, *USP18*, and *RIG-I/IPS-1* ratio, which were significantly correlated with *RIG-I* and *ISG15*.
†rs8099917 TT and rs12979860 CC.
‡rs8099917 TG and rs12979860 CT.

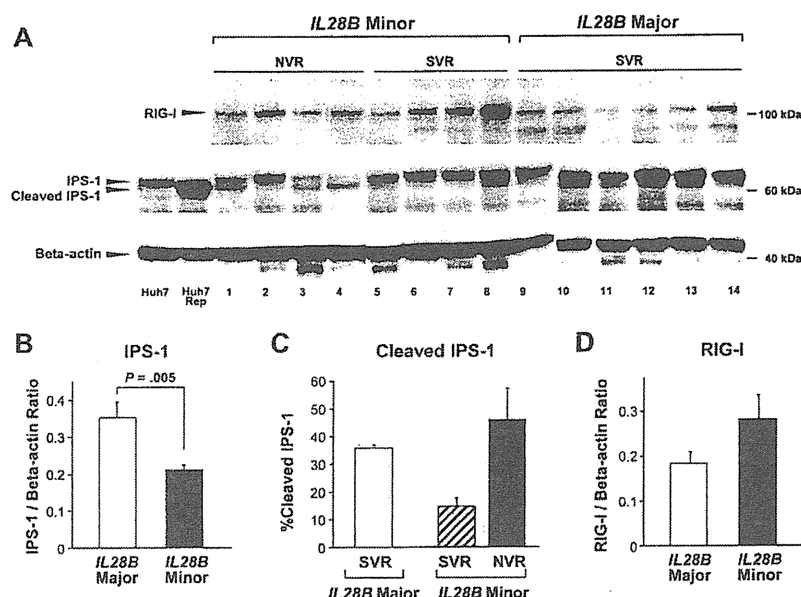

Fig. 5. (A) Western blotting for IPS-1 and RIG-I protein expression levels. Eight lanes contain samples from *IL28B* minor patients (lanes 1-8) and six lanes contain samples from *IL28B* major patients (lanes 9-14). Four lanes contain samples from nonvirological responders (NVR, lanes 1-4) and 10 lanes contain samples from sustained virological responders (SVR, lanes 5-14). Specific bands for RIG-I, full-length IPS-1, cleaved IPS-1, and β-actin are indicated by arrows. Naive Huh7 cells were used for a positive control for full-length IPS-1 (lane Huh7), and cells transfected with HCV-1b subgenomic replicon (Reference #20) were used for a positive control for cleaved IPS-1 (lane Huh7 Rep). (B) Total IPS-1 protein expression levels normalized to β-actin according to *IL28B* genotype. Error bars indicate standard error. *P*-value was determined by Mann-Whitney *U* test. (C) Percentage of cleaved IPS-1 products in total IPS-1 protein according to treatment responses stratified by *IL28B* genotype. Error bars indicate standard error. (D) RIG-I protein expression levels normalized to β-actin according to *IL28B* genotype. Error bars indicate standard error.

expression, total hepatic IPS-1 protein expression was significantly lower in *IL28B* minor patients than in *IL28B* major patients (Fig. 5B). With regard to *IL28B* minor patients, the percentage of cleaved IPS-1 protein in total IPS-1 in SVR was lower than that in NVR (Fig. 5C). In contrast to IPS-1 protein expression, hepatic RIG-I protein expression was higher in *IL28B* minor patients than that in *IL28B* major patients (Fig. 5D).

Discussion

In the present study we found that the baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the genetic variation of *IL28B* and final virological outcome in CH-C patients treated with PEG-IFNα/RBV combination therapy. Although the relationship between the *IL28B* minor allele and NVR in PEG-IFNα/RBV combination therapy is evident, mechanisms responsible for this association remain unknown. *In vitro* studies have suggested that cytoplasmic viral sensors, such as RIG-I and MDA5, play a pivotal role in the regulation of IFN production and augment IFN production through an amplification circuit.[7,8] Our results indicate that expressions of *RIG-I* and *MDA5* and a related amplification system may be up-regulated by endogenous IFN at a higher baseline level in *IL28B* minor patients. However, HCV elimination by subsequent exogenous IFN is insufficient in these patients, as reported,[19] suggesting that *IL28B* minor patients may have adopted a different equilibrium in their innate immune response to HCV. Our data are further supported by recent reports of an association between intrahepatic levels of IFN-stimulated gene expression and PEG-IFNα/RBV response as well as with *IL28B* genotype.[21-23]

In contrast to cytoplasmic viral sensor (*RIG-I, MDA5*, and *LGP2*) and modulator (*ISG15* and *USP18*) expression, the adaptor molecule (*IPS-1*) expression was significantly lower in *IL28B* minor patients. Moreover, western blotting further confirmed IPS-1 protein downregulation in *IL28B* minor patients by revealing decreased protein levels. Because IPS-1 is one of the main target molecules of HCV evasion,[9,18]

transcriptional and translational *IPS-1* expression are probably suppressed by HCV with resistant phenotype, which may be more adaptive in *IL28B* minor patients than in *IL28B* major patients. When we analyzed the proportion of full-length or cleaved IPS-1 to the total IPS-1 protein in a subgroup of *IL28B* minor patients, cleaved IPS-1 product was less dominant in SVR than in NVR, whereas uncleaved full-length IPS-1 protein was more dominant in SVR than in NVR. Therefore, the ability of HCV to evade host innate immunity by cleaving IPS-1 protein and/or host capability of protection from IPS-1 cleavage is probably responsible for the variable treatment responses in *IL28B* minor patients.

Our results indicated a close association between *IL28B* minor patients with higher γ-GTP level and higher frequency of HCV core double mutants, which are known factors for NVR. In contrast, no significant association was observed between *IL28B* genotype and age, gender, or liver fibrosis, which are also known to be unfavorable factors for virological response to PEG-IFNα/RBV. Therefore, certain factors other than the *IL28B* genotype may independently influence virological response. To elucidate whether gene expression involving innate immunity independently associates with a virological response from the *IL28B* genotype, we performed further analysis in a subgroup and conducted a multivariate regression and ROC analyses. Our multivariate and ROC analyses demonstrate that higher expressions of *RIG-I* and *ISG15* as well as a higher ratio of *RIG-I/IPS-1* are independently associated with NVR, and quantification of these values is more useful in predicting final virological response to PEG-IFNα/RBV than determination of *IL28B* genotype in each individual patients. However, the SVR rates in our patients were similar among *IL28B* genotypes, which suggests more SVR patients with the *IL28B* minor allele were included in the present study than those in the general CH-C population. Hence, our data did not necessarily exclude the possibility of the *IL28B* genotype in predicting NVR, although our multivariate analysis could not identify the *IL28B* minor allele as an independent factor for NVR. Interestingly, an association between *IL28B* genotype and expressions of *RIG-I* and *ISG15* as well as *RIG-I/IPS-1* expression ratio is still observed even in patients with the same subgroup of virological response (Fig. 3).

In the present study, although hepatic *IFNλ* expression was observed to be higher in *IL28B* minor and NVR patients, it was not statistically significant. Because *IL28B* shares 98.2% homology with *IL28A*, our primer could not distinguish the expression of *IL28B* from that of *IL28A*, and moreover, we could not specify which cell expresses *IFNλ* (i.e., hepatocytes or other immune cells that have infiltrated the liver). Therefore, the precise mechanisms underlying *IL28B* variation and expression of *IFNλ* in relation to treatment response need further clarification by specifying type of IFNλ and uncovering the producing cells.

In the present study we included genotype 1b patients because it is imperative to designate a virologically homogenous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have reported that the *RIG-I/IPS-1* ratio was significantly higher in NVR with HCV genotype 2.[19] However, our preliminary results indicated that baseline hepatic *RIG-I* and *ISG15* expression and the *RIG-I/IPS-1* expression ratio is not significantly different among *IL28B* genotypes in patients infected with genotype 2 (Supporting Figure). This may be related to the rarity of NVR with HCV genotype 2 and the lower effect of *IL28B* genotype on virological responses in patients infected with HCV genotype 2.[24] The association among treatment responses in all genotypes, the different status of innate immune responses, and *IL28B* genotype needs to be examined further.

Differences in allele frequency for *IL28B* SNPs among the population groups has been reported. The frequency of *IL28B* major allele among patients with Asian ancestry is higher than that among patients with European and African ancestry.[25] Because *IL28B* polymorphism strongly influences treatment responses within each population group,[5] our data obtained from Japanese patients can be applied to other population groups. However, the rate of SVR having African ancestry was lower than that having European ancestry within the same *IL28B* genotype.[5] Hence, further study is required to clarify whether this difference among the population groups with the same *IL28B* genotype could be explained by differences in expression of genes involved in innate immunity.

In a recent report, an SVR rate of telaprevir with PEG-IFNα/RBV was only 27.6% in *IL28B* minor patients.[26] Because new anti-HCV therapy should still contain PEG-IFNα/RBV as a platform for the therapy, our findings regarding innate immunity in addressing the mechanism of virological response and predicting NVR remain important in this new era of directly acting anti-HCV agents, such as telaprevir and boceprevir.

In conclusion, this clinical study in humans demonstrates the potential relevance of the molecules involved in innate immunity to the genetic variation

of *IL28B* and clinical response to PEG-IFNα/RBV. Both the *IL28B* minor allele and higher expressions of *RIG-I* and *ISG15* as well as higher *RIG-I/IPS-1* ratio are independently associated with NVR. Innate immune responses in *IL28B* minor patients may have adapted to a different equilibrium compared with that in *IL28B* major patients. Our data will advance both understanding of the pathogenesis of HCV resistance and the development of new antiviral therapy targeted toward the innate immune system.

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