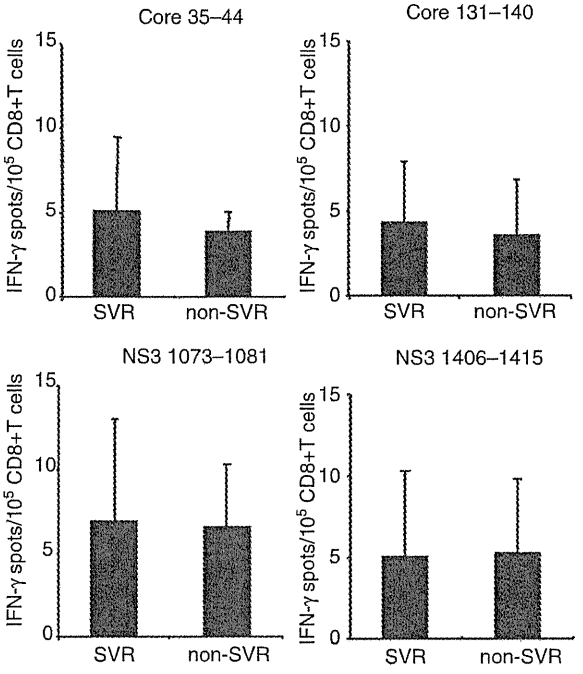

Figure 3 Comparison of the frequencies of hepatitis C virus (HCV)-specific CD8+ T cells in pre-treated HCV patients between sustained virologic response (SVR) and non-SVR. HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-treated HCV patients were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT). We analyzed the association between the HCV-specific CD8+ T cell responses and the achieving of SVR. SVR: patients who were observed SVR, non-SVR: patients who were not observed SVR.

SVR.[1,12] We also examined the association between cEVR and early elevation of HCV-specific CD8+ T cell responses. The frequencies of CD8+ T cell responses against all four HCV derived peptides in pre-IFN patients were not significantly different between cEVR and non-cEVR (Tatsumi T, unpublished data). In cEVR patients, the frequencies of CD8+ T cell responses against three HCV peptides $Core_{35-44}$, $Core_{131-140}$, $NS3_{1406-1415}$) increased significantly 4 weeks after the starting treatment and those against $NS3_{1073-1081}$ peptide tended to increase although these were not significant. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-cEVR patients (Tatsumi T, unpublished data). The cEVR results were almost similar to those of the SVR results. Although we could not evaluate the HCV RNA levels at 4 week after starting treatment, the cEVR results suggested that early elevation of the frequencies of HCV-specific CD8+ T cell responses might reflect the decrease of viral load of HCV.

CD8+ CTL activities in pre-treated HCV patients have been reported to be very low.[7,18,19] Consistent with the previous observations, the frequencies of HCV specific CD8+ T cell in pre-treated patients were also low in our study. The frequencies of HCV-specific CD8+ T cells in pre-treated patients were not associated with the HCV viral load and the serum ALT levels of patients before treatment. Several reports demonstrated that the baseline presence of HCV-specific CTLs prior to treatment was associated with viral clearance.[7,18] However, the frequencies of HCV-specific CD8+ T cells in pre-treated patients were not associated with the achievement of SVR in our study. In previous other reports, whole PBMC isolated from treated patients were used to evaluate the antiviral activity of HCV-specific CD8+ T cells. In our study, enriched CD8+ T cells obtained by magnetic sorting methods were used to enhance the sensitivity for the detection of HCV-specific CD8+ T cells. Both ELISPOT and staining with tetramers/pentamers could be applied for immunological monitoring for peptide-specific CTLs.[20] ELISPOT can detect activated functional CTLs, and tetramers/pentamers staining can detect peptide-specific CTLs.[20] In our study, we assessed the HCV-specific CD8+ T cell responses by IFN-γ ELISPOT, which is the most well-established methods and has already applied for immunological monitoring in cancer patients.[11] Recently perforin- or granzyme B-ELISPOT assays have also been reported. However, due to limitations in cell numbers of PBMC isolated from HCV patients, we were unable to apply another system of immunological monitoring and test other functional molecules. If we can apply these ELISPOT assays, we could directly evaluate the cytotoxic activity of HCV-specific CTLs.

In our study, the frequencies of HCV-specific CD8+ T cells in pre-treated patients were similar between SVR and non-SVR patients. In contrast, significant increase of the frequencies of HCV-specific CD8+ T cells between pre-IFN and IFN-4week could be observed in SVR patients, but not in non SVR patients. Caetano et al. evaluated the HCV-specific CD8+ T cells by HLA class I pentamers specific for the one HCV-Core epitope and one NS3 epitope which were same as we used.[16] They demonstrated that the increase of the frequencies of HCV-specific CTLs at 1 month after starting treatment was mainly due to terminally differentiated cells as well as, to a lesser extent, central memory cells in SVR patients and, in contrast, the increase of HCV-specific

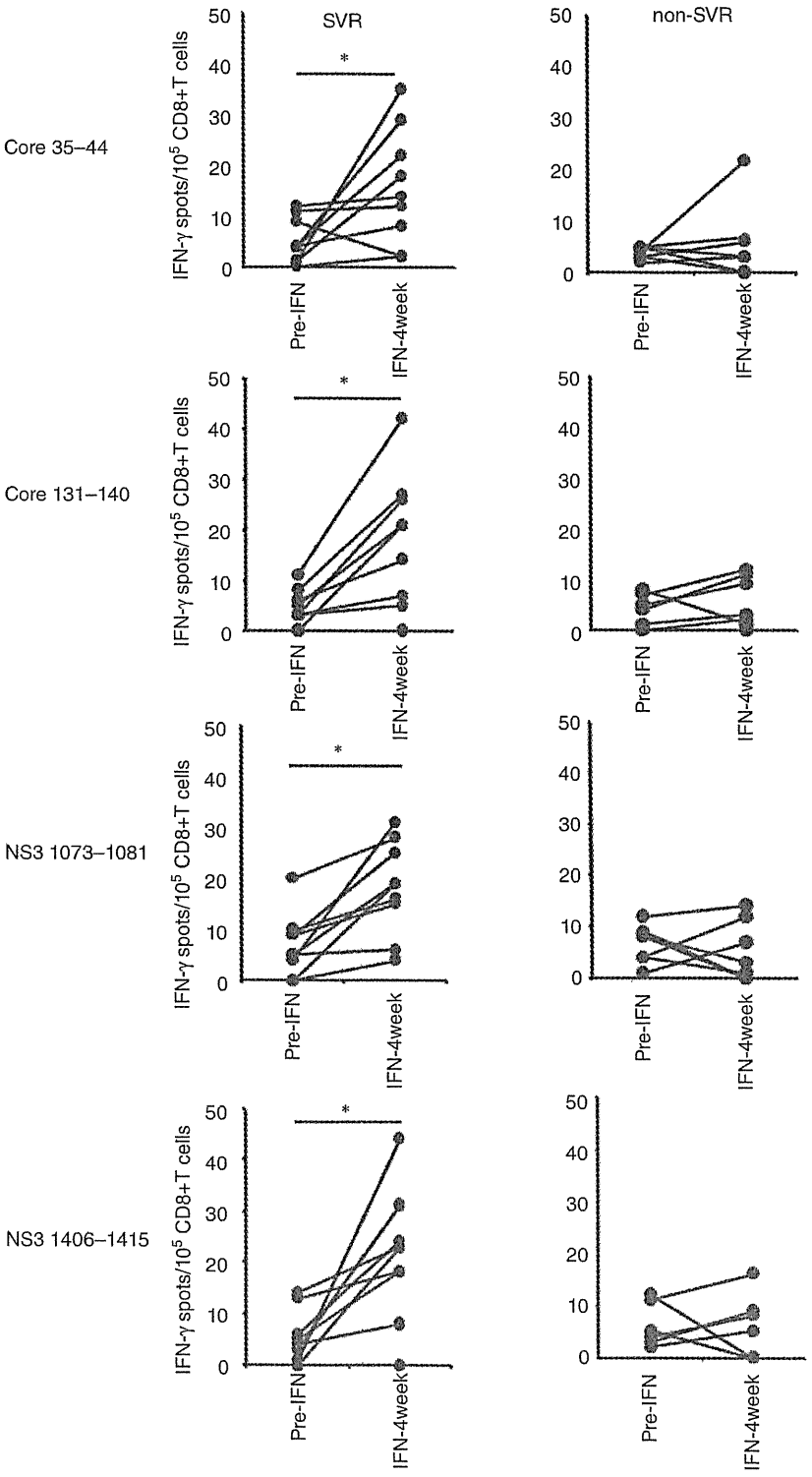

Figure 4 Analysis of the association of the change of hepatitis C virus (HCV)-specific CD8+ T cell responses between pre-IFN and IFN-4week chronic hepatitis C (CH-C) patients with the achieving sustained virologic response (SVR). Peripheral blood CD8+ T cells were isolated from pre-IFN and IFN-4week patients. HCV-specific CD8+ T cell responses were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. We analyzed the association of HCV-specific CD8+ T cell responses in treated CH-C patients with the achieving SVR. Each symbol within a panel represents the response of an individual donor to the indicated HLA-A2-presenting HCV Core or NS3 protein-derived peptides. The treated patients were divided into two groups; SVR group and non-SVR group. $^*P < 0.05$.

pre-terminally differentiated CD8+ T cells was also observed in non-SVR patients.[16] These results suggested that CTLs maturation efficiently occurred in SVR patients. HCV or HCV-gene products have been reported to inhibit the maturation pathway of CTLs.[5,21] Thus the decrease of viral load during this combination therapy may induce CTL maturation.

We demonstrated that the achievement of SVR in this combination therapy was associated with the early elevation of HCV-specific CD8+ T cell responses, but not with the pre-treated levels of HCV-specific CD8+ T cell responses. These results suggested, at least, that the enhancement of HCV-specific CD8+ T cell responses might play critical roles in the second slope of viral clearance by this combination therapy. The increasing frequencies of HCV-specific CD8+ T cells have also been reported to be associated with SVR during the combination therapy by evaluating with pentamers of HCV-specific peptides.[16] Ribavirin has immunomodulatory effect with a switch from Th2 to Th1 cytokine profile.[22] The combined use of pegIFNα and ribavirin might have more immunomodulatory effect to generate HCV specific CTLs. However, even now, this should be elucidated to develop better treatment of chronic hepatitis C.

Although CTL responses to HCV are multi-specific,[13,23] we and others tested only small part of the known CTL epitopes of HCV, which do not comprise all potential HLA A2-restricted CTL epitopes of HCV. HCV may have mutated and escaped from the CTL responses to the corresponding epitopes in the chronically infected patients. The epitopes used in our study have been applied to the detection of HCV-specific CTLs in several other previous studies,[5,15,16] which support the usefulness of the selected epitopes. Our results demonstrated that the increases of the frequencies of CD8+ T cells against four synthesized peptides were associated with the antiviral activity of this combination therapy. Thus the selected epitopes used in our experiments were probably stable, at least, during the 4 weeks after starting treatment.

In spite of recent progress for HCV treatment, there remains significant room for improvement. To date, a variety of viral factors and host factors that correlate with SVR in the combination therapy have been noted. Recently, in addition to viral factors and host factors, response and adherence to treatment have been noted.[2] To establish the better treatment, the detail mechanism of HCV elimination should be elucidated. In the present study, we demonstrated that early enhancement of HCV-specific CD8+ T cell responses was associated with the achieving SVR in this combination therapy. These suggest that activation of antiviral CTLs might be involved in the elimination of HCV. The early elevation of HCV-specific CTL responses in treated HCV patients may be a candidate for predicting SVR in this combination therapy.

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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

T. Miyagi and S. Shimizu contributed equally to this work and share the first authorship.

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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

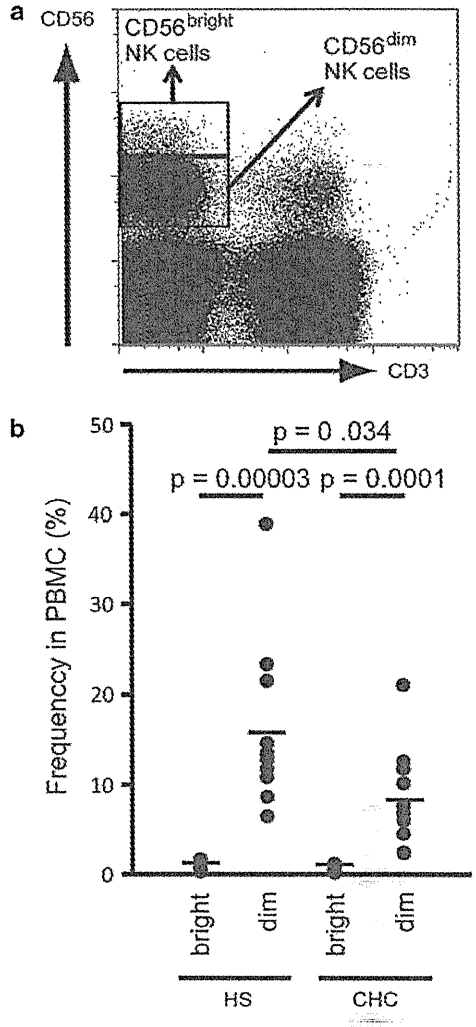

Fig. 1 Frequency of natural killer (*NK*) cell subsets in peripheral blood mononuclear cells (*PBMCs*). Frequencies of $CD56^{bright}$ NK and $CD56^{dim}$ NK cell subsets in PBMCs were evaluated by flow cytometry. PBMCs were obtained from patients with chronic hepatitis C virus (HCV) infection (*CHC*) ($n = 9$) 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

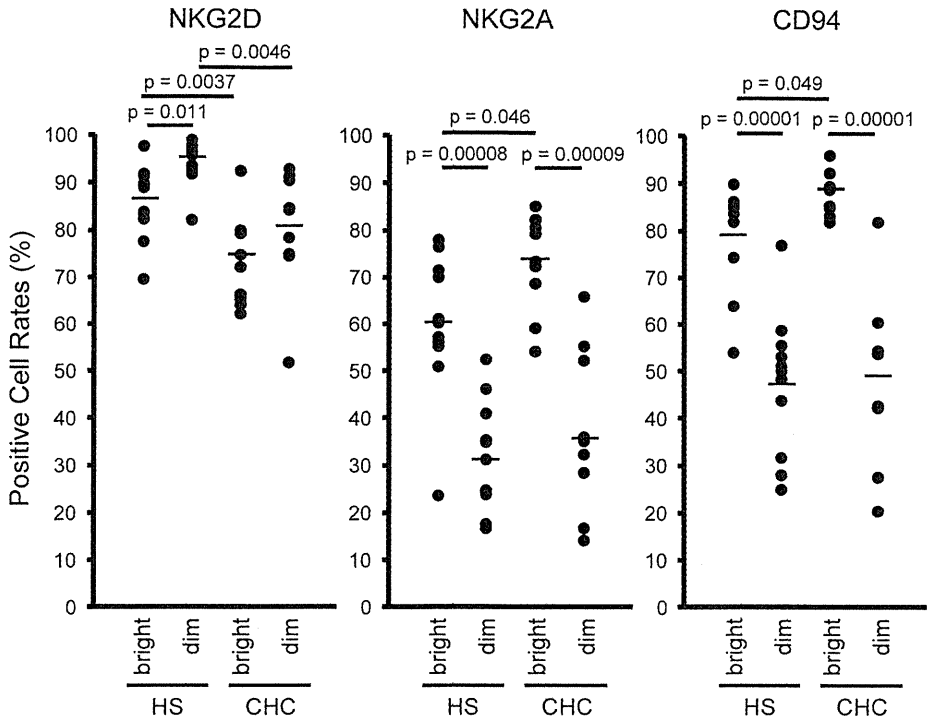

Fig. 2 NK receptor expression on NK cell subsets. The expression of NK activating or inhibitory receptors, NKG2D or NKG2A and CD94, respectively, on $CD56^{bright}$ NK cell subset (bright) and $CD56^{dim}$ NK cell subset (dim) was evaluated by flow cytometry with isotype control staining, electronically 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).

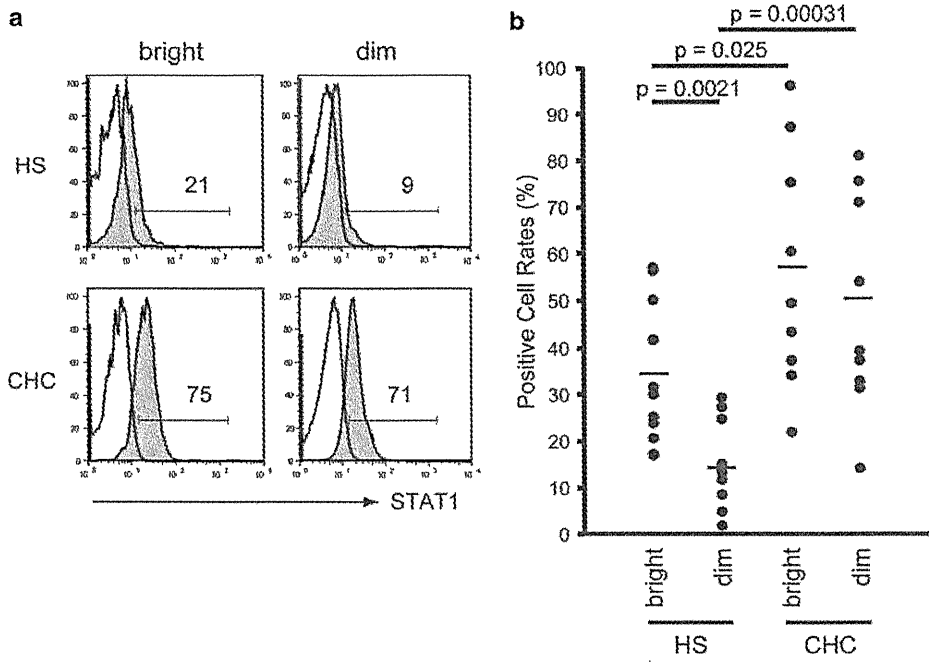

Fig. 3 Signal transducer and activator of transcription 1 (*STAT1*) expression in NK cell subsets. Intracellular STAT1 expression levels in $CD56^{bright}$ NK cell subset (bright) and $CD56^{dim}$ NK cell subset (dim) were evaluated by flow cytometry with isotype control staining, electronically gating 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]

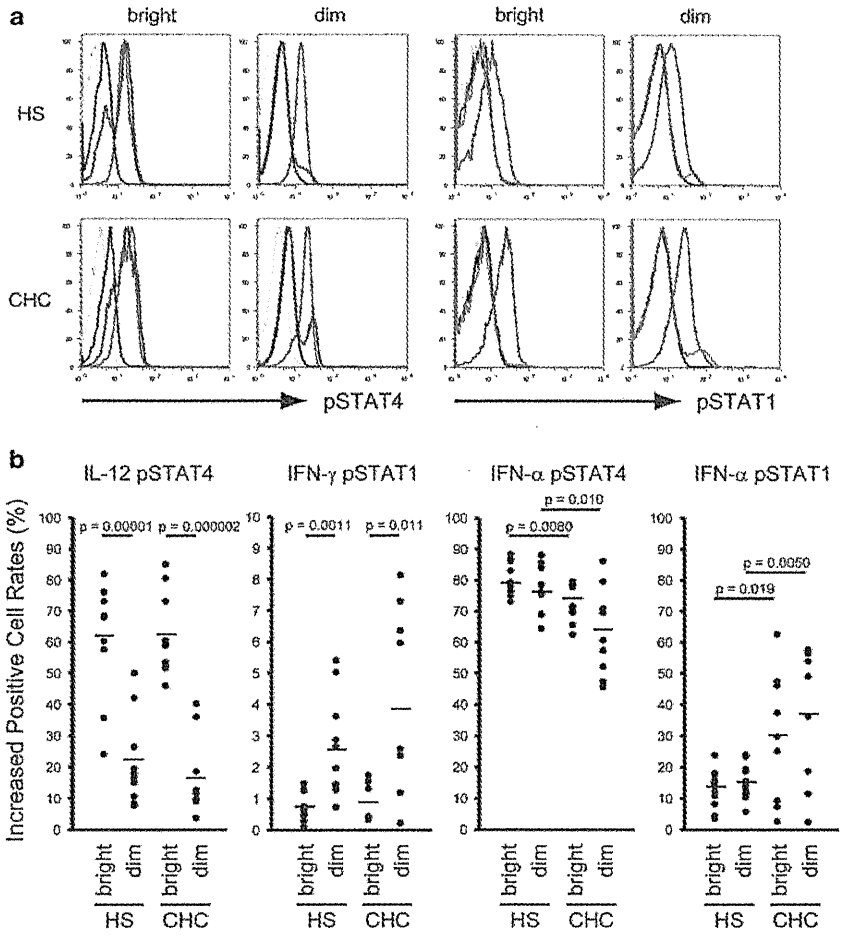

Fig. 4 Activation of STAT1/4 occurring in response to interleukin-12 (*IL-12*), interferon-γ (*IFN-γ*), or IFN-α in NK cell subsets. Phosphorylated STAT1 (*pSTAT1*) and pSTAT4 protein levels were evaluated by flow cytometry with isotype control staining. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). Prepared PBMCs were unstimulated or stimulated with natural IFN-α, IFN-γ, or IL-12 for 90 min in vitro, and then collected. pSTAT1 and pSTAT4 protein levels in $CD56^{bright}$ NK (bright) and $CD56^{dim}$ NK (dim) cell subsets were evaluated by flow cytometry, electronically gating on CD56 bright $CD3^-$ cells and CD56 dim $CD3^-$ cells. **a** Representative histograms of a patient and a healthy subject (*HS*) are shown. *Green lines* show staining of IFN-α-stimulated cells with isotype control. *Purple lines* show staining of unstimulated cells with the antibody. *Red*, *orange*, and *blue lines* show staining of IL-12-, IFN-γ- and IFN-α-stimulated cells, respectively, with the antibody. **b** Positive cell rates were determined based on staining with isotype controls. Increased positive cell rates were determined by subtracting the positive cell rate of unstimulated cells from those of stimulated cells. Comparisons of pSTAT1/4 level in response to IFN-α, IFN-γ, or IL-12 between bright and dim subsets in a subject group or between CHC and HS in a subset are shown as increased pSTAT1/4 positive cell rate with the statistically significant p values. Each *circle* represents individual data. *Horizontal bars* represent means. Statistical significance was analyzed using the unpaired Student's t-test

reported that the hepatic gene expression level in a subset of ISGs, including STAT1, was greater in CHC patients than in normal subjects. Sarasin-Filipowicz et al. [32] showed that the gene expression level in a subset of ISGs in CHC patients was greater in whole liver, including hepatocytes and nonparenchymal cells such as lymphocytes, than in PBMC, and suggested that chronic HCV infection had stronger local effects on the IFN system in the liver than in PBMC. Also, Tateno et al. [33] showed that the gene expression level of STAT1 in liver-infiltrating lymphocytes was about twofold greater than that in hepatocytes in CHC patients. Considering these reports, we speculate that the NK cell subsets in the liver as well as in the peripheral blood of CHC patients might display a high level of STAT1 expression. Whether our findings in peripheral blood could be applied to the liver in CHC patients requires further investigation. We also examined whether our findings with CHC patients would be observed in CHB patients. Unlike in the CHC patients, the CHB patient expression levels of STAT1 in either $CD56^{bright}$ or $CD56^{dim}$ subsets was not significantly higher than that in the HS, which would be consistent with the report of the

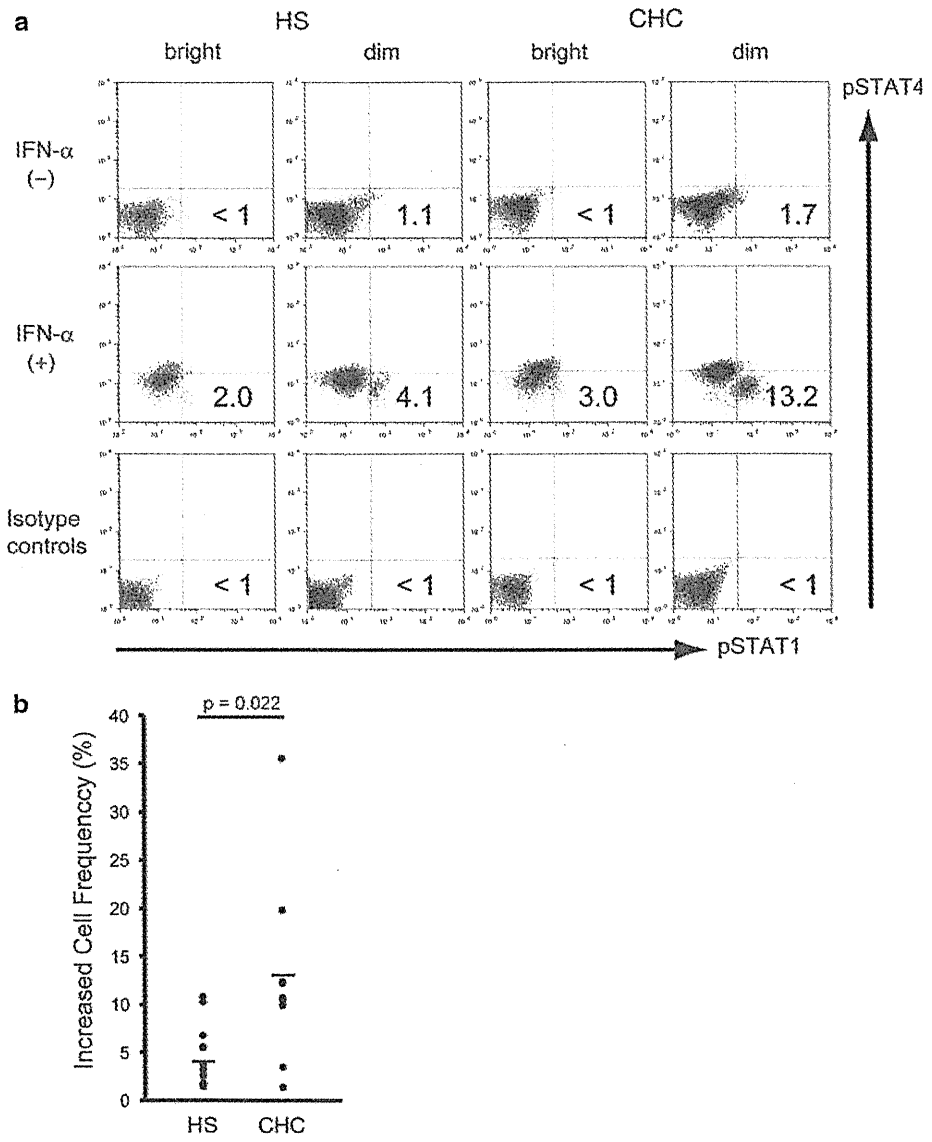

Fig. 5 Relationship between STAT1/4 phosphorylation occurring in response to IFN-α in NK cell subsets. pSTAT1 and pSTAT4 protein levels were simultaneously evaluated by flow cytometry with isotype control staining. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). Prepared PBMCs were unstimulated or stimulated with natural IFN-α for 90 min in vitro, and then collected. pSTAT1 and pSTAT4 protein levels in $CD56^{bright}$ NK (bright) and $CD56^{dim}$ NK (dim) cell subsets were evaluated by flow cytometry, electronically gating on CD56 bright $CD3^-$ cells and CD56 dim $CD3^-$ cells. **a** Representative dot plots of untreated or IFN-α treated cells stained with antibody or treated cells stained with isotype controls from a patient and a healthy subject (*HS*) are shown. *Numbers* are frequencies of gated cells that strongly phosphorylated STAT1 but weakly phosphorylated STAT4 in the corresponding subsets. **b** Increased cell frequency was determined by subtracting the gated cell frequency of unstimulated cells from those of stimulated cells. Comparisons of the increased cell frequency of the high-pSTAT1 population in response to IFN-α stimulation in the $CD56^{dim}$ NK cell subset between CHC and HS are shown with the statistically significant p value. Each *circle* represents individual data. *Horizontal bars* represent means. Statistical significance was analyzed using the unpaired Student's t-test

STAT1 signaling pathway being less activated in CHB than in CHC [34].

Lines of evidence have shown that $CD56^{dim}$ NK cells, but not $CD56^{bright}$ NK cells, decrease in number in peripheral blood in patients with CHC [12, 14, 15, 35]. In agreement with these reports, we observed a lower frequency of $CD56^{dim}$ NK cells, but not of $CD56^{bright}$ NK cells, in the CHC patients than in the HS (Fig. 1b). Although we observed significant up-regulation of STAT1 expression in both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells, the magnitude of the up-regulation of STAT1 expression in the CHC patients, compared with that in the

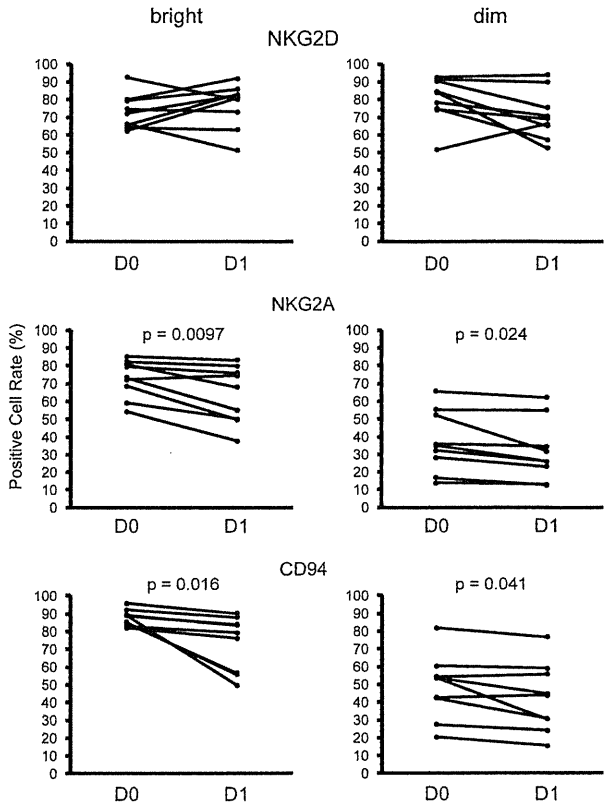

Fig. 6 Regulation of NK receptor expression in response to IFN-α-based therapy. The expression of NK activating or inhibitory receptors, NKG2D or NKG2A and CD94, respectively, on $CD56^{bright}$ NK (bright) and $CD56^{dim}$ NK (dim) cell subsets was evaluated by flow cytometry with isotype control staining, electronically gating on CD56 bright $CD3^-$ cells and CD56 dim $CD3^-$ cells. PBMCs were derived from patients treated with IFN-α-based therapy ($n = 9$) before (*D0*) and 1 day after (*D1*) the initiation of the therapy. Positive cells (positive cell rate) were determined based on isotype control staining. The changes in the NK receptor expression levels between D0 and D1 are shown as positive cell rates with the statistically significant p values. Each *circle* represents individual data. Statistical significance was analyzed using the paired Student's t-test

HS, was clearly greater in $CD56^{dim}$ NK cells than in $CD56^{bright}$ NK cells (Fig. 3b). Considering that STAT1 transmits the anti-proliferative effects induced by IFN-α [36–38], the greater up-regulation of STAT1 in $CD56^{dim}$ NK cells, compared with that of $CD56^{bright}$ NK cells, might have resulted in the significantly reduced frequency in $CD56^{dim}$ NK cells but not in $CD56^{bright}$ NK cells. Further study is required to examine this.

The most prolific producer of IFN-γ is the $CD56^{bright}$ NK cell rather than the $CD56^{dim}$ NK cell [4, 5]. In the present study, we found that $CD56^{bright}$ NK cells responded to IL-12 to phosphorylate STAT4 much more than $CD56^{dim}$ NK cells (Fig. 4a, b). IL-12 is one of the strongest stimulators of IFN-γ production from NK cells, which is transmitted by STAT4 phosphorylation [1, 3, 39, 40]. The preferential activation of STAT4 in $CD56^{bright}$ NK cells, compared with $CD56^{dim}$ NK cells, might be one of the underlying mechanisms by which $CD56^{bright}$ NK cells, compared with $CD56^{dim}$ NK cells, are armed to produce IFN-γ. On the other hand, we found that $CD56^{dim}$ NK cells responded to IFN-γ to phosphorylate STAT1, while $CD56^{bright}$ NK cells hardly did so (Fig. 4a, b). Moreover, some of the $CD56^{dim}$ NK cells responded to IFN-α to more strongly phosphorylate STAT1 than $CD56^{bright}$ NK cells (Fig. 5a). The $CD56^{dim}$ NK cells are strongly cytolytic armed effector cells [4, 5]. IFN-α or IFN-γ is one of the strongest inducers of the cytolytic function of NK cells, which is transmitted by STAT1 phosphorylation [1, 3, 38, 41]. Thus, the predominant activation of STAT1 in $CD56^{dim}$ NK cells, compared with $CD56^{bright}$ NK cells, might be one of the underlying mechanisms by which $CD56^{dim}$ NK cells become armed with a strong cytotoxic function. The differences in cytokine response to activate STAT molecules between these NK cell subsets might lead to the differences in their armed functions, such as cytolysis and cytokine production.

Ahlenstiel et al. [42] have recently reported that chronic exposure to HCV-induced IFN-α rendered NK cells with a functional polarization toward a cytotoxic phenotype, but without an increase in IFN-γ production. Moreover, Oliviero et al. [16] showed that NK cells from CHC patients were of a predominantly activating phenotype and that these phenotypic changes were associated with enhanced cytotoxic activity and defective IFN-γ production. These reports may be associated with our finding that NK cells, including $CD56^{bright}$ and $CD56^{dim}$ subsets, from the CHC patients displayed a high level of STAT1 expression (Fig. 3). Cytotoxic molecules such as perforin and granzyme, as well as STAT1, are among the ISGs [28, 41]. A high level of STAT1 in NK cells, particularly in $CD56^{dim}$ NK cells that are armed with a cytolytic function, in CHC patients might correspond to a high level of cytotoxic molecules in NK cells, resulting in enhanced cytotoxic activity. Indeed, the frequency of the population that strongly phosphorylated STAT1 upon IFN-α stimulation in $CD56^{dim}$ NK cells was significantly higher in the CHC patients than in the HS (Fig. 5b). This population might be highly armed cells with a cytolytic function. On the other hand, it has been reported that the STAT1 expression level in NK cells was correlated negatively with the activation of STAT4 to produce IFN-γ in response to IFN-α in NK cells [22, 24]. A high level of STAT1 in NK cells, particularly in $CD56^{bright}$ NK cells that are armed to produce IFN-γ, might cause defective IFN-γ production in the NK cells of patients with CHC.

Recent studies have demonstrated that a higher level of ISGs in hepatocytes as well as in PBMCs before IFN-α-based therapy is associated with resistance to this therapy

[32, 43]. We have also reported that a small number of CHC patients treated with IFN-α-based therapy revealed a tendency, in those who had a higher level of STAT1 (which is one of the ISGs) in the total NK cell population, to not respond well to the therapy in the early phase, such as in week 8 after its initiation [24]. In the present study, we did not observe a significant correlation between the STAT1 expression level in the NK cell subsets and the sensitivity to IFN-α based therapy, but we did find a tendency of those who had a higher level of STAT1 in the NK cell subsets to not respond well to the therapy in the early phase, such as in week 8 after its initiation (T. Miyagi et al. unpublished data). The number of evaluated patients, however, was small. More data on treated patients will be required to accurately evaluate the relationship between the STAT1 expression level in the NK cell subsets and the therapy outcome.

We have recently reported that NKG2D expression on NK cells could be down-regulated by the soluble major histocompatibility complex class I-related chain A (MICA), which was increased in patients with CHC compared with healthy controls [44]. In the present study, NKG2D expression levels on both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells from the CHC patients were significantly lower than those from the HS (Fig. 2). Thus, the lower NKG2D expression on either $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells in patients with CHC might be caused by the increased soluble MICA. In response to IFN-α treatment in vivo, the expression of NKG2A/CD94 was down-regulated in both subsets in the CHC patients. In vitro stimulation of NK cells with IFN-α did not down-regulate or up-regulate the messenger RNA expression of NKG2A/CD94 in NK cells (T. Miyagi et al. unpublished data). Thus, the lower expression of NKG2A/CD94 might be modulated not directly but indirectly by in vivo IFN-α treatment.

In the present study, we investigated how the NK cell subsets differed in frequency, phenotype, and cytokine response, and also how chronic HCV infection modified these differences. $CD56^{bright}$ NK cells had a relatively higher level of intracellular STAT1 expression than $CD56^{dim}$ NK cells in the HS. Both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells from the CHC patients displayed remarkably higher levels of STAT1 expression than those from the HS, without any significant differences between these subsets. Upon in vitro stimulation with cytokines such as IL-12, IFN-γ, and IFN-α, $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells phosphorylated STAT1/4 differently. These differences between the NK cell subsets in frequency, phenotype, and cytokine response were partly altered in the CHC patients, suggesting their possible association with the persistence of HCV infection and the resistance to IFN-α based therapy. These observations suggest the possibility of cellular or molecular targets for the treatment of chronic HCV infection.

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Alterations in microRNA expression profile in HCV-infected hepatoma cells: Involvement of miR-491 in regulation of HCV replication via the PI3 kinase/Akt pathway

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ABSTRACT

The aim of this study was to investigate the role of microRNA (miRNA) on hepatitis C virus (HCV) replication in hepatoma cells. Using miRNA array analysis, miR-192/miR-215, miR-194, miR-320, and miR-491 were identified as miRNAs whose expression levels were altered by HCV infection. Among them, miR-192/miR-215 and miR-491 were capable of enhancing replication of the HCV replicon as well as HCV itself. HCV IRES activity or cell proliferation was not increased by forced expression of miR-192/miR-215 or miR-491. Investigation of signaling pathways revealed that miR-491 specifically suppressed the phosphoinositol-3 (PI3) kinase/Akt pathway. Under inhibition of PI3 kinase by LY294002, the suppressive effect of miR-491 on HCV replication was abolished, indicating that suppression of HCV replication by miR-491 was dependent on the PI3 kinase/Akt pathway. miRNAs altered by HCV infection would then affect HCV replication, which implies a complicated mechanism for regulating HCV replication. HCV-induced miRNA may be involved in changes in cellular properties including hepatocarcinogenesis.

1. Introduction

Hepatitis C virus (HCV) is a major causative agent of liver diseases worldwide. Elimination of HCV fails in about 80% of infected patients, which leads to chronic hepatitis, liver cirrhosis, and subsequent development of hepatocellular carcinoma [1]. Combination therapy of pegylated-interferon-α and ribavirin results in sustained clearance of serum HCV-RNA in only ~50% of patients [2,3]. To improve therapeutic efficacy of the virologic response rate, drugs inhibiting the functions of HCV proteins such as NS3, NS5A, and NS5B, are currently under development. Although a number of studies have clarified the mechanisms of the effect of HCV on infected cells or the role of host factors on regulation of HCV replication, there remains much to be investigated.

MicroRNAs (miRNAs) were identified as a population of small RNAs, modulating translation by binding to sites of antisense complementarity in 3′ untranslated regions of target mRNA [4]. With respect to regulation of HCV replication, the relevance of several miRNAs has been recently reported. miR-122, a hepatocyte-specific miRNA, was identified as a positive regulatory factor for HCV replication by binding to two sites in the HCV genome [5]. Each of the interferon-β-induced miRNAs, miR-196, miR-296, miR-351, miR-431, and miR-448, has a partially complementary sequence to HCV, resulting in suppression of HCV replication [6]. Thus, a miRNA with homology to the HCV sequence is likely to have the ability to regulate HCV. Another possible mechanism of miRNA regulation of HCV replication is the targeting of some cellular gene involved in HCV replication. miR-141 was shown to suppress DLC-1 leading to efficient HCV replication [7]. Although some miRNAs were shown to be capable of regulating HCV replication, details of the relationship between miRNAs and HCV replication are still largely unknown.

In the present study, we performed miRNA array analysis to identify miRNA(s) altered by HCV infection in Huh7, a hepatoma cell line. We further investigated whether HCV-regulated miRNA could, in turn, affect HCV replication. As a result, we were able to identify five miRNAs: miR-192 and its homolog miR-215 and miR-194 as upregulated miRNAs and miR-320 and miR-491 as downregulated miRNAs. Among them, miR192/miR-215 and miR-491 enhanced HCV replication in HCV replicon cells as well as in cell culture-infectious HCV (HCVcc)-infected cells. miR-192/miR215 and miR-491 did not increase cell proliferation or HCV internal ribosome entry site (IRES) activity, suggesting that these were not the reasons for increased HCV replication. Further investigation revealed that miR-491 suppressed the PI3 kinase/Akt

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pathway suggesting that this could be responsible for augmentation of HCV replication by miR-491.

2. Materials and methods

2.1. Cells, antibodies

The hepatoma-derived cell line Huh7 was maintained in DMEM supplemented with 10% FCS. The HCV subgenomic cell line Huh-RepSI, harboring HCV-N (genotype 1b), was previously described [8]. Antibodies to phospho-ERK (Thr202/Tyr204), Akt, phospho-Akt (Ser473) were purchased from Cell Signaling Technology. An antibody to β-actin (A-5441) was from Sigma–Aldrich. A mouse monoclonal antibody to HCV core protein (C7-50) was obtained from Affinity BioReagents. A mouse monoclonal antibody to HCV NS5A (clone 388) was from Meridian Life Science, Inc. LY294002, a PI3 kinase inhibitor, was obtained from Calbiochem.

2.2. Immunoblot analysis

Total cellular protein was extracted with lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium vanadate, 50 mM NaF, and protease inhibitor cocktail (Nacalai Tesque, Japan) in phosphate-buffered saline. Protein samples were separated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad). After blocking, the membrane was probed with specific primary antibodies, followed by further incubation with a secondary antibody conjugated with horseradish peroxidase (GE Healthcare). Proteins were visualized using ECL Western blot detection reagents (GE Healthcare) and exposure to film.

2.3. miRNA transfection

Synthesized miRNAs, miR-192, miR-194, miR-215, miR-320, miR-491, and negative control miRNA were purchased from Thermo Fisher Scientific. Cells (2×10^5 per well) were seeded into 6-well plates, transfected with miRNA at a concentration of 10 nM using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instruction. After incubation for 2 days, the cells were harvested and assayed by immunoblot or real-time RT-PCR analysis.

2.4. Dual luciferase assay

We used a dicistronic plasmid, pRLHL, to investigate the effects of miRNAs on HCV IRES (Fig. 2A) [9]. Huh7 cells (1×10^6 cells in a 10-cm dish) were transfected with 10 μg of pRLHL using FuGene6 (Roche). After 24 h, the cells were seeded into 24-well plates (5×10^4 cells per well) and transfected with miRNA or negative control at a concentration of 10 nM as described above. After incubation for 2 days, cells were lysed, and assayed for HCV IRES-dependent firefly luciferase activity and cap-dependent renilla luciferase activity using the Dual Luciferase Reporter Assay System (Promega).

2.5. Cell culture-infectious HCV

HJ3-5(YH/QL) is a chimeric cell culture-infectious virus with a genome consisting of the core to NS2 sequence of genotype 1a (H77) virus placed within the background of the genotype 2a JFH1 virus, and containing compensatory mutations in E1 (Y361H) and NS3 (Q1251L) [10]. Virus stock (10^7 focus-forming units (FFU)/ml) was prepared as described previously [11].

For HCV infection, Huh7 cells (2×10^5 per well) were seeded into 6-well plates. After overnight incubation, the medium was replaced with 1 ml medium containing 4×10^5 FFU virus (the infection was carried out at an m.o.i. of ~2). After 12 h incubation, the cells were washed with PBS and re-fed with normal culture medium. At 5 days after inoculation with the virus, total RNA was obtained from the cells using Trizol (Invitrogen).

2.6. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells with RNAeasy (QIAGEN). The RNA, 1 μg, was reverse transcribed with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) in a 20 μl reaction, then 1 μl of the reaction was subjected to real-time PCR assay using TaqMan Gene Expression Assays (Applied Biosystems).

2.7. Cell proliferation assay

Cell proliferation was assessed by WST-1 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulpho-phenyl]-2H-tetrazolium, monosodium salt) assay according to the manufacturer's suggested protocol (Nacalai Tesque). Briefly, Huh7 cells (1×10^4 per well) were seeded into 96-well flat-bottom plates, transfected with synthesized miRNA or negative control as above, and cultured in DMEM containing 10% FBS. WST-1 reagent, 10 μl, was added to each well, the cells were incubated at 37 °C for 1 h, and absorbance at 450 nm was measured using a spectrophotometer.

2.8. miRNA array analysis

To screen for miRNA affected by HCV infection, we performed microarray analysis using *mir*Vana miRNA Bioarray V9.2 (Ambion), which carries genes for a total 633 kinds of miRNAs containing 471 human genes, 380 mouse genes and 238 rat genes. Using the flash PAGE system (Ambion), miRNA was purified from 22 μg total RNA extracted from HCVcc-infected cells or mock-infected cells. The purified miRNA samples from HCVcc-infected cells and mock-infected cells were labeled with Cy3 and Cy5, respectively, using mirVana miRNA Labeling kit (Ambion) and CyeDye Mono-Reactive Dye Pack (GE Healthcare Biosciences). The labeled miRNA was hybridized to the array for ~16 h at 42 °C. After hybridization, the array was washed with Low Stringency Wash (Ambion) once and High Stringency Wash (Ambion) twice. Next, the array was dried with centrifugation at 600*g* for 3 min and scanned with GenePix 4000B scanner (Axon Instruments, CA, USA). The signal data were calculated with an Array-Pro Analyzer ver. 4.5 (Media Cybernetics, Inc.). The array data were normalized by global normalization using the Microarray Data Analysis Tool (Filgen, Inc.).

3. Results

3.1. Identification of miRNAs regulated by HCV infection

Huh7 cells were infected with HCVcc at ~2 m.o.i. After incubation for 5 days, total RNA was extracted from the cells followed by purification with small RNA and miRNA array analysis. A portion of the cells was subjected to immunofluorescence analysis for staining of HCV core protein to verify that more than 90% of the cells were infected with HCV. The ratio of Cy3 intensity to Cy5 intensity was calculated and alteration of the miRNA expression profile was analyzed. A ratio of more than 1.5-fold increase/decrease was considered to be altered. To exclude miRNAs with low expression levels, those with a net intensity of Cy3 and Cy5 of more than 1000 were picked out. As a result, the miRNAs of miR-192, miR-194, miR-320, and miR-491 were identified as altered miRNAs (Table 1). miR-192 and miR-194 were up-regulated by HCV infection,

Table 1
miRNAs altered by HCV infection.

miRNA	Intensity				Sequence
	Cy3 (HCV)	Cy5 (mock)	Net	Cy3/Cy5	
miR-192	987.90	607.05	1594.95	1.63	CUGACCUAUGAAUUGACAGCC
miR-194	793.48	498.00	1291.48	1.59	UGUAACAGCAACUCCAUGUGGA
miR-215	156.21	69.39	225.60	2.25	AUGACCUAUGAAUUGACAGAC
miR-320	897.44	1401.93	2299.37	0.64	AAAAGCUGGGUUGAGAGGGCGAA
miR-491	925.38	2495.47	3420.85	0.37	AGUGGGGAACCCUUCCAUGAGGA

and miR-320 and miR-491 were down-regulated. In addition, miR-215, whose net expression while relatively low, was also studied in the subsequent investigation as an upregulated miRNA because it is considered to be a cousin of miR-192 (see their homologous sequences in Table 1). miR-215 showed a high induction level, and miR-192 and miR-215 were reported to have common induction mechanisms and target genes [12,13].

3.2. Regulation of HCV replication by miRNAs

Next, we checked whether the miRNAs were capable of regulating HCV replication. To assess this, we transfected Huh-RepSI, a HCV subgenomic replicon cell line, with synthesized miRNAs, and then monitored HCV RNA abundance and NS5A protein abundance using real-time RT-PCR and immunoblot analysis, respectively. Among the five miRNAs tested, miR-192/miR-215 and miR-491 significantly increased replicon abundance (Fig. 1A and B), while miR-194 and miR-320 did not show any significant change. HCV subgenomic replicon RNA contains the NS3 through NS5B region, which is required for genome RNA replication, but not for virus particle production. To confirm that the effect of the miRNAs was reproducible in a system equipped with the entire HCV life cycle, we used Huh7 cells infected with HCVcc. As expected, HCV abundance was upregulated by the three miRNAs in the HCVcc-infected cells similarly to HCV replicon cells (Fig. 1C and D). In addition, the HCV strain used in the experiment was a chimera of genotype 1a (H77, core to NS2) and genotype 2 (JFH-1, NS3 to NS5B) [10]. In particular, the genotype of the replication machinery of the virus (namely, NS3 to NS5B) was JFH-1. This differed from that of Huh-RepSI (HCV-N, genotype 1b) [8], which suggests that the enhancing effect of miR-192/miR-215 and miR-491 on HCV genome replication was not genotype-specific.

3.3. Effect of miRNAs on HCV IRES, cell proliferation

Since miR-192/miR-215 and miR-491 were shown to be capable of enhancing HCV replication, we next tried to elucidate how they regulate it. First, we examined whether the miRNAs can regulate HCV IRES activity. In this experiment, we transfected replicon cells with a dicistronic vector, pRLHL [9], which contained the firefly luciferase gene driven by HCV IRES and the renilla luciferase gene translated in a cap-dependent manner which was used as a control of general translational activity (Fig. 2A). After 24 h, the miRNAs were transfected, then luciferase activities induced by HCV IRES and cap translation were measured at 2 days after

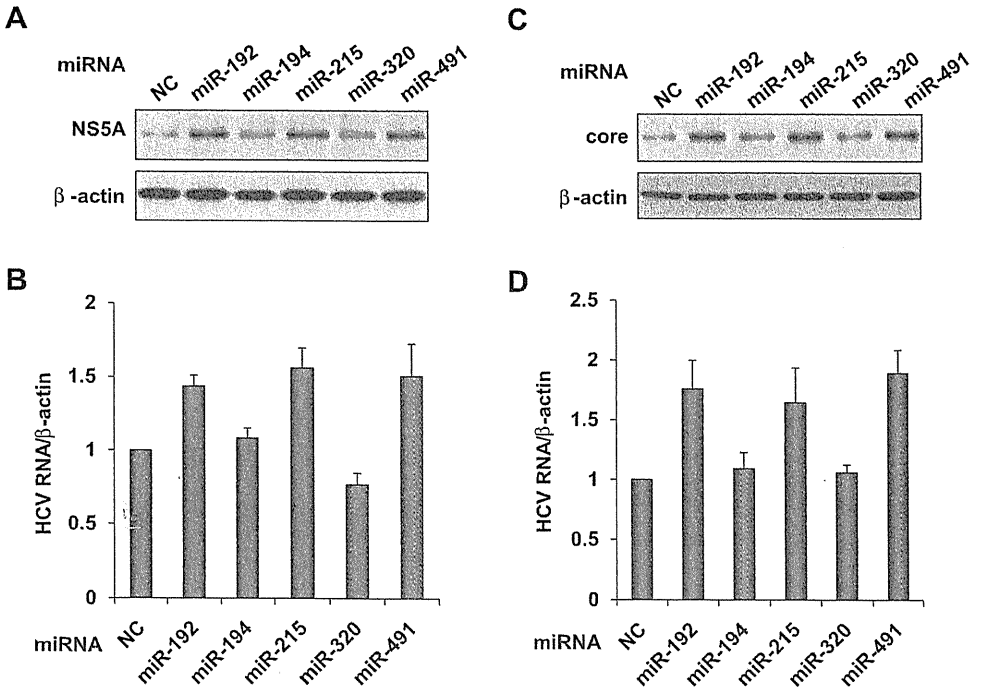

Fig. 1. Regulation of HCV replicon or HCVcc abundance by miRNAs. Cells of Huh-RepSI, a HCV subgenomic replicon, were transfected with synthesized miRNAs and assayed for NS5A protein expression (A) or HCV RNA abundance (B). HCVcc-infected Huh7 cells were transfected with synthesized miRNAs and assayed for core protein expression (C) or HCV RNA abundance (D). NC: negative control miRNA.

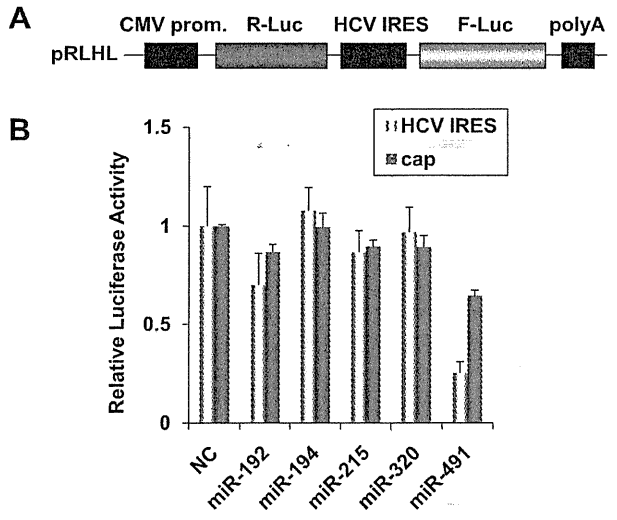

Fig. 2. Regulation of HCV IRES and cap-dependent translation by miRNAs. Huh-RepSI cells were transfected with a dicistronic vector, pRLHL (A), incubated for 24 h. The cells were seeded to 24-well plates and transfected with the miRNAs. After further incubation for 2 days, the cells were harvested and assayed for dual luciferase activity (B).

transfection (Fig. 2B). In this assay, activation of IRES was determined by the ratio of IRES-dependent luciferase activity to cap-dependent luciferase activity. Interestingly, none of the miRNAs could increase the HCV IRES activity. miR-491 suppressed cap-dependent translation and showed more suppression of HCV IRES activity. Thus, these results indicated that there was some mechanism upregulating HCV replication other than regulation of IRES activity.

Previous work demonstrated that HCV replication was affected by cell proliferation [14]. This led us to access the effects of the miRNAs on cell proliferation. Compared to negative control miRNA-transfected cells, however, none of the transfectants of the miRNAs, including those which increased HCV replication, revealed upregulation of cell proliferation, and miR-491 even suppressed it (Fig. 3). Therefore, regulation of cell proliferation was not the reason for the increase of HCV replication. The effect of miR-491 of suppressing cell growth was likely to be caused by inhibition of general translation as shown in Fig. 2B.

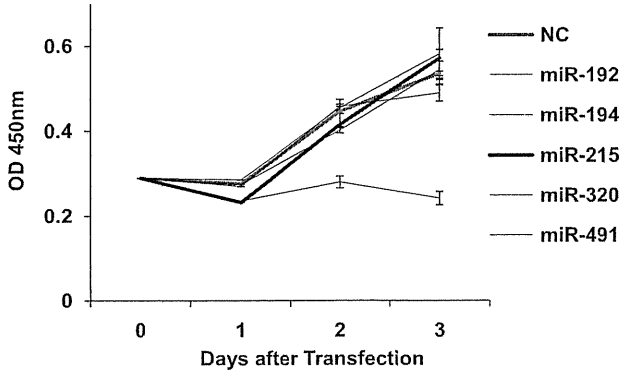

Fig. 3. Regulation of cell proliferation by miRNAs. Huh7 cells were seeded into 96-well plates, transfected with the miRNAs. At day 0, 1, 2, and 3 after transfection, the cells were subjected to WST-1 assay as described in Section 2.

3.4. Effect of miRNAs on intracellular signaling

To clarify the mechanism of the regulation of HCV replication, we next focused our investigation on intracellular signaling pathways. Previous studies have reported that HCV replication is regulated by intracellular signaling pathways, such as ERK [15], p38 [8], PI3 kinase/Akt [11], and smad [16], in addition to JAK/STAT. Since transfection of the miRNAs had no effect on the JAK/STAT signaling pathway (data not shown), we examined the phosphorylation of ERK and Akt. Because both showed a suppressing effect on HCV replication, suppression of the pathway was anticipated in cells in which HCV replication was enhanced. As shown in Fig. 4A, phosphorylation of Akt at Ser-473 was markedly suppressed in the cells transfected with miR-491, while no significant inhibition of ERK activity was observed. To further investigate the relevance of the PI3 kinase/Akt pathway to miR-491-induced upregulation of HCV replication, we used LY294002, a PI3 kinase inhibitor. When the PI3 kinase pathway was blocked by this reagent, the HCV RNA level was enhanced up to 2-fold. miR-491 transfection also resulted in an increase of HCV abundance, though the effect was less

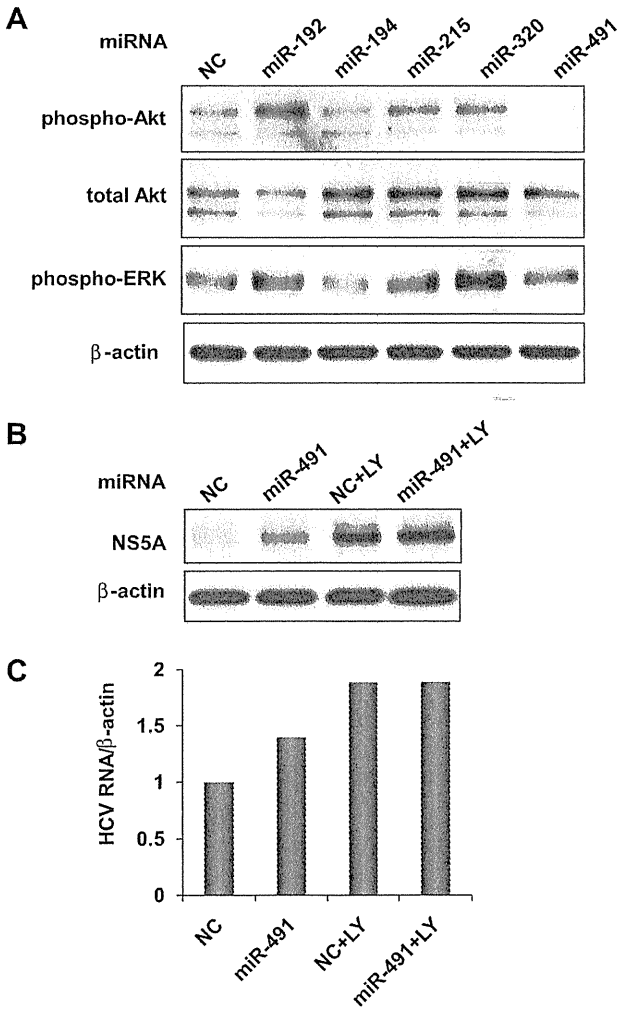

Fig. 4. Involvement of Akt suppression in miR-491-mediated upregulation of HCV replication. (A) Immunoblot analysis of miRNA-transfected HCV replicon cells using antibodies to Akt, phospho-Akt, phospho-ERK and β-actin. (B and C) HCV replicon cells were transfected with miR-491 or treated with Akt inhibitor, and assayed for NS5A protein abundance (B) or HCV RNA abundance (C). LY: LY294002.

than that of LY294002, presumably because of incomplete inhibition of Akt. When miR-491 transfected cells were cultured in the presence of LY294002, the HCV replication level was enhanced to the same extent as that in the LY294002-treated cells with negative control miRNA. Since no additive effect of miR-491 was observed under strong blockade of the PI3 kinase/Akt pathway, inhibition of this pathway was likely to be responsible for the miR-491-induced upregulation of HCV replication.

4. Discussion

In the present study, we tried to identify the miRNA(s) affected by HCV infection and establish how they influence HCV replication. Five miRNAs, miR-192, miR-194, miR-215, miR-320, and miR-491, were identified as HCV-regulated miRNAs by miRNA array analysis. Three upregulated miRNAs, miR-192, miR-194, and miR-215, were previously identified as p53-inducible miRNAs [12,13]. Two miRNA clusters which encode identical miR-194 sequences (i.e., the miR-194-2/miR-192 cluster on chromosome 11 and the miR-194-1/miR-215 cluster on chromosome 1) contain two closely related miRNAs, miR-192 and miR-215, suggesting that their expressions are regulated similarly which led to their simultaneous identification. miR-192/miR-194/miR-215 are known to act as tumor-suppressing miRNAs by inducing cell cycle arrest [12]. In Huh7 cells, however, the p53 function is believed to be abolished by a point mutation at codon 220. Therefore, the upregulation of miR-192/miR-194/miR-215 was likely to be exerted in a p53-independent manner. Since miR-192 and miR-194 are considered to be substantially expressed in human liver tissue [17] and there are several reports about the suppression of p53 function by HCV (reviewed in Ref. [18]), the result may not necessarily be the same if the investigation is conducted in human hepatocytes or in cells with intact p53 activity.

The downregulated miRNAs, miR-320 and miR-491, are considered to be relevant to carcinogenesis. miR-320 induces G1 arrest and suppresses cell proliferation by targeting CDK6 [19], CD71 [20], IGF1 [21] and induces apoptosis by suppressing Bcl-2 and Mcl-1 [22]. miR-491 is also capable of inducing apoptosis by targeting Bcl-xL [23], which is often upregulated in HCC tissues [24]. In this study, we showed that miR-491 inhibited the PI3 kinase/Akt pathway, which is one of the important pathways leading to cancerous properties. Importantly, miR-320 was identified as one of the significantly repressed miRNAs in CH-B, CH-C, and HCC compared with normal liver tissue [25]. Although the details of the relevance of miR320 and miR-491 to hepatocarcinogenesis have not yet been clarified, as these two miRNAs have a tendency to suppress genes related to carcinogenesis, their downregulation in HCV-infected cells may play some role in hepatocarcinogenesis.

Thus far, several miRNAs have been reported to regulate HCV replication. miR-122 was shown to be a direct activating factor for HCV replication [5], but alteration of this miRNA was not observed in response to HCV infection in this study. IFN-β-induced miRNAs, miR-196, miR-296, miR-351, miR-431 and miR-448, have been identified as anti-HCV miRNAs [6]. These miRNAs are able to regulate HCV replication by direct interaction with HCV genome RNA. In the case of miR-192/miR-215, there are several sites in the HCV genome sequence which show weak homology to the miRNAs (data not shown). Although the possibility of miR-192/miR-215 binding to the HCV genome and regulating replication cannot completely be excluded, this seems unlikely because the homologous sequence to miR-192/miR-215 cannot be found in the UTR region like miR-122 and direct binding to RNA usually suppresses the RNA function for protein synthesis. There is, however, a very rare case of miR-122-mediated facilitation of HCV replication by binding to two sites within the HCV genome.

Although the mechanism of miR-491-mediated suppression of the PI3 kinase pathway is not clear, it was speculated that some gene involved in Akt activation was the target of miR-491. However, the candidate of the target gene was not clearly found in the list of putative target genes of miR-491 revealed by in silico analysis. We tried to evaluate the mRNA levels of upstream genes of Akt, such as the genes which belong to the family of PI3 kinase, PTEN, growth factor receptors, using the RT-PCR method, but none of them was affected by miR-491 (data not shown). Nevertheless, investigation of target genes of miR-491 should be of interest for the field of oncology because here we have shown that miR-491 suppresses Akt, which is a factor closely related to various types of cancer via cell survival. Also, it has been demonstrated that miR-491 can induce apoptosis by ablating Bcl-xL [23]. Indeed, our observation that cell viability was significantly suppressed by forced expression of miR-491 presumably via decrease of Akt signaling suggests the anti-oncogenic feature of miR-491. Further study of the mechanism of miR-491, its target genes, and expression pattern in cancer tissue remain to be performed.

In conclusion, we showed altered expression profiles of miRNAs by HCV infection, and some of them were capable of regulating HCV replication, which may represent a complicated mechanism of HCV replication. A number of studies have demonstrated regulation of many cellular factors by miRNAs, which results in modulation of cellular functions including cell growth, apoptosis, cellular stresses, metabolism, and carcinogenesis. The miRNAs identified in this study may also be involved in changes in the phenotype of HCV-infected cells.

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