

Figure 2 The progression of analyses in the present study and population structure of each analysis.

both negative for HBeAg and whose serum HBV DNA was lower than 3.0 log copies/mL at NA cessation. Table 1 shows the comparison of clinical and virological backgrounds between the 53 relapse and 32 non-relapse patients using univariate analysis. Age and gender distributions were similar between the groups. Approximately 75% of the 85 patients had HBV genotype C, but the distribution of genotypes did not differ between the groups. Approximately 90% of patients were being treated with LVD alone at the time of discontinuation, compared with 6% of patients being given ETV. The median duration of NA treatment was about two times longer in patients without relapse. Levels of both HBsAg

and HBcrAg were significantly lower in non-relapse patients than in relapse patients at the time of NA discontinuation. The difference between serum HBsAg was also significant at the initiation of NAs, but not that of HBcrAg. As only patients with HBV DNA lower than 3.0 log copies/mL were analyzed, the majority of these cases showed levels below the 2.6 log copies/mL lower detection limit of the Amplicor assay at NA discontinuation. We therefore also tested HBV DNA with a TaqMan assay, which had a higher sensitivity than the Amplicor assay, in 43 patients whose serum samples were available. The prevalence of patients having a negative detection signal did not differ between the two groups. The number of

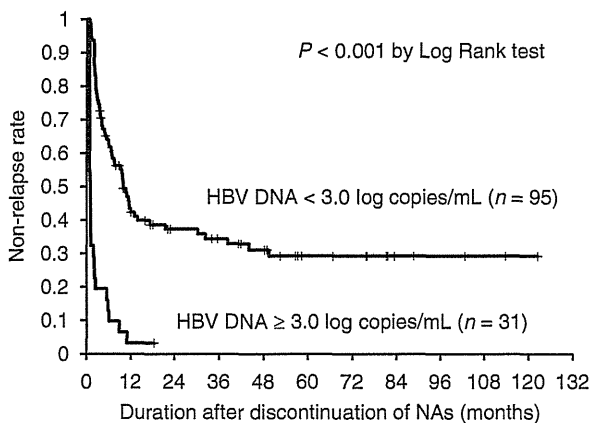


Figure 3 Comparison of non-relapse rates using the Kaplan-Meier method between 31 patients with serum hepatitis B virus (HBV) DNA equal to or higher than 3.0 log copies/mL and 95 patients with serum HBV DNA lower than 3.0 log copies/mL at the time of nucleos(t)ide analog (NA) discontinuation.

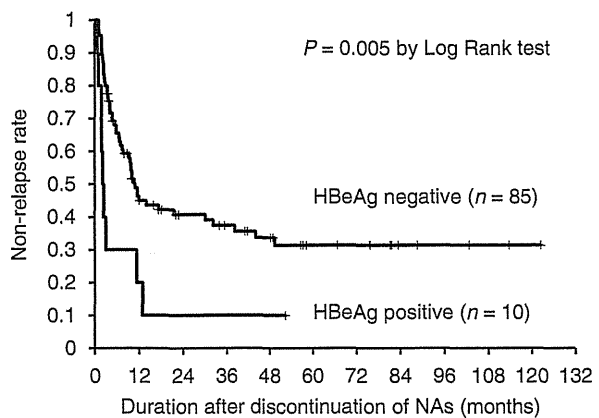


Figure 4 Comparison of non-relapse rates using the Kaplan-Meier method between 10 patients with detectable hepatitis B e antigen (HBeAg) and 85 patients without detectable HBeAg at the time of nucleos(t)ide analog (NA) discontinuation.

**Table 1** Comparison of clinical and virological backgrounds between patients with and without relapse of hepatitis at initiation and discontinuation of nucleos(t)ide analogs (NAs)

Background	Non-relapse patients (n = 32)	Relapse patients (n = 53)	P-value
At initiation of NAs			
Age (years)†	47 (17–75)	48 (26–74)	>0.2
Gender (M : F)	23:9	32:21	>0.2
ALT (IU/L)†	183 (9–1182)	187 (20–2052)	>0.2
Genotype (A : B : C : UD)	1:2:21:8	0:3:44:6	0.193
HBeAg (positive)‡	11 (34%)	16 (30%)	>0.2
HBV DNA			
Amplicor assay (log copies/mL)†	6.2 (<2.6–>7.6)	6.5 (<2.6–>7.6)	0.099
HBsAg (log IU/mL)†	2.7 (0.1–4.3)	3.3 (1.6–3.9)	0.018
HBcrAg (log U/mL)†	5.2 (<3.0–>6.8)	5.6 (<3.0–>6.8)	>0.2
At discontinuation of NAs			
Age (years)†	50 (21–78)	49 (26–79)	>0.2
NAs (LVD : LVD+ADV : ETV : ADV)	28:1:3:0	50:0:2:1	>0.2
Duration of NA treatment (months)†	36 (4–129)	17 (4–84)	0.007
Follow-up period after discontinuation of NAs (months)†	45 (6–123)	12 (1–111)	0.002
ALT (IU/L)†	16 (7–38)	20 (9–65)	0.002
HBV DNA			
Amplicor assay (log copies/mL)†	<2.6 (<2.6–2.9)	<2.6 (<2.6–2.9)	>0.2
TaqMan assay (negative signal)‡	5 (23%) (n = 22)	3 (14%) (n = 21)	>0.2
TaqMan assay (negative or positive signal)‡	13 (59%) (n = 22)	13 (62%) (n = 21)	>0.2
HBsAg (log IU/ml)†	2.0 (<-1.5–4.3)	3.1 (0.6–4.0)	0.001
HBcrAg (log IU/mL)†	3.4 (<3.0–4.9)	4.3 (<3.0–>6.8)	0.003

†Data are expressed as the median (range)

‡Data are expressed as a positive number (%)

ADV, adefovir dipivoxil; ALT, alanine aminotransferase; ETV, entecavir; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; LVD, lamivudine; UD, undetermined.

patients with a negative detection signal or a positive signal also did not vary significantly. The follow-up period after discontinuation of NAs was significantly shorter in patients with relapse than in those without because formal follow-up ended once patients relapsed. The median period of follow-up was 45 months in patients without relapse.

Multivariate analyses revealed that a shorter duration of NA treatment and higher levels of HBsAg and HBcrAg at discontinuation were significantly associated with the occurrence of hepatitis relapse (Table 2). The cut-off

values that showed the highest significance by ROC analysis were 1.9 log IU/mL for HBsAg (AUC = 0.707,  $P = 0.001$ ), 4.0 log U/mL for HBcrAg (AUC = 0.692,  $P = 0.003$ ), and 16 months (AUC = 0.674,  $P = 0.007$ ) for treatment duration.

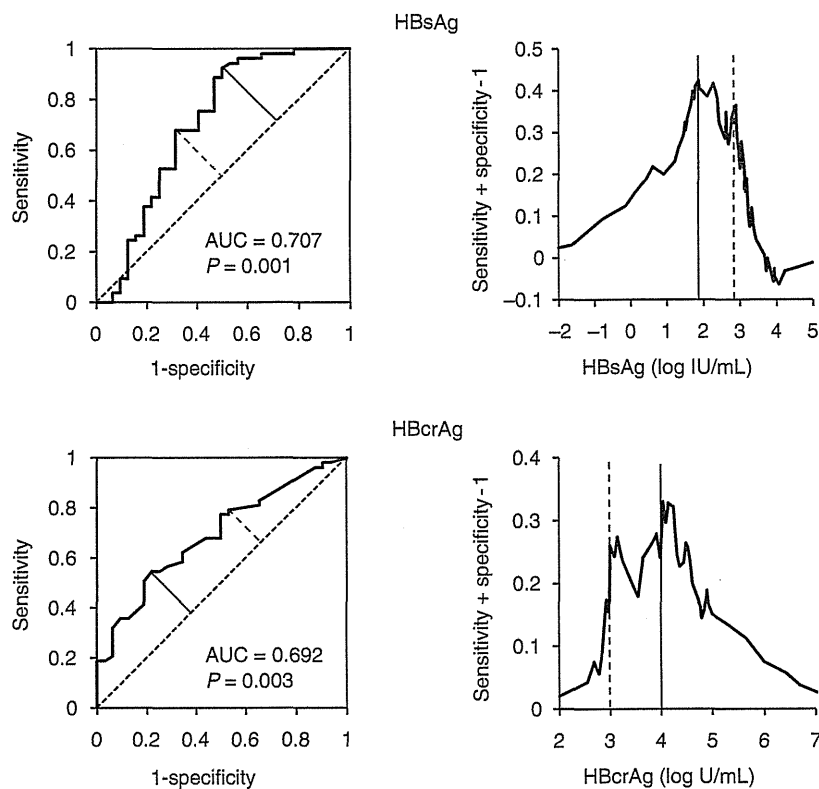
### Model for predicting relapse of hepatitis using levels of HBsAg and HBcrAg

The existence of a second cut-off value was suggested by ROC analysis for both of HBsAg (2.9 log IU/mL) and HBcrAg (3.0 log IU/mL) to discriminate between

**Table 2** Multivariate analysis of factors associated with relapse of hepatitis after discontinuation of nucleos(t)ide analogs (NAs)

Factor	Hazard ratio	95%CI	P-value
HBsAg at discontinuation $\geq 1.9$ log IU/mL	5.21	1.87–14.55	0.002
HBcrAg at discontinuation $\geq 4.0$ log U/mL	2.20	1.25–3.87	0.006
Duration of NA treatment $\geq 16$ months	0.54	0.31–0.93	0.027

CI, confidence interval; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen.



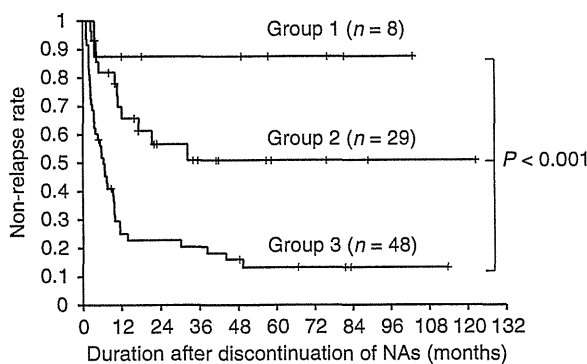
**Figure 5** Receiver operating characteristic curve (ROC) analysis of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg. Short diagonal lines indicate main inflection points and short broken diagonal lines indicate second inflection points. Vertical lines indicate actual values of antigens that correspond to the main inflection points and vertical broken lines indicate actual values of antigens that correspond to the second inflection points.

patients with and without relapse (Fig. 5). Thus, we set cut-off values as 1.9 and 2.9 log IU/mL for HBsAg and 3.0 and 4.0 log U/mL for HBcrAg in our model for predicting hepatitis relapse.

We tentatively defined three groups using the sum of the scores for HBsAg and HBcrAg levels at the time of NA discontinuation for our model. Conversions were made by assigning a score of 0 for an HBsAg level lower than 1.9 log IU/mL, 1 for a level from 1.9 to 2.8 log IU/mL, and 2 for a level equal to or higher than 2.9 log IU/mL. HBcrAg was scored as 0 for a level lower than 3.0 log U/mL, 1 for a level from 3.0 to 3.9 log U/mL, and 2 for a level equal to or higher than 4.0 log U/mL. Overall, group 1 consisted of patients with a total score of 0, group 2 of patients with a total score of 1 or 2, and group 3 of patients with a total score of 3 or 4.

Patients whose HBV DNA was lower than 3.0 log copies/mL and in whom HBeAg was negative at the time of NA discontinuation were assigned to one of the three groups. Figure 6 shows the comparison of non-relapse rates among the three groups using Kaplan–Meier analysis, which differed significantly. The non-relapse rate was approximately 90% in group 1, as low as 10% in

group 3, and intermediate in group 2. When factors associated with relapse were analyzed in group 3 patients, an age of over 40 years at the time of discontinuation was calculated as a significant factor (hazard



**Figure 6** Comparison of non-relapse rates using the Kaplan–Meier method among three groups classified by the sum of the scores of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels at the time of nucleos(t)ide analog (NA) discontinuation.

ratio = 5.25, range 2.37–11.65,  $P < 0.001$ ). No significant factors were associated with relapse in group 2 patients.

## DISCUSSION

THE EUROPEAN ASSOCIATION for the Study of the Liver recommends continuation of NA treatment until HBsAg is cleared.<sup>25</sup> Liu *et al.* came to a similar conclusion in their study of chronic hepatitis B patients treated with LVD.<sup>14</sup> Indeed, the clearance of HBsAg is a reliable marker for the safe discontinuation of NAs, but the rate of patients who can clear HBsAg is relatively low (1–3%/year).<sup>26–28</sup> Thus, additional factors associated with relapse of hepatitis B after discontinuation of NAs were analyzed in the present study to better identify candidates who could achieve drug-free status. Such studies are relatively few, possibly because patients who discontinue NAs prematurely often experience severe complicating relapse and hepatic failure.<sup>9</sup> Although prospective studies are desirable to obtain accurate results, retrospective studies, such as ours, are also necessary to minimize the risk of adverse complications.

Since HBV cannot be completely eradicated in hosts, the primary goal in treating chronic hepatitis B is to convert symptomatic patients into inactive carriers in whom HBeAg is negative (usually anti-HBe-positive), serum HBV DNA is low, and serum ALT is normal.<sup>1,2,18,29</sup> Thus, we set the clinical conditions of a successful discontinuation of NAs as serum HBV DNA level below 4.0 log copies/mL and ALT below 30 IU/L following NA cessation. Patients who satisfy these conditions are not recommended for treatment by the Japanese guidelines for hepatitis B,<sup>18</sup> and it is also widely accepted that the risk of developing cirrhosis or complicating hepatocellular carcinoma is very low in such patients.<sup>30,31</sup> We used our cohort's mean and maximal values of HBV DNA and ALT for relapse analyses. Mean values were useful for evaluating relapse of hepatitis as a whole since parameter levels often fluctuated after discontinuation, and maximal values were used to evaluate relapse in a real-time fashion during the follow-up period. It is noteworthy that the mean and maximal values correlated very closely for both HBV DNA and ALT. The mean HBV DNA value of 4.0 log copies/mL corresponded to the maximal HBV DNA value of 5.7 by ROC analysis, and similarly the mean ALT value of 30 IU/L corresponded to the maximal ALT value of 79 IU/L. Thus, relapse of hepatitis B was judged to occur when serum ALT became higher than 79 IU/L or when serum HBV DNA surpassed 5.7 log copies/mL after the time of NA discontinuation.

Such criteria may also be useful for physicians to detect relapse at an early phase and avoid the occurrence of severe reactivation or unnecessary discontinuation of NAs.

It is generally understood that patients with a higher level of HBV DNA at the time of NA discontinuation are likely to relapse, but this cut-off value has not been analyzed sufficiently. Our findings using ROC analysis showed that patients with levels lower than 3.0 log copies/mL have a good possibility to achieve successful discontinuation. The presence of HBeAg is also generally accepted as a reliable factor to predict relapse of hepatitis. Our study showed that patients with detectable HBeAg at the time of NA discontinuation were likely to relapse, even if their HBV DNA levels were lower than 3.0 log copies/mL. Therefore, we next analyzed additional factors associated with a relapse of hepatitis after discontinuation of NAs by selecting patients who met both of these criteria.

Nucleos(t)ide analog treatment produces a rapid decrease in serum HBV DNA by suppressing reverse transcription of pregenomic HBV RNA. However, the key intrahepatic HBV replicative intermediate, covalently closed circular DNA (cccDNA), tends to remain and is capable of reinitiating replication once NAs are ceased.<sup>32</sup> Measurement of HBV cccDNA has been reported to be useful for monitoring and predicting responses to antiviral treatments.<sup>33</sup> However, its measurement is difficult in the clinical setting as it requires a liver biopsy. Due to the mechanism of action of NAs mentioned above, serum HBV DNA does not reflect intrahepatic HBV cccDNA in patients undergoing NA treatment.<sup>34</sup> To address this, quantitative measurement of HBV antigens has been reported to be useful for predicting the effect of antiviral treatment in patients with chronic hepatitis B. Although HBsAg is usually used as a serum marker for the diagnosis of HBV infection, several groups have shown that HBsAg levels can also be reflective of the response to peg-interferon in chronic hepatitis B.<sup>28,35,36</sup> The HBcrAg assay measures serum levels of HB core and e antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these two denatured antigens. Since the assay measures all antigens transcribed from the pre-core/core gene, it is regarded as core-related.<sup>37</sup> Serum HBcrAg has been reported to accurately reflect intracellular levels of HBV cccDNA even during NA treatment,<sup>24,34,38</sup> and was found to be useful for identifying patients who were likely to show relapse of hepatitis after the discontinuation of NAs.<sup>39,40</sup> It is possible that levels of HBsAg and HBcrAg have different roles in

monitoring antiviral effects because the transcription of these two antigens are regulated by alternative enhancer-promoter systems in the HBV genome.<sup>3</sup> Therefore, we analyzed both of these antigens to elucidate their ability to predict relapse of hepatitis after discontinuation of NAs.

Multivariate analysis demonstrated that levels of HBsAg and HBcrAg at the time of NA discontinuation were independent factors significantly associated with relapse of hepatitis. Thus, we believe these factors can also be applied for predicting relapse in patients whose HBV DNA is lower than 3.0 log copies/mL and whose HBeAg is negative at NA discontinuation. HBV DNA levels were further analyzed using a highly sensitive assay based on real-time polymerase chain reaction (PCR). However, even the level of a negative signal did not ensure successful discontinuation of NAs. The results obtained here indicate that the combined use of HBV-related antigens are useful makers for monitoring the effect of anti-viral treatment in ways different from HBV DNA. Finally, since prolonged NA administration was also a significant factor associated with safe discontinuation, physicians are advised to continue patient treatment for at least 16 months for the best possible outcome.

From our data, a tentative model for predicting relapse of hepatitis after discontinuation of NAs was constructed using levels of HBsAg and HBcrAg at discontinuation. A negative result for HBeAg and HBV DNA lower than 3.0 log copies/mL at the time of NA discontinuation are the essential conditions in this system. Levels of HBsAg and HBcrAg were each converted into scores from 0 to 2 partly because two cut-off values were needed for each antigen and partly because a scoring system may be more convenient for clinical use. The sum of the two scores, which ranged from 0 to 4, was used to prospect relapse. We found that group 1 patients who had a low score (0) could be recommended to discontinue NAs because nearly 90% of this group achieved successful discontinuation. Further analysis of factors associated with relapse are needed for group 2 patients who had middle range scores (1 or 2), since the odds of achieving successful discontinuation were approximately 50%. Continuation of NA treatment is recommended for group 3 patients having high scores (3 or 4) because nearly 90% of this group relapsed. However, this recommendation may be reconsidered in patients younger than 40 years; such cases tended to have a lower relapse rate in group 3. It is also noteworthy that relapse occurred mainly during the first and second years following NA discontinuation in

all groups, similarly to a report by Liu *et al.*<sup>14</sup> Thus, clinicians should be vigilant in the early phase after discontinuation.

This study has several limitations. The patients who discontinued NAs were recruited retrospectively, and thus the decision to halt NA treatment was made by individual physicians without uniformly established criteria. Based on this, prospective studies are required to confirm our results. Furthermore, as over 90% of the patients we enrolled had genotype C and over 90% of cases were treated with LVD until discontinuation, the results obtained here can not be applied directly to other HBV genotypes or other types of NAs.

In conclusion, the present study showed that maximal levels of serum ALT and HBV DNA were useful for defining relapse patients after discontinuation of NAs. Along with serum HBV DNA of less than 3.0 log copies/mL and negative serum HBeAg, serum levels of HBsAg and HBcrAg at the time of NA discontinuation were able to predict relapse of hepatitis B and should therefore be considered when establishing uniform guidelines regarding the safe withdrawal of NA treatment. To this end, NA administration of more than 16 months is advisable to achieve successful discontinuation.

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## Case Report

## Early detection of interstitial pneumonia by monitoring KL-6 in a chronic hepatitis C patient undergoing pegylated interferon and ribavirin therapy

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A 58-year-old woman with chronic hepatitis C developed interstitial pneumonia (IP) while undergoing pegylated interferon (PEG IFN)- $\alpha$ -2a and ribavirin (RBV) therapy. Serum levels of sialylated carbohydrate antigen KL-6 (KL-6), a known marker of disease activity in fibrosing lung disorders, had been regularly measured once a month for early detection of IP, and had begun rising noticeably from 12 weeks to 540 U/mL at 33 weeks of treatment. On examination, remarkable fine crackles were detected by dorsal auscultation and bilateral ground-glass opacities and reticular shadows were depicted

by computed tomography. The patient successfully recovered from her early-stage pneumonia by immediate discontinuation of therapy, which indicates that regular monitoring of serum KL-6 may be effective for avoidance of IP progression induced by PEG IFN and RBV therapy.

**Key words:** interstitial pneumonia, KL-6, pegylated interferon, ribavirin

## INTRODUCTION

PEGYLATED INTERFERON (PEG IFN)- $\alpha$ -2a combined with ribavirin (RBV) has become one of the gold standards for hepatitis C virus (HCV) treatment.<sup>1</sup> However, side-effects are observed in almost 80% of patients receiving this therapy. Pulmonary toxicity in patients undergoing HCV treatment is rare, especially interstitial pneumonia (IP) induced by PEG IFN and RBV therapy. Sialylated carbohydrate antigen KL-6 (KL-6) is a mucinous high-molecular weight glycoprotein expressed on type 2 pneumonocytes that is a

useful marker for the clinical diagnosis of interstitial lung diseases and the evaluation of disease activity.<sup>2</sup> It was reported that the sensitivity, specificity and diagnostic accuracy for KL-6 were 93.9%, 96.3% and 95.7%, respectively, for interstitial lung diseases.<sup>3</sup>

Herein, we describe a patient who avoided progression to severe IP induced by PEG IFN- $\alpha$ -2a and RBV therapy by regularly measuring serum levels of KL-6.

## CASE REPORT

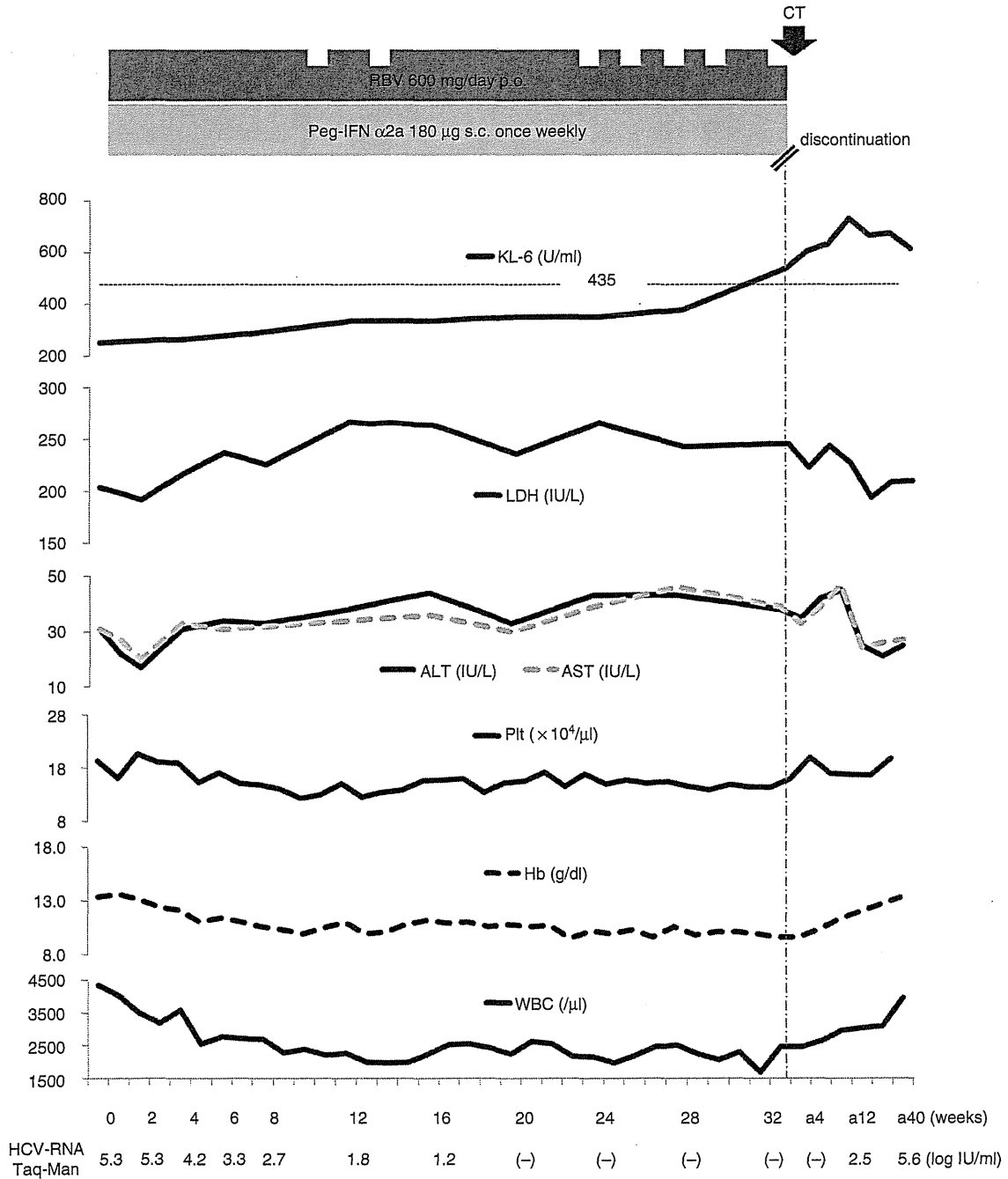
A 58-YEAR-OLD Japanese woman was referred to our hospital by her primary care physician for treatment of HCV likely stemming from a blood transfusion 33 years prior during childbirth. Her serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) had been consistently greater than 30 IU/L. Chronic hepatitis was histologically proven by liver tissue biopsy, which revealed scores for periportal bridging necrosis, intralobular degeneration and focal necrosis, and fibrosis of 1 each

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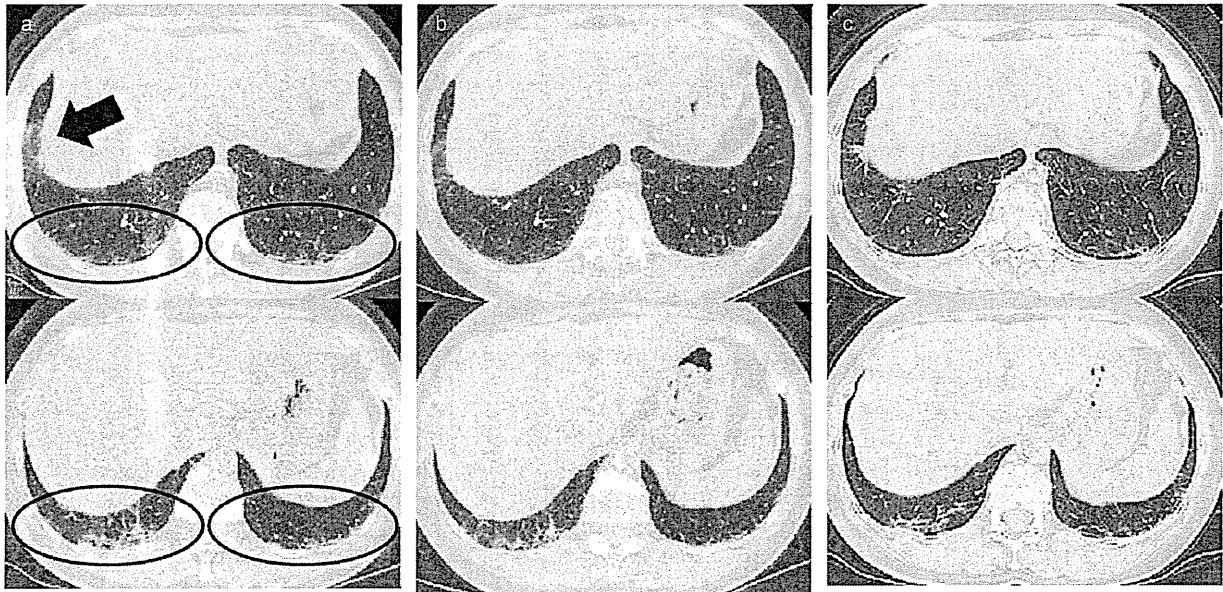
Conflicts of interest: The authors indicate no potential conflicts of interest.

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**Figure 1** Clinical course of the present case. Figure shows the time course of white blood cell count (WBC), hemoglobin level (Hb), platelet count (Plt), serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), lactate dehydrogenase (LDH), serum level of KL-6 and serum hepatitis C virus (HCV) RNA by Taq-Man assay. Serum levels of C-reactive protein have been <0.03 mg/dL during the clinical course (data not shown). Shaded areas indicate the doses of pegylated interferon (PEG IFN)- $\alpha$ -2a and ribavirin (RBV). KL-6 began to rise noticeably from 12 weeks and reached 540 U/mL (upper limit of normal range, 435) after 33 weeks of combination therapy. Computed tomography (CT) performed at 33 weeks revealed findings compatible with interstitial pneumonia (IP) (Fig. 2a). PEG IFN and RBV treatment was immediately discontinued. The patient avoided development of severe IP without additional therapy.



**Figure 2** (a) Computed tomography (CT) performed at 33 weeks of treatment with pegylated interferon (PEG IFN)- $\alpha$ -2a combined with ribavirin (RBV) shows bilateral ground-glass opacities (black arrow) and reticular shadows (black circles) located in the peripheral and dorsal areas of the lungs. (b) The shadows were diminished slightly 22 weeks after discontinuation of PEG IFN and RBV therapy. (c) The patient's prior pulmonary condition depicted by abdominal CT taken 2 years prior to treatment showed negligible reticular interstitial shadows at the base of the lungs, indicating that the patient might have had mild underlying chronic interstitial pneumonia.

and a portal inflammation score of 3, with 20% fatty deposition as assessed by the Knodell hepatitis activity index (HAI) classification.<sup>4</sup> Her single nucleotide polymorphism of interleukin 28B at rs8099917<sup>5,6</sup> was G/G. She neither smoked nor habitually consumed alcohol. No history of pulmonary disease, frequent respiratory infection or autoimmune disease was noted.

The patient was started on combination treatment of regular doses of PEG IFN- $\alpha$ -2a 180  $\mu$ g s.c. once weekly and RBV 600 mg/day p.o. (Chugai Pharmaceutical, Tokyo, Japan). Serum HCV RNA was decreased to an undetectable level by Taq-Man assay 20 weeks after the initiation of therapy (Fig. 1). She showed occasional mild hematopenia, resulting in periodic dose reductions of RBV to 400 mg/day (Fig. 1). Serum levels of KL-6 were 251 U/mL (normal range, 105–435) at treatment onset and were regularly measured once a month. KL-6 began to rise noticeably from 12 weeks and reached 540 U/mL after 33 weeks of therapy, at which time peripheral white blood cell count and serum levels of C-reactive protein and lactate dehydrogenase were 1690/ $\mu$ L with neutrophils of 900/ $\mu$ L,

0.03 mg/dL and 249 IU/L (normal range, 114–220), respectively. Although clinical symptoms of dry cough, fever and dyspnea were undetectable, fine crackles could be heard during auscultation of her back. Computed tomography (CT) revealed bilateral patchy ground-glass opacities around the dorsal area of the lungs (Fig. 2a). No evidence of congestive heart failure was demonstrated by cardiac ultrasonography. PEG IFN and RBV treatment was immediately discontinued due to suspicions of complicating IP. She was observed carefully without any further medication. Serum levels of KL-6 continued increasing until 4 weeks after cessation of therapy to 731 U/mL and then decreased gradually, but did not return to pretreatment levels at her final medical checkup 10 months after cessation of therapy. Accordingly, she was monitored by regular follow up at our outpatient clinic for further symptoms of IP and ultimately avoided development of severe IP. Serum HCV RNA became detectable by Taq-Man assay 12 weeks after discontinuation of PEG IFN and RBV therapy, but CT performed at 22 weeks after halting combination treatment depicted that the patchy ground-glass opacities had diminished slightly

**Table 1** Published cases of interstitial pneumonia complicating pegylated interferon (PEG IFN) and ribavirin combination therapy in English-language published work

Case	Age (years)	Sex	Genotype	Fibrosis	Tobacco	Type of PEG IFN	PEG IFN ( $\mu\text{g}/\text{week}$ )	Ribavirin (mg/day)	Onset (weeks)	Clinical symptoms	Therapies	Result
1 <sup>8</sup>	48	Female	2b	F3	N.D.	$\alpha 2a$	N.D.	N.D. + A	6	Fever, cough	Discontinuation and steroids	Resolved
2 <sup>9</sup>	72	Male	N.D.	N.D.	N.D.	$\alpha 2b$	1.5/kg	800	16	Dyspnea	Discontinuation and steroids	Resolved
3 <sup>10</sup>	49	Male	N.D.	Cirrhosis*	Former	$\alpha 2b$	150	1200	2	Cough, dyspnea	Discontinuation and steroids	Death <sup>§</sup>
4 <sup>11</sup>	71	Female	2 and 4	N.D.	Never	$\alpha 2a$	180	800	6	Cough, shortness of breath	Discontinuation	Resolved
5 <sup>12</sup>	51	Male	1a	F3*	N.D.	$\alpha 2b$	100	1200	5	Fever, dry cough, dyspnea	Discontinuation and steroids	Death #
6 <sup>13</sup>	58	Female	1b	F3*	Never	$\alpha 2b$	1.5/kg	1000	12	Dyspnea	Discontinuation and inhalation steroids	Resolved
7 <sup>14</sup>	47	Female	2b	Cirrhosis**	Never	$\alpha 2b$	100	800	8	Dry cough, dyspnea	Discontinuation and steroids	Resolved
8 <sup>15</sup>	43	Female	1b	Cirrhosis*	N.D.	$\alpha 2b$	120	800	48	Dyspnea, cough, fever	Discontinuation and steroids	Death <sup>§</sup>
9 <sup>16</sup>	68	Male	1b	N.P.	N.D.	$\alpha 2a$	100	800	36	Exertional dyspnea	Discontinuation and steroids	Resolved
10 <sup>17</sup>	51	Male	3	F2*	N.D.	$\alpha 2b$	150	800	4	Dry cough	Discontinuation and steroids	Resolved
Our case	58	Female	1b	F1	Never	$\alpha 2a$	180	600	33	None	Discontinuation	Resolved

\*clinically diagnosed without histopathology; \*METAVIR score<sup>21</sup>; \*\*histologically demonstrated as probable cirrhosis; <sup>§</sup>death from acute respiratory distress syndrome and multi-organ failure; <sup>§</sup>, death from progressive hypoxemia induced by interstitial pneumonia; <sup>§</sup>, death from acute cholestatic hepatitis.

+A, Amantadine; N.D., not described; N.P., not performed.

(Fig. 2b). Abdominal CT taken 2 years prior to therapy depicted negligible reticular interstitial shadows at the base of the lungs (Fig. 2c), indicating that the patient might have been complicated with mild chronic IP at treatment onset.

## DISCUSSION

**M**ANY SIDE-EFFECTS of PEG IFN and RBV therapy have been reported, such as hematological disorders, flu-like symptoms, neuropsychiatric disturbances, ophthalmological disorders, glucose metabolism disruption, autoimmune disease exacerbation, sarcoidosis, dermatological complications, hair loss and thyroid dysfunction; almost all of which can be managed with supportive care. It was also reported that respiratory tract symptoms, including a non-productive cough and shortness of breath, may occur,<sup>7</sup> and that the etiology of dyspnea and other respiratory symptoms is usually attributed to anemic severity. A total of 10 cases that were complicated with IP during combination therapy have been reported to date (Table 1).<sup>8–17</sup> Among them, no relationships with regards to age, sex or type of PEG IFN were apparent. In addition, IP arose at any stage of treatment or hepatic fibrosis, and was unrelated to present or former use of tobacco. Clinical symptoms included fever, cough and dyspnea in almost all cases, although these are non-specific as physical findings in patients with IP. However, crackles may be present despite the absence of abnormalities in chest X-rays; clinicians are advised to auscult the base of the lungs along the posterior axillary line when diagnosing for IP, as crackles may be audible in this location at disease onset, as presented in this case.

The mechanism of IP related to PEG IFN and RBV remains elusive, but is considered to be related to pathophysiological and immunomodulatory causes. One of the main contributing factors to IP is the direct toxicity of the HCV treatment to the lungs. Another possibility is indirect mechanisms acting via immunological pathways, such as T-cell abnormalities.<sup>18</sup> We also cannot exclude the involvement of HCV itself in the pathogenesis of IP induced by IFN therapy because no such reports have been found for patients treated for hepatitis B virus. IP associated with IFN monotherapy<sup>19</sup> or PEG IFN monotherapy<sup>8</sup> has been reported to date. However, RBV monotherapy has never been reported as the cause of IP because it is always given in conjunction with IFN or PEG IFN for treatment of HCV. Thus, PEG IFN, and not RBV, seems to have been the primary cause of IP.

Interstitial pneumonia developing during HCV treatment requires prompt detection and immediate discontinuation of PEG IFN and RBV therapy<sup>8–17</sup> due to a reported mortality rate of 7%.<sup>17</sup> Notably, three cases treated with PEG IFN and RBV died from associated IP despite being treated with corticosteroids after immediate IFN discontinuation.<sup>10,12,15</sup>

Serum KL-6 is a sensitive marker of disease activity in fibrosing lung disorders.<sup>2,3</sup> It was reported that KL-6 levels gradually increased from pretreatment levels when retrospectively measured every 12 weeks during a 48-week treatment course in chronic hepatitis C patients treated with PEG IFN and RBV therapy,<sup>20</sup> although no patients developed IP in the cohort. Nonetheless, changes in serum KL-6 may provide useful information to assess early suspicions of IP, especially if accompanied by other diagnostic findings such as clinical examination or CT. In this case, a progression to severe IP could be prevented by discontinuation of combination therapy through monthly monitoring of serum KL-6. Her continuously elevated serum KL-6 has necessitated regular follow up, and may be related to the persistent pulmonary shadows in chest CT.

In conclusion, clinicians should bear IP in mind as a complication during PEG IFN and RBV combination therapy. Measurement of serum KL-6 is advised to detect and avoid progression of IP at an early stage.

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## Original article

Serum interleukin (IL)-10 and IL-12 levels and *IL28B* gene polymorphisms: pretreatment prediction of treatment failure in chronic hepatitis C

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**Background:** Both *IL28B* gene polymorphisms and serum levels of interleukin (IL)-10, IL-12p40 and IL-18 have been reported to affect the outcome of natural and pegylated interferon and ribavirin-treated HCV infection.

**Methods:** To clarify their association and predictive value in treatment outcome of genotype 1 HCV-infected patients, we measured pretreatment serum IL-10, IL-12p40 and IL-18 levels using multiplex assays and determined *IL28B* gene polymorphisms (rs 8099917) in 52 cases with chronic hepatitis C.

**Results:** High baseline levels of IL-10 ( $P<0.001$ ) and low levels of IL-12p40 ( $P<0.001$ ) were significantly associated with a non-virological response (NVR) in our cohort. The *IL28B* polymorphism was tested and TT, TG or GG genotypes were found in 60%, 38% and 2% of patients,

respectively, with corresponding NVR rates of 10%, 60% and 100% ( $P<0.001$ ). Serum cytokine levels were significantly correlated with *IL28B* gene polymorphisms. When serum IL-10 levels were stratified at 5.0 pg/ml, NVR rates were 50% versus 0% ( $P=0.004$ ) for the TT genotype and 87% versus 0% ( $P=0.001$ ) for the TG or GG genotypes. Similarly, low IL-12p40 levels were associated with an NVR in patients with TG or GG genotypes ( $P=0.006$ ). In multivariate analysis, high IL-10, low IL-12p40 and *IL28B* TG or GG genotypes were independently associated with an NVR.

**Conclusions:** Serum IL-10 and IL-12p40 levels in combination with *IL28B* genotype, especially G-allele carriage, are strong predictive markers of an NVR to HCV treatment with pegylated interferon and ribavirin.

## Introduction

Chronic HCV infection often develops into chronic hepatitis leading to liver cirrhosis and/or hepatocellular carcinoma [1–3]. The successful eradication of HCV, defined as a sustained virological response (SVR), is therefore considered important. Despite recent advances, however, approximately 50% of patients with genotype 1 HCV infection do not achieve an SVR by conventional pegylated interferon (PEG-IFN) and ribavirin therapy [4,5].

It is considered beneficial to predict the response of patients with genotype 1 HCV and high viral load to PEG-IFN and ribavirin therapy before commencement of treatment because therapy can be long, costly and have many side effects. To date, many predictive factors have been reported for treatment response. Regarding viral factors, substitutions at core amino

acids 70 and 91 [6] or the IFN sensitivity determining region (ISDR) have been reported [7]. Concerning host factors, Ge *et al.* [8] recently identified single nucleotide polymorphisms (SNPs) located 5' to the *IL28B* gene that affect response to combination therapy using a genome-wide association study. Similarly, three other groups independently reported that these SNPs are associated with the effectiveness of combination treatment [9–11]. Thomas *et al.* [12] also reported that the same SNPs are associated with spontaneous clearance of HCV.

Interleukin (IL)-28A, IL-28B and IL-29 gene products belong to the IFN- $\lambda$  family. These cytokines are functionally considered to be IFNs, but have been reported to be structurally related to the IL-10 family, which include IL-10, IL-22 and IL-26, and the

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

Characteristic	All (n=52)	VR (n=36)	NVR (n=16)	P-value
Age, years	58 (17–74)	57 (17–72)	60 (45–74)	0.781
Male, n (%)	24 (46)	18 (50)	6 (38)	0.404
Body mass index, kg/m <sup>2</sup>	23 (18–30)	24 (18–30)	22 (19–29)	0.115
White blood cell count, cells/ $\mu$ l	4,470 (1,980–7,890)	4,810 (1,980–7,890)	3,700 (2,270–5,180)	0.007
Haemoglobin, g/dl	14.7 (12–18)	15.0 (13–18)	14.1 (12–16)	0.094
Platelet count, 10 <sup>4</sup> / $\mu$ l	17.5 (8–30)	17.9 (8–30)	16.7 (9–27)	0.420
ALT, IU/l	75 (22–389)	68 (24–389)	91 (22–357)	0.663
AST, IU/l	58 (20–288)	49 (20–218)	78 (25–288)	0.092
HCV RNA, 10 <sup>5</sup> IU/ml	21 (1.1–>50)	20 (1.1–>50)	18 (2.9–>50)	0.469
Core aa 70 (Arg70/Gln70/ND), n	30/21/1	23/12/1	7/9/0	0.139
Core aa 91 (Leu91/Met91/ND), n	37/14/1	26/9/1	11/5/0	0.463
ISDR of NS5A (wild/mutant), n	44/8	29/7	15/1	0.218
rs8099917 allele (TT/TG/GG), n	31/20/1	28/8/0	3/12/1	<0.001

Data are mean (range) unless indicated otherwise. aa, amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ISDR, interferon-sensitivity determining region; NVR, non-virological response; VR, virological response.

IFN- $\lambda$  family. The ligand-binding chains for IL-22, IL-26 and IFN- $\lambda$  are distinct from that used by IL-10. However, all of these cytokines use a common second chain, IL-10 receptor-2, to assemble their active receptor complexes. Thus, IL-10 receptor-2 is a shared component in at least four distinct class II cytokine-receptor complexes [13]. Although IL-10 was originally described as a cytokine synthesis inhibitory factor [14,15], recent studies have demonstrated that IL-10 produced by Th17 cells restrains the pathological effects of Th17 [16,17]. Furthermore, increased IL-10 levels are associated with a high risk of inefficient HCV clearance and resistance to IFN treatment [18–21]. Our recent study showed that low serum IL-10 levels as well as high IL-12p40 and IL-18 levels at baseline were independent predictive factors for a SVR to combination therapy [22]. Therefore, in the present study, we investigated the association between treatment outcome and the influence of *IL28B* genotype and serum cytokine levels in combination therapy.

## Methods

### Subjects

A total of 52 consecutive treatment-naïve patients with genotype 1 chronic hepatitis C were included in this study. Diagnosis of chronic hepatitis C was based on the following criteria as reported previously [23]: presence of serum HCV antibodies and detectable viral RNA, absence of detectable hepatitis B surface antigen and antibody to HIV and exclusion of other causes of chronic liver disease. No patients had a history of or developed decompensated cirrhosis or hepatocellular carcinoma. The baseline characteristics of the patients are shown in Table 1.

### Laboratory testing

Antibodies to HCV were measured in serum samples via third-generation enzyme-linked immunosorbent assays (EIA-3; Abbott Laboratories, North Chicago, IL, USA). Serum levels of HCV RNA were determined using the Cobas Amplicor assays (sensitivity 50 IU/ml; Roche Diagnostic Systems, Tokyo, Japan). HCV genotypes were determined using INNO-LiPA HCV II (Innogenetics, Gent, Belgium). All patients in our test cohort were infected with genotype 1b. Alanine aminotransferase, aspartate aminotransferase and other relevant biochemical tests were performed using standard methods [24].

### Antiviral therapy and definition of treatment outcome

All patients received bodyweight-adjusted PEG-IFN- $\alpha$ 2b (PegIntron; Schering-Plough KK, Tokyo, Japan;  $\leq 45$  kg, 60  $\mu$ g/dose; 46–60 kg, 80  $\mu$ g/dose; 61–75 kg, 100  $\mu$ g/dose; 76–90 kg, 120  $\mu$ g/dose;  $\geq 91$  kg, 150  $\mu$ g/dose), and ribavirin (Rebetol; Schering-Plough KK;  $\leq 60$  kg, 600 mg/day; 61–80 kg, 800 mg/day;  $\geq 81$  kg, 1,000 mg/day) for 48 weeks.

The response to therapy categories were defined 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 non-virological response (NVR) was defined as a decrease in HCV RNA of  $< 2$  log copies/ml at week 12 and detectable HCV RNA during the treatment course.

### Detection of amino acid substitutions in the core and NS5A regions

Core region and ISDR were determined by direct sequencing after amplification by reverse transcription and PCR as reported previously [22]. Amino acids at

positions 70 and 91 of the core region identical to the reference sequence HCV-J D90208 [25] were considered wild type [6]. The number of amino acid substitutions in the ISDR was defined as in Enomoto *et al.* [7].

#### Detection of serum IL-10, IL-12p40 and IL-18

Serum IL-10, IL-12p40 and IL-18 were quantified using Luminex® Multiplex Cytokine Kits (Procarta Cytokine assay kit; Panomics Inc., Fremont, CA, USA) for serum samples obtained before the start of treatment as reported previously [22]. All collected samples were immediately stored at -70°C prior to testing.

#### Genotyping of *IL28B*

Genomic DNA was isolated from the whole blood of patients using QuickGene-800 (Fujifilm, Tokyo, Japan). The concentration of genomic DNA was adjusted to 10–15 ng/μl for the TaqMan SNP genotyping assay. Genotyping of *IL28B* SNP (rs 8099917) was performed with a TaqMan 5' exonuclease assay using primers supplied by Applied Biosystems (Carlsbad, CA, USA). Probe fluorescence signals were detected with a TaqMan assay for real-time PCR (7500 Real Time PCR System; Applied Biosystems) according to the manufacturer's instructions.

The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine and all patients provided written informed consent.

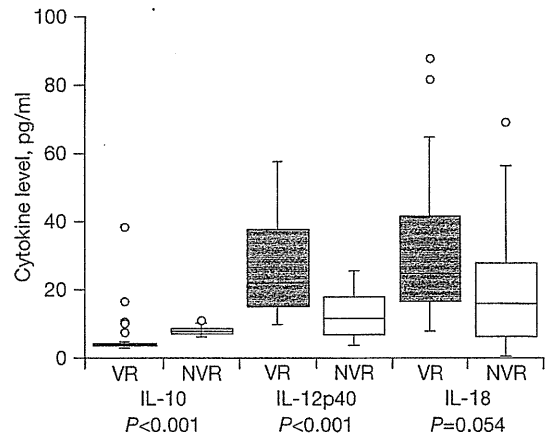
#### Statistical analyses

The Mann–Whitney U test was used to analyse continuous variables. The  $\chi^2$  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 used. A *P*-value of  $\leq 0.05$  was considered statistically significant. To predict treatment outcome, we analysed receiver operating characteristic (ROC) curves for serum levels of IL-10, IL-12p40 and IL-18. Optimal cutoff values were chosen as serum cytokine levels with the highest diagnostic accuracy, that is, when the sum of the false-negative and false-positive rates was minimized. The respective overall diagnostic values were expressed using the area under the curve (AUC). Multivariate analysis was performed using a logistic regression model with stepwise method. Statistical analyses were performed using PASW Statistics 18.0J (IBM, Tokyo, Japan).

## Results

Treatment outcome in patients with chronic hepatitis C Of the 52 patients receiving PEG-IFN and ribavirin therapy, 22 (42%) achieved an SVR. Among the 30 remaining patients, 14 had a relapse and 16 had an NVR. Before treatment, the median white blood cell

Figure 1. Detection of serum cytokines related to treatment outcome



Boxes represent the IQR of the data, lines across the boxes indicate the median values and the hash marks above and below the boxes indicate the 90th and 10th percentiles, respectively for each group. Open circles indicate outliers. Serum interleukin (IL)-10, IL-12p40 and IL-18 levels were detected in 36 patients with a virological response (VR) and 16 patients without. NVR, non-virological response.

count in the virological response group was significantly higher than that in the NVR group (Table 1). Haemoglobin value (15.4 versus 14.1 g/dl; *P*=0.021) was significantly higher in the SVR group compared to the NVR group as well. Substitutions in the ISDR and of aa70 and aa91 in the core region were not associated with treatment outcome.

#### Effects of antiviral therapy on serum cytokine levels

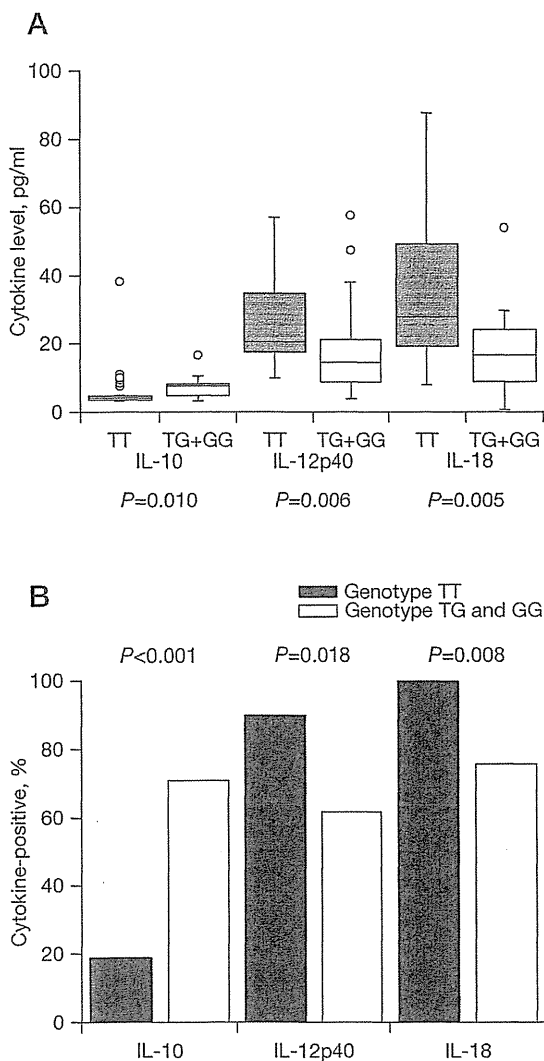
Serum samples obtained prior to antiviral therapy were examined for the presence of IL-10, IL-12p40 and IL-18 by multiplex assays. NVR patients showed significantly higher baseline IL-10 concentrations (8.1 pg/ml) than virological responders (4.1 pg/ml; *P*<0.001; Figure 1). The median baseline serum levels of IL-12p40 (22.1 versus 11.7 pg/ml; *P*<0.001) and IL-18 (24.8 versus 16.0 pg/ml; *P*=0.054) were higher in patients who achieved a virological response than in those with an NVR (Figure 1). Furthermore, serum IL-10 level (4.0 versus 8.1 pg/ml; *P*<0.001) was significantly lower and serum IL-12p40 (25.0 versus 11.7 pg/ml; *P*<0.001) and IL-18 (31.6 versus 16.0 pg/ml; *P*=0.010) levels were significantly higher in the SVR group compared with the NVR group. We also analysed whether pretreatment serum cytokines were correlated with time to clearance of HCV RNA. Serum baseline IL-10 level was significantly lower in patients who eradicated HCV RNA 12 weeks after the start of treatment (*P*=0.002).



*IL28B* genotype and treatment outcome

Among the 52 patients studied for rs8099917, 31 had the TT genotype (60%), 20 had the TG genotype (38%) and 1 had the GG genotype (2%). Responses to combination therapy for rs8099917 are shown in Table 1. Overall SVR rates in patients with the TT genotype (16/31, 53%) and with the TG or GG genotypes (6/21, 29%) were not significantly different ( $P=0.09$ ).

Figure 2. Serum cytokines related to *IL28B* gene polymorphisms



(A) Boxes represent the IQR of the data, lines across the boxes indicate the median values and the hash marks above and below the boxes indicate the 90th and 10th percentiles, respectively, for each group. Open circles indicate outliers. Serum interleukin (IL)-10, IL-12p40 and IL-18 were detected in 31 patients with the TT *IL28B* genotype and 16 patients with the TG or GG genotypes. (B) The prevalence of high serum IL-10, IL-12p40 and IL-18 levels in 31 patients with the TT *IL28B* genotype and in 16 patients with the TG or GG genotypes.

However, NVR rates in patients with either TG or GG (13/21, 62%) were significantly higher than in those with TT only (3/31, 10%;  $P<0.001$ ).

Association of *IL28B* genotype and serum cytokine levels

Median serum IL-10 levels were significantly higher in patients with the TG or GG genotypes (7.7 pg/ml) compared to those with TT (4.1 pg/ml;  $P=0.010$ ; Figure 2A). Conversely, patients with TT had significantly higher median IL-12p40 (20.6 versus 14.5 pg/ml;  $P=0.006$ ) and IL-18 (27.9 versus 16.6 pg/ml;  $P=0.005$ ) levels than patients with TG or GG (Figure 2A).

ROC curve analyses were performed to determine the optimal threshold values of serum cytokines for predicting treatment outcome among the 16 NVR patients and 36 cases with a virological response in our cohort (Figure 3). The optimal threshold value of IL-10 was identical to the 5.0 pg/ml that we had reported in a prior study [22]. The cutoff values for IL-12p40 and IL-18 were 12.1 pg/ml and 6.4 pg/ml, respectively. The calculated AUC for IL-10, IL-12p40 and IL-18 was 0.89 (95% CI 0.77–0.96), 0.81 (95% CI 0.67–0.90) and 0.67 (95% CI 0.52–0.79), respectively, as shown in Figure 3.

The presence of high IL-10 levels ( $\geq 5.0$  pg/ml) was significantly greater among patients with TG or GG genotypes (71%, 15 of 21) than among those with TT (19%, 6 of 31;  $P<0.001$ ; Figure 2B). High IL-12p40 levels ( $\geq 12.1$  pg/ml) were significantly less prevalent ( $P=0.018$ ) among patients with TG or GG (62%, 13 of 21) than among those with TT (90%, 28 of 31). High IL-18 levels ( $\geq 6.4$  pg/ml) were found in 100% (31 of 31) of patients with TT but only 76% (16 of 21) patients with TG or GG ( $P=0.008$ ).

Predicting treatment outcome by serum cytokine levels in combination with *IL28B* genotype

The NVR prediction rate by serum IL-10 in combination with rs8099917 genotype is shown in Figure 4. In patients with TT, a significantly higher proportion of patients with high serum IL-10 levels (50%, 3 of 6) showed an NVR than patients with low IL-10 (0%, 0 of 25;  $P=0.004$ ). Similarly, an NVR was significantly more likely in high versus low IL-10 levels (87%, 13 of 15 versus 0%, 0 of 6;  $P=0.001$ ) in patients with TG or GG (Figure 4A).

NVR rates by serum IL-12p40 levels and IL-18 levels in combination with rs8099917 genotype are shown in Figure 4B and 4C. Among patients with the TT genotype, the NVR rate did not differ between low and high IL-12p40 levels (0% versus 11%;  $P=0.729$ ) or IL-18 levels (0% versus 10%). In cases with TG or GG genotypes, the NVR rate was significantly higher for low IL-12p40 levels compared with high IL-12p40 levels (100% versus 38%;  $P=0.006$ ). Patients with low serum

IL-18 had a higher NVR rate, but this difference was not statistically significant (100% versus 50%;  $P=0.063$ ).

#### Factors associated with an NVR to PEG-IFN and ribavirin therapy

All factors found to be associated with an NVR were evaluated for independence in multivariate analysis. Genotype TG or GG (OR 10.43, 95% CI 1.73–62.96;  $P=0.011$ ), serum IL-10 levels  $\geq 5.0$  pg/ml (OR 1.21, 95% CI 1.03–1.41;  $P=0.018$ ) and IL-12p40 levels  $\geq 17.4$  mg/dl (OR 0.84, 95% CI 0.72–0.97;  $P=0.020$ ) were all independent predictive factors of an NVR.

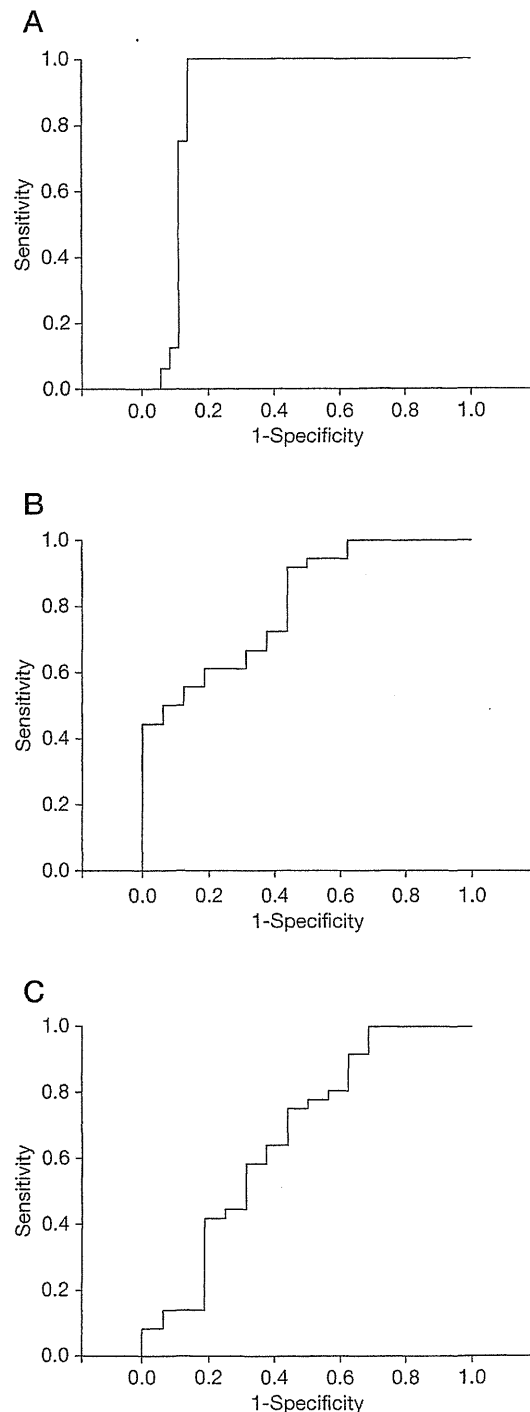
### Discussion

This study examined the *IL28B* (rs8099917) genotype and serum levels of IL-10, IL-12p40 and IL-18 in patients with chronic hepatitis C to assess their predictive value in treatment outcome with PEG-IFN and ribavirin. The key findings were as follows: *IL28B* G-allele carriers were associated with an NVR to PEG-IFN and ribavirin therapy in patients infected with HCV genotype 1, consistent with recent findings; *IL28B* genotype was associated with baseline serum IL-10, IL-12p40 and IL-18 levels; in carriers of an *IL28B* G-allele, NVR rates were high (80–100%) and associated with increased IL-10 and decreased IL-12p40 and IL-18 levels, thus providing new predictive markers of an NVR in PEG-IFN and ribavirin therapy; and *IL28B* genotype, high serum IL-10 levels, and low serum IL-12p40 levels were all independent factors related to an NVR in multivariate analyses.

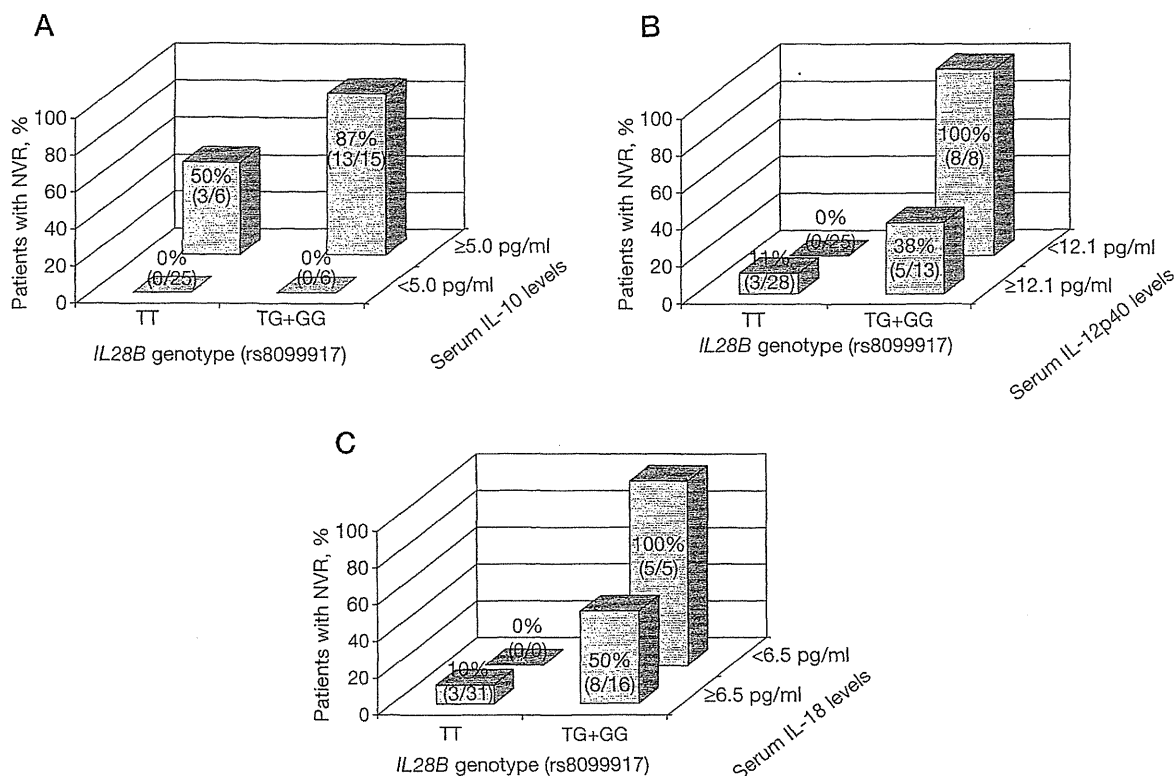
*IL28B* gene polymorphisms have recently been linked to the outcome of HCV infection during spontaneous and treatment-induced elimination of HCV [8–10,12]. In particular, carriage of a G-allele at the *IL28B* gene SNP (rs8099917) is associated with an NVR to PEG-IFN and ribavirin therapy in Japanese patients infected with HCV genotype 1 [10]. This finding was confirmed in our cohort with NVR rates of 62% with GT or GG genotypes versus 10% with TT genotypes ( $P<0.001$ ). Therefore, detection of the *IL28B* genotype is a useful marker to predict the outcome of PEG-IFN and ribavirin therapy in patients with chronic hepatitis C. Data for *IL28B* SNP in healthy subjects were not available for this study.

IFN- $\lambda$  produces an antiviral state by triggering a cascade through the JAK-STAT pathway that up-regulates IFN-stimulated genes. *IL28B* binds to a distinct receptor that may up-regulate a different set of IFN-stimulated genes [26,27]; the precise role of IFN- $\lambda$  in controlling multiple viral infections, including HCV, is currently under way. Further studies are also needed on how SNPs affect the function of *IL28B* and other cytokines.

Figure 3. Receiver-operating characteristic curves for serum cytokine levels on treatment outcome



The areas under the curve for (A) interleukin (IL)-10, (B) IL-12p40 and (C) IL-18 were 0.89, 0.81 and 0.67, respectively. All areas under the curve values were significantly higher than a 0.50 non-predictive value ( $P<0.01$  for all comparisons). IL-10 was predictive of a non-response. IL-12p40 and IL-18 were predictive of a virological response.

Figure 4. Non-virological response rate determined by serum cytokine levels and *IL28B* gene genotype

The prevalence of a non-virological response (NVR) in patients with high or low serum (A) interleukin (IL)-10, (B) IL-12p40 and (C) IL-18 levels according to *IL28B* genotype.

A strong association between high IL-10, low IL-12p40 and low IL-18 levels and an NVR to PEG-IFN and ribavirin therapy was found in this study, which is consistent with previous studies [22,28–30]. In ROC curve analyses, AUCs were high, especially for IL-10 (AUC=0.89) and IL-12p40 (AUC=0.81), confirming that these cytokines are strong predictive markers for an NVR. This study showed a strong correlation between the *IL28B* genotype and serum IL-10, IL-12p40 and IL-18 levels at baseline. Most strikingly, all patients who had low pretreatment IL-10 levels achieved a virological response regardless of *IL28B* genotype. By contrast, among patients with high IL-10 levels (≥5.0 pg/ml), NVR rates were 87% in *IL28B* G-allele carriers and 50% for the *IL28B* TT genotype. Additionally, all *IL28B* G-allele carriers showed an NVR when pretreatment serum IL-12p40 and IL-18 levels were <12.1 pg/ml and <6.5 pg/ml, respectively. It is unclear how serum IL-10, IL-12p40 and IL-18 are associated with an NVR to antiviral therapy in patients with chronic hepatitis C. Although IL-10 was originally described as a cytokine synthesis

inhibitory factor, recent studies have demonstrated that IL-10 produced by Th17 cells restrains the pathological effects of Th17 [31]. Production of IL-12p40 is directed towards the elimination of intracellular pathogens and viruses because IL-12p40 is a proinflammatory cytokine that promotes the differentiation of Th1 cells, suppresses Th2 function and amplifies the cytotoxicity of cytotoxic T-lymphocytes and natural killer cells [32]. Megjugorac *et al.* [33] reported that IL-29-treated plasmacytoid dendritic cells inhibiting production of IL-13, IFN- $\gamma$  and IL-10 by allogeneic T-cells were consistent with a role for this cytokine in plasmacytoid dendritic cell maturation and activation. Very recently, another report has been published demonstrating that IL-29 enhances IL-12p40 by macrophages and that IL-29 pretreatment primes the activation of macrophages induced by IFN- $\gamma$  [34]. However, the association between IL-28B and such cytokines has not been studied. To explain this relationship, further studies are needed to clarify whether a direct or indirect interaction exists between pretreatment levels of these cytokines and *IL28B* genotype.

Although other predictive factors of PEG-IFN and ribavirin therapy have been reported, including core amino acid 70 and 91 and ISDR mutations [6,7], no such significant associations were found here, possibly because of our study population size, which indicates that other factors may be more significant in predicting treatment outcome. However, multivariate analysis confirmed that *IL28B* G-allele, high IL-10 and low IL-12p40 levels were significant predictors of an NVR in patients with PEG-IFN and ribavirin therapy in this study. Hence, *IL28B* G-allele carriers combined with high IL-10 and/or low IL-12 may require alteration of treatment dose, duration, or regimen with a new antiviral drug.

In conclusion, serum IL-10, IL-12p40 and IL-18 levels are associated with *IL28B* genotype in patients with genotype 1 chronic hepatitis C. Pretreatment serum IL-10 and IL-12p40 levels with *IL28B* GT or GG genotypes are particularly useful for predicting an NVR to PEG-IFN and ribavirin therapy. The clinical significance of *IL28B* genotyping combined with baseline serum IL-10 and IL-12p40 levels to predict an NVR warrants further prospective validation.

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## Disclosure statement

The authors declare no competing interests.

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