Table 2 *P*-values for each genotype before and after propensity score matching, according to HCV-RNA disappearance from week 5 to week 8

	P-values for genotype 2a				*P*-values for genotype 2b			
	HCV-RNA (–)		HCV-RNA (+)		HCV-RNA (–)		HCV-RNA (+)	
	Before	Adjusted	Before	Adjusted	Before	Adjusted	Before	Adjusted
Age (years)	0.29	0.26	0.74	0.11	0.82	0.71	0.51	0.06
Sex	0.25	0.7	0.04	0.06	0.22	1	0.37	0.32
BW	0.38	0.34	0.90	0.17	0.41	0.26	0.33	0.18
BMI	0.35	0.1	0.17	0.41	0.81	0.53	0.31	0.42
Liver tissue	0.07	0.5	0.58	0.54	0.36	0.71	0.17	0.2
Prior IFN treatment	0.02	0.32	1	0.58	0.13	1	0.042	1
WBC	0.23	0.38	0.02	0.06	0.73	0.74	0.71	0.19
Neut	0.12	0.37	0.4	0.07	0.71	0.62	0.99	0.72
Hgb	0.41	0.52	0.28	0.50	0.72	0.49	0.53	0.74
PLT	0.25	0.35	0.87	0.06	0.20	0.29	0.70	0.22
AST	0.17	0.17	0.97	0.49	0.47	0.91	0.77	0.20
ALT	0.07	0.36	0.79	0.49	0.14	0.47	0.59	0.74
γ-GTP	0.28	0.21	0.60	0.38	0.20	0.54	0.42	0.43
HCV-RNA	0.84	0.56	0.6	0.12	0.86	0.70	0.29	0.06
T-chol	0.31	0.22	0.27	0.31	0.96	0.75	0.75	0.19
TG	0.61	0.41	0.46	0.48	0.29	0.06	0.22	0.22
IL28B	0.64	0.32	—	—	0.70	0.17	0.55	1

γ-GTP, γ-glutamyl transpeptidase; ALT, alanine aminotransferase; AST, serum aspartate aminotransferase; BMI, body mass index; BW, body weight; HCV, hepatitis C virus; Hgb, hemoglobin; IFN, interferon; Neut, neutrophils; PLT, platelet count; T-chol, total cholesterol; TG, triglyceride; WBC, white blood cell.

Underlined values were significantly different factors between each groups.

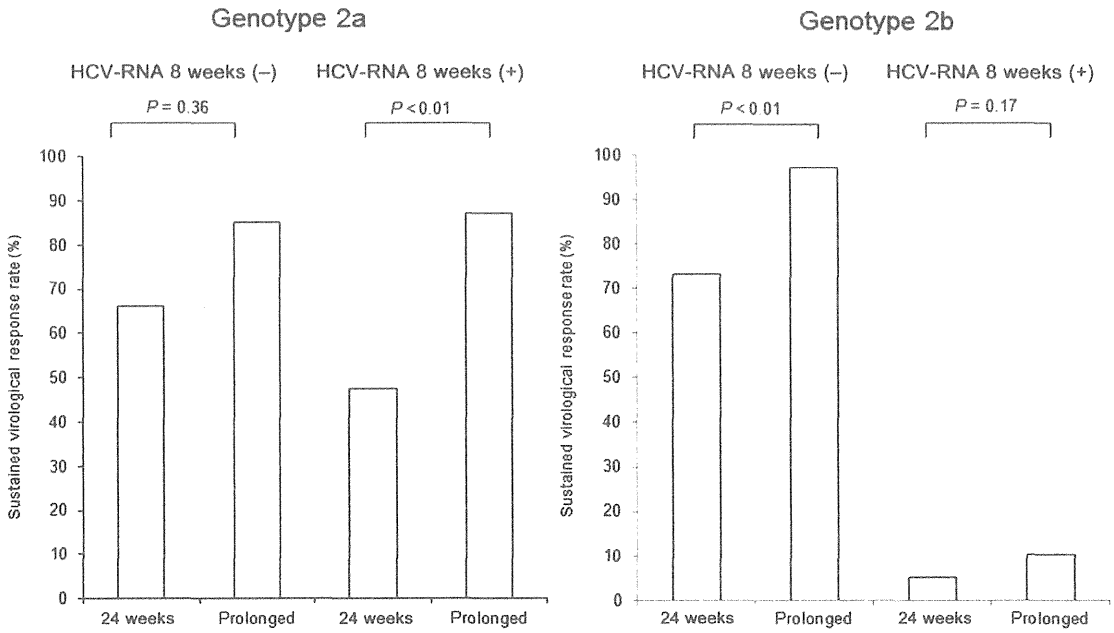

Figure 3 The estimator of sustained virological response rate according to the time point of hepatitis C virus (HCV)-RNA disappearance. Patients who were positive for HCV-RNA at week 4 of therapy were divided into two groups: negative (HCV-RNA 8 weeks [–]) and positive (HCV-RNA 8 weeks [+]) for HCV-RNA at 8 weeks of therapy. Clinical and virological factors between the 24-week and prolonged therapy groups were adjusted by propensity score analysis and the sustained virological response (SVR) rate calculated by inverse probability weighted estimation. In the case of genotype 2a HCV-RNA 8 weeks (+) patients and genotype 2b HCV-RNA 8 weeks (–) patients, prolonged therapy was effective ($P < 0.05$ by Kolmogorov–Smirnov test). However, for genotype 2b HCV-RNA 8 weeks (+) patients, there was no significant difference between the 24-week and prolonged therapy groups ($P = 0.17$).

mutations in the N-terminal region of IRRDR (aa 2332–2357; referred to as IRRDR/N[2b]) was significantly associated with RVR.[21] *In vitro* studies showed that there was no difference in IFN susceptibility between genotypes 2a and 2b,[22] while others have reported otherwise.[23] We suspect that this discrepancy may have arisen because of differences in the viral sequences investigated. It was reported that viral load and rs8099917 were independent predictive factors for SVR of PEG/RBV in genotype 2b-inected patients but not for genotype 2a-infected patients.[24,25] In our study, we found no association between IL28B single nucleotide polymorphism (SNP)s and SVR for either genotype. There was no significant difference in SVR rate among genotype 2b, TT group, or TG/GG group (24-week therapy 41/58 *vs* 10/18 $P = 0.23$; prolonged therapy 16/28 *vs* 4/6 $P = 0.13$). There was no significant difference in SVR rate among genotype 2a, TT group, or TG/GG group (24-week therapy 56/75 *vs* 16/20 $P = 0.62$; prolonged therapy 27/36 *vs* 9/10 $P = 0.31$).

There are additional factors associated with the SVR of genotype 1 patients. Greater histological fibrosis of the liver prior to therapy[26] lowers the SVR rate,[27] while lower triglyceride and higher low-density lipoprotein cholesterol levels were reported to be associated with higher rates of SVR. Steatosis is a significant independent predictor of SVR, and statin use is expected to improve SVR rates.[28] Obesity impedes the lymphatic system, where pegylated IFN is first absorbed,[29] and it was reported that obesity may be a significant independent predictor of poor SVR. Body mass index was also reported to be an independent risk factor for non-response to antiviral therapy.[30] Additionally, vitamin D tends to increase the rate of SVR for genotype 1, 2–3 treatment-naïve patients.[31] Most reported factors relating to SVR are specific to genotype 1, and little analysis of genotype 2 patients has been performed; hence, covariates should be chosen carefully. In the current analysis, age, sex, viral load, and history of antiviral therapy were included to control for the influence of confounding covariates. Hgb was also used as a covariate in the comparison of genotype 2b, as Hgb levels were significantly different for genotype 2b patients in the 24-week PEG/RBV group and the prolonged PEG/RBV group ($P = 0.047$ in Table 1). The factors described above have the potential to be included as covariates, but many more cases are required to perform propensity score analysis. It was difficult to include additional covariates in our examination because of the small number of patients and insufficient data, which may have limited the interpretation of our results.

In the case of genotype 2b-infected patients with undetectable HCV-RNA at 8 weeks, an increase in therapy duration to 48 weeks may be required to achieve a SVR, and it may also be applicable to genotype 2a-infected patients with detectable HCV-RNA at 8 weeks. However, as is the case for genotype 2b-infected patients with detectable HCV-RNA at 8 weeks, due consideration should be taken when ceasing treatment.

Conclusion

Virological response to PEG/RBV differs between HCV genotype 2a- and 2b-infected patients. For genotype 2a, prolonged therapy beyond 24 weeks increased the SVR rate in patients who remained HCV-RNA positive at week 8 of therapy. Conversely, prolonged therapy increased the SVR rate in for genotype 2b patients who had become HCV-RNA negative by week 8 of therapy.

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KIR3DL1-HLA-Bw4 combination and *IL28B* polymorphism predict response to Peg-IFN and ribavirin with and without telaprevir in chronic hepatitis C

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ABSTRACT

Natural killer cells play a key role in the immune control of viral infections. Killer immunoglobulin-like receptors (KIRs) regulate natural killer cell activation and inhibition through the recognition of their cognate HLA class I ligands. We assessed the predictive factors of a sustained virological response (SVR) in 200 Japanese patients with chronic genotype 1b hepatitis C who were treated with telaprevir (TVR), pegylated-interferon-α2b (PEG-IFN), and ribavirin (RBV) triple therapy (92 patients) or PEG-IFN/RBV therapy alone (108 patients). Sixteen KIR genotypes, HLA-A, -B and -C ligands, and an interleukin (IL) 28B polymorphism (rs8099917) were analyzed. We observed that triple therapy, white blood cell count, hemoglobin value, hepatitis C viral load, a rapid virological response (RVR), *IL28B* TT genotype, and *KIR3DL1-HLA-Bw4* genotype were associated with an SVR. In multivariate regression analysis, we identified an RVR ($P < 0.000001$; odds ratio [OR] = 20.95), the *IL28B* TT genotype ($P = 0.00014$; OR = 5.53), and *KIR3DL1-HLA-Bw4* ($P = 0.004$, OR = 3.42) as significant independent predictive factors of an SVR. In conclusion, *IL28B* and *KIR3DL1/HLA-Bw4* are independent predictors of an SVR in Japanese patients infected with genotype 1b HCV receiving TVR/PEG-IFN/RBV or PEG-IFN/RBV therapy.

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. More than half of patients with acute HCV infections progress to chronic hepatitis, which leads to liver cirrhosis and/or hepatocellular carcinoma (HCC) in at least 20% of cases [1]. HCC is a leading cause of death from malignant neoplasms in Japan [2]. Since approximately 70–80% of Japanese HCC patients are infected with HCV, viral eradication is considered important to decrease the incidence of HCC. Interferon (IFN)-based therapy can reduce HCV to undetectable levels and improve prognosis. The primary aim of antiviral therapy in HCV patients is a

Abbreviations: CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HLA, human leukocyte antigen; IL, interleukin; KIR, killer immunoglobulin-like receptors; OR, odds ratio; PEG-IFN, pegylated-interferon-α2b; RBV, ribavirin; RVR, rapid virological response; SVR, sustained virological response; TVR, telaprevir.

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sustained virological response (SVR), which is defined as undetectable serum HCV RNA 24 weeks after completion of therapy. Telaprevir (TVR) is an effective HCV non-structural 3/4A protease inhibitor that has recently been approved for the treatment of chronic hepatitis C genotype 1 in Japan. Triple therapy that combines TVR, pegylated-IFN-α2b (PEG-IFN), and ribavirin (RBV) treatment has achieved SVR rates of 70–80% [3,4].

Recent studies have highlighted that polymorphisms in the interleukin (IL) 28B gene are associated with spontaneous and treatment-induced resolution of HCV infection [5–8]. Similarly, killer immunoglobulin-like receptors (KIRs) and their human leukocyte antigen (HLA) class I ligands have also been implicated in spontaneous and treatment-based disease resolution [9–13]. Accordingly, combinations of IL28B polymorphisms and KIR genotypes have been studied by our own laboratory and others with regard to disease treatment and resolution [14–17]. However, it has not yet been elucidated whether polymorphisms of these innate immune genes are associated with virological response to TVR/PEG-IFN/RBV triple therapy. The objective of this study was to clarify whether *KIR-HLA* interactions, in addition to an *IL28B* polymorphism, would influence the outcome of TVR/PEG-IFN/RBV or PRG-IFN/RBV therapy in Japanese patients with chronic hepatitis C.

2. Materials and methods

2.1. Subjects

A total of 200 patients with chronic hepatitis C were enrolled in this study. All subjects were treated at Shinshu University Hospital or one of its affiliated hospitals. The clinical and demographic characteristics of our cohort are shown in Table 1. The diagnosis of chronic hepatitis C was based on previously reported criteria [18] of (1) the presence of serum HCV antibodies and HCV RNA $\geqslant$ 5.0 log IU/mL; (2) the 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 the Cobas TaqMan HCV test (Roche Diagnostic Systems, Tokyo, Japan). The linear dynamic range of the assay was 1.2–7.8 log IU/mL, and undetectable samples were defined as negative. All patients in our cohort were infected with HCV genotype 1b as determined by sequence analysis. Alanine aminotransferase, aspartate aminotransferase, and other relevant biochemical tests were performed using standard methods [19].

Ninety-two patients received a 12-week triple therapy regimen that included TVR (Telavic; Mitsubishi Tanabe Pharma, Osaka, Japan; 1500–2250 mg/day), PEG-IFN (Pegintron; MSD KK, Tokyo, Japan; 1.5 μg/kg of body weight weekly by subcutaneous injection), and RBV (Rebetol; MSD KK; 600–1000 mg/day according to body weight) followed by a 12-week course of dual therapy composed of PEG-IFN and RBV. The remaining 108 patients received PEG-IFN and RBV treatment for 48 weeks, as described previously [20]. No patients from our prior study were included in this cohort [21].

Patients achieving a sustained HCV response were identified as those whose serum HCV RNA was undetectable 24 weeks after completing therapy. A rapid virological response (RVR) was defined as undetectable HCV RNA at week 4 of treatment. Patients not attaining an SVR, who included non-responders and relapsers, were regarded as treatment failures.

This study was approved by the ethics 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.

2.2. HLA, KIR, and IL28B (rs8099917) genotyping

Genomic DNA was isolated from whole blood samples using QuickGene-800 assays (Fujifilm, Tokyo, Japan). We genotyped *HLA-A*, *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) that was based on PCR sequence-specific oligonucleotide probes [22]. *KIR* genes were divided into distinct group A and group B haplotypes based on centromeric as well as telomeric regions of the *KIR* locus. *KIR* genotypes were then identified according to the definition established by Cooley et al. [23]. Briefly, centromeric *AA* genotypes contained *KIR2DL3* but not *KIR2DL2* or *KIR2DS2*, centromeric *AB* genotypes contained *KIR2DL3* with *KIR2DL2* and/or *KIR2DS2*, and centromeric *BB* genotypes contained *KIR2DL2* and/or *KIR2DS2* but not *KIR2DL3*. Meanwhile, telomeric *AA* genotypes contained *KIR3DL1* and *KIR2DS4* but not *KIR3DS1* or *KIR2DS1*, telomeric *AB* genotypes contained *KIR3DL1* and *KIR2DS4* with *KIR3DS1* and/or *KIR2DS1*, and telomeric *BB* genotypes lacked *KIR3DL1* and/or *KIR2DS4*. Genotyping of an *IL28B* SNP (rs8099917) was performed using an ABI TaqMan allelic discrimination kit and the ABI7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA) [24]. 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.

2.3. 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 KIR-HLA combination data using the number of comparisons performed on our primary factors of

Table 1
Clinical features of patients with chronic hepatitis C with and without a sustained virological response.

Characteristic	All (n = 200)	SVR (n = 126)	Non-SVR (n = 74)	P
Age (yrs)	61 (53–65)	60 (52–65)	62 (55–67)	0.076
Male	109 (55)	71 (56)	38 (51)	0.493
Triple therapy	92 (46)	74 (59)	18 (24)	2.0×10^{-6}
White blood cells (/μL)	4445 (3788–5578)	4870 (3888–5730)	4275 (3670–5190)	0.022
Hemoglobin (g/dL)	14.4 (13.4–15.4)	14.4 (13.7–15.7)	14.1 (13.1–15.1)	0.010
Platelet count (10^4/μL)	15.9 (13.0–19.3)	16.3 (13.6–19.3)	15.5 (12.1–19.2)	0.163
Serum alanine aminotransferase (IU/L)	44 (29–68)	40 (26–64)	46 (33–70)	0.147
HCV RNA (log IU/mL)	6.5 (6.0–6.8)	6.4 (5.9–6.7)	6.5 (6.2–6.8)	0.027
IL-28 TT genotype	134 (67)	102 (81)	32 (43)	4.4×10^{-8}

Data are expressed as median (interquartile range) or n (%) as appropriate.
SVR, sustained virological response.

Table 2
Frequency of *HLA-Bw* and *-C* alleles in 126 patients with a sustained virological response (SVR) and 74 patients with a non-SVR to antiviral therapy for chronic hepatitis C.

	SVR (n = 126)	Non-SVR (n = 74)	P (Pc)
Genotype			
Bw4/Bw4	15 (12%)	7 (10%)	0.594
Bw4/Bw6	61 (48%)	23 (31%)	0.017 (0.17)
Bw6/Bw6	50 (40%)	44 (60%)	0.011 (0.11)
C1/C1	104 (83%)	65 (88%)	0.318
C1/C2	22 (17%)	9 (12%)	

Data are expressed as n (%).
SVR, sustained virological response.

interest in Table 2 (i.e., 5 combinations × 2 comparisons between two groups = 10 tests). 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 their median value. Statistical analyses were performed using SPSS software version 21.0 J (IBM, Tokyo, Japan). We evaluated synergy between IL28B and KIR-HLA using the method described by Cortina-Borja et al. [25].

3. Results

3.1. Patient characteristics and treatment outcome

Of the 200 patients who received antiviral therapy, 126 (63%) achieved an SVR. The remaining 74 patients were considered to be non-responders: 39 relapsed, 33 were null responders, and 2 experienced viral breakthrough. Before treatment, median white blood cell count (4870 vs. 4275 /μL, P = 0.022) and hemoglobin value (14.4 vs. 14.1 g/dL, P = 0.010) in the SVR group were significantly higher than in the non-SVR group (Table 1). Median HCV RNA level (6.4 vs. 6.5 log IU/mL, P = 0.027) was significantly lower in the SVR group compared with the non-SVR group. Patients who were administered triple therapy had a significantly higher SVR rate (59% [74/126] vs. 24% [18/74], $P = 2.0 \times 10^{-6}$; OR = 4.43, 95% CI = 2.34–8.39). An RVR was also strongly associated with an SVR (66% [83/126] vs. 10% [7/74], $P = 9.7 \times 10^{-15}$; OR = 18.48, 95% CI = 7.81–43.71). The RVR and SVR rates in patients treated with TVR/PEG-IFN/RBV were 78% (72/92) and 80% (74/92), respectively. In contrast, these rates were 17% (18/108) and 48% (52/108), respectively, in patients treated with PEG-IFN/RBV.

3.2. HLA class I allele frequencies and KIR genotypes in patients with chronic hepatitis C

We first examined for associations between *HLA-B* and *-C* alleles and response to antiviral therapy. The frequency of the *HLA-B*51:01* allele in patients with an SVR was higher than in patients with a non-SVR (10% [24/252] vs. 3% [4/148], P = 0.017 [Pc = 0.49]; OR = 3.79, 95% CI = 1.29–11.15). Conversely, the *HLA-B*15:01* allele was less frequently found in responders (5% [12/252] vs. 10% [15/148], P = 0.039 [Pc = 1.13]; OR = 0.44, 95% CI = 0.20–0.98). No specific *HLA-A* or *-C* alleles were detected in our cohort.

Next, we searched for differences in the distribution of *HLA-Bw4* and *HLA-C1* allele frequencies between SVR and non-SVR patients (Table 2). The frequency of *HLA-Bw4Bw6* in responders was higher than in non-responders (48% [61/126] vs. 31% [23/74], P = 0.017 [Pc = 0.17]; OR = 2.08, 95% CI = 1.14–3.81). In contrast, patients with the *HLA-Bw6* homozygote had a higher non-SVR rate (40% [50/126] vs. 59% [44/74]; P = 0.011 [Pc = 0.11]; OR = 0.45, 95% CI = 0.25–0.81). Overall, *HLA-Bw4* was significantly associated with an SVR among patients (60% [76/126] vs. 41% [30/74], P = 0.007, OR = 2.23, 95% CI = 1.24–4.00). No remarkable allelic frequencies were seen for *HLA-C1*.

With respect to *KIR* genes, no associations between the 16 genes examined and treatment outcome were observed (Fig. 1). *KIR* gene profiles were classified based on centromeric and telomeric regions of the *KIR* A and B haplotypes (Cen-A/B and Tel-A/B). When we compared the Cen-A/B and Tel-A/B frequencies between the SVR and non-SVR groups, no significant differences were apparent (Table 3).

3.3. HLA and KIR compound genotypes and antiviral response of HCV

To determine the effect of HLA/KIR genotypes on possible associations with an SVR, we analyzed combinations of activating or inhibitory *KIRs* and their *HLA* ligands. Among the combinations of *KIR2DL1-HLA-C2*, *KIR2DL2-HLA-C1*, *KIR2DL3-HLA-C1*, *KIR3DL1-HLA-Bw4*, and *KIR3DL2-HLA-A3* and *-A11*, only the frequency of the inhibitory *KIR3DL1* receptor and its *HLA-Bw4* ligand was remarkably higher in responders than in non-responders (58% [73/126] vs. 39% [29/74], P = 0.010 [Pc = 0.10]; OR = 2.14, 95% CI = 1.19–3.84) (Table 4). When stratified for patients treated with TVR/PEG-IFN/RBV or PEG-IFN/RBV, although *KIR3DL1-HLA-Bw4* was significantly associated with an SVR in patients treated with PEG-IFN/RBV (69%

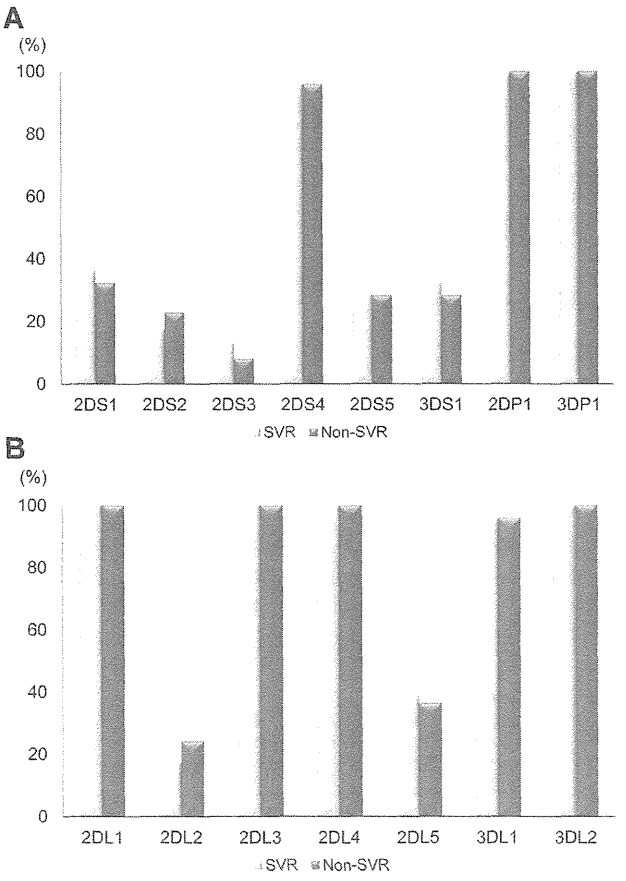

Fig. 1. Frequency of 16 KIR genes in 126 patients with a sustained virological response (SVR) and 74 patients with a non-SVR to antiviral therapy for chronic hepatitis C.

Table 3
Frequencies of centromeric and telomeric KIR genotypes in 126 patients with a sustained virological response (SVR) and 74 patients with a non-SVR to antiviral therapy for chronic hepatitis C.

	SVR (n = 126)	Non-SVR (n = 74)	P
Genotype			
AA	71 (56%)	36 (49%)	0.292
Bx	55 (44%)	38 (51%)	
Centromere motif			
AA	104 (83%)	56 (76%)	0.241
AB	22 (18%)	18 (24%)	
Telomere motif			
AA	80 (64%)	50 (68%)	0.827
AB	41 (33%)	21 (28%)	
BB	5 (4%)	3 (4%)	

Data are expressed as n (%).
SVR, sustained virological response.

Table 4
Frequencies of KIR-HLA receptor-ligand pairs in 126 patients with a sustained virological response (SVR) and 74 patients with a non-SVR to antiviral therapy for chronic hepatitis C.

KIR-HLA receptor-ligand pair	SVR (n = 126)	Non-SVR (n = 74)	P (Pc)
KIR2DL1-HLA-C2	22 (18%)	9 (12%)	0.833
KIR2DL2-HLA-C1	19 (15%)	17 (23%)	0.161
KIR2DL3-HLA-C1	104 (83%)	65 (88%)	0.318
KIR3DL1-HLA-Bw4	73 (58%)	29 (39%)	0.010 (0.10)
KIR3DL2-HLA-A3 and -A11	32 (25%)	20 (27%)	0.800

Data are expressed as n (%).
SVR, sustained virological response.

Table 5
Logistic regression analysis of variables contributing to a sustained virological response to antiviral therapy.

Factor	Odds ratio	95% CI	P
RVR	20.95	7.68–57.11	<0.000001
IL28B TT genotype	5.53	2.30–13.32	0.00014
KIR3DL1-HLA-Bw4	3.42	1.50–7.83	0.004

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

[36/52] vs. 38% [21/56], $P = 0.001$ [$Pc = 0.01$]; OR = 3.75, 95% CI = 1.69–8.34), no association between *KIR3DL1-HLA-Bw4* and triple therapy was observed (50% [37/74] vs. 44% [8/18], $P = 0.672$).

3.4. Association of a sustained virological response with KIR-HLA and IL28B

The SVR rate in patients with the *IL28B* TT genotype was significantly higher than in those with the TG or GG genotype (81% [102/126] vs. 43% [32/74], $P = 4.4 \times 10^{-8}$; OR = 5.58, 95% CI = 2.94–10.58) (Table 1).

We next evaluated several factors found in association with an SVR to antiviral therapy for independence by logistic regression analysis. A total of 126 responders were compared with 74 non-responders by means of a forward stepwise likelihood ratio logistic regression method. An RVR ($P < 0.000001$; OR = 20.95, 95% CI = 7.68–57.11), the *IL28B* TT genotype ($P = 0.00014$; OR = 5.53, 95% CI = 2.30–13.32), and *KIR3DL1-HLA-Bw4* ($P = 0.004$; OR = 3.42, 95% CI = 1.50–7.83) were all identified as independent parameters that significantly influenced an SVR (Table 5).

As the frequency of the *IL28B* TT genotype along with *KIR3DL1-HLA-Bw4* in SVR group was significantly higher than in non-SVR group (46% [58/126] vs. 18% [13/74]; $P = 4.9 \times 10^{-5}$ [$Pc = 3.9 \times 10^{-4}$]; OR = 4.00, 95% CI = 2.00–8.01) (Table 6), we applied a recently described test to evaluate for synergistic effects between these genetic factors [25]. Based on logistic regression analysis, this method evaluated whether the observed ORs of the 2 independent factors were greater combined than separately. We observed an absence of synergy between the 2 favorable factors of *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* in the SVR population (synergy factor = 0.68, 95% CI = 0.19–2.48; $P_{synergy} = 0.560$), which confirmed that they were indeed independent of each other. Moreover, when stratified for each treatment regimen, patients who achieved an SVR with the PEG-IFN/RBV regimen had a significantly higher frequency of *IL28B* TT and *KIR3DL1-HLA-Bw4* compared with those who did not (50% vs. 18%, $P = 0.00040$ [$Pc = 0.0032$], OR = 4.60, 95% CI = 1.92–11.02). For triple therapy, the frequency of the *IL28B* TG/GG genotype without *KIR3DL1-HLA-Bw4* was significantly higher in non-responders (39% vs. 9%, $P = 0.0018$ [$Pc = 0.014$], OR = 0.16, 95% CI = 0.05–0.56).

4. Discussion

Natural killer (NK) cells are a subset of lymphocytes that can interact directly with virus-infected cells as well as activate dendritic cells and secrete Th1-type cytokines to augment antiviral cytotoxic T-cell responses. The NK cell response is controlled by multiple activating and inhibitory receptors. It is thought that the net inhibitory or activating signal derived from these receptors determines whether or not the NK cell is activated. KIR molecules are known to interact with their HLA class I ligands to modulate NK cell activity. The ligands for KIR2DL are HLA-C alleles, which are classified as C group 1 (C1) if the amino acid at position 80 is asparagine or C group 2 (C2) if lysine occupies that position. The inhibitory KIR2DL2 and -2DL3 recognize the C1 allotype, while KIR2DL1 recognizes C2 allotypes [26]. KIR3DL1 recognizes HLA-B Bw4 allotypes, particularly those with an isoleucine at position 80 [27].

Our data showed that KIR3DL1 and its HLA-Bw4 ligand were associated with an SVR following antiviral therapy that included TVR/PEG-IFN/RBV triple therapy in Japanese patients with chronic hepatitis C. In combination with a prior study by our group [21], our findings demonstrate a favorable influence of these genes in patients achieving an SVR with IFN-based treatment. As almost one half of the Japanese population have the functional *KIR3DL1-HLA-Bw4* combination, this inhibitory receptor-ligand interaction

Table 6
Frequencies of IL28 genotype and KIR3DL1/HLA-Bw4 combinations in 126 patients with a sustained virological response (SVR) and 74 patients with a non-SVR to antiviral therapy for chronic hepatitis C.

IL28B	KIR3DL1/HLA-Bw4	SVR (n = 126)	Non-SVR (n = 74)	P (Pc)
TT	+/+	58 (46%)	13 (18%)	4.9×10^{-5} (3.9×10^{-4})
TT	Other	44 (35%)	19 (26%)	0.174
TG/GG	+/+	15 (12%)	16 (22%)	0.067
TG/GG	Other	9 (7%)	26 (35%)	4.9×10^{-7} (3.9×10^{-6})

Data are expressed as n (%).
SVR, sustained virological response.

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 [28]. Such cells may be less strongly controlled by inhibitory signals than other NK cells, more easily activated by viral infection, and more readily promoted for cytolysis and IFN-gamma production. On the contrary, although *KIR2DL3-HLAC1* has been associated with treatment-induced and spontaneous HCV eradication in Caucasians [9,11,16], our data showed no association of this gene with the response to treatment for HCV infection.

In multivariate analysis, we witnessed that an RVR, the *IL28B* TT genotype, and *KIR3DL1-HLA-Bw4* were independent factors related to an SVR in patients treated with anti-viral therapy with and without TVR. This confirmed that RVR and IL28B genotype were strong predictors of an SVR to triple therapy in the Japanese population similarly to previous studies of HCV treatment with PEG-IFN/RBV only [7,24]. Furthermore, SVR frequencies were positively correlated with a combination of the *IL28B* TT genotype and *KIR3DL1-HLA-Bw4* ($P = 0.000049$), but we did not observe that they were acting synergistically. The calculation of a synergy factor allows for differentiation between a true synergistic interaction and an apparent one. The synergy factor is designed to be robust for small sizes, even when individual cells are zero. The result of this analysis complemented the findings obtained by logistic regression that the combination of the 2 independent factors had no significant advantage over each factor in isolation for an SVR.

In conclusion, the present study showed significant independent associations of an RVR, the *KIR3DL1-HLA-Bw4* combination, and *IL28B* with an SVR to interferon-based therapy, including TVR triple therapy, in Japanese patients with genotype 1 HCV.

Author contributions

TU and MO conceived and designed the experiments. YK and YN performed the experiments. TU performed the statistical analysis and wrote the first draft. TU, SW, HM, AM, SS, TK, SM, SJ, MK, AM, AK, MK, MT, KY, KK, and ET provided the specimens and clinical data of the patients. All authors contributed to further drafts, and have read and approved the final manuscript.

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BRIEF COMMUNICATION

Genetic polymorphism in *IFNL4* and response to pegylated interferon-α and ribavirin in Japanese chronic hepatitis C patients

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Key words
hepatitis C virus; interferon-λ 4; IL28B; pegylated interferon

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Abstract

A genetic polymorphism of the newly discovered interferon-λ 4 (*IFNL4*) gene was associated with hepatitis C virus (HCV) clearance in individuals of African ancestry. To assess whether a dinucleotide variant of *IFNL4* (ss469415590) also affected treatment outcome of antiviral therapy in Japan, we genotyped 213 patients with chronic genotype 1 HCV infection and 176 healthy subjects. The ΔG allele was associated with treatment failure [odds ratio (OR) 4.73, $P = 0.019$], as was the IFL3 rs8099917 single nucleotide polymorphism (SNP) (OR 5.06, $P = 0.068$). The correlation between ss469415590 and rs8099917 was high ($r^2 = 0.92$, $D' = 0.98$). Multivariate analysis revealed that the rs8099917 SNP was independently associated with treatment failure (OR 5.28, $P = 0.009$). Therefore, ss469415590 may be another predictive marker of antiviral therapy outcome in the Japanese population.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. Chronic HCV infection often develops into chronic hepatitis, leading to liver cirrhosis and/or hepatocellular carcinoma (HCC) (1, 2). As approximately 70–80% of Japanese HCC patients are infected with HCV (3), the successful eradication of HCV, defined as a sustained virological response (SVR), is considered important to decrease the incidence of HCC. Approximately 50% of Japanese patients with genotype 1 HCV infection do not achieve an SVR by conventional pegylated interferon-α (PEG-IFN) and ribavirin (RBV) therapy. Although the addition of a direct-acting antiviral agent to this regimen has increased response rate (4), reliable markers are needed to better predict treatment outcome.

The strongest genetic factors associated with patient response to PEG-IFN and RBV therapy and spontaneous clearance are single nucleotide polymorphisms (SNPs) around *IFNL3* (*IL28B*) [reviewed in (5)]. In particular, the rs8099917 SNP has been shown as a good predictive marker for the response to PEG-IFN and RBV therapy in Japanese patients (6–8). Prokunina-Olsson et al. (9) recently discovered a new transiently induced region that harbors a dinucleotide variant ss469415590 (TT or ΔG) between *IFNL3* and *IFNL2*. ss469415590 (ΔG) is a frameshift variant that

creates a novel gene, designated as *IFNL4*, that encodes the interferon-λ4 protein (IFNL4) having moderate similarities to IFNL3. *IFNL4* is transcribed and translated in primary hepatocyte cell lines by the double-stranded RNA virus analog polyinosinic:polycytidylic acid, causing upregulation of interferon-stimulated genes in these cells and replication reduction of an HCV subgenomic replicon (10). In addition, ss469415590 is strongly associated with HCV clearance in African-Americans (9), but this polymorphism has not yet been investigated in the Japanese population. The objective of this study was to determine the prevalence of this *IFNL4* SNP among the Japanese and assess whether it influenced the treatment outcome of PEG-IFN and RBV therapy in patients with chronic hepatitis C.

We recruited 213 treatment-naïve patients with chronic hepatitis C and 176 healthy subjects. Patients were seen at Shinshu University Hospital or affiliated hospitals. Controls were hospital staff volunteers who had indicated the absence of any major illness on a standard questionnaire. The racial background of all subjects was Japanese. Diagnosis of chronic hepatitis C was based on the following criteria, as reported previously (11): (1) presence of serum HCV antibodies and detectable viral RNA; (2) absence of detectable hepatitis B surface antigen and antibody in the human immunodeficiency virus; and (3) exclusion of other causes of chronic liver disease. No patient had a history of, or developed, decompensated cirrhosis or HCC. The baseline characteristics of the patients are shown in Table 1. The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine (No. 302) and all participants provided written informed consent.

Antibodies to HCV were measured in serum samples via third-generation Abbott HCV EIA-3 assays (Abbott Laboratories, Abbott Park, IL). Serum levels of HCV RNA were determined using Cobas Amplicor assays (sensitivity: 50 IU/ml; Roche Diagnostic Systems, Tokyo, Japan). All patients in our test cohort were infected with genotype 1b. Relevant biochemical tests were performed using standard methods (12).

Patients received bodyweight-adjusted doses of PEG-IFNα-2b (PegIntron, MSD K.K., Tokyo, Japan) and RBV (Rebetol, MSD K.K.) for 48 weeks, as reported previously (13). Response to therapy was categorized as follows: an SVR was defined as undetectable serum HCV RNA 24 weeks after completing therapy. Relapse was defined as a reappearance of serum HCV RNA after treatment in patients whose HCV RNA level was undetectable during or at the completion of therapy. A nonresponse was defined as a decrease in HCV RNA of <2 log copies/ml at week 12 and detectable HCV RNA during the treatment course.

Genomic DNA was isolated from whole blood samples by phenolic extraction of sodium dodecyl sulfate-lyzed and proteinase K-treated cells, as described previously (14). Genotyping of the rs8099917 SNP (T/G) was performed using an ABI TaqMan allelic discrimination kit and the ABI7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA) (8). Exon 1 of the *IFNL4* gene was amplified by the polymerase chain reaction (PCR) in the presence of Takara Taq™ (Takara Bio Inc., Otsu, Japan) with the sense primer (5′-CATTGCCTTCCCTGGGATCCTAAC-3′) and the antisense primer (5′-GGACCCCTTGGGACAGGAAC-3′). The sizes of the amplified DNA fragments (333 or 334 bp) were confirmed by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. PCR products were directly sequenced with a BigDye Terminator Cycle Sequencing Reaction Kit using an ABI 3100 DNA sequencer. We assessed the rs4803221 (C/G), ss469415590 (TT/ΔG), rs73555604 (G/A), and rs150891559 (G/C) SNPs in this study.

Statistical analyses were performed using PASW Statistics 21.0J software (IBM, Tokyo, Japan). The Mann–Whitney *U*-test was used to analyze continuous variables, whereas the chi-squared test with Yate's correction was used for the analysis of categorical data. In cases where the number of subjects was <5, Fisher's exact test was employed. The Hardy–Weinberg equilibrium test was performed for each SNP between control and patient groups. Pairwise linkage disequilibrium pattern, haplotype block structure, and haplotype frequency analysis were assessed for all SNPs by the block definition by Gabriel

Table 1 Demographic and clinical characteristics of patients with chronic hepatitis C

Characteristic	All ($n=213$)	VR ($n=162$)	NVR ($n=51$)	*P* value
Age (years)[a]	60 (24–80)	60 (24–80)	59 (39–75)	0.859
Male, *n* (%)	66 (58)	50 (58)	16 (57)	0.926
White blood cell count (/μl)[a]	4300 (1870–8610)	4490 (1870–8610)	4055 (2000–8240)	0.076
Hemoglobin (g/dl)[a]	14.3 (9.0–18.2)	14.4 (9.0–18.2)	13.9 (10.9–16.4)	0.049
Platelets (10^4/μl)[a]	15.4 (7.7–33.6)	15.7 (7.7–33.6)	13.3 (7.7–29.2)	0.236
ALT (IU/l)[a]	46 (14–389)	45 (14–389)	48 (19–323)	0.353
AST (IU/l)[a]	43 (17–246)	41 (17–231)	43 (19–246)	0.297
HCV RNA (10^5 IU/ml)[a]	18 (1.1–51)	19 (1.1–51)	14 (1.5–51)	0.411
rs8099917 allele (TT/TG/GG)	144/64/5	124/36/2	20/28/3	<0.001
ss469415590 allele (TT/TT TT/ΔG ΔGΔG)	142/65/6	122/37/3	20/28/3	<0.001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; VR, virological response; NVR, null virological response.
[a]Median (range).

et al. (15) and were based on a 95% confidence interval (CI) of D' with HAPLOVIEW version 4.2 software (16). We plotted r^2 values. A P value of ≤ 0.05 was considered to be statistically significant. Association strength was estimated by calculating the odds ratio (OR) and 95% CI.

Of the 213 patients receiving PEG-IFN and RBV therapy, 105 (49%) achieved an SVR. Among the 108 patients not reaching an SVR, 57 relapsed and 51 did not respond to therapy. Pre-treatment values for median hemoglobin were significantly higher in the virological response (VR) group compared with the null virological response (NVR) group (Table 1).

We genotyped the rs8099917 SNP and four other SNPs in *IFNL4* (rs150891559, rs73555604, ss469415590, and rs4803221) in 213 patients with chronic hepatitis C and 176 healthy subjects. As rs150891559 and rs73555604 were homozygous for the major allele in all patients and controls, we focused on the rs8099917, ss469415590, and rs4803221 SNPs in this study. The observed genotype frequencies for patients and controls were all in Hardy–Weinberg equilibrium. Among the 213 patients ($2n = 426$), the three SNPs (rs4803221, ss469415590, and rs8099917) showed strong linkage disequilibrium (LD) ($r^2 = 0.88–0.96$) with each other and exhibited haplotypes as follows: haplotype 1: G/TT/T; $n = 348$ (81.7%); haplotype 2: C/ΔG/G; $n = 73$ (17.1%); haplotype 3: G/ΔG/T; $n = 4$ (0.9%); and haplotype 4: G/TT/G; $n = 1$ (0.2%). Among the 176 healthy subjects ($2n = 352$), haplotypes 1 and 2 were seen in 323 (91.8%) and 29 (8.2%) of cases, respectively. The frequencies of haplotypes 1 and 2 showed significant differences between patients with chronic HCV infection and healthy subjects ($P = 0.049$ and $P = 0.025$, respectively). The statistical power of this study was 0.90 and therefore sufficient for analysis.

The G allele frequency of rs8099917 (33.3% *vs* 12.3%; $P = 0.011$, OR = 3.55) and the ΔG allele frequency of ss469415590 (37.3% *vs* 13.3%; $P = 0.044$, OR = 3.27) were significantly higher in the NVR group than in virological responders (Table 2). The overall frequency of the TG or GG genotype for rs8099917 was 60.8% and more common in subjects with an NVR than in those showing a virological response (60.8% *vs* 23.5%; $P = 0.068$, OR = 5.06). TT/ΔG or ΔG/ΔG was also significantly associated with an NVR (60.8% *vs* 24.7%; $P = 0.019$, OR = 4.73). Interestingly, 5 of 213 patients had a rare linkage between rs8099917 and ss469415590 (haplotypes 3 and 4), but showed differing responses to PEG-IFN and RBV therapy. Among the four patients with haplotype 3 (T/ΔG/G), three achieved an SVR and one experienced an NVR. The other patient with haplotype 4 (G/TT/C) exhibited an NVR.

We next evaluated several factors apparently associated with an NVR to PEG-IFN and RBV therapy for independence by multivariate analysis. Only the rs8099917 SNP (TG or GG) was found to be an independent risk factor (OR = 5.28, 95% CI: 2.53–11.01, $P = 0.009$).

In this study, we investigated the frequency of the ss469415590 SNP in Japanese patients with type 1 chronic hepatitis C and analyzed its association with the outcome of PEG-IFN and RBV therapy. As it has been suggested that ss469415590 ΔG/TT is in complete linkage disequilibrium with the minor allele [G] of rs8099917 in individuals of Chinese ancestry, our data were able to confirm that the correlation between ss469415590 and rs8099917 was extremely high ($r^2 = 0.92$, $D' = 0.98$), such that only 5 (2.3%) of 213 individuals were ruled out.

Our data showed that the frequency of haplotype 1 (rs4803221, ss469415590, and rs8099917: G/TT/T) was higher in healthy subjects than in those chronic hepatitis C. These frequencies were very similar to those in published (6) and HapMap data from Japan. Since the major IL28B alleles have been associated with spontaneous clearance of HCV infection (17), carriers of haplotype 1 may have a decreased risk of chronic HCV infection.

As proposed by Prokunina-Olsson et al., ss469415590 [ΔG] was highly associated with treatment outcome in Japanese patients (OR = 4.73, $P = 0.019$). We observed a stronger association for rs8099917 (OR = 5.06) than for ss469415590 with an NVR, although this difference did not reach statistical significance. In multivariate analysis of our cohort,

Table 2 Association of rs8099917 and ss469415590 SNPs with response to PEG-IFN and RBV therapy[a]

SNP	NVR ($n = 51$)	VR ($n = 162$)	OR (95% CI)	P	Controls ($n = 176$)
rs8099917					
G allele	33.3	12.3	3.55 (2.09–6.02)	0.011	8.2
T allele	66.7	87.7			91.8
T/G or G/G	60.8	23.5	5.06 (2.59–9.88)	0.068	16.5
T/T	39.2	76.5			83.5
ss469415590					
ΔG allele	33.3	13.3	3.27 (1.94–5.51)	0.044	8.2
TT allele	66.7	86.7			91.8
TT/ΔG or ΔG/ΔG	60.8	24.7	4.73 (2.43–9.20)	0.019	16.5
TT/TT	39.2	75.3			83.5

CI, confidence interval; NVR, null virological response; OR, odds ratio; SNP, single nucleotide polymorphism; VR, virological response.
[a]All values are expressed as percentages.

rs8099917 [G] was an independent factor related to an NVR in Japanese patients treated with PEG-IFN and RBV therapy, and treatment response was correlated with the rs8099917 SNP, but not ss469415590, in five patients with varying SNPs. Hence, our results indicate that although rs8099917 remains a powerful predictor of PEG-IFN and RBV therapy in the Japanese, ss469415590 may be an effective marker as well.

Although our data support that ss469415590 [ΔG] is strongly associated with treatment failure in chronic hepatitis C, the mechanism remains unknown by which ss469415590 [ΔG] and the IFN-λ4 protein might cause impaired HCV clearance. Prokunina-Olsson et al. (9) showed that IFN-λ4 may pre-active the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway and limit further activation by type-I and type-III IFNs. Furthermore, Bibert et al. (18) have attributed the effect of IFNL4ΔG to decreased induction of IFNL3 and IP-10 mRNA, which rely on the ss469415590 genotype.

In summary, this report demonstrates an association between the dinucleotide variant ss469415590 and treatment response to PEG-IFN and RBV therapy for HCV genotype 1 infections in the Japanese population. Further studies on this polymorphism will more clearly elucidate the mechanism of antiviral response.

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Conflict of Interest

Drs TU and ET are currently conducting research sponsored by MSD. All other authors have declared no conflicting interests.

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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

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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

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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.

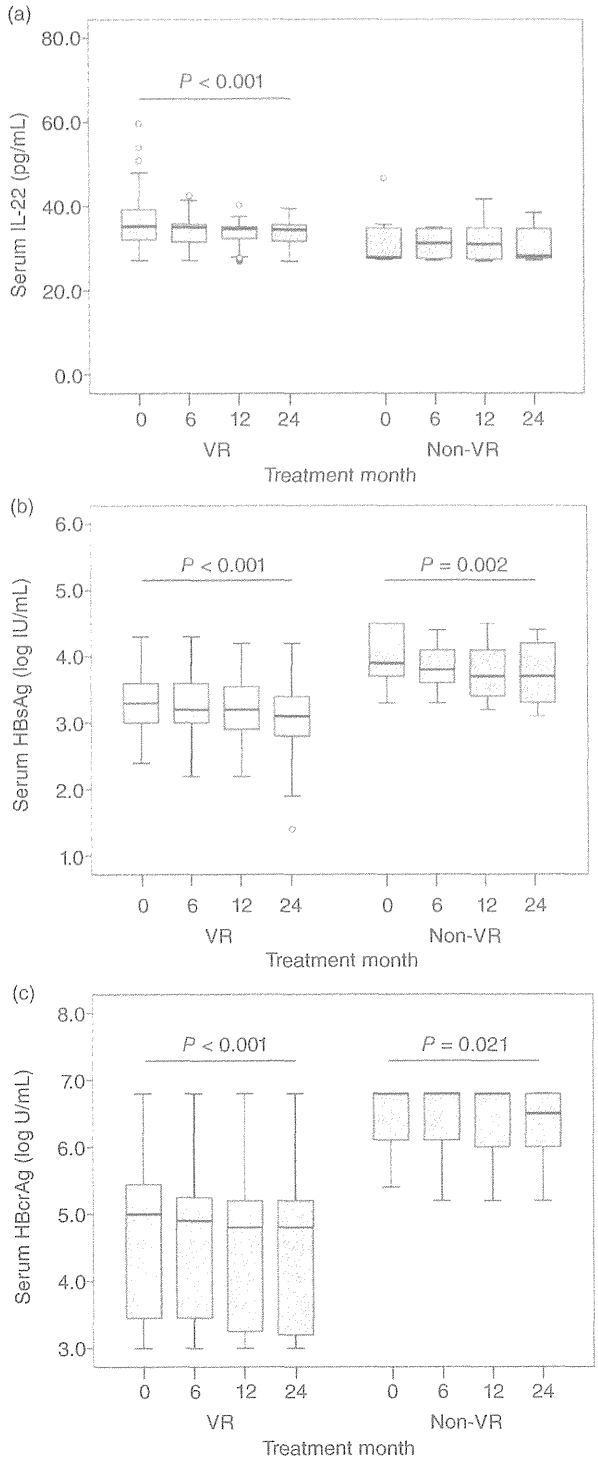

Figure 1 Comparison of serum (a) IL-22, (b) HBsAg and (c) HBcrAg levels during entecavir therapy in the VR ($n = 39$) and non-VR ($n = 9$) groups. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The harsh marks above and below the boxes indicate 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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