Table 2 Comparison of host and viral factors between patients with and without HBeAg negative hepatitis in 19 patients whose ALT levels were normal at 2 years after SC

Clinical characteristics	HBeAg negative hepatitis		*P*
	Present ($n = 4$)	Absent ($n = 15$)	
Age at SC (years)†	41 (30–43)	37 (23–65)	0.549
Sex (male : female)	2:2	8:7	1.000
Follow-up period (years)†	9.1 (8.3–14.1)	12.2 (3.2–23.1)	0.610
Laboratory data at SC			
Albumin (g/dL)†	4.3 (3.8–4.3)	4.3 (3.7–4.7)	0.364
Bilirubin (mg/dL)†	1.0 (1.0–1.3)	0.8 (0.5–1.3)	0.083
Platelets (/μL)†	14.9 (13.3–16.4)	16.9 (9.6–22.5)	0.667
Events during follow-up period			
Initiation of NUC therapy‡	3 (75)	2 (13)	0.037
Development of HCC‡	1 (25)	1 (7)	0.386

†Data are expressed as median (range).
‡Data are expressed as number of patients (%).
ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; NUC, nucleoside/nucleotide analog; SC, seroconversion.

DISCUSSION

ALTHOUGH ACTIVE HEPATITIS usually subsides following HBeAg SC, it recurs in a considerable proportion of patients several years afterwards. Hsu *et al.*[5] followed 283 patients with HBeAg SC for a median follow-up period of 8.6 years and observed that ALT elevation of over twice the upper limit of normal occurred in 94 patients (33%). Of these, 68 (72%) were considered to have HBeAg negative hepatitis B because HBV DNA was detectable without the reappearance of HBeAg at the time of ALT elevation. HBeAg negative hepatitis is a major health concern because its occurrence is closely associated with progression to cirrhosis and development of HCC,[9–12] and thus prediction of its onset is important. Hsu *et al.*[5] found that patients with more frequent acute exacerbations of hepatitis before HBeAg SC and those with cirrhosis at the time of HBeAg SC had a higher risk of developing HBeAg negative hepatitis. Although significant, these factors were insufficient to accurately predict the occurrence of the disease.[26–30] Therefore, we analyzed several additional factors, including HBV DNA, HBsAg and HBcrAg levels, as well as viral mutations that halt HBeAg production.

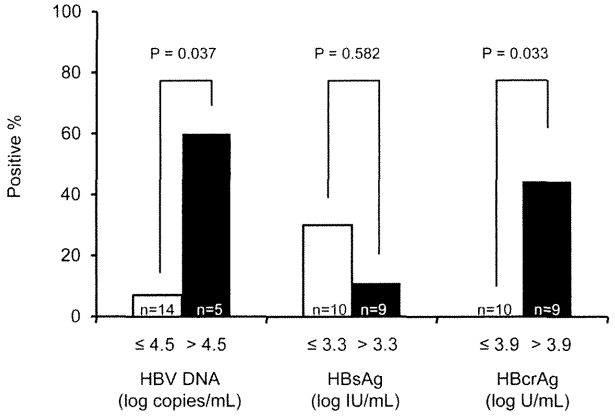

Figure 4 Occurrence of hepatitis B e-antigen (HBeAg) negative hepatitis is compared among patients using higher and lower levels of corresponding markers at 2 years after seroconversion (SC). The cut-off value for each marker was determined by receiver-operator curve analysis. HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

In the present study, we found that the majority of patients with HBeAg SC achieved normalization of ALT within 2 years following SC, after which such normalization became relatively rare. Abnormal ALT was determined using the distribution of integrated ALT level from 2 years after SC to the end of follow up, which clearly showed the existence of two groups. We defined patients with an abnormal integrated level of ALT as having HBeAg negative hepatitis because this abnormality tended to persist and was preceded by HBV DNA elevation. Our result also conferred the important realization that ALT abnormality within 2 years after SC may not necessarily indicate the occurrence of HBeAg negative hepatitis, which has a poor prognosis. NUC

therapy was not available for patients with chronic hepatitis B in Japan when our subjects began follow up. Hence, the natural history of SC has been evaluated in this cohort. Follow up stopped in this study when NUC therapy was commenced. Currently, we perform NUC therapy on patients with HBe negative hepatitis based on age and ALT activity, as advised by the Ministry of Health, Labor and Welfare.[17]

Many host and viral factors were also analyzed to predict the occurrence of HBeAg negative hepatitis in the current study. Host factors, including age and sex, did not differ between the groups with and without HBeAg negative hepatitis, but changes in median ALT level around SC clearly differed between the two groups. Specifically, ALT level did not decrease even after SC in patients with HBeAg negative hepatitis, while it normalized during the SC period in those without. Viral factors were analyzed at several time points around SC. Among them, median HBcrAg level clearly differed between the groups; HBcrAg showed a steep decrease around the SC period in patients without HBeAg negative hepatitis, while it exhibited a significantly slower decline in those with. Similarly to earlier reports, median levels of HBV DNA and HBsAg showed some differences between the two groups, but these were not remarkable when analyzed chronologically. Negative results were also seen in the analyses of PC and BCP mutations. Multivariate analysis showed that abnormal ALT level at 2 years after SC was the only significant factor to predict the occurrence of HBeAg negative hepatitis among the factors analyzed. Because patients with normal ALT had maintained that level for at least 1 year, this result may indicate that continuous normalization of ALT is rare in patients with HBeAg negative hepatitis after SC and that ALT abnormality is associated with higher levels of HBcrAg and HBV DNA.

Because ALT level was closely related to the occurrence of HBeAg negative hepatitis, we next analyzed for predictive factors in patients whose ALT level was normal (<31 IU/L) at 2 years after SC. We observed that increased HBV DNA and HBcrAg levels at 2 years after SC were significant factors for predicting the occurrence of HBeAg negative hepatitis, but that HBsAg level was not. Single or combined monitoring use of HBV DNA and HBcrAg levels may therefore be useful to predict the recurrence of hepatitis in patients whose ALT level normalizes following HBeAg SC. However, further studies are required to verify this in the clinical setting.

Whereas HBsAg is a serum marker commonly used for the diagnosis of HBV infection, HBcrAg assays measure serum levels of HBc, HBe and the 22-kDa precore antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these three denatured antigens.[31] Because the latter assay measures all antigens transcribed from the precore/core gene, it is regarded as core-related.[21] It has been suggested that viral antigen levels, including those of HBsAg and HBcrAg, are differently associated with HBV activity from HBV DNA and ALT levels, and thus are useful for predicting the future activity of hepatitis B. For example, HBcrAg level was seen to predict hepatitis relapse after discontinuation of NUC therapy,[32,33] and HBsAg level has been reportedly associated with the response to pegylated interferon therapy differently from HBV DNA.[34,35] Both antigen levels are believed to be related to intracellular levels of HBV cccDNA. However, it is possible that levels of HBsAg and HBcrAg have different roles in monitoring viral activity because the transcription of these two antigens is regulated by alternative enhancer–promoter systems in the HBV genome.[1] The serum level of HBcrAg was more useful than that of HBsAg to predict the occurrence of HBeAg negative hepatitis in the present study. This difference may be attributed to the fact that the production of all antigens that constitute HBcrAg is regulated by the same system as that of HBeAg, while the production of HBsAg is not.

Lastly, it is reasonable to presume that the PC and BCP mutations which halt HBeAg production are associated with integrated values of ALT elevation because the disease is essentially caused by HBV containing these mutations.[8,10] However, the prevalence of either mutation did not differ between the groups at any time point during the study. Our results showed that almost all patients had PC and/or BCP mutations, especially after SC, and implied that the existence of these mutations alone was not sufficient for developing ALT elevation. HBV genotype is also closely associated with HBeAg SC,[36] but we could not include genotype as a factor because our entire cohort was genotype C.

A recent review by Papatheodoridis *et al.*[37] showed that histologically significant liver disease is rare in HBeAg negative patients with persistently normal ALT based on stringent criteria and serum HBV DNA of 20 000 IU/mL or less. They suggest that such individuals can be considered as true inactive HBV carriers, who require continued follow up rather than liver biopsy or immediate therapy. On the contrary, liver biopsy samples obtained from eight of our patients with HBeAg negative hepatitis having elevated ALT levels after SC revealed necroinflammatory activity. Hence, it remains controversial if histological findings are important for diagnosis of HBeAg negative hepatitis.

This study has the main limitations of a retrospective design and a small cohort size. However, our findings from careful extended follow up indicate that ALT abnormality after 2 years from SC can be considered to be HBeAg negative hepatitis, and that HBcrAg and HBV DNA levels may be useful for predicting the long-term outcome of patients who achieve HBeAg SC and ALT normalization.

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Original Article

Serum levels of interleukin-22 and hepatitis B core-related antigen are associated with treatment response to entecavir therapy in chronic hepatitis B

Sadahisa Okuhara,[1] Takeji Umemura,[1] Satoru Joshita,[1] Soichiro Shibata,[1] Takefumi Kimura,[1] Susumu Morita,[1] Michiharu Komatsu,[1] Akihiro Matsumoto,[1] Kaname Yoshizawa,[1] Yoshihiko Katsuyama,[2] Masao Ota[3] and Eiji Tanaka[1]

[1]Department of Medicine, Division of Hepatology and Gastroenterology, [3]Department of Legal Medicine, Shinshu University School of Medicine, and [2]Department of Pharmacy, Shinshu University Hospital, Matsumoto, Japan

Aim: We sought to clarify the associations between serum cytokines and chemokines, hepatitis B surface antigen (HBsAg), hepatitis B core-related antigen (HBcrAg), and hepatitis B virus (HBV) DNA and response to entecavir therapy in chronic hepatitis B.

Methods: We analyzed six cytokines (interleukin [IL]-2, IL-6, IL-10, IL-12p70, IL-21 and IL-22) and five chemokines (CCL2, CCL3, CXCL9, CXCL10 and CXCL11) before and at 6, 12 and 24 months during entecavir therapy in 48 chronic hepatitis B patients. Quantitative measurement of HBsAg, HBcrAg and HBV DNA was performed. A virological response (VR) was defined as serum HBV DNA of less than 2.1 log copies/mL by treatment month 24.

Results: Thirty-nine patients (81%) achieved a VR. Serum IL-6 ($P = 0.031$), CXCL-9 ($P = 0.002$), and CXCL-10 ($P = 0.001$) were high in chronic HBV and correlated positively with transaminases and bilirubin. Before treatment, elevated IL-22 ($P = 0.031$) and lower HBsAg ($P = 0.001$) and HBcrAg ($P < 0.001$), but not HBV DNA, were associated with a favorable treatment outcome. In multivariate analysis, high IL-22 (hazard ratio = 13.67, $P = 0.046$) and low HBcrAg (hazard ratio = 10.88, $P = 0.048$) were independently associated with a VR. The levels of IL-22 ($P < 0.001$), HBsAg ($P < 0.001$), and HBcrAg ($P < 0.001$) all decreased from baseline to 24 months of treatment in virological responders.

Conclusion: Serum IL-22 and HBcrAg are predictive markers of a VR to entecavir therapy in patients with chronic hepatitis B.

Key words: entecavir, hepatitis B core-related antigen, hepatitis B surface antigen, hepatitis B virus, interleukin-22

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is the primary cause of cirrhosis and hepatocellular carcinoma (HCC) and is one of the major causes of death globally.[1,2] Because high plasma HBV DNA concentrations and quantitative hepatitis B surface antigen (HBsAg) levels are associated with progression to cirrhosis and development of HCC,[3,4] viral suppression by means of nucleoside/nucleotide analog therapy has shown clinical benefits via a reduction in hepatic decompensation and lower HCC rates.[5–7]

Cytokines and chemokines are involved in cell-mediated and humoral immune responses as well as in antiviral activity, viral clearance, apoptosis and fibrogenesis. As the control of cytokine production is highly complex and their effects widespread throughout multiple regulatory networks, it would seem that screening for multiple biomarkers may best clarify the immunopathogenesis of this disease and predict responses to antiviral therapy. Our previous studies have shown that several cytokines and chemokines are associated with treatment outcome in patients with chronic hepatitis C using bead-based multiplex immunoassays.[8–10] Although other reports have demonstrated an association between individual cytokines and clinical outcome in subjects with HBV,[11–18] the

Correspondence: Dr Takeji Umemura, Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. Email: tumemura@shinshu-u.ac.jp
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relationship between multiple cytokines and chemokines and response to nucleoside/nucleotide analog therapy in chronic hepatitis B patients has not yet been examined in the Japanese population.

The objective of this study is to determine which cytokines and chemokines in chronic hepatitis B are related to the clinical and virological characteristics of hepatitis and how they affect the HBV response to entecavir (ETV) treatment.

METHODS

Subjects

WE ENROLLED 48 consecutive patients with chronic hepatitis B in this study. All patients were treatment naïve at the time of commencing ETV at a daily dose of 0.5 mg for a duration of at least 24 months. Clinical and laboratory data of the patients were analyzed at baseline and at months 6, 12 and 24 of therapy. Chronic hepatitis B was based on HBsAg positivity for at least 6 months. No patients had a history of organ transplantation, decompensated cirrhosis, HCC or the concurrent use of immunomodulatory drugs or corticosteroids. Patients who were co-infected with the hepatitis C virus (HCV) or who exhibited evidence of other liver diseases, such as primary biliary cirrhosis, autoimmune hepatitis, alcoholic liver disease and non-alcoholic liver disease, were excluded from this study. A group of 10 healthy individuals negative for HBV and HCV serology and normal transaminase levels was used as the control. All patients and subjects were negative for antibodies to HIV type 1. The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine. All patients provided written informed consent.

Laboratory testing

Hepatitis B surface antigen, hepatitis B e-antigen (HBeAg), anti-HBe, anti-HCV and anti-HIV-1 were determined using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan).[19] Serum levels of HBV DNA were quantified using the COBAS TaqMan HBV Test v2.0 (Roche Diagnostics, Tokyo, Japan) that had a dynamic range of 2.1–9.0 log copies/mL. Quantitative measurement of HBsAg was performed using an HISCL HBsAg assay based on the chemiluminescence enzyme immunoassay (CLEIA; Sysmex, Kobe, Japan) which had a quantitative range of −1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range. Serum HB core-related antigen (HBcrAg) levels were measured using a CLEIA-based HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio, Tokyo, Japan). We expressed HBcrAg level in terms of log U/mL with a quantitative range set at 3.0–6.8 log U/mL. HBV genotypes were determined using commercially available ELISA kits (HBV GENOTYPE EIA; Institute of Immunology). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and other relevant biochemical tests were performed using standard methods.[20]

Definitions

A virological response (VR) was defined as a HBV DNA level that was undetectable by real-time polymerase chain reaction (<2.1 copies/mL) at 24 months. A virological breakthrough was defined as an increase in HBV DNA level by 1 log copies/mL or more above nadir while on treatment following an initial decline to 2 log copies/mL or more.

Detection of cytokines and chemokines

Six cytokines (interleukin [IL]-2, IL-6, IL-10, IL-12p70, IL-21 and IL-22) and five chemokines (CCL2/MCP-1, CCL3/MIP-1α, CXCL9/MIG, CXCL10/IP-10 and CXCL11/I-TAC) were quantified using Luminex Multiplex Cytokine Kits (Procarta Cytokine Assay Kit) for serum samples obtained before the start of treatment and at weeks 24, 48 and 96 as reported previously.[8,9] These markers had been implicated in HBV pathogenesis in earlier reports.[11–16,18] All collected samples were immediately stored at −70°C and remained in storage until testing.

Statistical analysis

The Mann–Whitney *U*-test and Kruskal–Wallis test were used to analyze continuous variables where appropriate. The Friedman test was employed to evaluate changes in serum cytokine levels over time. Spearman's rank correlation coefficients were adopted to evaluate the relationship between pairs of markers. The χ^2-test with Yates's correction was used for the analysis of categorical data. In cases where the number of subjects was less than five, we employed Fisher's exact test. $P < 0.05$ was considered statistically significant. To predict treatment outcome, cut-off points for continuous variables were decided by receiver–operator curve (ROC) analysis with Youden's index. Factors attaining a *P*-value of less than 0.1 in univariate analysis were evaluated by multivariate analysis using a stepwise logistic regression model. These

included age, HBe positivity, platelets, and levels of HBsAg, HBcrAg, HBV DNA and IL-22 before treatment. Statistical analyses were carried out using SPSS software version 21.0J (IBM Japan, Tokyo, Japan).

RESULTS

Baseline clinical characteristics of patients

THE CLINICAL PROFILE of the experimental patient cohort is shown in Table 1. Among our 48 patients with chronic hepatitis, 39 (81%) achieved a VR at 24 months. A VR was attained in 11 of 20 HBeAg positive patients (55%) and in all 28 HBeAg negative patients (100%). One patient (5%) demonstrated HBeAg seroclearance through to month 24, but did not attain HBeAg seroconversion. No patient experienced a virological breakthrough.

The median age of patients achieving a VR was significantly higher than that of patients who did not (55 vs 37 years; $P = 0.031$) (Table 1). In contrast, viral responders had significantly lower median HBsAg (3.3 vs 3.9 log IU/mL; $P = 0.001$) and HBcrAg (5.0 vs 6.8 log U/mL; $P < 0.001$) levels than non-responders. We found no significant differences between patient groups with regard to sex, HBV genotype, or albumin, AST, ALT, bilirubin or platelet levels. When stratified by HBeAg positivity, HBsAg level only was significantly associated with a VR (3.2 vs 3.9 log IU/mL; $P = 0.003$). When we compared HBeAg positive and negative patients, median HBV DNA and HBcrAg levels, but not HBsAg, were significantly higher in HBeAg positive patients (Table S1).

Detection and quantification of serum markers in patients with chronic hepatitis B and controls

Serum samples obtained prior to ETV therapy were examined for the presence of six cytokines and five chemokines by multiplex assays. As shown in Table 2, the median baseline serum concentrations of IL-6 (6.5 vs 5.8 pg/mL; $P = 0.031$) and three chemokines (CCL2 [39.3 vs 31.5 pg/mL; $P = 0.022$], CXCL9 [329.2 vs 127.8 pg/mL; $P = 0.002$] and CXCL10 [217.1 vs 58.7 pg/mL; $P = 0.001$]) were significantly higher in patients with chronic hepatitis B than in healthy controls. When we subdivided patients into HBeAg positive or anti-HBe positive groups, no significant differences in the median concentrations of any cytokine or chemokine were seen, including IL-22 (Table S1).

Effect of ETV therapy on serum cytokine levels

The median levels of serum cytokines and chemokines in our cohort are shown in Table 3. Among our patients, the median baseline serum IL-22 concentration was significantly higher in virological responders than in non-responders (35.3 vs 27.8 pg/mL; $P = 0.031$) (Fig. 1a). No other cytokines or chemokines were associated with

Table 1 Demographic and clinical characteristics of 48 patients with chronic hepatitis B

Characteristics	Total, $n = 48$	VR (+), $n = 39$	VR (−), $n = 9$	P
Age, years	55 (24–81)	55 (24–81)	37 (26–67)	**0.031**
Male, n (%)	33 (69)	29 (74)	4 (44)	0.18
HBeAg positive, n (%)	20 (42)	11 (28)	9 (100)	**<0.001**
HBV genotype C, n (%)	45 (94)	37 (95)	8 (89)	1.00
HBV DNA (log copies/mL)	6.6 (2.7 to >9.1)	6.4 (2.7 to >9.1)	8.0 (3.9 to >9.1)	0.06
HBsAg (log IU/mL)	3.4 (−1.2 to 4.5)	3.3 (−1.2 to 4.3)	3.9 (3.3–4.5)	**0.001**
HBcrAg (log U/mL)	5.2 (3.0–6.8)	5.0 (3.0–6.8)	6.8 (5.4–6.8)	**<0.001**
Albumin (mg/dL)	4.2 (2.3–5.3)	4.2 (3.1–5.3)	4.2 (2.3–4.5)	0.80
AST (IU/L)	48 (15–1476)	51 (15–1476)	36 (28–358)	0.82
ALT (IU/L)	49 (9–2021)	63 (9–2021)	56 (29–954)	0.74
Bilirubin (mg/dL)	0.8 (0.3–3.1)	0.8 (0.3–3.1)	0.7 (0.5–1.0)	0.33
Platelet (/μL)	16.3 (8.0–28.9)	15.2 (8.0–28.9)	19.5 (11.9–27.7)	0.053

Continuous variables are expressed as median values (range).
Bolded figures indicate statistical significance.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

Table 2 Serum cytokines and chemokines in patients with chronic hepatitis B and healthy subjects

Cytokine/chemokine	Patients	Controls	*P*-value
IL-2	2.3 (0–4.9)	2.1 (1.9–2.4)	0.42
IL-6	6.5 (2.7–19.1)	5.8 (5.8–6.5)	**0.031**
IL-10	1.1 (0.0–26.8)	1.4 (1.3–1.6)	0.49
IL-12p70	12.9 (0.1–22.0)	12.9 (12.8–12.9)	0.50
IL-21	12.5 (5.0–1916.5)	11.5 (10.5–253.5)	0.68
IL-22	34.9 (27.2–75.7)	33.6 (32.3–39.0)	0.47
CCL2	39.3 (23.8–8118.8)	31.5 (26.7–39.3)	**0.022**
CCL3	4.8 (0.0–651.8)	7.0 (5.0–9.9)	0.25
CXCL9	329.2 (89.8–18 758.9)	127.8 (107.5–874.3)	**0.002**
CXCL10	217.1 (18.6–3594.3)	58.7 (24.7–859.5)	**0.001**
CXCL11	40.8 (0.7–553.8)	25.8 (12.9–90.3)	0.23

Continuous variables are expressed as median values (range) (pg/mL).
Bolded figures indicate statistical significance.
IL, interleukin.

a VR. When stratified by HBeAg positivity, serum IL-22 and IL-6 levels in the VR group were significantly higher than those in the non-VR group (35.3 vs 31.2 pg/mL [$P = 0.046$] and 6.9 vs 6.1 pg/mL [$P = 0.031$], respectively).

Several clinical findings (HBV DNA, HBsAg, HBcrAg, albumin, AST, ALT, bilirubin and platelet) at baseline were examined for their correlation with serum cytokines or chemokines in patients with chronic hepatitis B. Serum IL-6, CXCL9, CXCL10 and CXCL11 were all positively correlated with values for AST, ALT and bilirubin, but were negatively correlated with serum HBsAg (Table 4). CXCL9, CXCL10 and CXCL11 were also significantly correlated with each other (data not shown). There was a negative correlation between HBsAg and AST, ALT and bilirubin (data not shown).

Prediction of VR in patients with chronic hepatitis B

We performed ROC analysis to determine the optimal cut-off values for serum IL-22, HBsAg and HBcrAg in predicting a VR for chronic HBV infection with the values obtained from the 39 patients who achieved a VR and the nine who did not. The selection of optimal cut-off point values was based on the IL-22, HBsAg and HBcrAg levels at which accuracy was maximal. Optimal cut-off value, sensitivity, specificity, positive predictive value, negative predictive value and calculated area

Table 3 Serum cytokines and chemokines in treatment outcome to antiviral therapy

Cytokine/chemokine	VR	Non-VR	*P*-value
IL-2	2.3 (0.0–4.9)	3.1 (0.0–3.3)	0.60
IL-6	6.8 (2.7–19.1)	6.1 (4.3–12.5)	0.22
IL-10	0.6 (0.0–26.8)	1.5 (0.0–5.0)	0.86
IL-12p70	12.9 (0.1–22.0)	12.9 (1.2–18.0)	0.74
IL-21	12.2 (5.0–1916.5)	19.9 (5.9–27.8)	0.70
IL-22	35.3 (27.2–75.7)	27.8 (27.3–46.7)	**0.031**
CCL2	40.8 (24.4–118.8)	34.8 (23.8–60.3)	0.13
CCL3	4.5 (0.0–651.8)	6.5 (2.7–22.9)	0.57
CXCL9	322.5 (115.4–18 758.9)	353.6 (89.8–1545.1)	0.60
CXCL10	206.3 (29.1–3594.3)	294.2 (18.6–2240.7)	0.94
CXCL11	39.9 (0.7–553.8)	48.8 (12.6–428.2)	0.80

Continuous variables are expressed as median values (range) (pg/mL).
Bolded figure indicates statistical significance.
IL, interleukin; VR, virological response.

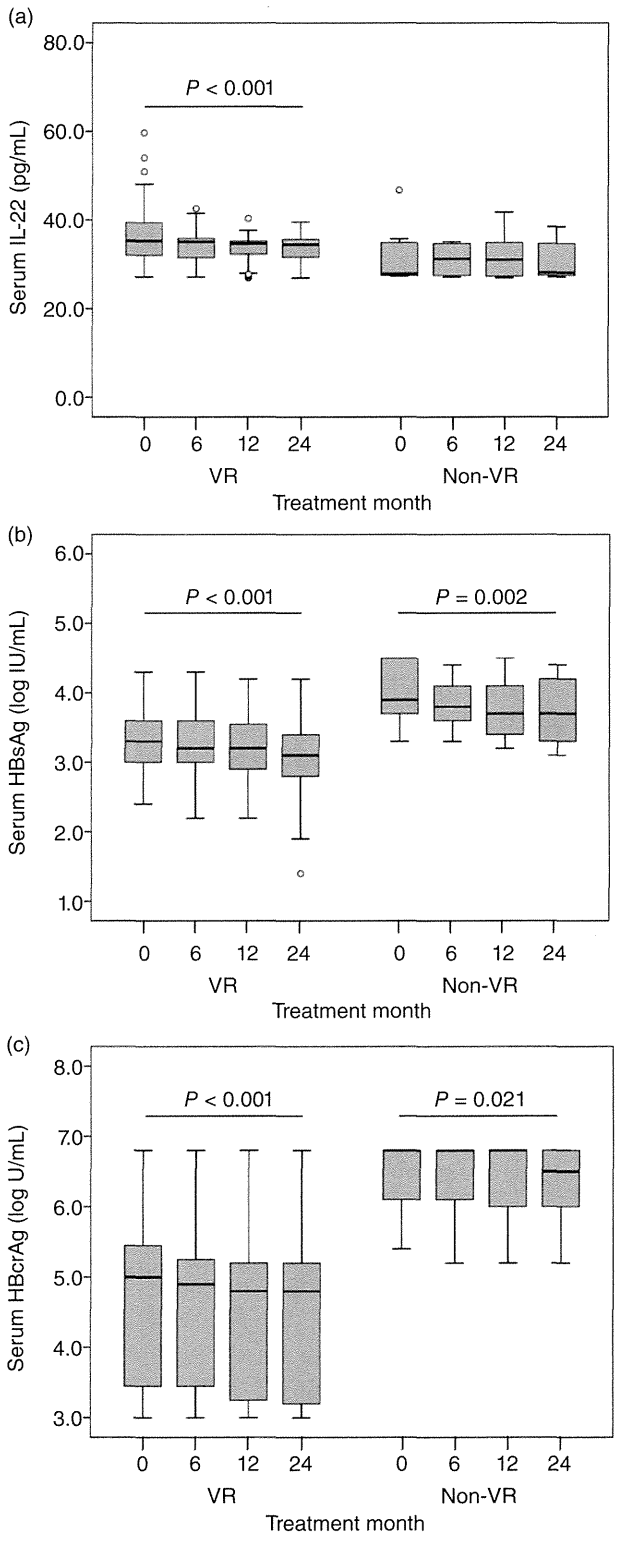

Figure 1 Comparison of serum (a) IL-22, (b) HBsAg and (c) HBcrAg levels during entecavir therapy in the VR ($n = 39$) and non-VR ($n = 9$) groups. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The harsh marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. IL, interleukin; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis core-related antigen; VR, virological response.

under the curve (AUC) values for each parameter are listed in Table 5. The AUC values were consistently high and ranged between 0.731 (IL-22) and 0.858 (HBcrAg).

Several factors found in association with a VR to ETV therapy were evaluated for their independence by multivariate analysis. We determined that IL-22 of 27.8 pg/mL or more (hazard ratio [HR] = 13.67 [95% confidence interval [CI] = 1.05–178.11], $P = 0.046$) and HBcrAg of 5.7 log U/mL or less (HR = 10.88 [95% CI = 1.02–115.44], $P = 0.048$) were independent factors related to a VR. HBsAg did not have a significant independent association in this study ($P = 0.071$).

Serum cytokine and chemokine changes during treatment

Longitudinal analysis of IL-22, HBsAg and HBcrAg levels was carried out at 6, 12 and 24 months after the initiation of therapy and showed significant gradual reductions in IL-22 ($P < 0.001$, Friedman test), HBsAg ($P < 0.001$) and HBcrAg ($P < 0.001$) in samples collected from patients who achieved a VR (Fig. 1). We noted a higher median serum IL-22 concentration at month 6 in the VR group than in the non-VR group ($P = 0.012$), and there were significant differences at each time point for HBsAg (6 months, $P = 0.002$; 12 months, $P = 0.006$; and 24 months, $P = 0.004$) and HBcrAg (6 months, $P < 0.001$; 12 months, $P < 0.001$; and 24 months, $P < 0.001$) between responders and non-responders.

DISCUSSION

IN THE PRESENT study, we measured the levels of six cytokines and five chemokines in patients with chronic hepatitis B and analyzed their association with ETV therapy outcome using a bead-array multiplex immunoassay system. Four of our observations are noteworthy and require further comment. First, serum IL-6, CCL2, CXCL9 and CXCL10 concentrations were

Table 4 Correlation between cytokines, chemokines and clinical parameters

		IL-2	IL-6	IL-10	IL-12	IL-21	IL-22	CCL2	CCL3	CXCL9	CXCL10	CXCL11
HBV DNA	*r*	0.08	0.01	0.10	0.06	0.08	0.17	−0.13	0.01	−0.13	−0.10	0.20
	P	0.61	0.97	0.51	0.69	0.61	0.25	0.39	0.95	0.39	0.50	0.18
HBsAg	*r*	−0.99	−0.35	−0.14	0.22	−0.08	−0.05	−2.5	0.02	−0.78	−0.61	−0.32
	P	0.51	**0.015**	0.35	0.14	0.61	0.74	0.09	0.89	**<0.001**	**<0.001**	**0.025**
HBcrAg	*r*	0.04	0.05	−0.16	0.24	0.18	0.14	−0.13	0.14	−0.14	−0.15	0.11
	P	0.79	0.76	0.29	0.11	0.21	0.35	0.40	0.33	0.36	0.31	0.45
Albumin	*r*	0.17	0.02	0.17	−0.02	0.05	−0.02	0.12	0.08	0.13	−0.09	0.02
	P	0.25	0.91	0.24	0.89	0.75	0.88	0.40	0.60	0.39	0.53	0.91
AST	*r*	0.05	0.40	0.11	−0.11	−0.03	0.14	0.13	−0.07	0.78	0.75	0.36
	P	0.72	**0.004**	0.45	0.47	0.83	0.33	0.39	0.66	**<0.001**	**<0.001**	**0.013**
ALT	*r*	0.02	0.42	0.12	−0.11	−0.06	0.16	0.10	−0.08	0.69	0.71	0.46
	P	0.91	**0.003**	0.40	0.44	0.70	0.28	0.52	0.57	**<0.001**	**<0.001**	**0.001**
Bilirubin	*r*	−0.03	0.36	0.07	0.08	−0.03	0.13	0.27	−0.12	0.33	0.65	0.35
	P	0.83	**0.012**	0.64	0.58	0.84	0.38	0.07	0.42	**0.023**	**<0.001**	**0.015**
Platelet	*r*	0.08	0.12	0.15	−0.09	0.13	0.25	−0.05	0.19	0.31	0.04	0.13
	P	0.57	0.42	0.33	0.55	0.38	0.09	0.74	0.20	**0.033**	0.82	0.39

Bolded figures indicate statistical significance.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IL, interleukin; *r*, Spearman's rank correlation.

higher in patients with chronic hepatitis B than in healthy subjects. Second, serum IL-22 concentration before treatment was significantly higher in patients achieving a VR to ETV therapy. In contrast, responders had lower serum levels of HBsAg and HBcrAg at baseline. Third, IL-22, HBsAg and HBcrAg decreased during treatment and remained low in patients with a VR. Fourth, serum IL-6, CXCL9, CXCL10 and CXCL11 were positively correlated with serum values of AST, ALT and bilirubin, but were negatively correlated with HBsAg.

Interleukin-6 is a well-recognized multifunctional cytokine that may reflect more active hepatic necroinflammation and be associated with chronic HBV infection severity. As in previous studies,[18,21] serum IL-6 was significantly higher in the HBV-infected group than in healthy controls and was positively correlated with such clinical parameters as transaminases and bilirubin. Hence, our data support that IL-6 is strongly associated with the severity of liver diseases.

CXCL9, CXCL10 and CXCL11 appear to be particularly important in chronic HCV infection by promoting the development of intrahepatic inflammation that leads to fibrogenesis.[22,23] These chemokines are also significantly elevated in patients with necroinflammatory activity of acute and chronic hepatitis C.[24,25] In our study, serum CXCL9 and CXCL10 were higher in patients with chronic HBV infection than in healthy individuals, which was in agreement with a previous

Table 5 Optimal cut-off value, sensitivity, specificity, AUC, and predictive value of serum IL-22, HBsAg and HBcrAg at baseline of treatment in 48 patients with chronic hepatitis B

	Cut-off value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
IL-22	27.8 pg/mL	56 (21–86)	90 (76–97)	0.731 (0.533–0.929)	90	56
HBsAg	3.6 log IU/mL	78 (40–97)	77 (61–89)	0.838 (0.704–0.971)	44	94
HBcrAg	5.7 log U/mL	89 (52–100)	82 (67–93)	0.858 (0.754–0.962)	53	97

All AUC values were significantly higher than a 0.50 non-predictive value ($P < 0.01$ for all comparisons). Cut-off values were determined by constructing receiver–operator curves.
AUC, area under the curve; CI, confidence interval; HBcrAg, hepatitis core-related antigen; HBsAg, hepatitis B surface antigen; IL, interleukin; NPV, negative predictive value; PPV, positive predictive value.

report.[12] Moreover, the serum CXCR3-associated chemokines CXCL9, CXCL10 and CXCL11 were all well correlated with serum values of AST, ALT and bilirubin. Because we observed a significant correlation between these chemokines and IL-6, our findings suggest that CXCR3-associated chemokines may too contribute to necroinflammatory activity in chronic HBV infection. However, there were insufficient histological data in our study to assess whether IL-6 and CXCR3-associated chemokines were correlated with degree of fibrosis, in addition to a lack of biochemical evidence of inflammation. We furthermore showed a striking negative association between HBsAg concentration and levels of IL-6 and CXCR3-associated chemokines. As HBsAg was also negatively correlated with transaminases and bilirubin, this HBsAg decline may be linked to increased immunological activity.

Interestingly, this study demonstrated a beneficial role of IL-22 in achieving a VR during ETV therapy. IL-22 is an IL-10 family cytokine that is important for the modulation of tissue responses during inflammation and is expressed by many types of lymphocytes of both the innate and adaptive immune systems, most notably T-helper 17 cells, γδ T cells, natural killer cells and lymphoid tissue inducer-like cells. The IL-22 receptor is highly expressed on hepatocytes.[26,27] At present, several studies support a protective role of IL-22 in the prevention of hepatocellular damage, although there is evidence indicating dual protective and pathogenic roles for this cytokine in the liver.[17,28–30] Some groups have examined the association between IL-22 and liver fibrosis in humans and mice.[31,32] In one report, tumor-infiltrating lymphocytes in HCC exhibited elevated IL-22 expression, and these IL-22$^+$ lymphocytes promoted tumor growth and metastasis in mice.[33] Although human patients with chronic hepatitis B show increased percentages of T-helper 17 cells in the peripheral blood and liver and an increased concentration of IL-22 in the serum,[14,34] there have been no reports on treatment outcome in patients with chronic HBV infection during ETV therapy. In our study, IL-22 levels decreased over time in both the VR and non-VR groups, but they were consistently higher in the VR group. This difference in IL-22 levels between the two groups further supports the possibility that IL-22 may be important for the activation of immune cells that contribute to viral control. When stratified by HBe positivity, although IL-22 was still significantly associated with a VR, the number of patients was only 20 in this study. Further research is needed to clarify the association between IL-22 and treatment response.

Lastly, we uncovered that lower baseline serum HBsAg and HBcrAg levels were associated with a VR. HBcrAg assays measure serum levels of HB core, e and 22-kDa precore antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these three denatured antigens.[35] Because this assay measures all antigens transcribed from the precore/core gene, it is regarded as core related.[36] The AUC values for baseline HBsAg and HBcrAg levels were high at 0.838 and 0.858, respectively. Several studies have shown that HBsAg is useful for the management of ETV therapy,[37,38] whereby an HBsAg decline is most profound in patients losing HBeAg detectability during treatment.[39] HBeAg positivity was also significantly associated with treatment outcome in the present study. However, because HBcrAg, but not HBsAg or HBeAg, was an independent factor related to a VR in multivariate analysis, our results indicated that serum HBcrAg quantitation may offer clinicians a new tool in predicting treatment outcome in HBV infection. Further investigation of large cohorts must be done to validate the significance of our findings.

With a VR at 12 months established as a parameter, 38 patients (79%) achieved this event. Serum IL-22, HBsAg and HBcrAg levels were all still significantly associated with a VR at 12 months. AUC values were as high as between 0.737 (IL-22) and 0.878 (HBcrAg). Furthermore, ALT normalization was achieved in 40 (83%) and 42 (88%) patients at 12 and 24 months, respectively. Although lower median pretreatment levels of HBsAg and HBcrAg were significantly associated with ALT normalization, there was no such statistically significant relation for IL-22 (data not shown).

In summary, a cytokine (IL-6) and several chemokines (CCL2, CXCL9 and CXCL10) were seen to be elevated in patients with chronic hepatitis B. Our results indicate that serum IL-6 and CXCR3-associated chemokines are correlated with liver injury, serum IL-22 is a useful biomarker for predicting a VR to ETV therapy, and a lower level of serum HBcrAg is related to a favorable response to antiviral therapy.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

Table S1 Demographic, clinical characteristics, and serum cytokines and chemokines in patients with hepatitis B e-antigen (HBeAg) positive and hepatitis B e-antigen (HBeAg) negative patients.

KIR, *HLA*, and *IL28B* Variant Predict Response to Antiviral Therapy in Genotype 1 Chronic Hepatitis C Patients in Japan

Yuichi Nozawa[1☯], Takeji Umemura[1*☯], Satoru Joshita[1], Yoshihiko Katsuyama[2], Soichiro Shibata[1], Takefumi Kimura[1], Susumu Morita[1], Michiharu Komatsu[1], Akihiro Matsumoto[1], Eiji Tanaka[1], Masao Ota[3*]

1 Division of Hepatology and Gastroenterology, Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan, **2** Department of Pharmacy, Shinshu University Hospital, Matsumoto, Japan, **3** Department of Legal Medicine, Shinshu University School of Medicine, Matsumoto, Japan

Abstract

Natural killer cell responses play a crucial role in virus clearance by the innate immune system. Although the killer immunoglobulin-like receptor (KIR) in combination with its cognate human leukocyte antigen (HLA) ligand, especially *KIR2DL3-HLA-C1*, is associated with both treatment-induced and spontaneous clearance of hepatitis C virus (HCV) infection in Caucasians, these innate immunity genes have not been fully clarified in Japanese patients. We therefore investigated 16 KIR genotypes along with *HLA-B* and *-C* ligands and a genetic variant of interleukin (IL) 28B (rs8099917) in 115 chronic hepatitis C genotype 1 patients who underwent pegylated-interferon-α2b (PEG-IFN) and ribavirin therapy. *HLA-Bw4* was significantly associated with a sustained virological response (SVR) to treatment (P = 0.017; odds ratio [OR] = 2.50,), as was the centromeric A/A haplotype of *KIR* (P = 0.015; OR 3.37). In contrast, SVR rates were significantly decreased in patients with *KIR2DL2* or *KIR2DS2* (P = 0.015; OR = 0.30, and P = 0.025; OR = 0.32, respectively). Multivariate logistic regression analysis subsequently identified the *IL28B* TT genotype (P = 0.00009; OR = 6.87, 95% confidence interval [CI] = 2.62 - 18.01), *KIR2DL2/HLA-C1* (P = 0.014; OR = 0.24, 95% CI = 0.08 - 0.75), *KIR3DL1/HLA-Bw4* (P = 0.008, OR = 3.32, 95% CI = 1.37 - 8.05), and white blood cell count at baseline (P = 0.009; OR = 3.32, 95% CI = 1.35 - 8.16) as independent predictive factors of an SVR. We observed a significant association between the combination of *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* in responders (P = 0.0019), whereas *IL28B* TT along with *KIR2DL2-HLA-C1* was related to a non-response (P = 0.0067). In conclusion, combinations of *KIR3DL1/HLA-Bw4*, *KIR2DL2/HLA-C1*, and a genetic variant of the *IL28B* gene are predictive of the response to PEG-IFN and ribavirin therapy in Japanese patients infected with genotype 1b HCV.

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* E-mail: tumemura@shinshu-u.ac.jp (TU); otamasao@shinshu-u.ac.jp (MO)

☯ These authors contributed equally to this work.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. Chronic HCV infection often develops into chronic hepatitis, which may progress to liver cirrhosis and/or hepatocellular carcinoma (HCC)[1]. HCC is a leading cause of death from malignant neoplasms in Japan[2]. Since approximately 70% of Japanese HCC patients are infected with HCV, the successful eradication of this virus, defined as a sustained virological response (SVR), is considered important to decrease the incidence of HCC.

Natural killer (NK) cells are key components of the innate antiviral immune response that are controlled by a balance of activation and inhibitory receptors. NK cell activation receptors include C-type lectin-like receptors (NKG2C, NKG2D, and NKG2E), natural cytotoxicity receptors (NKp30, NKp44, and NKp46), and CD16, while known inhibitory receptors include killer cell immunoglobulin-like receptors (KIRs) and the CD94/NKG2 family, which also contains a C-type lectin-like receptor (NKG2A) [3,4]. Sixteen *KIR* genes and pseudogenes have been identified that are encoded by a family of genes located on human chromosome 19q13.4. One particular feature of *KIR*s is their substantial genetic diversity. Some inhibitory *KIR*s

recognize human leukocyte antigen (HLA) class I molecules as their ligands; *KIR2DL1* recognizes *HLA*-C group 2 (*HLA-C2*) allotypes having lysine at amino acid position 80, whereas *KIR2DL2* and KIR2DL3 recognize *HLA-C* group 1 (*HLA-C1*) allotypes having asparagine at amino acid position 80 [5]. *KIR2DL2* and *KIR2DL3* also recognize HLA-B*4601 acquiring the-C1 epitope by gene conversion [6]. Furthermore, *KIR3DL1* recognizes subsets of *HLA*-A and *HLA*-B allotypes having the -Bw4 epitope determined by amino acid positions 77-83 [7].

It has been well documented that certain KIR-HLA receptor-ligand combinations are associated with susceptibility to infectious diseases, such as HCV, as well as with disease progression and treatment response [8-15]. Recent reports have also identified a relationship between interleukin (IL) 28B gene polymorphisms and treatment and spontaneous resolution of HCV infection[16-19]. Dring et al. observed that the presence of *IL28B* gene polymorphisms and *KIR* genotypes synergized to increase the risk of chronic HCV infection[20], although this finding is under debate[21]. Suppiah et al. [22] recently reported that genotyping for *IL28B*, *HLA*-C, and *KIR* genes was useful for predicting HCV treatment response in patients of European descent. As these gene associations have not yet been studied in the Japanese population, we evaluated whether HLA-KIR interactions, in addition to an *IL28B* polymorphism, would influence the outcome of pegylated-interferon-α (PEG-IFN) and ribavirin therapy in Japanese patients with chronic hepatitis C.

Materials and Methods

Ethics statement

This study was approved by the ethical committee of Shinshu University School of Medicine, Matsumoto, Japan, and written informed consent was obtained from all participants. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Subjects

One hundred and fifteen consecutive IFN-treatment-naïve patients with chronic hepatitis C were enrolled in this study. All subjects were seen at Shinshu University Hospital or one of its affiliated hospitals. The clinical and demographic characteristics of our cohort are shown in Table 1. Diagnosis of chronic hepatitis C was based on previously reported criteria [23]: 1) presence of serum HCV antibodies and detectable viral RNA; 2) absence of detectable hepatitis B surface antigen and antibody to the human immunodeficiency virus; and 3) exclusion of other causes of chronic liver disease or a history of decompensated cirrhosis or HCC. Serum levels of HCV RNA were determined using Cobas Amplicor assays (sensitivity: 50 IU/mL; Roche Diagnostic Systems, Tokyo, Japan). HCV genotypes were determined using INNO-LiPA HCV II kits (Innogenetics, Gent, Belgium). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests were performed using standard methods[24]. Liver fibrosis was assessed using the AST to platelet ratio index (APRI) in this study. APRI has been recognized as a noninvasive test to estimate the degree of liver fibrosis in chronic liver disease with HCV infection[25]. APRI was calculated for all study subjects as follows: AST/upper limit of normal (45 IU/L) × 100/platelet count (10^9/L). Patients received PEG-IFN-α2b (Pegintron; MSD KK, Tokyo, Japan; 1.5 μg/kg of body weight by subcutaneous injection once per week) and ribavirin (Rebetol; MSD KK; 600-1000 grams daily, according to body weight) for 48 weeks, as described previously[26]. Patients achieving a sustained HCV response were defined as those whose serum HCV RNA was undetectable 24 weeks after completing therapy. Patients who did not meet this criterion, who included non-responders and relapsers, were regarded as treatment failures.

Table 1. Clinical features of sustained and non-sustained virological response patients with chronic hepatitis C.

Characteristic	All	SVR	Non-SVR	*P*
	(n = 115)	(n = 56)	(n = 59)	
Age (yr)	60 (24 - 80)	59 (25 - 80)	60 (24 - 75)	0.43
Male	66 (57)	34 (61)	32 (54)	0.48
Alanine aminotransferase (IU/L)	46 (17 - 389)	48 (17 - 389)	45 (17 - 309)	0.81
Aspartate aminotransferase (IU/L)	43 (17 - 246)	42 (17 - 231)	43 (17 - 246)	0.49
White blood cells (/μL)	4410 (2280 - 8240)	4740 (2700 - 8170)	4070 (2280 - 8240)	0.011
Hemoglobin (g/dL)	14.4 (9.2 - 18.2)	15.1 (11.0 - 18.2)	13.9 (9.2 - 17.4)	0.002
Platelet count (10^4/μL)	15.9 (6.7 - 33.6)	16.6 (8.3 - 26.2)	15.6 (6.7 - 33.6)	0.30
APRI	0.89 (0.21 - 5.40)	0.59 (0.22 - 5.40)	0.66 (0.21 - 5.06)	0.41
HCV RNA ($\log_{10}$ IU/mL)	6.4 (5.0 - 7.3)	6.1 (5.0 - 6.8)	6.5 (5.0 - 7.3)	< 0.001

Data are expressed as median (range) or n (%) as appropriate. SVR, sustained virological response; HCV, hepatitis C virus

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HLA, KIR, and IL28B (rs8099917) Genotyping

Genomic DNA was isolated from whole blood samples using QuickGene-800 assays (Fujifilm, Tokyo, Japan). We genotyped *HLA*-B, *HLA*-C, and *KIR* using a Luminex multi-analyzer profiling system with a LAB type® HD and KIR SSO genotyping kit (One Lambda, Inc., Canoga Park, CA), which is based on PCR sequence-specific oligonucleotide probes[27]. Subjects were identified as having the B/x or A/A genotype as defined previously[28]. Genotypes for the centromeric (*Cen*) and telomeric (*Tel*) parts of the *KIR* locus were determined according to the presence or absence of one or more B haplotype-defining *KIR* genes. Thus, *Cen-A1* and *Tel-A1* were the centromeric and telomeric motifs, respectively, of the canonical *A KIR* haplotype in the present study, *Cen-B1* and *Cen-B2* were alternative centromeric motifs of common *B KIR* haplotypes, and *Tel-B1* was the common telomeric motif of *B* haplotypes[29]. For much of this analysis, *Cen-B1* and *-B2* were grouped together as *Cen-B*, whereas *Cen-A1* was shortened to *Cen-A* and *Tel-A1* to *Tel-A*, as reported

previously[30,31]. Genotyping of an *IL28B* SNP (rs8099917) was performed using a TaqMan 5' exonuclease assay with primers supplied by Applied Biosystems[32]. Probe fluorescence signals were detected using a TaqMan assay for Real-Time PCR (7500 Real Time PCR System, Applied Biosystems) according to the manufacturer's instructions.

Statistical Analysis

The Mann-Whitney *U* test was employed to analyze continuous variables. Pearson's chi-squared test was used for the analysis of categorical data. We adopted Fisher's exact test when the number of subjects was less than 5. The Bonferroni correction for multiple testing was applied to our data of KIR-HLA combinations using the number of comparisons performed by our primary factors of interest in Table 2 (i.e., 8 tests = 4 combinations × 2 comparisons between two groups). A P value of < 0.05 was considered to be statistically significant. Association strength was estimated by calculating the odds ratio (OR) and 95% confidence interval (CI). Our model was checked by regression diagnostic plots to verify normality, linearity of data, and constant variance. Stepwise logistic regression analysis with a forward approach was performed to identify independent factors associated with an SVR after continuous variables were separated into 2 categorical variables by each median value. Statistical analyses were performed using SPSS software version 21.0J (IBM, Tokyo, Japan). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to determine the reliability of the predictors of therapy response.

Results

Patient Characteristics and Treatment Outcome

All patients in our test cohort were infected with HCV genotype 1b. Of the 115 patients receiving PEG-IFN-α2b and ribavirin therapy, 56 (49%) achieved an SVR. The remaining 59 patients were non-responders, 28 of whom experienced a relapse and 31 who were null responders. The median white blood cell count (P = 0.011) and hemoglobin value (P = 0.002) in the SVR group were significantly higher than those in the non-SVR group prior to treatment. HCV viral load at baseline was significantly associated with treatment outcome (P < 0.001).

Association of HLA and KIR with a Sustained Virological Response

We first determined the frequency of *HLA-Bw* and *HLA-C* alleles in SVR and non-SVR patients (Figure 1). The frequency of *HLA-Bw4Bw6* in responders was significantly higher than that in non-responders (55% [31/56] vs. 36% [21/59]; P = 0.033; OR = 2.24, 95% CI = 1.06 - 4.75). Conversely, patients with the *HLA-Bw6* homozygote had a higher non-SVR rate (32% [18/56] vs. 54% [32/59]; P = 0.017; OR = 0.40, 95% CI = 0.19 - 0.85). Overall, *HLA-Bw4* was associated with an SVR among patients (68% [38/56] vs. 46% [27/59]; P = 0.017; OR = 2.50, 95% CI = 1.17 - 5.35). The frequencies of HLA-C were not statistically significant. We further checked whether particular HLA-Bw or HLA-C alleles were beneficial to treatment outcome. The HLA-B*35:01 allele was more frequently found in patients with an SVR than in those without (13% [15/102] vs. 4% [5/118]; P = 0.014 [Pc = 0.36]; OR = 3.49, 95% CI = 1.23 - 9.97).

Table 2. Frequency of *IL28B* genotype, *KIR3DL1*/*HLA-Bw4*, and *KIR2DL2*/*HLA-C1* combinations in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

KIR3DL1/ HLA-Bw4	*KIR2DL2/HLA-C1*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
+/+	+/+	5 (9%)	7 (12%)	0.61	
+/+	Other	31 (55%)	19 (32%)	0.012 (0.1)	2.61 (1.22 - 5.58)
Other	+/+	1 (2%)	10 (17%)	0.014 (0.12)	0.09 (0.01 - 0.72)
Other	Other	19 (34%)	23 (39%)	0.57	
IL28B	*KIR3DL1/ HLA-Bw4*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
TT	+/+	27 (48%)	13 (22%)	0.003 (0.024)	3.29 (1.47 - 7.39)
TT	Other	17 (30%)	14 (24%)	0.42	
TG/GG	+/+	9 (16%)	13 (22%)	0.42	
TG/GG	Other	3 (5%)	19 (32%)	0.00062 (0.0005)	0.12 (0.03 - 0.43)
IL28B	*KIR2DL2/ HLA-C1*	SVR	Non-SVR	P (Pc)	OR (95% CI)
		(n = 56)	(n = 59)		
TT	Other	38 (68%)	18 (31%)	0.000062 (0.0005)	4.81 (2.19 - 10.58)
TT	+/+	6 (11%)	9 (15%)	0.47	
TG/GG	Other	12 (21%)	24 (41%)	0.026 (0.21)	0.40 (0.17 - 0.91)
TG/GG	+/+	0 (0%)	8 (14%)	0.013 (0.1)	-

Data are expressed as n (%).

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The distribution of *KIR* genes and their association with treatment outcome are shown in Figure 2. No statistically significant differences were found for any allele combination apart from *KIR2DL2* and *KIR2DS2*; patients with these genes had significantly decreased SVR frequencies compared with those without (P = 0.015 [Pc = 0.48]; OR = 0.30, 95% CI = 0.11 - 0.82 and P = 0.025 [Pc = 0.8]; OR = 0.32, 95% CI = 0.12 - 0.90, respectively).

KIR genotype profiles were determined by the presence or absence of each *KIR* locus in patients (Figure 3). Since strong linkage disequilibrium is a prominent feature in the *KIR* region, *KIR* gene profiles were classified based on *Cen* and *Tel* motifs. When we evaluated SVR according to genotype and *Cen* and *Tel* frequencies, we observed that virologic clearance with *Cen-A/A* was significantly higher than that without (54% [50/92] vs.

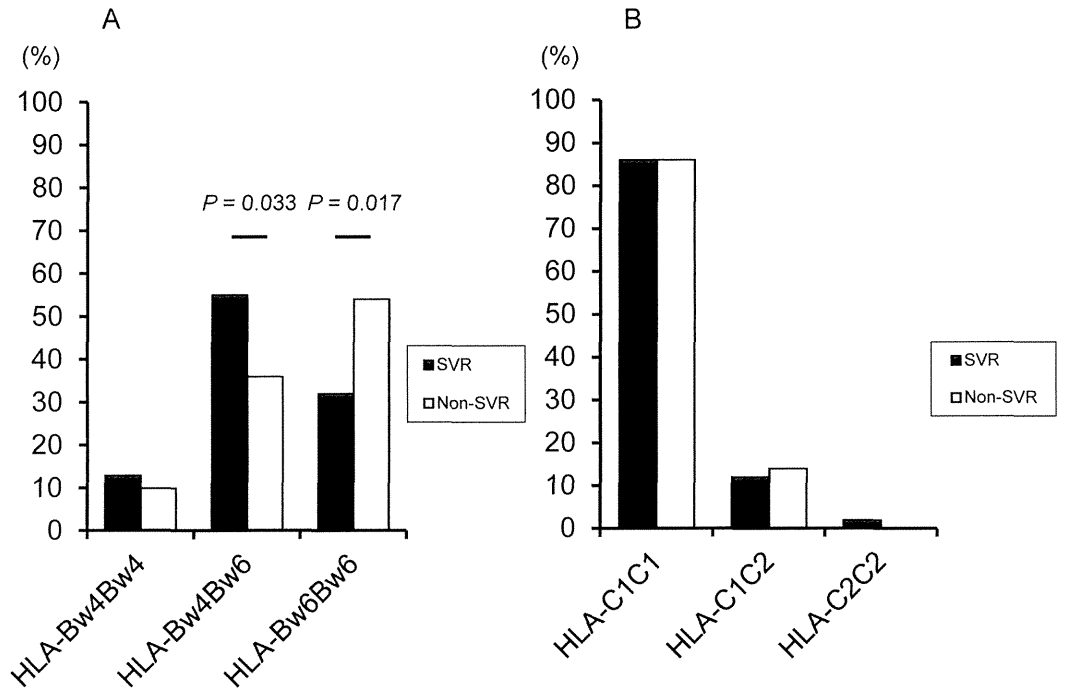

Figure 1. Frequency of *HLA-Bw* and -C alleles in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

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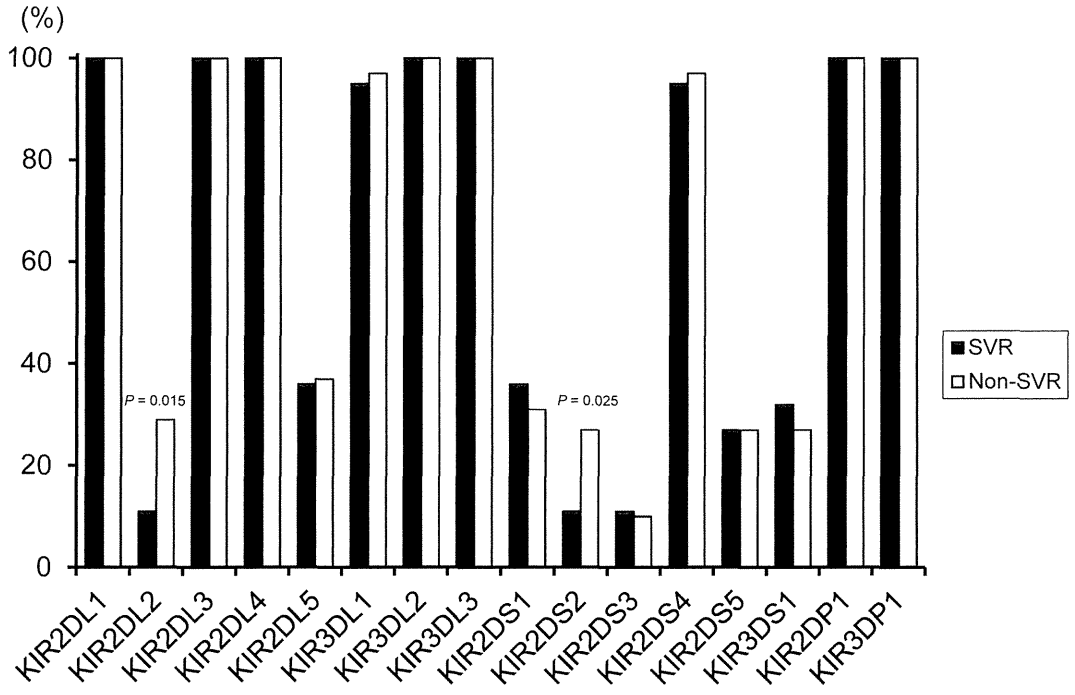

Figure 2. Frequency of each *KIR* gene in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C.

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26% [6/23], $P = 0.015$; OR = 3.37, 95% CI = 1.22 - 9.33). There were no significant differences regarding *AA* genotype and *Tel*.

We next analyzed combinations of activation/inhibitory *KIR*s and their *HLA* ligands for possible associations with an SVR. Among the combinations of *KIR3DL1-HLA-Bw4*, *KIR2DL2-HLA-C1*, and KIR2DL1-*HLA*-C2, patients who carried the inhibitory *KIR3DL1* receptor and its ligand *HLA-Bw4* had a significantly higher response rate than those without *KIR3DL1* or *HLA-Bw4* (58% [36/62] vs. 38% [20/53]; $P = 0.030$ [*Pc* = 0.12]; OR = 2.29, 95% CI = 1.08 - 4.84). In contrast, the *KIR2DL2-HLA-C1* combination resulted in a significantly lower SVR rate (26% [6/23] vs. 54% [50/92]; $P = 0.015$ [*Pc* = 0.06]; OR = 0.30, 95% CI = 0.11 - 0.82). Although several studies have found that *KIR2DL3-HLA-C1* carriers are associated with treatment-induced and spontaneous clearance of HCV in Caucasians, no such association was found in our cohort (data not shown).

Patients with *KIR3DL1-HLA-Bw4* but without *KIR2DL2-HLA-C1* had a higher SVR rate (55% [31/56] vs. 32% [19/59]; $P = 0.012$ [*Pc* = 0.1]; OR = 2.61, 95% CI = 1.22 - 5.58) (Table 2). Conversely, the frequency of the *KIR2DL2-HLA-C1* positive, but *KIR3DL1-HLA-Bw4* negative condition was significantly higher in non-responders (17% [10/59] vs. 2% [1/56]; P = 0.014 [*Pc* = 0.12]; OR = 0.09, 95% CI = 0.01 - 0.72).

Prediction of a Sustained Virological Response by KIR-HLA and IL28B

Examination of the *IL28B* rs8099917 SNP in our cohort revealed significant differences in SVR frequencies. The SVR rate in patients with the *IL28B* TT genotype was significantly higher in those with TG or GG genotypes (62% [44/71] vs. 27% [12/44], $P = 0.0003$; OR = 4.35, 95% CI = 1.92 - 9.85). In subjects with *IL28B* TT and *KIR3DL1-HLABw4*, virologic clearance was significantly increased over other combinations (68% [27/40] vs. 39% [29/75]; $P = 0.003$ [*Pc* = 0.024]; OR 3.29, 95% CI = 1.47 - 7.39).

We next evaluated several factors found in association with an SVR to PEG-IFN and ribavirin therapy for independence by logistic regression analysis. Fifty-six responders were compared with 59 non-responders by means of a forward stepwise likelihood ratio logistic regression method; estimated OR coefficients, 95% CI, and *P* values are summarized in Table 3 for the variables that remained in equation at the last step. *IL28B* TT genotype ($P = 0.00009$; OR = 6.87, 95% CI = 2.62 - 18.01), *KIR2DL2-HLA*-C1 ($P = 0.014$; OR = 0.24, 95% CI = 0.08 - 0.75), white blood cell count ≥ 4410/μL ($P = 0.009$; OR = 3.32, 95% CI= 1.35 - 8.16), and *KIR3DL1-HLA-Bw4* ($P = 0.008$; OR = 3.32, 95% CI = 1.37 - 8.05) were all identified as independent parameters that significantly influenced an SVR.

The frequency of the *IL28B* TT genotype with *KIR3DL1-HLA-Bw4* in responders was significantly higher than in non-responders (48% [27/56] vs. 22% [13/59]; $P = 0.003$ [*Pc* = 0.024]; OR = 3.29, 95% CI = 1.47 - 7.39) (Table 2). Patients with the *IL28B* TT genotype without *KIR2DL2-HLA-C1* had a significantly higher SVR rate (68% [38/56] vs. 31% [18/59]; $P = 0.000062$ [*Pc* = 0.0005]; OR = 4.81, 95% CI = 2.19 - 10.58). The frequency of a non-SVR was significantly higher in patients with the *IL28B* non-TT genotype both with and without

KIR profile	Geno type	Cen motif	Tel motif	3DL3	2DS2	2DL2	2DL3	2DP1	2DL1	3DP1	2DL4	3DL1	3DS1	2DL5	2DS3	2DS5	2DS1	2DS4	3DL2	SVR (n = 56)	Non-SVR (n = 59)
1	AA	A/A	A/A	+	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+	34 (60.7)	28 (47.5)
2	Bx	A/A	A/B	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	9 (16.1)	10 (16.9)
3	Bx	A/B	A/A	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	2 (3.6)	8 (13.6)
4	Bx	A/A	A/B	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	4 (7.1)	2 (3.4)
5	Bx	A/A	B/B	+	-	-	+	+	+	+	+	-	+	+	-	+	+	-	+	3 (5.4)	1 (1.7)
6	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	1 (1.8)	2 (3.4)
7	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	1 (1.8)	2 (3.4)
8	Bx	A/B	A/A	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	0 (0.0)	3 (5.1)
9	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	1 (1.8)	0 (0.0)
10	Bx	A/B	A/B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1 (1.8)	0 (0.0)
11	Bx	A/B	B/B	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	1 (1.8)	0 (0.0)
12	Bx	A/B	A/A	+	-	+	+	+	+	+	+	+	-	-	-	-	-	+	+	0 (0.0)	1 (1.7)
13	Bx	A/A	A/A	+	-	-	+	+	+	+	+	+	-	+	+	-	-	+	+	0 (0.0)	1 (1.7)

Figure 3. *KIR* gene profile frequencies in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C. Numerical data represent the number of individuals (%). The presence of *KIR* genes is indicated by gray shading. Cen, centromeric; Tel, telomeric.

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Table 3. Logistic regression analysis of variables contributing to a sustained virological response to pegylated interferon and ribavirin.

Factor	Odds ratio	95% confidence interval	*P*
IL28B TT genotype	6.87	2.62 - 18.01	0.00009
KIR2DL2/HLA-C1	0.24	0.08 - 0.75	0.014
White blood cells ≥ 4410/μL	3.32	1.35 - 8.16	0.009
KIR3DL1/HLA-Bw4	3.32	1.37 - 8.05	0.008

Only variables achieving statistical significance ($P < 0.05$) in multivariate logistic regression analysis are shown.

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KIR2DL2-HLA-C1 (14% [8/59] vs. 0% [0/8]; $P = 0.013$ [*Pc* = 0.1] and 41% [24/59] vs. 21% [12/56]; $P = 0.026$ [*Pc* = 0.21]; OR = 0.40, 95% CI = 0.17 - 0.91, respectively). The ability to predict an SVR by *IL28B* genotype and *KIR3DL1-HLA-Bw4* and *KIR2DL2-HLA-C1* was next evaluated. Corresponding values for sensitivity, specificity, PPV, and NPV are listed in Table S1 in File S1. A combination of the *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* demonstrated high predictive specificity (78%), as did the combination of *IL28B* TT genotype and *KIR2DL2-HLA-C1* (86%).

Lastly, we analyzed combinations of the three factors of *IL28B* genotype, *KIR3DL1-HLA-Bw4*, and *KIR2DL2-HLA-C1* for prediction of treatment outcome (Table S2 in File S1). The frequencies of *IL28B* TT, *KIR2DL2-HLA-C1*-negative, with and without *KIR3DL1-HLA-Bw4* were significantly higher among responders (38% [21/56] vs. 19% [11/59]; P = 0.024 [Pc = 0.29]; OR = 2.62, 95% CI = 1.12 - 6.12 and 30% [17/56] vs. 12% [7/59]; P = 0.015 [Pc = 0.18]; OR = 3.24, 95% CI = 1.22 - 8.57, respectively).

Discussion

The present study examined *HLA*, *KIR*, and *IL28B* gene variant associations with an SVR following PEG-IFN and ribavirin therapy in Japanese patients with chronic hepatitis C. We found a significant association of *HLA-Bw* alleles with treatment outcome, although the frequency of *HLA-C* alleles did not differ significantly between responders and non-responders. Functional analyses have demonstrated that NK cells in *HLA-C1C1* subjects exhibit a more rapid and stronger antiviral response that those in *HLA-C2C2* subjects due to differing responses of *HLA-C*-inhibited NK subsets[33]. *HLA-C2C2* homozygousity is strongly associated with treatment failure in HCV patients of European ancestry [11,22], but we could not assess its role in our study because this genotype was found in only 1 of 115 patients.

We uncovered a significant association between the presence of *KIR2DL2* or *KIR2DS2* and lower SVR rates. Several reports have shown that *KIR2DL3-HLAC1* in Caucasians [11,22] and *KIR2DL5* in Brazilians [34] are associated with treatment outcome of antiviral therapy. Since our results showed no such statistical significances, these conflicting interpretations may reflect differences in patient selection, genetic background, sample size, and/or treatment regimen. Further studies are required to clarify this discrepancy in the Japanese population.

A study by Dring et al. examined *KIR* haplotypes in patients with HCV infection and showed that a centromeric *KIR* haplotype was increased in chronic HCV infection as compared with resolved cases [20]. We therefore determined *KIR* haplotypes and *Cen-A/B* and *Tel-A/B* in our patients as well, and found an interesting association between *Cen-A/A* and an SVR to antiviral therapy (P = 0.015; OR 3.37). Since *Cen-A/B* is determined by *KIR2DL3* and *KIR2DS2* and/or *KIR2DL2*, this finding is consistent with our results demonstrating a relationship between *KIR2DS2* and *KIR2DL2* genotypes and treatment failure.

The most significant finding in this study was the association between KIR-HLA receptor-ligand pairings and treatment outcome in chronic hepatitis C. Among the inhibitory KIR-HLA receptor-ligand pairs, patients with *KIR3DL1-HLA-Bw4* exhibited a significantly higher SVR rate when compared to those without this pair (P = 0.03; OR 2.29). Conversely, virologic clearance in patients with *KIR2DL2-HLA-C1* was significantly lower than in those without (P = 0.015; OR = 0.30). Stratification analysis of the 4 groups of *KIR3DL1-HLA-Bw4* (presence or absence) and *KIR2DL2-HLA-C1* (presence or absence) revealed a higher frequency of responders with *KIR3DL1-HLA-Bw4* presence, *KIR2DL2-HLA-C1* absence compared with those possessing *KIR2DL2-HLA-C1* presence, *KIR3DL1-HLA-Bw4* absence (62% vs. 9%; P = 0.0044; OR =16.32). When these KIR-HLA pairs were both either positive or negative, SVR rates were similar at 42% and 45%, respectively. Together with the results of logistic regression analysis, we clearly showed that *KIR3DL1-HLA-Bw4* was positively associated with an SVR (OR = 3.32) and that *KIR2DL2-HLA-C1* had a negative association (OR = 0.24) with treatment outcome. As almost one half of the Japanese population have the functional *KIR3DL1-HLA-Bw4* combination, this inhibitory receptor-ligand interaction is potentially important in understanding NK cell diversification. The NK-cell surface expression of KIR3DL1 is higher in individuals having Bw4 than in those lacking it [35]. Therefore, these cells might be more weakly controlled by inhibitory signals than other NK cells, more easily activated by viral infection, and more readily promoted for cytolysis and IFN-gamma production.

This study confirmed that the *IL28B* TT genotype is a strong predictor of an SVR in Japanese patients[18,32]. Furthermore, SVR frequencies were positively correlated with a combination of the *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* (P = 0.0019) and negatively associated with the *IL28B* TT genotype and *KIR2DL2-HLA-C1* (P = 0.0067). These combinations were also highly specific for virologic response prediction. In light of these findings, patients with poor expected treatment outcome may be advised to wait for the use of combinations of direct acting antiviral agents[36]. Akuta et al. reported that a combination of amino acid substitutions in the core region of HCV and *IL28B* genotype was a useful predictor of PEG-IFN, ribavirin, and telaprevir therapy results in Japan[37]. Since we could not collect sera before treatment for all patients, we were not able to assess the effect of amino acid substitutions in the HCV core region. Furthermore, interferon-free combinations of direct-acting antiviral agents have become an area of considerable clinical interest. Chu et al. have reported that IL28B genotype appears to affect early viral kinetics in patients with chronic hepatitis C receiving interferon-free treatment [38]. Recently, two groups have discovered *IFN* lambda *4* (*IFNL4*), a new gene that may account for associations of spontaneous and IFN-based treatment clearance of HCV [39,40]. The IFN-λ 4 protein is generated by individuals who carry the ΔG allele of the ss469415590 variant, and the presence of this protein is strongly associated with impaired clearance of HCV. Linkage disequilibrium is strong between the *IFNL4-ΔG* allele and the unfavorable rs12979860-T allele (*IL28B*) in subjects of European or Asian ancestry, whereas this linkage disequilibrium is moderate in individuals of African ancestry [39]. We have confirmed that the linkage disequilibrium between the *IFNL4-ΔG* allele and *IL28B* SNP (rs8099917) is high and that the *IFNL4-ΔG* allele is strongly associated with treatment failure of PEG-IFN and ribavirin therapy in patients with Japanese chronic hepatitis C [41]. Hence, the clinical impacts of HLA-KIR genetic variants, IL28B genotype, and the IFNL4 allele should be explored.

In conclusion, the present study showed significant associations of *KIR3DL1-HLA-Bw4, KIR2DL2-HLA-C1*, and *IL28B* combinations with an SVR to PEG-IFN and ribavirin therapy in Japanese patients with genotype 1 HCV. The clinical significance of *IL28B* genotyping combined with HLA/KIR pairs to predict treatment outcome warrants further validation for triple therapy.

Supporting Information

File S1. Table S1, Sensitivity, specificity, and predictive values of IL28B TT genotype and KIR3DL1/HLA-Bw4 or

KIR2DL2/HLA-C1 for a sustained virological response in 115 patients with chronic hepatitis C. Data are expressed as % (n). PPV, positive predictive value; NPV, negative predictive value. Table **S2**, Frequency of ***IL28B*** genotype and ***KIR3DL1/HLA-Bw4*** and ***KIR2DL2/HLA-C1*** combinations in 56 patients with a sustained virological response (SVR) and 59 patients with a non-SVR to pegylated interferon and ribavirin therapy of chronic hepatitis C. Data are expressed as n (%).
(DOC)

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Author Contributions

Conceived and designed the experiments: YN TU ET MO. Performed the experiments: YN TU YK MO. Analyzed the data: YN TU YK MO. Contributed reagents/materials/analysis tools: YN TU SJ YK SS TK SM MK AM ET. Wrote the manuscript: TU MO.

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