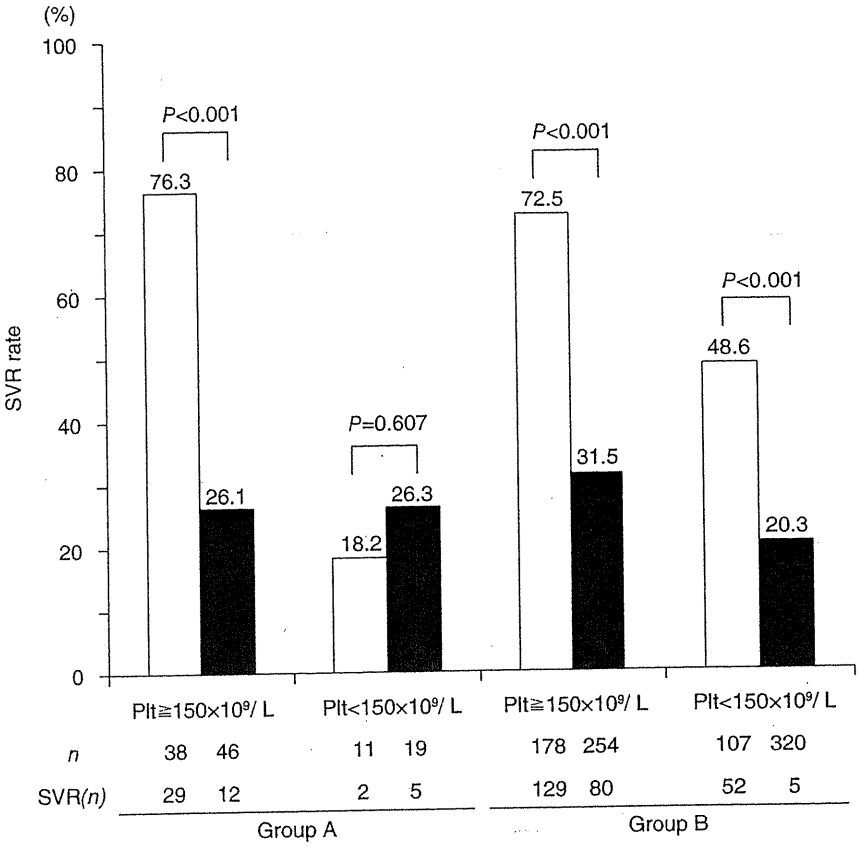

Figure 1 Comparison of the sustained virological response (SVR) rate and platelet count of patients who received the minimum acceptable dosage of pegylated interferon α-2b and ribavirin. In group A (alanine aminotransferase [ALT] <30 IU/L) patients whose platelet count was over 150×10^9/L, the SVR rate was significantly higher for those who received the minimum acceptable dosage than for those who did not (29 of 38, 76.3% vs. 12 of 46, 26.1%, $P < 0.001$). In group B (ALT ≧ 30 IU/L), the SVR rate was significantly higher for those who received the minimum acceptable dosage, with no relation to platelet count. The white column means an SVR rate of patients who received the minimum acceptable dosage. The black column means an SVR rate of patients who did not receive the minimum acceptable dosage.

patient received at least the minimum acceptable treatment dosage. In group A patients whose platelet count was over 150×10^9/L, the SVR rate was significantly higher for those who received at least the minimum acceptable dosage than for those who did not (29 of 38, 76.3% vs. 12 of 46, 26.1%, $P < 0.001$). In group B, the SVR rate was significantly higher for those who received the minimum acceptable dosage with no relation to platelet count (over 150×10^9/L: 129 of 178, 72.5% vs. 80 of 254, 31.5%, $P < 0.001$, under 150×10^9/L: 52 of 107, 48.6% vs. 65 of 320, 20.3%, $P < 0.001$) (Fig. 1). Further, in group A patients whose platelet count was over 150×10^9/L and who received at least the minimum acceptable dosage, the SVR rate was not significantly different by sex or age (under 65 men: 8/11, 72.7%, under 65 women: 15/20,75.0%, over 65 men: 1/1, 100%, over 65 women, 5/6, 83.3%). Furthermore, we compared the SVR rates of patients whose liver fibrosis was F2-4, and found no significant difference between groups A and B.

In a comparison of the SVR rate of patients with or without one or more previous courses of IFN plus RBV, there was no significant difference between groups A and B.

Background factors associated with SVR

To determine the relative weight of the background factors influencing SVR, both univariate and multivariate analyses were performed. Univariate analysis showed that age (<65 years old), homeostasis model assessment-insulin resistance (HOMA-IR) (<2) and total cholesterol (≧220 mg/dL) were significantly associated with SVR in the NALT group, butγGTP, HCVRNA level and LDL-C were not (Table 2). In the multivariate analysis, age (odds ratio [OR] 0.236, $P = 0.017$) and total cholesterol (OR 4.098, $P = 0.039$) were independent factors associated with an SVR in the NALT group (Table 3).

Change of ALT levels after the combination therapy of PEG-IFN α-2b plus RBV

After 6 months of the combination therapy, the mean ALT level of the group A patients who achieved an SVR

Table 2 Univariate analysis of background factors influencing a sustained virological response (SVR)

Factors	Group A (ALT < 30 IU/L) (n = 114)			Group B (ALT ≧ 30 IU/L) (n = 875)		
	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
Sex						
Men	1			1		
Women	2.186	0.949–5.038	0.066	0.682	0.515–0.902	0.007
Age (years)						
<65	1			1		
≧65	0.247	0.096–0.631	0.004	0.341	0.242–0.481	<0.001
Histological Staging						
F 0–1	1			1		
F 2–3	0.349	0.128–1.207	0.103	0.382	0.264–0.553	<0.001
Serum HCV RNA level (logIU/mL)						
<6	1			1		
≧6	0.486	0.198–1.192	0.115	0.449	0.317–0.636	<0.001
γGTP (IU/)						
<44	1			1		
≧44	0.523	0.196–1.394	0.195	0.407	0.306–0.541	<0.001
Albumin (mg/dL)						
≧3.5	1			1		
<3.5				0.169	0.072–0.398	<0.001
Platelet count ($\times 10^9$/L)						
≧150	1			1		
<150	0.312	0.121–0.805	0.886	0.422	0.317–0.561	<0.001
Hemoglobin (g/dL)						
≧14	1			1		
<14	1.304	0.564–3.016	0.534	0.703	0.533–0.928	0.013
Fasting plasma glucose (mg/dL)						
<95	1		1			
≧95	0.471	0.210–1.057	0.068	0.553	0.411–0.744	0.001
HbA1c (%)						
<6.4	1			1		
≧6.4				0.235	0.103–0.535	0.001
HOMA-IR						
<2	1			1		
≧2	0.156	0.052–0.466	<0.001	0.188	0.121–0.290	<0.001
Total cholesterol (mg/dL)						
<220	1			1		
≧220	3.462	1.051–11.396	0.041	1.394	0.732–2.653	0.312
Tryglyceride (mg/dL)						
<150	1			1		
≧150	1.00	0.267–4.533	0.895	0.747	0.453–1.234	0.255
HDL-C (mg/dL)						
<40	1			1		
≧40	3.182	0.605–16.725	0.172	1.065	0.623–1.822	0.817
LDL-C (mg/dL)						
<140	1			1		
≧140	1.067	0.090–12.706	0.959	0.985	0.402–2.410	0.973

ALT, alanine aminotransferase; CI, confidence interval; γ-GTP, γ-glutamyltranspeptidase; HDL-C, High density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessment-insulin resistance; LDL-C, Low density lipoprotein-cholesterol.

Table 3 Multivariate analysis of background factors influencing an sustained virological response (SVR) in normal alanine aminotransferase (NALT) patients

Factors	Odds ratio	95% CI	*P*-value
Age (years)			
<65	1		
≧65	0.236	0.072–0.771	0.017
HCV RNA (logIU/mL)			
<6	1		
≧6	0.391	0.131–1.167	0.092
Total cholesterol (mg/dL)			
<220	1		
≧220	4.098	1.077–15.591	0.039

CI, confidence interval.

decreased from 24.4 ± 3.4 IU/L to 16.3 ± 10.1 IU/L for the men and from 23.6 ± 3.5 IU/L to 14.1 ± 5.9 IU/L for the women. ALT-flare ups were observed for 34.0% (18 of 53) of the non-responsive group A patients. The mean ALT level was 63.6 ± 35.1 IU/L, and only three of these patients (16.7%) had serum ALT activity >100 IU/L (max 163 IU/L).

DISCUSSION

THIS IS THE first report of a large multicenter trial of the efficacy and safety of PEG-IFN α-2b plus RBV treatment of Japanese chronically infected HCV patients with NALT. A large randomized controlled trial of PEG-IFN α-2a 180 μg/week plus RBV at a fixed dose of 800 mg/day for American HCV patients with NALT reported an SVR rate of 40% for patients with genotype 1 treated for 48 weeks,[16] comparable to that achieved by patients with elevated ALT activity.[19,20] Our results were similar (37.8%), which indicates that Japanese NALT patients are suitable candidates for PEG-IFN α and RBV combination treatment.

Puoti *et al.*[17] reported that, for patients treated with PEG-IFN α-2a 180 μg/week plus an optimal RBV dosage (1000–1200 mg/day), the SVR rate was improved to 62% for HCV-1 NALT patients. In Japan, RBV taken orally at a daily dose of 600–1000 mg based on body weight is the recommended treatment of the Japanese Ministry of Health, Labor and Welfare. Thus, we are not able to use the same dose of RBV as used in the United States and European countries. On the other hand, Hiramatsu *et al.* have reported that maintaining a high dose (≧12 mg/kg/day) of RBV during the full treatment period could strongly suppress the relapse rate with chronic hepatitis C genotype 1 responding to α-2b plus RBV.[27] However, in their study, 165 (16.8%) of 984 patients who were enrolled discontinued the treatment because of adverse events or voluntary withdrawal, and 331 patients (33.6%) discontinued the treatment because of non-response. SVR in the intention-to-treat analysis was only 347 of 984 (35.3%), and the rate was similar to ours. Maintaining a higher dose of RBV results in higher rates of discontinuation due to adverse events, which leads to a decrease in SVR. Thus we feel it is best to reduce the dose of RBV. Therefore, we analyzed the SVR rates of our patients who were given less than the minimum acceptable dosage.

Our results indicate that taking at least the minimum acceptable dosage during treatment increased the SVR rate of NALT patients with genotype 1 by two to three times more than patients who did not take the minimum acceptable dosage. The current results confirm our previous study,[23,28] as well as indicate that receiving at least the minimum acceptable dosage is also very important for NALT patients to achieve SVR. The SVR rate was almost the same for patients taking a higher total dosage of RBV and those receiving the minimum acceptable dosage, and prescribing the minimum acceptable dosage would be safe and more cost effective than prescribing a higher dosage of RBV for NALT patients.

For HCV patients with NALT, Puoti *et al.*[17] stated that young patients without contraindications should take a combination therapy of PEG-IFN α plus RBV rather than to take a watchful-waiting strategy, we feel that older patients with NALT also may be acceptable candidates for PEG-IFN α plus RBV treatment. Moreover, results that the men over 65 years-of-age with elevated ALT had a lower SVR rate (36.4%) than those under 65 years (70.1%) indicate that it is necessary to treat the men with interferon at a younger age and before the exacerbation of ALT.

In this study, patients with NALT had milder histological disease than those with elevated ALT, which may be related to the higher rate of SVR in the NALT group.

Okanoue *et al.* reported that HCV carriers with ALT<30 IU/L and PLT counts >150 × 10^9/L were recommended to have follow up without antiviral treatment, because over 90% show normal or minimal liver damage with good prognosis from the point of view of the prevention of HCC.[29] Our data showed a higher SVR rate if NALT patients received at least the minimum acceptable dosage when liver fibrosis was not advanced. Therefore, from the point of view of eliminating HCV,

we feel that NALT patients also should receive PEG-IFN α plus RBV treatment if liver fibrosis is not advanced.

Further, our data demonstrated that total cholesterol could be useful for predicting which NALT patients will achieve SVR. These results showed that the total cholesterol level is inversely associated with liver fiborosis.[30,31] Therefore, serum total cholesterol might be helpful for a determination to treat NALT patients with PEG-IFN α-2b plus RBV, whether or not liver fibrosis is advanced, even when we cannot do liver biopsy. We feel that whether or not to initiate therapy should be decided not only by age and serum ALT level, but also by serum total cholesterol and the guidelines of AASLD as above mentioned.[12]

Although IFN α treatment for patients with NALT has been reported to cause ALT-flare ups after treatment,[32,33] we previously reported that the number of patients with elevated ALT levels in a 2-year follow up was not significantly different between patients treated with IFN α and untreated patients.[34] There has been only one report that PEG-IFN α-2a plus RBV combination treatment did not cause ALT flare-ups after treatment,[16] but the precise relationship remains to be elucidated. Our data indicated that the ALT flare up rate after treatment was 15.8%, and watching non-SVR patients carefully after treatment is important to check for ALT flare ups. Along with a report that over 60% of patients with NALT have an elevated ALT level at 3 years,[35] we considered that the PEG-IFN α plus RBV combination treatment is also safe for patients with NALT, although we must note that we did not follow up a full 2 years to observe the change of ALT levels.

This study has a limitation that liver biopsy was done only for about half of the enrolled patients and that we could not measure biomarkers of liver fibrosis such as hyaluronic acid, so we could not precisely estimate the liver fibrosis. However, because the present study was a large multicenter design, the findings are of great interest for clarifying the efficacy and safety of PEG-IFN α-2b plus RBV combination treatment for patients with NALT.

CONCLUSIONS

THE EFFICACY AND safety of PEG-IFN α-2b plus RBV combination therapy for patients with chronic HCV infection who have NALT is similar to that of patients with elevated ALT levels. These results indicate that patients with NALT are suitable candidates for treatment with PEG-IFN α-2b plus RBV.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Efficacy of pegylated interferon plus ribavirin combination therapy for hepatitis C patients with normal ALT levels: a matched case–control study

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Abstract
Background The antiviral effect of pegylated interferon (Peg-IFN) plus ribavirin combination therapy in chronic hepatitis C (CHC) patients with normal alanine aminotransferase (ALT) levels (N-ALT) has been reported to be equivalent to that for patients with elevated ALT levels (E-ALT). However, the actual antiviral effect in N-ALT patients remains obscure because efficacy can be overestimated in patients with an advantageous background.
Methods In this study, 386 patients were extracted, for a matched case–control study, from 1320 CHC patients treated with Peg-IFN alpha-2b plus ribavirin combination therapy; 193 N-ALT patients [116 with hepatitis C virus genotype 1 (HCV-1), 77 with HCV genotype 2 (HCV-2)] were matched with 193 E-ALT patients by a propensity score method using the variables of age, sex, IFN treatment history, body mass index, and platelet counts.
Results On multivariate analysis for sustained virological response (SVR) in N-ALT patients, younger age, low HCV RNA level at baseline, and HCV-2 were significant factors. The matched case–control study showed that the SVR rates of N-ALT patients were equivalent to those of E-ALT patients; at 49 and 40% in the HCV-1 group ($P = 0.146$), and 78 and 81% in the HCV-2 group ($P = 0.691$). However, in N-ALT patients with non-SVR, approximately 40% showed ALT elevation at 24 weeks post-treatment.
Conclusion Our findings indicate that the antiviral effect of Peg-IFN plus ribavirin therapy in N-ALT patients is comparable to that for E-ALT patients irrespective of their advantageous background; however, the application of this therapy for N-ALT patients, especially for those with HCV-1, should be considered carefully.

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Keywords Hepatitis C virus · Normal alanine aminotransferase · Pegylated interferon plus ribavirin combination therapy · Propensity score method · Matched case–control study

Introduction

In patients with hepatitis C virus (HCV) infection, alanine aminotransferase (ALT) levels fluctuate and sometimes biochemical remission is maintained. Approximately 20% of patients with normal ALT levels (N-ALT) show ALT elevation and fibrosis progression within 3–5 years [1–5], and consequently, 70–80% of N-ALT patients have mild to moderate fibrosis on liver biopsy. N-ALT patients have been excluded from conventional interferon (IFN) therapy, because their sustained virological response (SVR) rates on conventional IFN monotherapy have been reported to be only 6–15% [6–9], and ALT levels were noted to increase during or after treatment in 47–62% of the patients. The incidence of ALT flares has raised concerns regarding the risk of conventional IFN therapy compared with a small benefit. However, a large randomized controlled trial has demonstrated that combination therapy with pegylated interferon (Peg-IFN) and ribavirin produced SVR rates in N-ALT patients with chronic hepatitis C (CHC) that were comparable to those of patients with elevated ALT levels (E-ALT) [10]. Thus, such treatment is now being considered for N-ALT patients with CHC [11].

Comparison of the characteristics of N-ALT and E-ALT patients has shown that the mean age of N-ALT patients was lower than that of E-ALT patients, and females and HCV genotype 2 patients were predominant among N-ALT patients [4, 7, 12–17]. In the American Association for the Study of Liver Disease guideline, the pretreatment predictors of achieving SVR with Peg-IFN plus ribavirin combination therapy for CHC patients are HCV genotype 2 or 3 infection, low viral load (<600 KIU/ml), female gender, and age less than 40 years [11]. Considering these characteristics, N-ALT patients with CHC can be said to have an advantageous background, and their response to antiviral therapy, including Peg-IFN plus ribavirin combination therapy, can be overestimated. Therefore, patient background, especially factors affecting the treatment efficacy of the combination therapy, needs to be matched between study groups in order to compare the treatment efficacies in N-ALT patients with CHC and E-ALT patients with CHC accurately. In this study, we evaluated, by a matched case–control study approach, whether the antiviral efficacy in N-ALT patients with CHC, reported to be equal to that in E-ALT patients with CHC, could be obtained without their advantageous background, and whether the factors contributing to SVR in N-ALT patients were the same as those in E-ALT patients. In addition, ALT flares after treatment in N-ALT patients without SVR were examined.

Patients and methods

Patient selection and study design

The subjects were 1320 consecutive CHC patients, 1015 with HCV genotype 1 (HCV-1) and 305 with HCV genotype 2 (HCV-2) who had undergone combination therapy with Peg-IFN alpha-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (REBETOL; Schering-Plough) at standard doses for 48 weeks (patients with HCV-1) or for 24 weeks (patients with HCV-2) at 30 medical institutions participating in the Osaka Liver Forum between December 2004 and December 2007. Peg-IFN alpha-2b and ribavirin dosages were based on body weight according to the manufacturer's instructions and were modified based on the manufacturer's instructions according to the severity of adverse hematologic effects. In the 1 month preceding treatment, none of the patients had received any IFN formulations or other types of drugs for liver supporting therapy. Before starting treatment, all patients had positive anti-HCV and a detectable level of HCV RNA according to a polymerase chain reaction (PCR)-based assay (COBAS Amplicor HCV Monitor Test v2.0; Roche Diagnostics, Branchburg, NJ, USA). None of the patients showed evidence of dual infection with hepatitis B virus or human immunodeficiency virus, or other forms of liver diseases such as alcoholic liver disorder, autoimmune hepatitis, or drug-induced liver injury.

In this study, a normal serum ALT level was defined as ALT $\leq$30 IU/l at the start of the combination therapy, as, in the guidelines for treatment of hepatitis C in N-ALT patients in Japan, ALT levels of $\leq$30 IU/l are regarded as an indicator of no or little inflammation in the liver, and patients whose ALT levels are $\leq$30 IU/l are recommended to be followed without antiviral therapy, especially if the platelet count is $\geq 15 \times 10^4/mm^3$.

Among the 1320 consecutive CHC patients, the antiviral effect in 193 N-ALT patients (116 with HCV-1, 77 with HCV-2) was compared with that in 193 E-ALT patients (116 with HCV-1, 77 with HCV-2) who were matched by a propensity score method based on age, sex, IFN treatment history, body mass index (BMI), and platelet counts. BMI was calculated as weight (kg)/[height (m)]^2.

HCV RNA was determined at week 4, week 12, end of treatment (EOT), and 24 weeks after EOT. HCV RNA was also determined at week 24 for HCV-1 patients. HCV RNA was monitored by the PCR Amplicor method with a detection limit of 50 IU/ml. (COBAS Amplicor HCV v2.0;

Roche Diagnostics). Complete early virological response (cEVR) and end-of-treatment response (ETR) were defined as undetectable HCV RNA at week 12 and EOT, respectively.

Written informed consent was obtained from each patient, and the study protocol was reviewed and approved according to the ethical guidelines of the 2004 Declaration of Helsinki by institutional review boards at the respective sites.

Propensity score

Propensity score methods are used to create balanced covariates and reduce selection bias in a matched case–control study. Propensity scores were calculated using a multivariate logistic regression model that had ALT levels as a dependent variable and other covariates as independent variables, and the model was utilized for matching between the N-ALT patients with CHC (the case group) and the E-ALT patients with CHC (the control group). Data analyses were conducted using SAS, version 9.2 (SAS Institute, Cary, NC, USA).

Statistical analysis

Continuous variables are reported as the mean with standard deviation (SD) or median levels, while categorical variables are shown as the count and proportion. Statistical significance was assessed by Student's t test (mean), the Mann–Whitney U test (median), and the χ^2 test for independent samples, and the paired t test for paired samples. For all tests, two-sided P values were calculated, and the results were considered statistically significant if $P < 0.05$. Variables that achieved statistical significance on univariate analysis were subjected to multivariate logistic regression analysis. Stepwise and multivariate logistic regression models were used to explore the independent factors that could be used to predict SVR. Statistical analysis was performed using the SPSS program for Windows, version 15.0 J (SPSS, Chicago, IL, USA).

Results

Baseline characteristics of all CHC patients according to HCV genotype and ALT levels before matching

The baseline characteristics of 1320 patients at the commencement of combination therapy with Peg-IFN and ribavirin are shown in Table 1, according to HCV genotype and ALT levels before matching. Of the 116 N-ALT patients with HCV-1 there were 36 males and 80 females (69%), with a mean age of 54 ± 11 years. Eighty-five (73%) were IFN-naïve. In terms of liver histology, 66 (73%) patients had

Table 1 Demographic characteristics of patients with normal ALT and patients with elevated ALT

	HCV genotype 1			HCV genotype 2		
	Normal ALT ($n = 116$)	Elevated ALT ($n = 899$)	P value	Normal ALT ($n = 77$)	Elevated ALT ($n = 228$)	P value
Sex: male/female	36/80	512/387	<0.001	32/45	121/107	0.081
Age (years)	54 ± 11	56 ± 10	0.136	51 ± 13	52 ± 13	0.423
Body mass index (kg/m^2)	22.9 ± 3.1	23.3 ± 3.2	0.131	23.0 ± 2.9	23.3 ± 3.2	0.424
Past IFN therapy: naïve/experienced (relapser/non-responder)[a]	85/31 (18/5)	547/352 (131/154)	0.011	58/19 (9/4)	175/53 (21/10)	0.876
Histology (METAVIR)[b]						
Activity: 0–1/2–3	66/25	296/338	<0.001	43/6	69/94	<0.001
Fibrosis: 0–1/2–4	67/24	330/304	<0.001	42/7	101/62	0.002
HCV RNA (KIU/ml)[c]	1800	1700	0.793	2200	1100	<0.001
White blood cell (/mm^3)	5220 ± 1507	5137 ± 1582	0.595	5538 ± 1687	5338 ± 1725	0.377
Neutrophil (/mm^3)	2770 ± 1074	2595 ± 1078	0.108	3017 ± 1180	2688 ± 1230	0.047
Hemoglobin (g/dl)	13.6 ± 1.5	14.2 ± 1.4	<0.001	13.8 ± 1.6	14.2 ± 1.4	0.071
Platelet (×10^4/mm^3)	19.9 ± 5.7	16.2 ± 5.3	<0.001	20.5 ± 4.5	17.8 ± 5.8	<0.001
ALT (IU/l)	24 ± 5	88 ± 62	<0.001	22 ± 5	97 ± 67	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon

[a] Status was unknown in 8 patients in the normal ALT group and 67 in the elevated ALT group with HCV genotype 1; and in 6 in the normal ALT group and 22 in the elevated ALT group with HCV genotype 2

[b] Data missing in 25 patients in the normal ALT group and in 265 in the elevated ALT group with HCV genotype 1; and in 28 in the normal ALT group and in 65 in the elevated ALT group with HCV genotype 2

[c] Values are expressed as medians

mild activity (activity, 0–1) and 67 (74%).had mild fibrosis (fibrosis, 0–1) by the METAVIR system. Mean white blood cell counts, hemoglobin levels, and platelet counts were 5220 ± 1570 /mm^3, 13.6 ± 1.5 g/dl, and 19.9 ± 5.7 × 10^4/mm^3. In 899 E-ALT patients compared to N-ALT patients, the proportions of female and IFN-naïve patients were significantly lower, at 43% ($P < 0.001$) and 61% ($P = 0.011$), respectively. Higher scores for activity ($P < 0.001$) and fibrosis ($P < 0.001$) were observed in E-ALT patients. E-ALT patients had higher hemoglobin levels and lower platelet counts than N-ALT patients, at 14.2 ± 1.5 g/dl ($P < 0.001$) and 16.2 ± 5.3 × 10^4/mm^3 ($P < 0.001$), respectively. Mean ALT levels were 24 ± 5 IU/l in N-ALT patients and 88 ± 62 IU/l in E-ALT patients ($P < 0.001$).

Of the 77 N-ALT patients with HCV-2, 32 were males and 45, females (58%). Their mean age was 51 ± 13 years and 58 (75%) were IFN-naïve. In terms of liver histology, 43 (88%) patients had mild activity (activity, 0–1) and 42 (86%) had mild fibrosis (fibrosis, 0–1). Compared to the 228 E-ALT patients, the N-ALT patients had higher HCV RNA levels (median 2200 vs. 1100 KIU/ml, $P < 0.001$). Higher scores for activity ($P < 0.001$) and fibrosis ($P = 0.002$) were observed in E-ALT patients. Neutrophils and platelet counts in N-ALT patients were higher than those in E-ALT patients, at 3017 ± 1180 versus 2688 ± 1230 /mm^3 ($P = 0.047$) and 20.5 ± 4.5 × 10^4 versus 17.8 ± 5.8 × 10^4/mm^3 ($P < 0.001$), respectively. Mean ALT levels were 22 ± 5 IU/l in N-ALT patients and 97 ± 67 IU/l in E-ALT patients ($P < 0.001$).

Prognostic factors for SVR in the N-ALT patients

For all N-ALT patients (HCV-1, 116; HCV-2, 77), univariate analysis for factors associated with achieving SVR was performed for the following variables: sex, age, BMI, history of past IFN therapy, histology, baseline HCV RNA level, HCV genotype, white blood cell count, neutrophil count, hemoglobin level, platelet count, and ALT level (Table 2). The results indicated that age, fibrosis, baseline HCV RNA level, and HCV genotype contributed to SVR. Next, multivariate logistic regression analysis was performed for all N-ALT patients ($n = 193$), using these factors except for fibrosis, as there were many missing samples. The multivariate analysis showed that younger age [by 10-year increase: odds ratio (OR) 0.552; 95% confidence interval (CI) 0.404–0.756; $P < 0.001$] and lower baseline HCV RNA level (by 100-KIU/ml increase: OR 0.976; 95% CI 0.954–0.998; $P = 0.037$), as well as HCV genotype (genotype 2 vs. genotype 1: OR 3.724; 95% CI 1.859–7.463; $P < 0.001$) were independently associated with SVR (Table 3).

Table 2 Factors associated with SVR in patients with normal ALT—univariate analysis

Factor	SVR ($n = 117$)	Non-SVR ($n = 76$)	P value
Sex: male/female	43/74	25/51	0.645
Age (years)	50 ± 13	57 ± 9	<0.001
Body mass index (kg/m^2)	22.8 ± 3.3	23.1 ± 2.6	0.511
Past IFN therapy: naïve/experienced	88/29	55/21	0.737
Histology (METAVIR)[a]			
Activity: 0–1/2–3	67/14	42/17	0.148
Fibrosis: 0–1/2–4	69/12	40/19	0.022
HCV genotype: 1/2	57/60	59/17	<0.001
HCV RNA (KIU/ml)[b]	1700	2100	0.040
White blood cell (/mm^3)	5461 ± 1426	5170 ± 1798	0.213
Neutrophil (/mm^3)	2968 ± 1167	2709 ± 1032	0.126
Hemoglobin (g/dl)	13.7 ± 1.4	13.7 ± 1.6	0.970
Platelet (×10^4/mm^3)	20.4 ± 4.8	19.8 ± 5.8	0.388
ALT (IU/l)	23 ± 5	24 ± 5	0.384

SVR sustained virological response, *ALT* alanine aminotransferase, *IFN* interferon, *HCV* hepatitis C virus

[a] Data missing in 36 patients in the SVR group and in 17 in the non-SVR group

[b] Values are expressed as medians

Table 3 Factors associated with SVR in patients with normal ALT—multivariate analysis

Factor	Category	Odds ratio	95% CI	P value
Age	By 10 years	0.552	0.404–0.756	<0.001
HCV genotype	1/2	3.724	1.859–7.463	<0.001
HCV RNA	By 100 KIU/ml	0.976	0.954–0.998	0.037

The number of patients used for this multivariate analysis was 193 (SVR, $n = 117$; non-SVR, $n = 76$)

SVR sustained virological response, *ALT* alanine aminotransferase, *CI* confidence interval, *HCV* hepatitis C virus

Comparison of patient characteristics between patients with normal ALT and those with elevated ALT matched by a propensity score method

The baseline characteristics of CHC patients matched by a propensity score method at the commencement of combination therapy with Peg-IFN and ribavirin were compared between N-ALT patients and E-ALT patients (see Table 4). There were 116 CHC patients with HCV-1 in each of the groups of N-ALT and E-ALT patients. The two groups were well matched by propensity score methods and there was no significant difference, except in ALT values (mean value, N-ALT, 24 ± 5 IU/l vs. E-ALT, 78 ± 53 IU/l, $P < 0.001$). Similarly, with CHC patients with HCV-2, there were no significant differences, except for ALT levels (mean value, N-ALT, 22 ± 5 IU/l vs. E-ALT, 80 ± 58 IU/l, $P < 0.001$), activity scores [0–1, N-ALT, 88% (43/49) vs. E-ALT, 49% (25/51), $P < 0.001$], and HCV RNA levels (median value, N-ALT, 2200 KIU/ml vs. E-ALT, 1000 KIU/ml, $P < 0.001$).

Treatment efficacy of combination therapy with Peg-IFN and ribavirin in CHC patients

Antiviral effects of the combination therapy with Peg-IFN and ribavirin were evaluated by rapid virological response (RVR), cEVR, ETR, SVR, and relapse rates, as shown in Table 5. Among patients with HCV-1 in the N-ALT and E-ALT patients, respectively, RVR rates were 6% (6/98) and 6% (6/102), cEVR rates were 53% (62/116) and 43% (50/116), and ETR rates were 72% (84/116) and 58% (67/116) ($P = 0.019$). SVR and relapse rates in N-ALT patients were 49% (57/116) and 32% (27/84). These rates in E-ALT patients were 40% (46/116) and 31% (21/67). In the patients with HCV-2, RVR, cEVR, ETR, SVR, and relapse rates were 68% (41/60), 90% (69/77), 96% (74/77), 78% (60/77), and 19% (14/74) for N-ALT patients, and 62% (36/58), 91% (70/77), 91% (70/77), 81% (62/77), and 11% (8/70) for E-ALT patients, respectively. Comparisons between N-ALT and E-ALT patients with HCV-1 or HCV-2 showed no significant differences in RVR, cEVR, ETR, SVR, and relapse rates, except in ETR rates in patients with HCV-1.

Changes in ALT levels during combination therapy and follow-up periods in N-ALT patients with SVR and those with non-SVR

Changes in ALT levels in N-ALT patients during the combination therapy and follow-up periods were evaluated according to the treatment response (Fig. 1). In patients with HCV-1, the mean baseline ALT level in the SVR

Table 4 Comparison of characteristics between patients with normal ALT and patients with elevated ALT matched by a propensity score method

	HCV genotype 1			HCV genotype 2		
	Normal ALT ($n = 116$)	Elevated ALT ($n = 116$)	*P* value	Normal ALT ($n = 77$)	Elevated ALT ($n = 77$)	*P* value
Sex: male/female	36/80	32/84	0.564	32/45	30/47	0.742
Age (years)	54 ± 11	55 ± 11	0.746	51 ± 13	50 ± 13	0.742
Body mass index (kg/m^2)	22.9 ± 3.1	22.6 ± 2.9	0.536	23.0 ± 2.9	22.8 ± 2.9	0.780
Past IFN therapy: naïve/experienced (relapser/non-responder)[a]	85/31 (18/5)	80/36 (13/18)	0.469	58/19 (9/4)	57/20 (7/4)	0.853
Histology (METAVIR)[b]						
Activity: 0–1/2–3	66/25	49/35	0.056	43/6	25/26	<0.001
Fibrosis: 0–1/2–4	67/24	59/25	0.736	42/7	36/15	0.068
HCV RNA (KIU/ml)[c]	1800	1700	0.896	2200	1000	<0.001
White blood cell (/mm^3)	5220 ± 1507	5329 ± 1626	0.569	5538 ± 1687	5530 ± 1780	0.977
Neutrophil (/mm^3)	2770 ± 1074	2702 ± 1094	0.641	3017 ± 1180	2755 ± 1189	0.189
Hemoglobin (g/dl)	13.6 ± 1.5	13.7 ± 1.4	0.542	13.8 ± 1.6	14.0 ± 1.4	0.592
Platelet (×10^4/mm^3)	19.9 ± 5.7	19.4 ± 7.1	0.562	20.5 ± 4.5	20.6 ± 5.5	0.911
ALT (IU/l)	24 ± 5	78 ± 53	<0.001	22 ± 5	80 ± 58	<0.001

ALT alanine aminotransferase, *IFN* interferon, *HCV* hepatitis C virus

[a] Data unknown in 8 patients in the normal ALT group and in 5 in the elevated ALT group with HCV genotype 1; and in 6 in the normal ALT group and 9 in the elevated ALT group with HCV genotype 2

[b] Data missing in 25 patients in the normal ALT group and in 32 in the elevated ALT group with HCV genotype 1; and in 28 in the normal ALT group and 26 in the elevated ALT group with HCV genotype 2

[c] Values are expressed as medians

Table 5 Antiviral effect for patients with normal ALT and those with elevated ALT according to HCV genotype

		Normal ALT	Elevated ALT	*P* value
HCV genotype 1		$n = 116$	$n = 116$	
	Undetectable HCV RNA rate			
	At week 4 (RVR)[a]	6% (6/98)	6% (6/102)	1.000
	At week 12 (cEVR)	53% (62/116)	43% (50/116)	0.287
	At week 48 (ETR)	72% (84/116)	58% (67/116)	0.019
	Post-24 weeks (SVR)	49% (57/116)	40% (46/116)	0.146
	Relapse rate	32% (27/84)	31% (21/67)	0.916
HCV genotype 2		$n = 77$	$n = 77$	
	Undetectable HCV RNA rate			
	At week 4 (RVR)[b]	68% (41/60)	62% (36/58)	0.563
	At week 12 (cEVR)	90% (69/77)	91% (70/77)	0.723
	At week 24 (ETR)	96% (74/77)	91% (70/77)	0.191
	Post-24 weeks (SVR)	78% (60/77)	81% (62/77)	0.691
	Relapse rate	19% (14/74)	11% (8/70)	0.212

ALT alanine aminotransferase, *HCV* hepatitis C virus, *RVR* rapid virological response, *cEVR* complete early virological response, *ETR* end-of-treatment response, *SVR* sustained virological response

[a] Data missing in 18 patients in the normal ALT group and in 14 in the elevated ALT group with HCV genotype 1

[b] Data missing in 17 patients in the normal ALT group and in 19 in the elevated ALT group with HCV genotype 2

group ($n = 57$) was similar to that in the non-SVR group ($n = 59$) (mean ± standard error of the mean (SEM): SVR group, 24.5 ± 0.6 IU/l; non-SVR group, 24.2 ± 0.7 IU/l; $P = 0.694$). Transitions of ALT levels were not significantly different between SVR and non-SVR groups during the therapy. However, in the SVR group, the ALT level fell to 15.1 ± 0.7 IU/l at 24 weeks after treatment completion ($P < 0.001$, compared to the baseline level), while in the non-SVR group, higher ALT levels were observed after treatment compared to the baseline level; the ALT level rose to the peak value of 36.2 ± 3.6 IU/l at post-12 weeks ($P = 0.001$), and slightly fell to 31.3 ± 2.6 IU/l at post-24 weeks ($P = 0.007$) (Fig. 1a). In comparison with the SVR group, the non-SVR group showed significant differences in mean ALT levels at post-4, -12, and -24 weeks ($P = 0.002$, <0.001, and <0.001, respectively). At post-48 weeks in the non-SVR group, the ALT level was 30.4 ± 2.9 IU/l, which was still higher than the baseline level ($P = 0.025$).

Similarly, in patients with HCV-2, baseline ALT levels in the SVR group ($n = 60$) and the non-SVR group ($n = 17$) were equivalent (mean ± SEM; SVR, 21.8 ± 0.7 IU/l; non-SVR, 22.5 ± 1.1 IU/l; $P = 0.622$), and there was no significant difference in transitions of the ALT levels during therapy. However, after treatment, in the non-SVR group, ALT levels tended to rise in comparison with those at baseline; they rose to 74.9 ± 26.9 IU/l at post-12 weeks ($P = 0.068$) and fell to 35.7 ± 10.2 IU/l at post-24 weeks ($P = 0.196$). On the other hand, in the SVR group, ALT levels fell significantly, to 16.4 ± 1.3 IU/l at post-12 weeks ($P < 0.001$) and 15.2 ± 1.2 IU/l at post-24 weeks ($P < 0.001$) (Fig. 1b). Comparison of ALT levels between the SVR and non-SVR groups after treatment showed that mean ALT levels in the non-SVR group tended to be high at post-4, -12 and, -24 weeks ($P = 0.045$, 0.051, and 0.066, respectively). At post-48 weeks in the non-SVR group, the ALT level was 32.4 ± 8.9 IU/l, which tended to be high compared with the baseline ALT level, although no significant difference was found ($P = 0.248$).

Next, the ALT levels in N-ALT patients were examined according to the treatment response at 24 weeks after completion of the combination therapy. In HCV-1 patients with SVR, ALT levels remained below the upper limit of normal (ULN) for this study (<30 IU/l) in 55 (98%) patients, and ALT elevation <2 × ULN occurred in only one (2%) patient (ALT 32 IU/l). On the other hand, in patients with non-SVR, ALT levels remained stable in 34 (60%) patients but increased to <2 × ULN in 20 (35%) patients, and to ≥2 × ULN in 3 (5%) patients (ALT 62, 79, and 135 IU/l). Similarly, in HCV-2 patients with SVR, ALT levels remained stable in 56 (95%) patients, and ALT elevation rarely occurred [<2 × ULN, 2 (3%) patients; ≥2 × ULN, one (2%) patient (ALT 68 IU/l)]. In contrast, in patients with non-SVR, ALT levels remained normal in 10 (67%) patients but increased to <2 × ULN in 4 (27%) patients and to ≥2 × ULN in one (6%) patient (ALT 174 IU/l).

Discussion

N-ALT patients with CHC are known to show demographic and virological features associated with higher

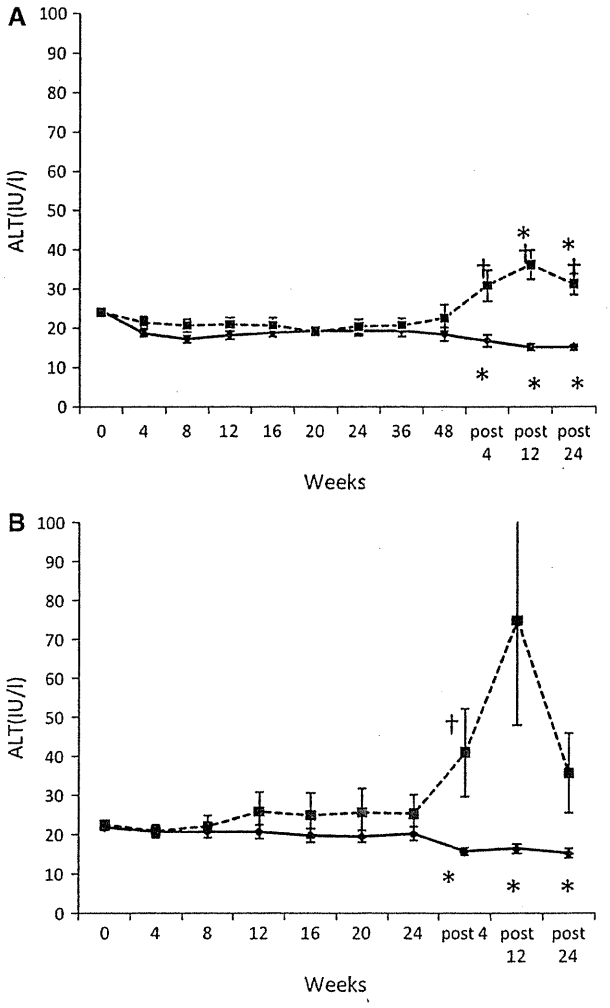

Fig. 1 Changes in serum alanine aminotransferase (*ALT*) levels (±standard error of the mean) according to response in patients with normal ALT levels with chronic hepatitis C treated with pegylated interferon and ribavirin. *Solid lines* show ALT levels in patients with a sustained virological response (SVR), and *dashed lines* show these levels in patients with a non-SVR. *Single-asterisks* denote a statistically significant difference ($P < 0.05$) in mean ALT levels between baseline and each time point of the follow-up period. *Daggers* denote a statistically significant difference between SVR and non-SVR groups. **a** Patients infected with hepatitis C virus genotype 1 (HCV-1). The number of patients was 57 in the SVR group and 59 in the non-SVR group. **b** HCV-2 patients. The number of patients was 60 in the SVR group and 17 in the non-SVR group

response rates to Peg-IFN and ribavirin combination therapy [4, 7, 12–17]. In the present study, N-ALT patients were younger and had higher platelet counts than E-ALT patients, thus giving N-ALT patients an advantage in antiviral efficacy in comparison with E-ALT patients in our cohort. However, the preponderance of females was greater in N-ALT patients with HCV-1 in this study, giving N-ALT patients a disadvantage. Accordingly, a direct comparison was made between these two patient groups after matching E-ALT patients with N-ALT patients using propensity score methods to reduce the bias due to differences in patient backgrounds. As a result, the efficacy of the combination therapy in N-ALT patients was revealed to be still equivalent to that in E-ALT patients, irrespective of their advantageous background. Moreover, in N-ALT patients with HCV-1, not only the ETR rate, but also the SVR rate tended to be higher than these rates in E-ALT patients (49% in N-ALT patients vs. 40% in E-ALT patients). Accordingly, N-ALT patients with HCV-1 can achieve a better treatment response in comparison with E-ALT patients, but further study is needed to clarify this.

In the present study, multivariate logistic regression analysis showed that achieving SVR was strongly influenced by HCV genotype and baseline HCV RNA level in N-ALT patients, which was consistent with findings of multicenter studies with E-ALT patients [18–21]. Therefore, decisions for treatment and the treatment regimen for N-ALT patients can mirror those recommended for E-ALT patients. The results of our multivariate analysis also revealed that patient age influenced the achievement of SVR in N-ALT patients. This offers support for the decision to offer antiviral treatment to younger N-ALT patients.

Among patients in our study who achieved SVR with the combination therapy, ALT levels after treatment decreased significantly, as shown in Fig. 1. However, approximately 40% of the non-SVR patients had increased ALT levels of up to $<2 \times$ ULN, and about 5% of patients had increased ALT levels of $\geq 2 \times$ ULN at 24 weeks after completion of the combination therapy, regardless of HCV genotype. When N-ALT patients are commencing the combination therapy, these patients should be told about the possibility of ALT exacerbation [6–9], although it is difficult to know whether this is drug-induced or due to the natural course. It is also difficult to state which patient characteristics make ALT elevation more likely to occur after the treatment.

Taking the findings obtained in the present study together, in N-ALT patients with HCV genotype 2, earlier treatment with Peg-IFN plus ribavirin combination therapy is desirable, as better efficacy was found for younger patients, with an SVR rate of approximately 80% being attained with this combination therapy, and few direct-acting antiviral agents (DAAs) have been developed for genotype 2. On the other hand, N-ALT patients with HCV genotype 1 should consider awaiting the DAAs, because SVR cannot be attained in about half of these patients, and the ALT level rises after treatment in about 40% of patients with non-SVR.

From the aspect of long-term prognosis, we need to verify, by prospective study, that viral eradication is really required for N-ALT patients because the incidence of hepatocellular carcinoma and liver-related mortality in

N-ALT patients has not been clarified. Deuffic-Burban et al. [22] calculated the impact of Peg-IFN plus ribavirin on morbidity and mortality in N-ALT patients using the Markov model and concluded that antiviral therapy in N-ALT patients would decrease morbidity and mortality rates. However, the treatment of N-ALT patients with CHC still remains an area of investigation, particularly with respect to the benefit-to-risk ratio of treatment. To help determine the indications for antiviral therapy in N-ALT patients, the liver histology should be evaluated before treatment. The presence of significant hepatic fibrosis (≥F2 by the METAVIR classification [23]) reflects continuous hepatic inflammation over a period of time and suggests a future risk of liver-related disease progression. Antiviral therapy may be appropriate for these patients. On the other hand, periodic follow up without antiviral therapy is recommended for patients in stages F0-1, because most of such patients show a low risk for progression to cirrhosis and the development of hepatocellular carcinoma [24].

This study had some limitations. First, the factors of viral mutation and host genetic mutation, which have been reported recently to affect the efficacy of Peg-IFN plus ribavirin combination therapy, could not be measured, and evaluation of the serum HCV RNA levels by a real-time PCR method, which is more sensitive to the measurement of serum HCV RNA levels, could not be done in the patients enrolled in this study, because we had few stored patient serum samples. Detailed examinations using the real-time PCR method in patients who are matched based on the factors of viral mutation and host genetic mutation as well as background factors will be needed for further study. Second, we excluded the factor of fibrosis from the multivariate analysis for factors associated with SVR in N-ALT patients, because data for fibrosis were lacking in 53 of the 193 patients in this study. Accordingly, the present study could not demonstrate whether fibrosis was associated with SVR in N-ALT patients. Finally, in this study, we investigated the antiviral efficacy of Peg-IFN plus ribavirin combination therapy for patients with N-ALT at the start of the therapy, not for patients with 'persistently' normal ALT. Accordingly, this study does not show the efficacy of this treatment in patients with persistently normal ALT. However, we believe that the results obtained in this study can be useful for pre-treatment prediction in outpatients who may not be followed by the reason of having normal ALT levels.

We have shown, in this matched case–control study using a propensity score method, that the therapeutic effect of combination therapy with Peg-IFN alpha-2b and ribavirin in N-ALT patients with CHC is comparable to that for E-ALT patients, irrespective of their advantageous background. Further work is needed to verify that HCV eradication can improve the prognosis of N-ALT patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Differential alteration of CD56^{bright} and CD56^{dim} natural killer cells in frequency, phenotype, and cytokine response in chronic hepatitis C virus infection

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Abstract
Background Natural killer (NK) cells play an important role in immune responses to virus infection. The cell population consists of CD56^{bright} (bright-subset) and CD56^{dim} (dim-subset) subsets that possess armed functions of cytokine production and cytolysis, respectively. How these subsets are involved in chronic hepatitis C virus infection (CHC) remains obscure.
Methods We investigated the frequency, phenotype, and cytokine response of these subsets in blood from CHC patients and healthy subjects (HS).
Results Dim-subset, but not bright-subset, showed lower frequency in the patients than in HS. Bright-subset from the patients more frequently expressed the NKG2A/CD94 inhibitory receptor than that from HS, while both subsets from the patients expressed lower levels of the NKG2D activating receptor. Both subsets from the patients displayed a significantly higher level of the signal transducer and activator of transcription (STAT) 1, compared with the HS. Upon stimulation with interferon-α, bright-subset activated less STAT4, required for interferon-γ production, and dim-subset activated more STAT1, required for cytolysis, in the patients than in HS.
Conclusions These results indicate alterations of NK cell subsets in frequency, phenotype, and cytokine response in CHC, which might be associated with the immune pathogenesis of CHC.

Keywords NK cells · CD56^{bright} · CD56^{dim} · HCV · Chronic hepatitis

Abbreviations

NK	Natural killer
IFN	Interferon
IL	Interleukin
HCV	Hepatitis C virus
CHC	Chronic hepatitis C virus infection
CHB	Chronic hepatitis B virus infection
PBMC	Peripheral blood mononuclear cell
STAT	Signal transducer and activator of transcription
pSTAT	Phosphorylated-signal transducer and activator of transcription
HS	Healthy subjects
ISG	Interferon-stimulated gene
MICA	Major histocompatibility complex class I-related chain A

Introduction

Natural killer (NK) cells play an important role in innate immune responses to a variety of viral infections by directly killing infected cells with cytotoxic molecules such

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as perforin and granzyme [1]. The cells also have great ability to secrete a key cytokine, interferon (IFN)-γ, which activates subsequent adaptive immune responses as well as inhibiting viral replication [1, 2]. The activation of NK cells is regulated by several cytokines, such as interleukin (IL)-12, IL-15, and IFN-α [1, 3]. It is also regulated by a balance of activating and inhibitory signals, respectively transmitted by activating and inhibitory receptors that recognize ligands on the cell surface of potential target cells [1, 3]. The NK cell population consists of two subsets in the context of the CD56 surface expression level: $CD56^{bright}$ and $CD56^{dim}$ subsets [4, 5]. It has been reported that $CD56^{dim}$ NK cells are strongly cytolytic armed effector cells, and that $CD56^{bright}$ NK cells are focused on the production of cytokines such as IFN-γ [4, 5]. However, how these subsets are distinguished in the context of immune responses to virus infections remains to be fully elucidated.

Hepatitis C virus (HCV) causes persistent infection in more than 70% of infected patients. Whereas some patients show a carrier-like state, most develop chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, which is why HCV infection is a worldwide health problem [6]. The administration of IFN-α is a well-established anti-viral therapy for HCV infection. More than 90% of patients with acute HCV infection respond to IFN-α-based therapy, while the response rate falls to around 50% for patients with chronic HCV infection (CHC) [7–10], suggesting a mechanism by which persistent HCV infection leads to resistance to IFN-α-based therapy. The NK cell number has been demonstrated to decrease in patients with CHC [11–16], while it is controversial whether NK cell functions are impaired in patients with CHC [17–19]. It thus remains unclear whether the perturbation of NK cells, such as that of $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells, is involved in the persistence of CHC as well as the resistance to therapy.

In the present study, we investigated how the NK cell subsets differ in frequency, phenotype, and response to cytokine stimulation. We also examined how chronic HCV infection modifies those differences. We found clear differences between the NK cell subsets in the response to cytokine stimulation as well as in the frequency and phenotype, which were altered in the CHC patients. This alteration might be associated with the immune pathogenesis of CHC.

Subjects, materials, and methods

Subjects

Eleven patients with CHC (HCV RNA genotype 1) and eleven healthy volunteers were enrolled in this study from October 2007 to March 2008. Flow cytometric analysis was carried out consecutively for these subjects. Since some of the flow cytometric data were lacking for two subjects in the patient group, they were excluded from the final analysis. The demographic data of these analyzed subjects are shown in Table 1. There was no significant difference in demographic variables (age/sex) between these two groups. The patients were histologically diagnosed as having mild or moderate chronic hepatitis and were evaluated for the degree of liver inflammation and fibrosis according to the METAVIR scoring system [20]. In addition, six patients with chronic hepatitis B virus infection (CHB) (male/female 4/2, age range (median) 31–63 years (38), alanine aminotransferase (ALT) range (median) 24–496 IU/l (70), hepatitis B virus (HBV)-DNA viral load range (median) 5.6 to >9.0 log copies/ml (7.3), hepatitis B envelope antigen (HBeAg)+/− 3/3) were enrolled in this study and consecutive flow cytometric analyses were performed. None of the subjects in the patient group displayed any evidence of other types of liver diseases. The study was approved by the Human Research Ethics Committee of Osaka University Hospital.

Isolation of peripheral blood mononuclear cell populations

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized peripheral blood by Ficoll–Hypaque density gradient centrifugation as described elsewhere [21].

Flow cytometric analysis

The staining of prepared cells was performed as described [21–26]. Briefly, for the NK receptor staining, cells were

Table 1 Characteristics of patients and subjects

	CHC	HS
Number	9	11
Sex: M/F	4/5	8/3
Age, years, range (median)	44–74 (60)	45–72 (50)
ALT, IU/l, range (median)	14–136 (43)	ND
Activity (A 0–1/2–3)	(7/2)	ND
Fibrosis (F 0–1/2–3)	(5/4)	ND
Viral load, kIU/ml, range (median)	130 to >5000 (3400)	ND
Viral genotype	1	ND

Serum hepatitis C virus (HCV) RNA levels were quantitatively determined using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 kIU/mL; Roche Diagnostics, Branchburg, NJ, USA)

CHC patients with chronic hepatitis C virus infection, *HS* healthy subjects, *ALT* alanine aminotransferase, *ND* not determined

stained with biotin-conjugated anti-CD56 antibody (B159), allophycocyanin-conjugated anti-CD3 antibody (UCHT1) and phycoerythrin-conjugated anti-NKG2D (1D11), phycoerythrin-conjugated anti-NKG2A (Z199) or fluorescein isothiocyanate-conjugated anti-CD94 antibody (HP-3D9), or the corresponding isotype control, followed by staining with peridinin chlorophyll protein-conjugated streptavidin (BD Biosciences, San Jose, CA, USA). For intracellular staining of the signal transducer and activator of transcription (STAT) or phosphorylated-STAT (pSTAT), cells were stained with biotin-conjugated anti-CD56 antibody (B159) and (1), (2) Alexa Fluor® 647-conjugated or (3), (4) fluorescein isothiocyanate-conjugated anti-CD3 antibody (UCHT1), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and cold pure methanol, and then stained with (1) phycoerythrin-conjugated anti-pSTAT1 (pY701) antibody (4a) and Alexa Fluor® 488-conjugated anti-pSTAT4 (pY693) antibody (38/p-Stat4) or (2) Alexa Fluor® 488-conjugated anti-pSTAT4 (pY693) antibody (38/p-Stat4), or (3) Alexa Fluor® 647-conjugated anti-pSTAT1 (pY701) antibody (4a) or (4) phycoerythrin-conjugated anti-STAT1 antibody (1/Stat1), or the corresponding isotype control, followed by staining with peridinin chlorophyll protein-conjugated streptavidin (BD Biosciences). All antibodies except for anti-NKG2A antibody (Beckman Coulter, Brea, CA, USA) were purchased from BD Biosciences. The stained cells were analyzed with a FACSCalibur (BD Biosciences), and the data were processed using the FlowJo program (Tree Star, Ashland, OR, USA).

In vitro stimulation of PBMCs

Prepared PBMCs were resuspended at 2×10^7 cells/ml in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Japan Bioserum, Hiroshima, Japan) for in vitro stimulation with cytokines. Natural human IFN-α, recombinant human IFN-γ, and IL-12 were used at concentrations of 1,000 IU/ml, 50 ng/ml, and 10 ng/ml, respectively. For cells left unstimulated, media were added. The cells were incubated at 37°C in 5% CO_2 for 90 min, and then collected for further analyses. Natural human IFN-α was a generous gift from Otsuka Pharma (Tokyo, Japan). Recombinant human IFN-γ and IL-12 were obtained from PBL Biomedical Laboratories (Piscataway, NJ, USA) and R&D Systems (Minneapolis, MN, USA), respectively.

Statistical analysis

The statistical significance of differences between two groups or that of changes in the indicated variables in response to IFN-α treatment was determined by applying an unpaired or paired Student's t-test, respectively. The statistical significance was defined as $p < 0.05$.

Results

Frequency of $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells

The NK cell number has been demonstrated to decrease in patients with CHC [11–16]. We examined the proportion of $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells in PBMCs from the CHC patients and those from the healthy subjects (HS). We defined CD 56^{bright} NK cells or $CD56^{dim}$ NK cells as CD56 bright CD3-negative cells or CD56 dim CD3-negative cells, respectively, by flow cytometry (Fig. 1a). The proportion of $CD56^{bright}$ NK cells was much lower than that of $CD56^{dim}$ NK cells in both the HS and patient groups (Fig. 1b). The proportion of $CD56^{dim}$ NK cells from the CHC patients was significantly lower than that from the HS, while that of $CD56^{bright}$ NK cells did not show a significant difference between these groups.

Expression level of activating or inhibitory NK receptors on $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells

The activation of NK cells is partly regulated by the balance of signals transmitted by activating and inhibitory NK receptors [1, 3]. We therefore examined the expression level of activating or inhibitory NK receptors such as NKG2D or NKG2A/CD94 on $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells, by flow cytometry. We found lower expression of NKG2D, an activating receptor, on $CD56^{bright}$ NK cells than on $CD56^{dim}$ NK cells (Fig. 2). In contrast, the expression of NKG2A/CD94, an inhibitory receptor, on $CD56^{bright}$ NK cells was higher than that on $CD56^{dim}$ NK cells. The expression levels of NKG2D on both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells from the CHC patients were significantly lower than those from the HS. However, the expression level of NKG2A/CD94 on $CD56^{bright}$ NK cells from the CHC patients was significantly higher than that from the HS, while that on $CD56^{dim}$ NK cells was not significantly different between these groups. In addition, no significant correlation was observed between the expression levels of the activating or inhibitory NK receptors and the level of viral load or the histological level of liver inflammation or fibrosis in the CHC patients (data not shown).

STAT1 expression levels in $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells

We have recently reported that NK cells displayed lower intracellular STAT1 expression than other immune cells

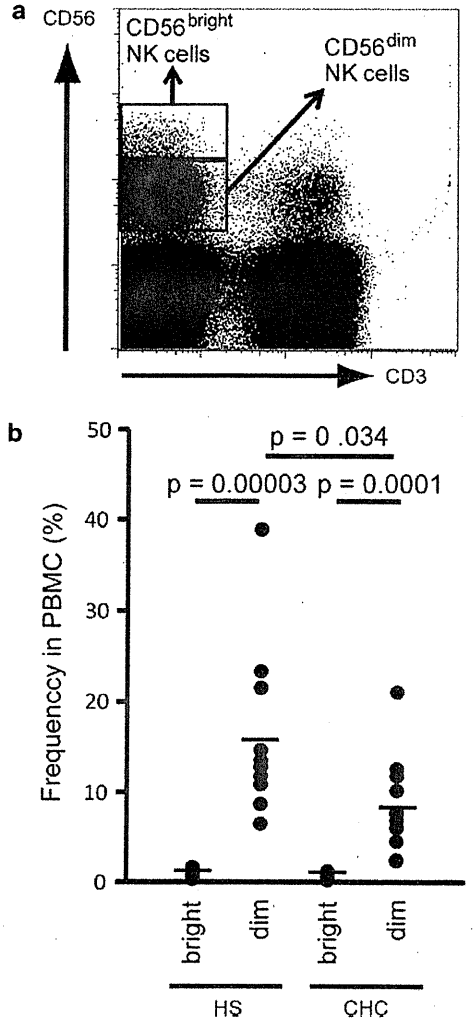

Fig. 1 Frequency of natural killer (*NK*) cell subsets in peripheral blood mononuclear cells (*PBMCs*). Frequencies of $CD56^{bright}$ NK and $CD56^{dim}$ NK cell subsets in PBMCs were evaluated by flow cytometry. PBMCs were obtained from patients with chronic hepatitis C virus (HCV) infection (*CHC*) ($n = 9$) and healthy subjects (*HS*) ($n = 11$). **a** $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells were defined as CD56 bright $CD3^-$ cells or CD56 dim $CD3^-$ cells, respectively, by flow cytometry. CD56 dim and CD56 bright were divided at a threshold where most CD3+ cells lost CD56 expression. **b** The frequency of $CD56^{bright}$ subset (bright) or $CD56^{dim}$ subset (dim) was evaluated by flow cytometry, electronically gating on CD56 bright $CD3^-$ cells or CD56 dim $CD3^-$ cells. Comparisons of the frequencies of those NK cell subsets in PBMCs between bright and dim subsets or between the CHC patients and the HS are shown with the statistically significant p values. Each *circle* represents data for an individual. *Horizontal bars* represent means. Statistical significance was analyzed using the unpaired Student's t-test

such as T cells or NKT cells, and that the level was higher in patients with CHC [24]. We therefore examined the expression level of STAT1 in the NK cell subsets, $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells. The expression level of STAT1 in $CD56^{bright}$ NK cells was clearly higher than that in $CD56^{dim}$ NK cells in the HS (Fig. 3a, b). The expression level of STAT1 in either $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells from the CHC patients was significantly higher than that from the HS, and no significant difference was observed in the STAT1 expression levels between $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells in the CHC patients. In addition, no significant correlation was observed between the STAT1 expression level in these NK cell subsets and the level of viral load or the level of liver inflammation or fibrosis in the CHC patients (data not shown). We also examined the expression level of STAT1 in the NK cell subsets from patients with CHB, which is also capable of causing chronic hepatitis. The expression level of STAT1 in either $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells in the CHB patients was modestly, but not significantly, higher than that of the HS (positive cell rate, mean ± SD; 35.0 ± 26.8% in $CD56^{bright}$ subset and 28.7 ± 18.1% in $CD56^{dim}$ subset in the CHB patients).

Activation of STAT1/4 occurring in response to cytokines in $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells

We have previously reported altered IFN-α-signaling in the total NK cell population from CHC patients [24]. We next examined the cytokine signaling in $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells. As IFN-α can phosphorylate both STAT1 and STAT4, IFN-γ can phosphorylate STAT1, and IL-12 can phosphorylate STAT4 in NK cells [1, 3], we evaluated the phosphorylation level of STAT1/4 that occurred in response to IFN-α, IFN-γ, or IL-12 in $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells.

In response to IL-12, the majority of $CD56^{bright}$ NK cells phosphorylated STAT4, while only some of the $CD56^{dim}$ NK cells did so (Fig. 4a). In response to IFN-γ, almost no $CD56^{bright}$ NK cells phosphorylated STAT1, while some of the $CD56^{dim}$ NK cells did so. These patterns were observed in both the HS and the patient group. Statistical analysis demonstrated that the phosphorylation level of STAT4 occurring in response to IL-12 in $CD56^{bright}$ NK cells was significantly higher than that in $CD56^{dim}$ NK cells in both the HS and the CHC patient group, while that of STAT1 occurring in response to IFN-γ in $CD56^{bright}$ NK cells was significantly lower than that in $CD56^{dim}$ NK cells in both groups (Fig. 4b). However, the phosphorylation level of STAT4 or STAT1, respectively, occurring in response to IL-12 or IFN-γ did not show any significant difference between these groups in either $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells.

In response to IFN-α, the whole population of $CD56^{bright}$ NK cells or CD 56^{dim} NK cells phosphorylated both STAT4 and STAT1 in both subject groups (Fig. 4a). Statistical analysis, however, demonstrated that

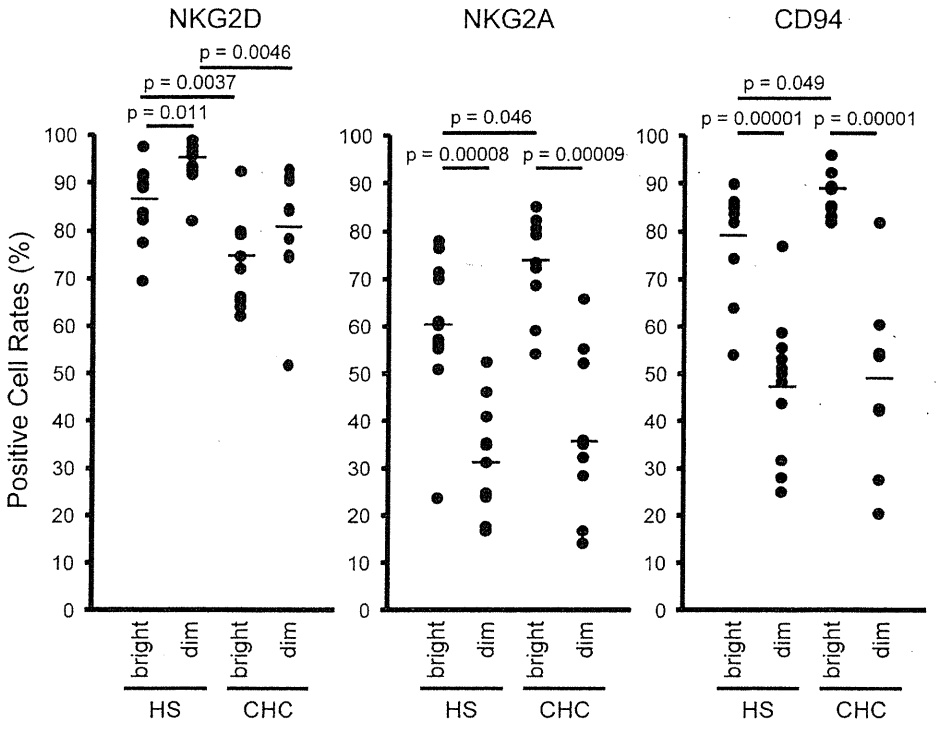

Fig. 2 NK receptor expression on NK cell subsets. The expression of NK activating or inhibitory receptors, NKG2D or NKG2A and CD94, respectively, on $CD56^{bright}$ NK cell subset (bright) and $CD56^{dim}$ NK cell subset (dim) was evaluated by flow cytometry with isotype control staining, electronically gating on CD56 bright $CD3^-$ cells and CD56 dim $CD3^-$ cells. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). Positive cells (positive cell rate) were determined based on isotype control staining. Comparisons of those NK receptor expression levels between bright and dim subsets in a subject group or between CHC and HS in a subset are shown as positive cell rates with the statistically significant p values. Each *circle* represents data for an individual. *Horizontal bars* represent means. Statistical significance was analyzed using the unpaired Student's t-test

the phosphorylation level of STAT4 occurring in response to IFN-α in the CHC patients was significantly lower than that in the HS in both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells (Fig. 4b). On the other hand, the phosphorylation level of STAT1 occurring in response to IFN-α in the CHC patients was significantly greater than that in the HS in both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells.

We next examined the relationship between STAT1 phosphorylation and STAT4 phosphorylation occurring in response to IFN-α in the NK cell subsets. Upon stimulation with IFN-α, the whole population of $CD56^{bright}$ NK cells phosphorylated both STAT1 and STAT4, while some of the $CD56^{dim}$ NK cells more strongly phosphorylated STAT1 but more weakly phosphorylated STAT4, compared with the remaining $CD56^{dim}$ NK cells, which more weakly phosphorylated STAT1 but more strongly phosphorylated STAT4 (Fig. 5a). Moreover, the frequency of the 'high-pSTAT1 population' in response to IFN-α in $CD56^{dim}$ NK cells in the CHC patient group was significantly greater than that in the HS group (Fig. 5a, b).

Regulation of NK receptor expression level on $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells occurring in response to IFN-α-based therapy in vivo

To examine whether $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells would respond differently to IFN-α treatment in vivo, we evaluated the frequency, the expression level of NK receptors, and the STAT1 expression level in $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells before and after the initiation of IFN-α-based therapy. The frequency of $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells did not show any significant change between before and 1 day after initiation of the therapy (data not shown). The expression levels of NKG2A/CD94 on both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells were significantly decreased in response to the therapy 1 day after its initiation (Fig. 6). On the other hand, the expression level of NKG2D on $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells did not show any significant change between before and 1 day after initiation of the therapy. The STAT1 expression levels in both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells were significantly increased in response to the therapy (data not shown).

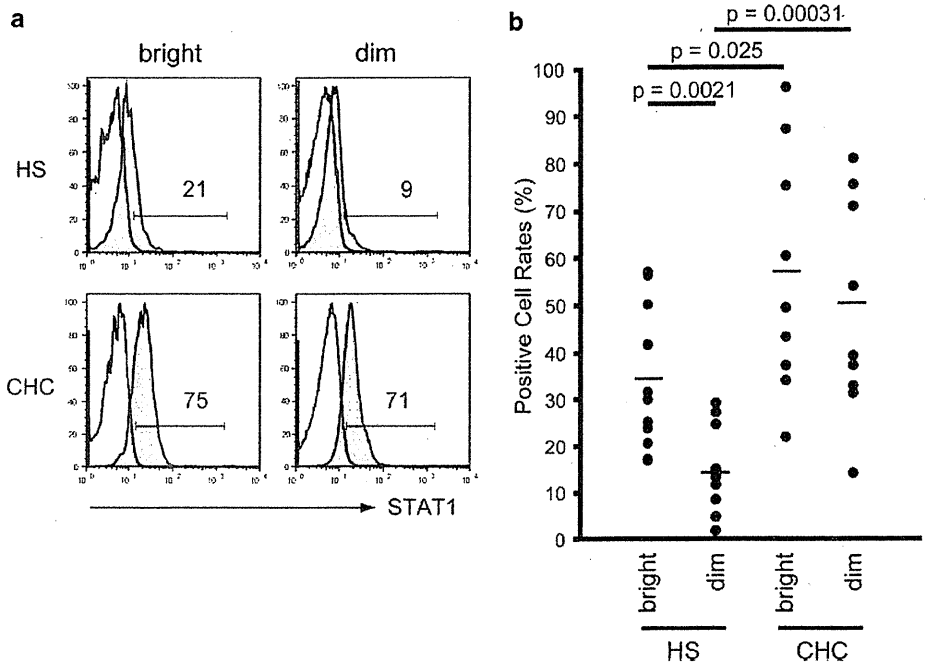

Fig. 3 Signal transducer and activator of transcription 1 (*STAT1*) expression in NK cell subsets. Intracellular STAT1 expression levels in $CD56^{bright}$ NK cell subset (bright) and $CD56^{dim}$ NK cell subset (dim) were evaluated by flow cytometry with isotype control staining, electronically gating on CD56 bright $CD3^-$ cells and CD56 dim $CD3^-$ cells. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). **a** Representative histograms from a patient and a healthy subject (*HS*) are shown. *Dotted lines* show staining with the isotype control. *Thick lines* with *shaded areas* show staining with the antibody. Numbers are percentages of positive cells (positive cell rate) determined based on isotype control staining. **b** Comparisons of STAT1 expression level between bright and dim subsets in a subject group or between CHC and HS in a subset are shown as positive cell rates with the statistically significant p values. Each *circle* represents data for an individual. *Horizontal bars* represent means. Statistical significance was analyzed using the unpaired Student's t-test

Discussion

In the present study, we found clear differences between $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells in their responses to cytokines, as well as the cell frequency and the surface expression level of the NK receptors. We also found some differences between these subsets in the alteration caused by chronic HCV infection. Of interest and novelty are the findings that the NK cell subsets displayed different intracellular STAT1 expression levels (Fig. 3) and responded differently to cytokine stimulation to lead to differences in the phosphorylation of STAT1/4 (Figs. 4, 5) and that some of the differences were altered in the CHC patients. Furthermore, both subsets showed alterations of IFN-α signaling in the CHC patients, compared with the HS (Fig. 4).

We have recently shown that NK cells from patients with CHC display a higher level of STAT1 expression than those from HS [24] and suggested that the up-regulation of STAT1 expression might result from a host response to HCV infection with IFN-α and/or IFN-γ production, because STAT1 itself is one of the IFN-stimulated genes (ISGs) whose expression is up-regulated by IFN-α or IFN-γ [27, 28], which has been reported to be detected in the sera of patients with CHC [29, 30]. The present study has shown that both NK cell subsets from the patients with CHC displayed a higher level of STAT1 expression than those from the HS (Fig. 3b); this might also have been induced similarly in both subsets by a host response to HCV infection. Since a host response to HCV infection would be associated with the liver inflammation and subsequent fibrosis, we examined whether the STAT1 expression level in these NK cell subsets could be correlated with the level of liver inflammation or fibrosis which had been histologically evaluated using liver biopsy samples. Although no significant correlation was observed between the STAT1 expression level in the $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells and the level of liver inflammation or fibrosis, there was a tendency of a higher level of inflammation or fibrosis being correlated with a higher level of STAT1 expression in NK cells, including $CD56^{bright}$ and $CD56^{dim}$ subsets, in our limited number of patients (T. Miyagi et al. unpublished data); further investigation should be done with a larger number of subjects. Another question that emerged was whether our findings in peripheral blood could be applied to the liver in CHC patients. Chen et al. [31]