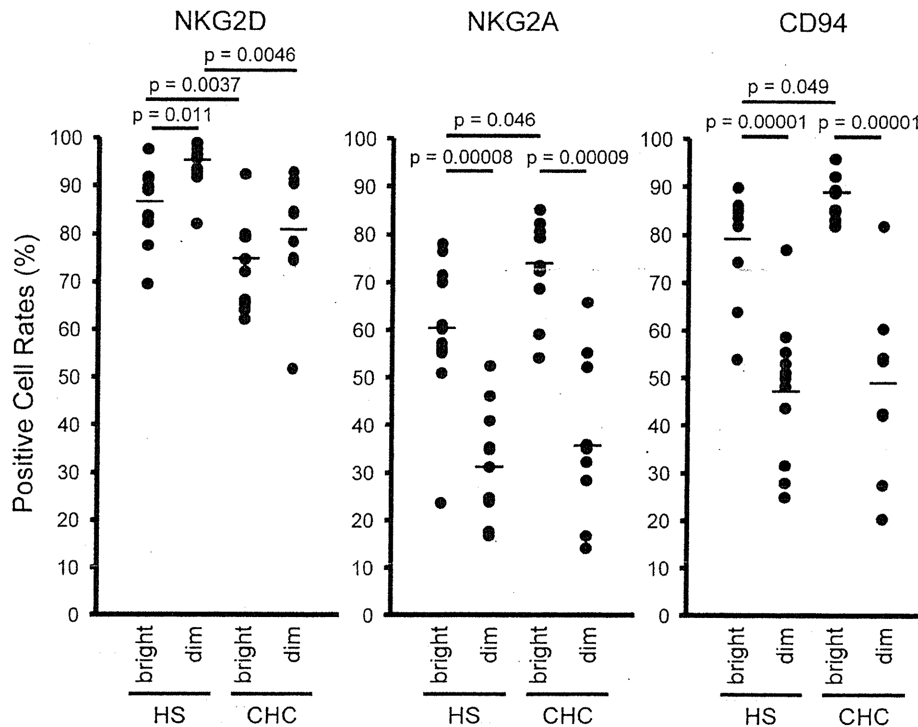


**Fig. 2** NK receptor expression on NK cell subsets. The expression of NK activating or inhibitory receptors, NKG2D or NKG2A and CD94, respectively, on CD56$^{bright}$ NK cell subset (bright) and CD56$^{dim}$ NK cell subset (dim) was evaluated by flow cytometry with isotype control staining, electronically 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-$\alpha$ 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-$\alpha$ 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-$\alpha$ in the NK cell subsets. Upon stimulation with IFN-$\alpha$, 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-$\alpha$ 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-$\alpha$-based therapy in vivo

To examine whether CD56$^{bright}$ NK cells and CD56$^{dim}$ NK cells would respond differently to IFN-$\alpha$ 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-$\alpha$-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).

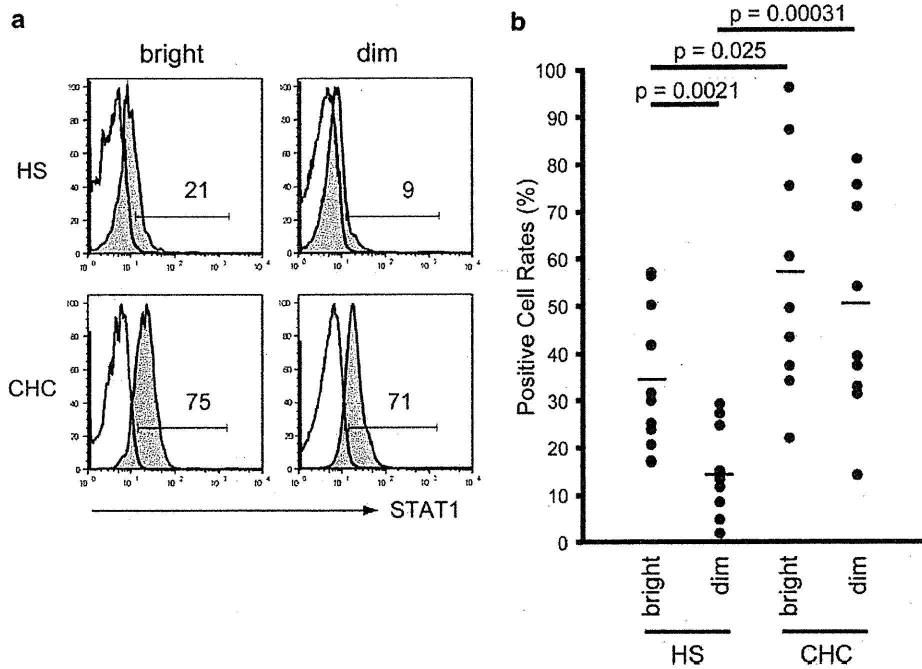


**Fig. 3** Signal transducer and activator of transcription 1 (*STAT1*) expression in NK cell subsets. Intracellular STAT1 expression levels in CD56$^{bright}$ NK cell subset (bright) and CD56$^{dim}$ NK cell subset (dim) were evaluated by flow cytometry with isotype control staining, electronically 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-$\alpha$ 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-$\alpha$ and/or IFN-$\gamma$ production, because STAT1 itself is one of the IFN-stimulated genes (ISGs) whose expression is up-regulated by IFN-$\alpha$ or IFN-$\gamma$ [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]

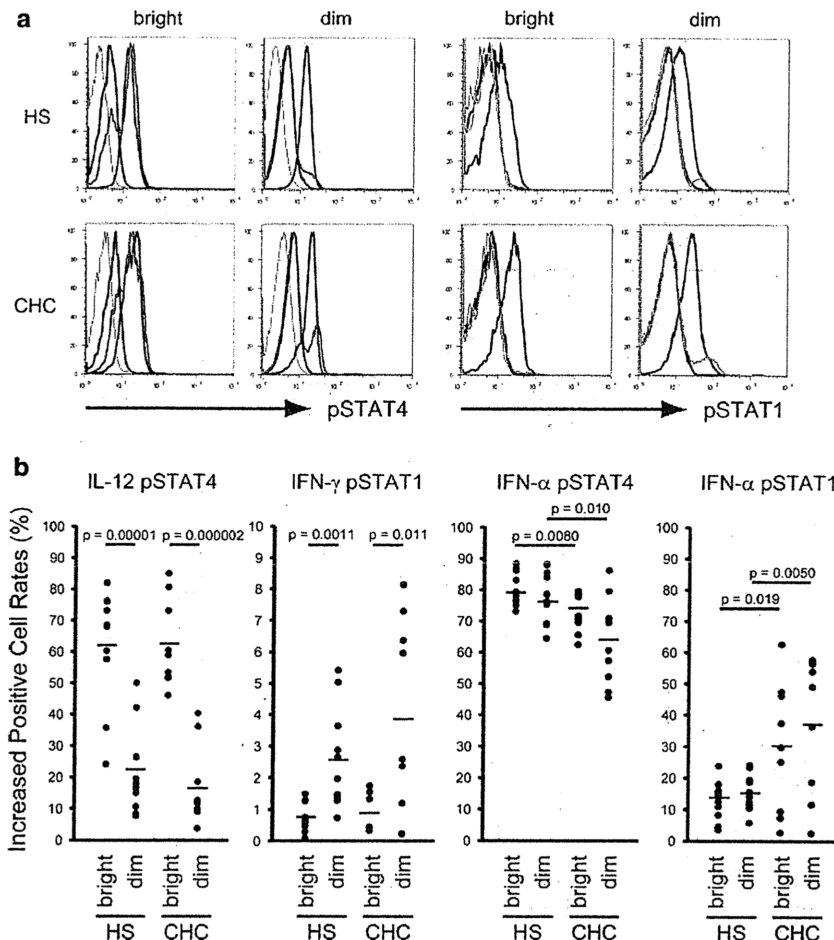


**Fig. 4** Activation of STAT1/4 occurring in response to interleukin-12 (*IL-12*), interferon-γ (*IFN-γ*), or IFN-α in NK cell subsets. Phosphorylated STAT1 (*pSTAT1*) and pSTAT4 protein levels were evaluated by flow cytometry with isotype control staining. PBMCs were derived from patients with chronic 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

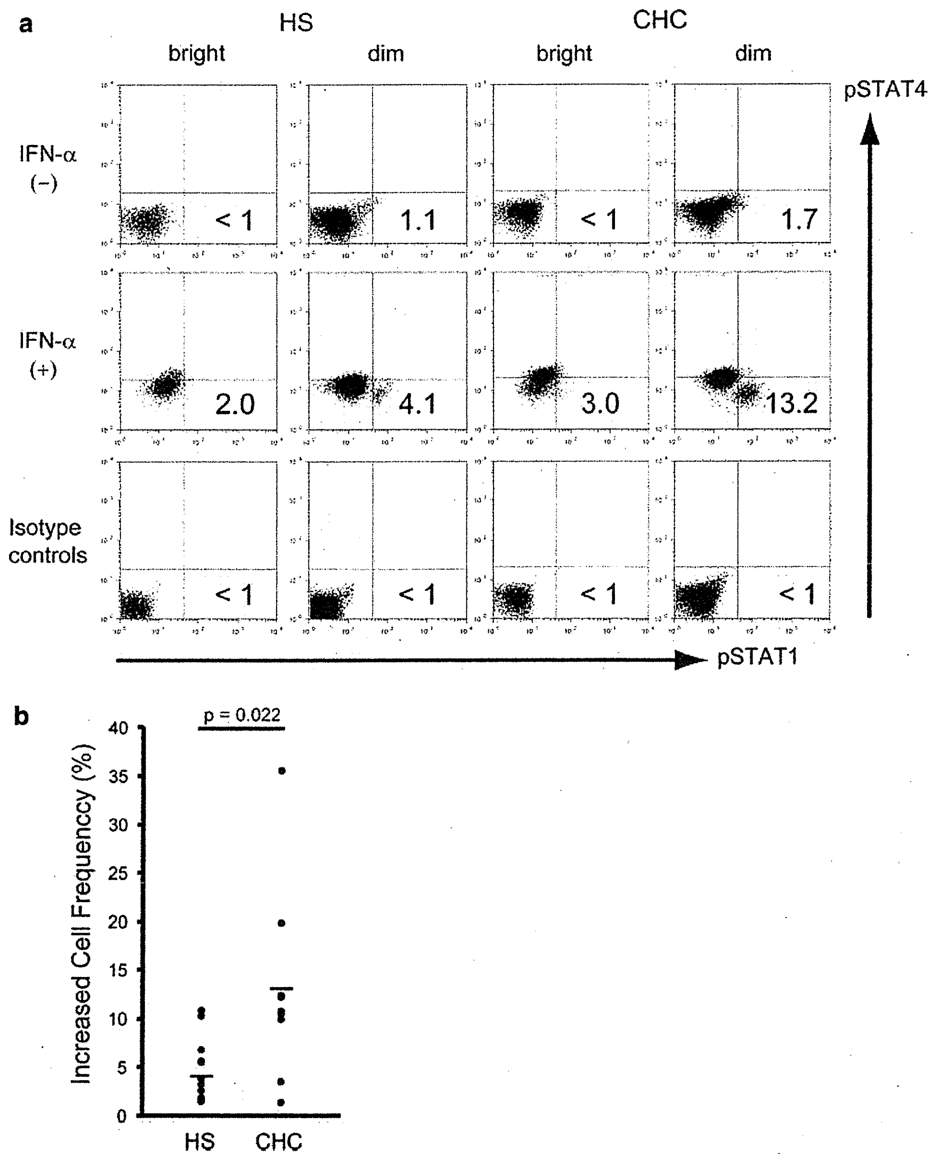


**Fig. 5** Relationship between STAT1/4 phosphorylation occurring in response to IFN-α in NK cell subsets. pSTAT1 and pSTAT4 protein levels were simultaneously evaluated by flow cytometry with isotype control staining. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy 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

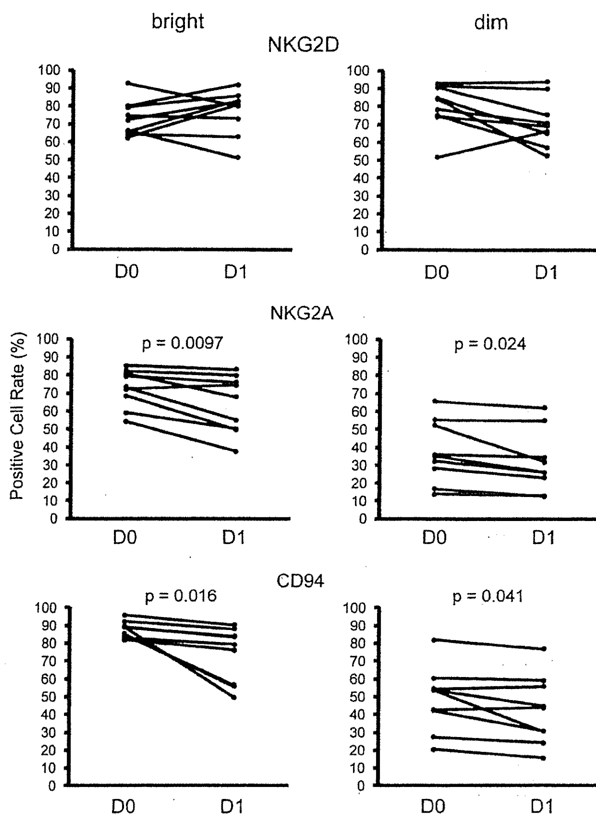


**Fig. 6** Regulation of NK receptor expression in response to IFN-α-based therapy. The expression of NK activating or inhibitory receptors, NKG2D or NKG2A and CD94, respectively, on CD56$^{bright}$ NK (bright) and CD56$^{dim}$ NK (dim) cell subsets was evaluated by flow cytometry with isotype control 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.

**Acknowledgments** This work was supported by Grants-in-aid for Scientific Research (to T. Takehara and T. Miyagi) and by a grant for the Global Centers of Excellence Program (to T. Miyagi) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-aid (to T. Takehara) from the Ministry of Health, Labour and Welfare of Japan.

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J Gastroenterol (2011) 46:944–952
DOI 10.1007/s00535-011-0403-0

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# The efficacy of extended treatment with pegylated interferon plus ribavirin in patients with HCV genotype 1 and slow virologic response in Japan

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Received: 27 December 2010 / Accepted: 23 March 2011 / Published online: 7 May 2011
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**Abstract**

*Background* Which patients with hepatitis C virus (HCV) genotype 1 can benefit from extended treatment with pegylated interferon (Peg-IFN) plus ribavirin is unknown, although the overall sustained virologic response (SVR) rate has been shown to improve in patients with a late virologic response (LVR), defined as detectable serum HCV RNA at week 12 and undetectable at week 24.

*Methods* Among 1163 chronic hepatitis C patients with genotype 1 treated with Peg-IFN plus ribavirin combination therapy, 213 patients with an LVR were examined in this study. In addition, we selected 81 patients of matched sex and age from each of the 48- and 72-week treatment groups, using the propensity score, to compare the efficacy of the two treatment durations.

*Results* With 72-week treatment, the timing of HCV RNA disappearance and the hemoglobin level at baseline

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showed a strong correlation with the SVR on multivariate analysis. Earlier HCV RNA disappearance was associated with a better SVR rate, regardless of the ribavirin dose (HCV RNA disappearance at week 16, 74%; at week 20, 52%; and at week 24, 31%, $p = 0.01$). The SVR rate with 72-week treatment was higher than that with 48-week treatment, irrespective of age, sex, or the platelet value, and, especially in aged patients (≥65 years old), the SVR rate increased markedly with 72-week treatment (48 weeks, 25% vs. 72 weeks, 56%; $p < 0.05$).
*Conclusions* An earlier response predicts a higher SVR rate in patients with an LVR given 72-week treatment. Extended treatment with Peg-IFN plus ribavirin for patients with an LVR improved the treatment efficacy, even for aged patients.

**Keywords** Chronic hepatitis C · Pegylated interferon and ribavirin combination therapy · Extended treatment · Aged patients

## Introduction

Long persistence of hepatitis C virus (HCV) infection can lead to the progression of liver fibrosis, causing liver cirrhosis and ultimately hepatocellular carcinoma (HCC) [1, 2]. Past studies have clearly shown alleviation of liver fibrosis, a reduced incidence of HCC, and markedly improved prognosis in patients in whom HCV has been successfully eradicated [3–9]. The currently recommended treatment for chronic hepatitis C is pegylated interferon (Peg-IFN) plus ribavirin therapy, which can improve antiviral efficacy for patients with chronic hepatitis C [10–16]. However, HCV still persists in approximately half of genotype 1 patients treated with Peg-IFN plus ribavirin [12–14, 16]. Accordingly, the treatment method needs to be well managed in order to maximize the virologic response.

For patients with HCV genotype 1, a high sustained virologic response (SVR) rate (73–81%) was found in patients who achieved an early virologic response (EVR), defined as undetectable serum HCV RNA at week 12. However, an SVR was attained at a low rate (14–44%) in patients with a late virologic response (LVR; defined as detectable serum HCV RNA at week 12 and undetectable at week 24), because of a high relapse rate [13, 16–24]. For the treatment strategy, drug dosages and durations of treatment can be modified by considering individual patient situations. We have reported a dose-dependent effect of ribavirin on reducing the relapse rate for patients responding to Peg-IFN plus ribavirin therapy [17, 18]. However, this effect was limited to patients with an EVR and sufficient efficacy was not observed in patients with an LVR, who should be treated not only with a high dose of ribavirin, but also for a longer duration.

For patients with an LVR, previous studies have verified that extended therapy (72-week treatment) can improve the SVR rate (38–60%) compared to standard 48-week therapy (18–36%) by reducing the relapse rate [19, 20]. However, which group of patients with an LVR can benefit from extended therapy remains obscure. In general, in order to clarify the relationship between treatment duration and anti-viral effect, a randomized control trial (RCT) should be conducted in which patients are distributed into standard and extended-therapy groups. However, it is impossible, from an ethical perspective, to conduct an RCT in Japan, because some previous studies have already revealed the usefulness of extended therapy [19–23].

In the present study, we tried to identify the factors associated with SVR in patients with an LVR infected with HCV genotype 1 who received extended treatment. Furthermore, a case-control matched study was conducted in order to compare the effectiveness of the extended treatment with that of the standard treatment of Peg-IFN plus ribavirin therapy.

## Patients and methods

### Patients

The present study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. Among 1163 chronic hepatitis C patients with genotype 1 treated with Peg-IFN plus ribavirin combination therapy between December 2004 and June 2007, 213 patients with an LVR who completed the therapy with undetectable HCV RNA at the end of the treatment were enrolled in this study. All patients were Japanese, infected with HCV genotype 1, and having a viral load of more than $10^5$ IU/ml. The patients with an LVR continued combination therapy for 48 or 72 weeks according to the decision of the investigator at the participating clinical center. The patients treated for 46–52 weeks were classified as the 48-week treatment group and those who were treated for 68–78 weeks were classified as the 72-week treatment group. The baseline characteristics of all patients before matching are summarized in Table 1. In addition, we selected 81 patients of matched sex and age, using propensity scores, from each of the 48- and 72-week treatment groups.

Patients eligible for this study were negative for hepatitis B surface antigen and anti-human immunodeficiency virus. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcoholic liver disease, autoimmune hepatitis). This study was conducted according to the ethical guidelines of the

**Table 1** Baseline characteristics of patients with LVR according to treatment duration

| Factor | 48 weeks | 72 weeks | *p* value |
|---|---|---|---|
| Number of patients | 106 | 107 | |
| Age (years) | 56.6 ± 9.1 | 60.2 ± 7.8 | 0.002 |
| Sex: male/female | 51/55 | 38/69 | 0.07 |
| Body weight (kg) | 59.9 ± 11.5 | 59.2 ± 10.3 | 0.64 |
| History of IFN treatment: naïve/experienced | 64/42 | 69/38 | 0.57 |
| White blood cells (/mm$^3$) | 4908 ± 1389 | 4893 ± 1430 | 0.91 |
| Neutrophils (/mm$^3$) | 2455 ± 936 | 2503 ± 1042 | 0.91 |
| Red blood cells ($\times 10^4$/mm$^3$) | 438 ± 49 | 439 ± 38 | 0.48 |
| Hemoglobin (g/dl) | 13.9 ± 1.5 | 13.9 ± 1.4 | 0.76 |
| Platelets ($\times 10^4$/mm$^3$) | 17.0 ± 5.8 | 16.2 ± 5.7 | 0.21 |
| AST (IU/l) | 56 ± 34 | 56 ± 34 | 0.74 |
| ALT (IU/l) | 70 ± 50 | 68 ± 56 | 0.78 |
| Serum HCV RNA (KIU/ml)[a] | 1850 | 2400 | 0.03 |
| Histology (METAVIR)[b] | | | |
| Fibrosis, 0–2/3–4 | 75/7 | 62/17 | 0.03 |
| Activity, 0–1/2–3 | 49/33 | 45/34 | 0.75 |
| Peg-IFN dose (μg/kg/week)[c] | 1.47 ± 0.17 | 1.48 ± 0.7 | 0.21 |
| Ribavirin dose (mg/kg/day)[c] | 11.3 ± 1.7 | 11.5 ± 1.5 | 0.22 |
| HCV RNA negativity: 16/20/24 weeks[d] | 65/23/12 | 51/32/14 | 0.23 |

*LVR* late virologic response, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *IFN* interferon, *HCV* hepatitis C virus

[a] Data shown are median values

[b] 52 missing

[c] Initial dose

[d] The times of HCV RNA negativity were unknown in 6 patients with 48-week treatment and 10 patients with 72-week treatment

Declaration of Helsinki amended in 2008, and informed consent was obtained from each patient.

### Treatment

All patients received Peg-IFN alfa-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (Rebetol; Schering-Plough) for the duration of the study of 48 or 72 weeks. Peg-IFN alfa-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg), according to a standard treatment protocol for Japanese patients.

### Dose reduction

Dose modification followed, as a rule, the manufacturer's drug information according to the intensity of the hematologic adverse effects. The dose of Peg-IFN alfa-2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to <1500/mm$^3$, the neutrophil count declined to <750/mm$^3$ or the platelet (Plt) count declined to <8 × 10$^4$/mm$^3$, and the agent was discontinued if the WBC count declined to <1000/mm$^3$, the neutrophil count declined to <500/mm$^3$, or the Plt count declined to <5 × 10$^4$/mm$^3$. Ribavirin was also reduced, from 1000 mg to 600 mg, or from 800 mg to 600 mg, or from 600 mg to 400 mg, if the hemoglobin (Hb) level decreased to <10 g/dl, and it was discontinued if the Hb level decreased to <8.5 g/dl. Both Peg-IFN alfa-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. During this therapy, no iron supplements or hematopoetic growth factors, such as erythropoietin alfa or granulocyte–macrophage colony stimulating factor, were administered.

### Virologic assessment and definition of virologic response

The serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analyzed using the

COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/ml). LVR was defined as detectable serum HCV RNA at treatment week 12 and undetectable at treatment week 24; SVR was defined as the absence of detectable serum HCV RNA at 24 weeks after the the end of the treatment, and relapse was defined as the absence of detectable serum HCV RNA at the end of the treatment but detectable serum HCV RNA at 24 weeks after the end of the treatment.

### Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients were expressed as means ± standard deviation or median values. To analyze the relationship between baseline data and SVR, univariate analysis using the Mann–Whitney *U*-test or the $\chi^2$test, and multivariate analysis using logistic regression analysis were performed. The significance of trends in values was determined with the Mantel–Haenszel $\chi^2$test. A two-tailed $p$ value of <0.05 was considered significant. Statistical analysis was conducted with SPSS version 15.0J (SPSS, Chicago, IL, USA).

## Results

### Baseline characteristics and efficacy of treatment in patients with LVR according to treatment duration

Table 1 shows the baseline characteristics of the patients with LVR stratified according to treatment duration before matching. The patients given 72-week treatment were significantly older ($p = 0.002$), had higher HCV-RNA ($p = 0.03$), and included many with advanced liver fibrosis (METAVIR fibrosis score 3 or 4) ($p = 0.03$). Those with 72-week treatment tended to include many female patients compared to the patients given 48-week treatment ($p = 0.07$). Drug reductions due to side effects occurred with a higher frequency in the 72-week treatment group than in the 48-week treatment group; Peg-IFN, 48-weeks, 40% (42/106) versus 72-weeks, 55% (59/107); ribavirin, 48-weeks, 53% (56/106) versus 63% (67/107). However, the main reasons for reductions of both drugs were almost the same; Peg-IFN, 48-weeks, neutropenia ($n = 23$), thrombocytopenia ($n = 14$); 72-weeks, neutropenia ($n = 24$), thrombocytopenia ($n = 19$), general fatigue ($n = 4$); and ribavirin, 48-weeks, anemia ($n = 47$), general fatigue ($n = 3$); 72-weeks, anemia ($n = 51$), general fatigue ($n = 4$). The SVR rate with 72-week treatment was significantly higher than that with 48-week treatment (59%, 63/107 vs. 37%, 39/106, $p = 0.002$), due to less relapse after treatment.

### Factors associated with SVR for patients with LVR treated for 72 weeks

The baseline factors, including the timing of the HCV RNA disappearance, were assessed for association with SVR by univariate and multivariate logistic regression analyses in the 107 patients with 72-week treatment. Univariate analysis showed that factors significantly associated with SVR were age, sex, red blood cell count, Hb, and the timing of HCV RNA disappearance (Table 2A). The factors selected as significant by univariate analysis were evaluated by multivariate logistic regression analysis. The timing of HCV RNA disappearance and Hb at baseline were independent factors for SVR ($p = 0.002$, $p = 0.002$, respectively) (Table 2B).

### Baseline characteristics of matched patients with LVR

In order to reduce the selection bias among the LVR patients with 48- and 72-week treatment, a matched case–control study was performed; 81 patients were selected from each of the two treatment duration groups, by matching sex and age, using propensity scores. Baseline characteristics were about the same for the two groups, except for the red blood cell count and the progression stage of liver fibrosis (Table 3). In terms of age and sex, the mean age of the male patients was 57.2 ± 8.3 years in the 48-week treatment group and 58.5 ± 8.2 years in the 72-week treatment group, and the mean ages of the female patients were 59.9 ± 7.6 and 60.0 ± 8.5 years, respectively. The male–female ratio of patients more than 65 years old was similar for the two treatment duration groups (male/female, 8/16; 48-week treatment, 10/17; 72-week treatment). Those less than 65 years old were of the same proportion (54%, male/female, 26/31; 48-week treatment, 25/29; 72-week treatment).

### SVR rate among patients with LVR in relation to the factors at baseline and treatment duration

We analyzed the association between the SVR rate and baseline characteristics using the matched population. The SVR rate with 72-week treatment was significantly higher than that with 48-week treatment regardless of age (<65 years, 72 weeks, 63%, 34/54 vs. 48 weeks, 39%, 22/57, $p = 0.01$; ≥65 years, 72 weeks, 56%, 15/27 vs. 48 weeks, 25%, 6/24, $p < 0.05$) (Fig. 1a). For males, the SVR rate with 72-week treatment was 77% (27/35), which was significantly higher than that with 48-week treatment (38%, 13/34, $p = 0.001$). For females, the SVR rate with 72-week treatment tended to be higher than that with 48-week treatment (72 weeks, 48%, 22/46 vs. 48 weeks, 32%, 15/47, $p = 0.14$) (Fig. 1b). Among female patients

**Table 2** Factors associated with SVR among patients with 72-week treatment before matching

| Factor | SVR | Relapser | $p$ value |
|---|---|---|---|
| A. Univariate analysis | | | |
| Number of patients | 63 | 44 | |
| Age (years) | 58.8 ± 8.0 | 62.3 ± 7.2 | 0.02 |
| Sex: male/female | 28/35 | 10/34 | 0.03 |
| Body weight (kg) | 60.0 ± 10.0 | 58.2 ± 11.1 | 0.19 |
| History of IFN treatment: naïve/experienced | 38/25 | 31/13 | 0.31 |
| White blood cells (/mm$^3$) | 5021 ± 1474 | 4709 ± 1361 | 0.22 |
| Neutrophils (/mm$^3$) | 2621 ± 1046 | 2343 ± 1026 | 0.15 |
| Red blood cells ($\times 10^4$/mm$^3$) | 448 ± 39 | 426 ± 32 | 0.005 |
| Hemoglobin (g/dl) | 14.3 ± 1.3 | 13.3 ± 1.2 | 0.001 |
| Platelets ($\times 10^4$/mm$^3$) | 15.8 ± 5.3 | 16.7 ± 6.3 | 0.63 |
| AST (IU/l) | 56 ± 36 | 54 ± 32 | 0.68 |
| ALT (IU/l) | 71 ± 62 | 64 ± 45 | 0.33 |
| Serum HCV RNA (KIU/ml)[a] | 2400 | 2500 | 0.88 |
| Histology (METAVIR)[b] | | | |
| Fibrosis, 0–2/3–4 | 34/9 | 28/8 | 1.00 |
| Activity, 0–1/2–3 | 25/18 | 20/16 | 0.82 |
| Peg-IFN dose (μg/kg/week)[c] | 1.29 ± 0.30 | 1.28 ± 0.32 | 0.80 |
| Ribavirin dose (mg/kg/day)[c] | 9.7 ± 1.8 | 9.4 ± 2.1 | 0.57 |
| HCV RNA negativity: 16/20/24 weeks[d] | 39/15/4 | 12/17/10 | 0.001 |

| Factor | Category | Odds ratio | 95% CI | $p$ value |
|---|---|---|---|---|
| B. Multivariate analysis | | | | |
| Age | 1 year old | – | – | NS |
| Sex | male/female | – | – | NS |
| Red blood cells | $1 \times 10^4$/mm$^3$ | – | – | NS |
| Hemoglobin | 1 g/dl | 2.030 | 1.289–3.197 | 0.002 |
| HCV RNA negativity | 16/20/24 weeks | 0.751 | 0.633–0.890 | 0.001 |

*SVR* sustained virologic response, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *CI* confidence interval, *NS* not significant

[a] Data shown are median values

[b] 23 missing

[c] Mean doses throughout the treatment

[d] The times of HCV RNA negativity were unknown in 5 patients with 48-week treatment and 5 patients with 72-week treatment

more than 65 years old, the SVR rate with 72-week treatment increased with marginal significance (72 weeks, 53%, 9/17 vs. 48 weeks, 19%, 3/16, $p = 0.07$).

The SVR rate in patients with no to moderate fibrosis (METAVIR fibrosis score 0–2) was 58% (26/45) among patients with 72-week treatment, and this rate was significantly higher than that among patients with 48-week treatment (35%, 19/55) ($p = 0.03$). On the other hand, for patients with more advanced liver fibrosis (METAVIR fibrosis score 3 or 4), the SVR rate was 54% (7/13) among the patients with 72-week treatment and 33% (1/3) among those with 48-week treatment; the difference was not significant due to the small number of subjects. However, the SVR rate among the patients with a lower Plt value ($<12 \times 10^4$/mm$^3$ at baseline), which is indicative of advanced fibrosis, was significantly higher among the patients given 72-week treatment (61%, 14/23) than that among those given 48-week treatment (24%, 4/17) ($p = 0.03$) (Fig. 1c).

### SVR rate among patients with LVR in relation to the timing of HCV disappearance and treatment duration

We analyzed the association of the SVR rate with the timing of HCV RNA disappearance. The SVR rate among the patients with 72-week treatment was 74% (32/43) in patients with undetectable HCV RNA at week 16, 52% (13/25) at week 20, and 31% (4/13) at week 24, and the rates were higher than those among the patients with 48-week

**Table 3** Baseline characteristics of matched patients with LVR

| Factor | 48 weeks | 72 weeks | $p$ value |
|---|---|---|---|
| Number of patients | 81 | 81 | |
| Age (years) | 58.8 ± 8.0 | 59.4 ± 8.4 | 0.52 |
| Sex: male/female | 34/47 | 35/46 | 1.00 |
| Body weight (kg) | 58.9 ± 11.7 | 60.1 ± 11.0 | 0.46 |
| History of IFN treatment: naïve/experienced | 50/31 | 48/33 | 0.87 |
| White blood cells (/mm$^3$) | 4717 ± 1286 | 5020 ± 1516 | 0.19 |
| Neutrophils (/mm$^3$) | 2332 ± 926 | 2611 ± 1133 | 0.13 |
| Red blood cells (×10$^4$/mm$^3$) | 433 ± 44 | 445 ± 35 | 0.03 |
| Hemoglobin (g/dl) | 13.8 ± 1.3 | 14.1 ± 1.3 | 0.13 |
| Platelets (×10$^4$/mm$^3$) | 16.2 ± 5.3 | 16.2 ± 5.9 | 0.64 |
| AST (IU/l) | 56 ± 35 | 51 ± 27 | 0.63 |
| ALT (IU/l) | 68 ± 52 | 61 ± 37 | 0.88 |
| Serum HCV RNA (KIU/ml)[a] | 1900 | 2400 | 0.10 |
| Histology (METAVIR)[b] | | | |
| Fibrosis, 0–2/3–4 | 55/5 | 45/13 | 0.04 |
| Activity, 0–1/2–3 | 37/23 | 41/17 | 0.34 |
| Peg-IFN dose (μg/kg/week)[c] | 1.47 ± 0.19 | 1.48 ± 0.17 | 0.31 |
| Ribavirin dose (mg/kg/day)[c] | 11.4 ± 1.9 | 11.5 ± 1.5 | 0.57 |
| HCV RNA negativity: 16/20/24 weeks | 52/18/11 | 43/25/13 | 0.34 |

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase

[a] Data shown are median values

[b] 44 missing

[c] Initial dose

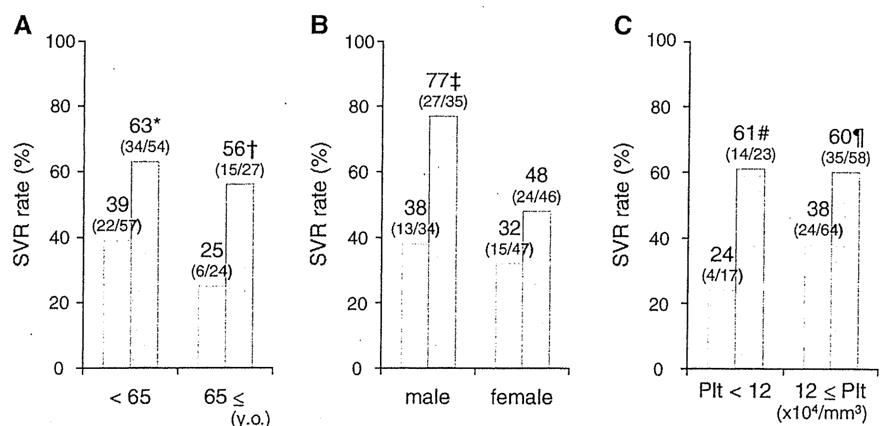


**Fig. 1** Sustained virologic response (*SVR*) rate according to baseline characteristics and treatment duration. **a** SVR rate according to age. **b** SVR rate according to sex. **c** SVR rate according to platelet counts. *Light gray shade bars* indicate 48-week treatment. *Dark gray shade bars* indicate 72-week treatment. *y.o.* Years old, *Plt* platelets. *$p = 0.014$, †$p = 0.045$, ‡$p = 0.001$, #$p = 0.027$, ¶$p = 0.018$ compared to 48-week treatment

treatment (48, 11, and 9%, respectively) (Fig. 2). Regardless of the timing of the HCV disappearance, the SVR rate was raised among the patients with 72-week treatment, and the timing of the HCV RNA disappearance showed a strong correlation with SVR among the patients with 72-week treatment ($p = 0.01$). We also assessed the association of the SVR rate according to ribavirin adherence and the timing of HCV RNA disappearance in LVR patients with each treatment duration (Table 4). Ribavirin adherence was distributed in two categories by mean value (ribavirin throughout the treatment, 9.5 mg/kg/day). Among the patients with 48-week treatment, the SVR rates of patients with higher doses of ribavirin (more than 9.5 mg/kg/day) was slightly higher than that of patients with lower doses of ribavirin (less than 9.5 mg/kg/day) in each of categories of timing of HCV RNA disappearance, but the difference was not significant. However, among the patients given less than 9.5 mg/kg/day, the SVR rate increased significantly in patients with 72-week treatment, compared with 48-week treatment, in patients with

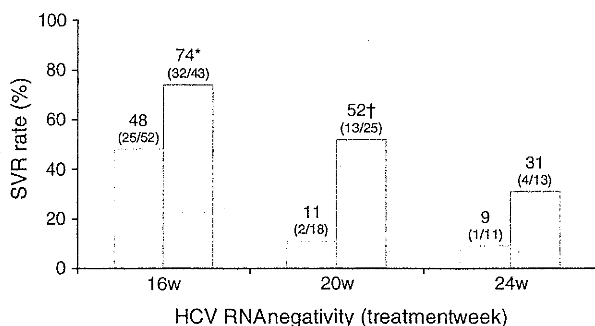


**Fig. 2** SVR rate according to timing of hepatitis C virus (*HCV*) RNA negativity. *Light gray shade bars* indicate 48-week treatment. *Dark gray shade bars* indicate 72-week treatment. $^{*}p = 0.012$, $^{\dagger}p = 0.009$, compared to 48-week treatment

**Table 4** SVR rate according to timing of HCV RNA negativity and ribavirin adherence

| | The timing of HCV RNA disappearance | | |
|---|---|---|---|
| | 16 weeks | 20 weeks | 24 weeks |
| Ribavirin <9.5 mg/kg/day | | | |
| 48-week treatment | 43% (9/21) | 0% (0/7) | 0% (0/6) |
| 72-week treatment | 75% (15/20) | 47% (7/15) | 25% (1/4) |
| *p* value | <0.05 | 0.05 | 0.40 |
| Ribavirin ≥9.5 mg/kg/day | | | |
| 48-week treatment | 52% (16/31) | 18% (2/11) | 20% (1/5) |
| 72-week treatment | 74% (17/23) | 60% (6/10) | 33% (3/9) |
| *p* value | 0.10 | 0.08 | 1.00 |

undetectable HCV RNA at week 16 (72 weeks, 75% vs. 48 weeks, 43%, $p < 0.05$), and increased with marginal significance in patients with undetectable HCV RNA at week 20 (72 weeks, 47% vs. 48 weeks, 0%, $p = 0.05$). Among the patients with undetectable HCV RNA at week 24, a significant difference was not observed because of the small number of patients in this category. For the patients given more than 9.5 mg/kg/day, the SVR rate with 72-week treatment tended to be higher than that with 48-week treatment, although the patient number was too small to reveal a benefit of extended treatment. As indicated above, the efficacy in patients with LVR given lower doses of ribavirin (less than 9.5 mg/kg/day) could be improved not by an increase in ribavirin dosage, but only by a longer treatment duration, irrespective of the category of timing of HCV RNA disappearance.

## Discussion

In order to raise the SVR in patients with HCV genotype 1 treated with Peg-IFN plus ribavirin combination therapy, two strategies are possible: one is the use of a higher dose of drugs and the other is a longer duration of therapy. With respect to drug dose, we have reported that Peg-IFN is dose-dependently correlated with EVR, and ribavirin is dose-dependently correlated with relapse in patients with an EVR [17, 18]. On the other hand, among patients with an LVR, maintaining a high dose of ribavirin (>12 mg/kg/day average dose ) did not lead to sufficient reduction of the relapse rate [18]. Thus, the SVR rate in patients with an LVR cannot be improved by a dose-increase strategy, and another treatment strategy, a longer duration of therapy, needs to be devised for patients with LVR in order to reduce the relapse rate.

Past studies have reported that extended therapy reduced the relapse rate. However, more consideration is needed to determine which group of patients can attain the desired effect by extended therapy. Eradication of serum HCV RNA is difficult in female or aged patients or patients with advanced liver fibrosis or a lower Plt count [17, 25], and these patients are considered to be mostly those with an LVR. Previously, we reported that patients more than 65 years old with an LVR showed a low SVR rate [25]. Therefore, in the present study, we tried to identify the group of patients for whom the SVR rate could be improved by extended therapy.

The factors associated with SVR in patients with extended therapy were evaluated by univariate and multivariate logistic regression analyses in the present study. As a result, the timing of HCV RNA disappearance was found to be a significant factor affecting SVR. This suggests that the earlier HCV RNA disappeared, the greater the SVR rate for 72-week treatment as well as 48-week treatment. Examination of the impact of ribavirin exposure on the SVR rate in patients with an LVR showed that, even if a high dose of ribavirin were given, the SVR rate did not show a significant increase among the patients with 48-week treatment, as previously reported [18]. However, the present study showed that an increase in the SVR rate was attained among the patients with 72-week treatment in each category of the timing of HCV RNA disappearance, especially in patients with lower doses of ribavirin. A similar result was found on stratified analysis for the timing of HCV RNA disappearance and Peg-IFN adherence (data not shown). That finding indicated that extend treatment is an effective strategy for LVR patients to increase the SVR rates, although the drug doses of Peg-IFN and ribavirin have been reported to affect the SVR rates in patients with an EVR. And the better efficacy of extended treatment was revealed to be limited to only those patients with earlier HCV RNA disappearance; they are good candidates for extended therapy. Further study is needed to determine whether more extended therapy; for example, 96-week treatment, would be effective for patients with later HCV RNA disappearance.

In the group with extended therapy in the present study, the Hb level at baseline was also significantly associated with SVR. We examined the relationship between Hb level at baseline and age and sex. The mean Hb levels at baseline according to age and sex were highest among male patients less than 65 years old (mean Hb, g/dl, male less than 65 years old; 15.2 ± 1.3, male more than 65 years old; 14.5 ± 0.9, female less than 65 years old; 13.5 ± 1.0, female more than 65 years old; 13.4 ± 1.0). The factors of age and sex were not selected as significant by multivariate analysis, but the Hb level at baseline did affect the SVR rate according to age and sex. In fact, among the patients with 72-week treatment in this study, the SVR rate among male patients less than 65 years old tended to be higher (84%, 21/25) than that of male patients more than 65 years old (60%, 6/10, $p = 0.19$), female patients less than 65 years old (45%, 13/29, $p < 0.01$), and female patients more than 65 years old (53%, 9/17, $p < 0.05$).

In this study, stratified analysis according to baseline factors revealed that extended therapy significantly improved the anti-viral effect, irrespective of age, sex, and Plt value. Especially, 48 weeks of standard treatment was insufficient for an anti-viral effect in aged or female patients, while extended therapy could significantly raise the SVR rate. It is of special clinical significance that extended therapy was found to be beneficial for aged patients, many of whom show an LVR. While the efficacy of extended therapy for patients with advanced liver fibrosis could not be proven in this study, it is conceivable that extended therapy could significantly raise the SVR rate in patients with a lower Plt value, which is indicative of advanced fibrosis. Further study is needed to clarify the efficacy of extended therapy for patients with advanced liver fibrosis.

The main limitation of this study is that it was not designed for randomization, and the treatment duration for patients with an LVR was decided by their physicians. Therefore, older female patients with more advanced liver fibrosis, for whom a poor treatment outcome was expected, tended to be treated for a longer period (72 weeks). However, considering the usefulness of extended therapy for patients with LVR reported in studies from the United States and Europe, there was an ethical issue against conducting an RCT in Japan which would have distributed the patients with an LVR into standard or extended therapy groups. Accordingly, we conducted a case-control study matched for age and sex, in order to compare the efficacy of 72-week treatment with that of 48-week treatment. Because it is known to be difficult to treat aged and female patients with HCV genotype 1 [25], these two factors of age and sex were chosen for minimal matching. As a result, the proportion of patients with advanced liver fibrosis (METAVIR fibrosis score 3 or 4) was not compensated for, and the selected patients in the 72-week treatment group included more patients with advanced liver fibrosis (who are difficult to treat) than the selected patients in the 48-week treatment group. Nevertheless, a higher SVR rate was obtained in the 72-week treatment group in comparison with 48-week treatment.

Recently, genetic polymorphism near the IL28B gene has been reported to be associated with the anti-viral effect of Peg-IFN plus ribavirin combination therapy [26–28]. Single-nucleotide polymorphisms (SNPs) of the IL28B gene are related to on-treatment response (rapid virologic response [RVR], EVR) and SVR [29]. However, no significant difference was observed for relapse after treatment between the major and minor types of IL28B SNPs, if HCV RNA disappeared at the same timing of the treatment [29]. Therefore, the same result as that in the present study may have been attained if the factors of IL28B SNPs had been included as evaluable factors. Further study is needed to examine the issue of the involvement of IL28B SNPs in the efficacy of 72-week Peg-IFN plus ribavirin therapy in patients with LVR.

In conclusion, our results have demonstrated that extended therapy for patients with LVR infected with HCV genotype 1 improved the SVR rate in all categories of patients, even for aged patients with an LVR. The timing of HCV RNA disappearance in patients with an LVR was a predictive factor for SVR and this suggests that response-guided therapy may be needed for later responders.

**Acknowledgments** Other institutions and participants in the Osaka Liver Forum are: Osaka General Medical Center, A Inoue; Higashiosaka City Central Hospital, S Iio; Itami City Hospital, Y Saji; Toyonaka Municipal Hospital, M Inada; Yao Municipal Hospital, H Fukui; Otemae Hospital, Y Doi; Osaka Medical Center for Cancer and Cardiovascular Diseases, K Katayama; Ashiya Municipal Hospital, T Kitada; National Hospital Organization Minami Wakayama Medical Center, K Fujimoto; Nishinomiya Municipal Central Hospital, H Ogawa; Saiseikai Senri Hospital, K Suzuki; Izumiotsu Municipal Hospital, S Yamagata; Osaka Kaisei Hospital, N Imaizumi; Kano General Hospital, S Kubota; Saso Hospital, M Nishiuchi; and Meiwa Hospital, Y Hayakawa. This work was supported by a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labour and Welfare of Japan, and Scientific Research from the Ministry of Education, Science, and Culture of Japan. All authors have no financial relationships relevant to this study.

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J Gastroenterol (2011) 46:1031–1037
DOI 10.1007/s00535-011-0409-7

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Efficacy of re-treatment with pegylated interferon plus ribavirin combination therapy for patients with chronic hepatitis C in Japan

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Received: 27 December 2010 / Accepted: 31 March 2011 / Published online: 3 May 2011
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## Abstract

*Background* It is still not known which patients with chronic hepatitis C who failed to respond to previous pegylated interferon (Peg-IFN) plus ribavirin therapy can benefit from re-treatment.

*Methods* Seventy-four patients (HCV genotype 1, $n = 56$, genotype 2, $n = 18$) were re-treated with Peg-IFN plus ribavirin.

*Results* On re-treatment, the sustained virologic response (SVR) rate was 41% for genotype 1 and 56% for genotype 2. With genotype 1, the factors associated with an SVR were previous treatment response and the serum hepatitis C virus (HCV) RNA level at the start of re-treatment. Patients with a ≥2-log decrease in HCV RNA at week 12 (partial early virologic response, p-EVR) in previous treatment had significantly higher SVR rates than those without these

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decreases ($p < 0.001$); no patient without a p-EVR in the previous treatment attained an SVR with re-treatment (0/16). All patients with <5 $\log_{10}$ IU/ml of HCV RNA at the start of re-treatment attained an SVR (6/6), while only 33% (15/45) of those patients with ≥5 $\log_{10}$ IU/ml of HCV RNA attained an SVR ($p < 0.01$). Among the patients with relapse in the previous treatment, those who attained an SVR on re-treatment required a longer duration of re-treatment than the duration of the previous treatment (re-treatment, 63.8 ± 13.0 weeks vs. previous treatment, 53.9 ± 13.5 weeks, $p = 0.01$).

*Conclusions* Re-treatment of genotype 1 patients should be limited to patients with a p-EVR in the previous treatment and a low HCV RNA level at the start of re-treatment. In re-treatment with Peg-IFN plus ribavirin, longer treatment duration can contribute to increasing the anti-viral effect.

**Keywords** Chronic hepatitis C · Pegylated interferon and ribavirin combination therapy · Re-treatment

## Introduction

Pegylated interferon (Peg-IFN) plus ribavirin combination therapy can improve anti-viral efficacy and is currently recommended as first-line therapy for chronic hepatitis C. However, hepatitis C virus (HCV) still persists in approximately half of the genotype 1 patients treated with Peg-IFN plus ribavirin [1–4], and the number of patients who fail to achieve a sustained virologic response (SVR) consequently increases over time.

Recently, the addition of a protease inhibitor to Peg-IFN plus ribavirin combination therapy has been reported to improve the anti-viral effect, but this triple therapy increases side effects, especially severe anemia [5–7]. In Japan, HCV carriers are 10–20 years older than those in the United States and European countries, and patients who are ineligible for triple therapy exist in large numbers due to their potential tendency of having anemia. On the other hand, re-treatment with Peg-IFN plus ribavirin is a possible choice, until triple therapy becomes commercially available, for patients who have failed to show an SVR to previous anti-viral therapy, and for patients who are ineligible for triple therapy. As for re-treatment with Peg-IFN plus ribavirin, there have been only a few studies of patients who failed to show an SVR to previous Peg-IFN plus ribavirin [8–11]. Although re-treatment with Peg-IFN plus ribavirin for patients who failed to respond to previous Peg-IFN plus ribavirin is not recommended in the practice guidelines of the American Association for the Study of the Liver (AASLD) [1], there are some patients who respond to re-treatment. However, it remains obscure in which patients eradication of HCV can be successfully attained by re-treatment with Peg-IFN plus ribavirin.

In the present study, we tried to determine which patients could benefit from re-treatment and to identify the factors associated with an SVR in re-treatment.

## Patients and methods

### Patients

The present study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. This study was conducted with 74 chronic hepatitis C patients (genotype 1, $n = 56$, genotype 2, $n = 18$) who had previously completed Peg-IFN α-2b plus ribavirin combination therapy but had failed to attain an SVR. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcoholic liver disease, autoimmune hepatitis), or coinfection with hepatitis B or anti-human immunodeficiency virus. This study was conducted according to the ethical guidelines of the Declaration of Helsinki amended in 2008, and informed consent was obtained from each patient.

### Treatment

For the previous treatment, Peg-IFN α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (Rebetol; Schering-Plough) was started between December 2004 and January 2008. For re-treatment with Peg-IFN plus ribavirin, Peg-IFN α-2a (Pegasys; Roche, Basel, Switzerland) plus ribavirin (Copegus; Roche) or Peg-IFN α-2b plus ribavirin was started between February 2006 and January 2009. In principle, as a starting dose, Peg-IFN was given once weekly at a dose of 180 μg of Peg-IFN α-2a and 1.5 μg/kg of Peg-IFN α-2b, and ribavirin was given at a total dose of 600–1000 mg/day based on body weight (for genotype 1, body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg; for genotype 2, body weight <60 kg, 600 mg; >60 kg, 800 mg), according to a standard treatment protocol for Japanese patients.

### Dose reduction and discontinuance

Dose modification followed, as a rule, the manufacturer's drug information on the intensity of the hematologic adverse effects. The Peg-IFN α-2a and α-2b doses were reduced to 50% of the assigned dose when the neutrophil count fell below 750/$mm^3$ or the platelet (Plt) count fell below $8 \times 10^4/mm^3$, and the agent was discontinued when the neutrophil count fell below 500/$mm^3$ or the Plt count fell below $5 \times 10^4/mm^3$. Ribavirin was also reduced from 1000 to 600, 800 to 600, or 600 to 400 mg when the

hemoglobin (Hb) was below 10 g/dl, and was discontinued when the Hb was below 8.5 g/dl. Both Peg-IFN and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. No iron supplement or hematopoietic growth factors, such as epoietin alpha or granulocyte–macrophage colony stimulating factor (G-CSF), were administered.

### Virologic assessment and definition of virologic response

The serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analyzed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/ml). A rapid virologic response (RVR) was defined as undetectable serum HCV RNA level at week 4, a partial early virologic response (p-EVR) was defined as more than a 2-log decrease in HCV RNA level at week 12 compared with the baseline, a complete EVR (c-EVR) was defined as undetectable serum HCV RNA at week 12, a late virologic response (LVR) was defined as detectable serum HCV RNA at week 12 and undetectable at week 24, and an SVR was defined as undetectable serum HCV RNA at 24 weeks after the end of the treatment. Relapse was defined as undetectable serum HCV RNA at the end of the treatment but a detectable amount after the end of the treatment. For both the previous treatment and this re-treatment, patients without a p-EVR or without clearance of HCV RNA at week 24 were considered to be showing non-response (NR) and had to stop treatment. A patient who attained HCV RNA negativity during the re-treatment continued to be treated for 48 or 72 weeks according to response-guided therapy and the decision of the investigator at the participating clinical center.

### Statistical analysis

Baseline data of the patients are expressed as mean ± SD or median values. In order to analyze the differences between baseline data or the factors associated with SVR, univariate analysis using the Mann–Whitney *U*-test or the $\chi^2$ test was performed. A two-tailed $p$ value of $<0.05$ was considered significant. The analysis was conducted with SPSS version 15.0J (SPSS, Chicago, IL, USA).

## Results

The baseline characteristics of the patients are summarized in Table 1. Of the 56 genotype 1 patients, 32 were relapsers and 24 showed NR to previous treatment. Among the relapsers, 15 had shown a c-EVR (58%, 15/26) and 29 a p-EVR (100%, 29/29) in the previous treatment. Of the 18 genotype 2 patients, 17 were relapsers and one had shown NR to the previous treatment. Among the relapsers, 5 had shown an RVR (42%, 5/12) in the previous treatment. In the previous treatment, all patients had received Peg-IFN α-2b plus RBV combination therapy. There were no significant differences among the baseline characteristics between the previous treatment and the re-treatment in

**Table 1** Baseline characteristics of patients and treatment factors in previous treatment and re-treatment

| | Genotype 1 | | | | | | Genotype 2 | |
|---|---|---|---|---|---|---|---|---|
| | All patients | | Previous treatment relapsers | | Previous treatment non-responders | | All patients | |
| Number of patients | 56 | | 32 | | 24 | | 18 | |
| Sex: male/female | 32/24 | | 19/13 | | 13/11 | | 11/7 | |
| | Previous treatment | Re-treatment | Previous treatment | Re-treatment | Previous treatment | Re-treatment | Previous treatment | Re-treatment |
| Age (years) | 57.6 ± 9.2 | 59.5 ± 9.4 | 57.8 ± 9.0 | 59.8 ± 9.4 | 57.3 ± 9.6 | 59.0 ± 9.5 | 57.4 ± 9.0 | 58.4 ± 1.7 |
| White blood cells (/mm$^3$) | 4909 ± 1404 | 4670 ± 1566 | 5117 ± 1276 | 4756 ± 979 | 4633 ± 1543 | 4545 ± 2178 | 5111 ± 1697 | 4412 ± 1744 |
| Red blood cells (×10$^4$/mm$^3$) | 435 ± 40 | 426 ± 52 | 444 ± 34 | 437 ± 36 | 4243 ± 46 | 412 ± 67 | 448 ± 36 | 447 ± 38 |
| Hemoglobin (g/dl) | 13.9 ± 1.2 | 13.5 ± 1.7 | 14.1 ± 1.1 | 13.8 ± 1.3 | 13.7 ± 1.3 | 13.1 ± 2.1 | 14.4 ± 1.2 | 14.2 ± 1.3 |
| Platelets (×10$^4$/mm$^3$) | 16.5 ± 6.1 | 17.5 ± 6.9 | 18.4 ± 6.6 | 19.1 ± 6.5 | 14.1 ± 4.4 | 15.2 ± 6.9 | 17.5 ± 6.3 | 16.2 ± 4.9 |
| AST (IU/l) | 58 ± 30 | 60 ± 45 | 55 ± 31 | 56 ± 44 | 61 ± 28 | 64 ± 47 | 52 ± 34 | 34 ± 13 |
| ALT (IU/l) | 74 ± 55 | 77 ± 74 | 73 ± 65 | 79 ± 80 | 74 ± 40 | 75 ± 66 | 65 ± 52 | 34 ± 18 |
| Serum HCV RNA (KIU/ml) | 1600 | 1100 | 1600 | 1100 | 1600 | 990 | 1300 | 690 |
| Peg-IFN type: α2a/α2b | 0/56 | 24/32 | 0/32 | 14/18 | 0/24 | 10/14 | 0/18 | 4/14 |

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *HCV* hepatitis C virus, *Peg-IFN* pegylated interferon

**Table 2** Factors associated with a sustained virologic response (SVR) in re-treatment with Peg-IFN plus ribavirin

| Factor | SVR | Non-SVR | $p$ value |
|---|---|---|---|
| Number of patients | 23 | 33 | |
| Age (years) | 59.5 ± 7.6 | 59.5 ± 10.5 | 0.55 |
| Sex: male/female | 16/7 | 16/17 | 0.17 |
| White blood cells (/mm$^3$) | 4778 ± 1022 | 4589 ± 1884 | 0.29 |
| Neutrophils (/mm$^3$) | 2446 ± 849 | 2291 ± 1486 | 0.21 |
| Hemoglobin (g/dl) | 13.6 ± 1.3 | 13.4 ± 1.9 | 0.73 |
| Platelets (×10$^4$/mm$^3$) | 18.2 ± 6.3 | 16.9 ± 7.3 | 0.28 |
| AST (IU/l) | 52 ± 33 | 65 ± 52 | 0.46 |
| ALT (IU/l) | 75 ± 61 | 79 ± 82 | 0.72 |
| Serum HCV RNA: <5log/5log≤ | 6/15 | 0/31 | <0.01 |
| Peg-IFN type: α2a/α2b | 7/16 | 17/16 | 0.27 |
| Peg-IFN dose (μg/kg/week) | | | |
| α2a | 2.64 ± 0.61 | 2.73 ± 0.72 | 0.90 |
| α2b | 1.18 ± 0.43 | 1.19 ± 0.34 | 0.90 |
| Ribavirin dose (mg/kg/day) | 8.6 ± 2.9 | 9.4 ± 2.7 | 0.28 |
| 1st treatment virologic response | | | |
| p-EVR; +/− | 22/0 | 14/16 | <0.001 |
| Relapse/NR | 20/3 | 12/21 | <0.001 |

*p-EVR* partial early virologic response, *NR* non-response

terms of peripheral blood cell counts, or the levels of aminotransaminases and serum HCV RNA at the start of treatment.

In genotype 1 patients, the HCV RNA negative rate on re-treatment was 54% (29/54) at week 12 and 71% (40/56) at week 24, and the SVR rate was 41% (23/56). The factors associated with SVR were assessed by univariate analysis for the following variables; age, gender, peripheral blood cell counts, aminotransferases, previous treatment response, serum HCV RNA level, the type of Peg-IFN in re-treatment, and drug adherence (Table 2). As a result, the factors of previous treatment response and serum HCV RNA level at the start of re-treatment were selected as being significant. In examining the efficacy of the re-treatment according to the previous treatment response, the relapsers in the previous treatment had a significantly higher HCV RNA negative rate at weeks 12 and 24 and a significantly higher SVR rate than those with NR in the previous treatment (Fig. 1a). Patients with a p-EVR in the previous treatment showed similar results, while no patient without p-EVR in the previous treatment attained an SVR on re-treatment (0/16) (Fig. 1b). Even among the patients without HCV RNA negativity in the previous treatment, if p-EVR had been attained in the previous treatment, 43% (3/7) of these patients attained an SVR on re-treatment. As for the serum HCV RNA level at the start of re-treatment, all patients with less than 5 $\log_{10}$ IU/ml of HCV RNA attained an SVR (6/6), and 33% (15/45) of those patients with more than 5 $\log_{10}$ IU/ml of HCV RNA attained an SVR ($p < 0.01$).

In examining the efficacy of re-treatment according to treatment duration, among the patients with c-EVR and without RVR on re-treatment, those who were re-treated for 72 weeks tended to attain higher SVR rates than those who were re-treated for 48 weeks (72 weeks, 75%, 9/12, vs. 48 weeks, 25%, 2/8, $p = 0.06$). On the other hand, 43% (3/7) of the patients with an LVR on re-treatment attained an SVR on re-treatment. Among the patients with relapse

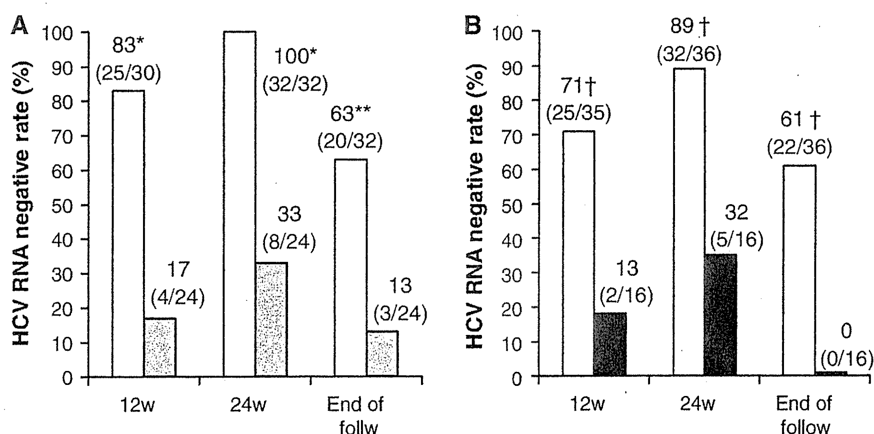


**Fig. 1** Virologic response on re-treatment according to previous treatment response. **a** Hepatitis C virus (HCV) RNA negative rate on re-treatment according to relapse or non-response in previous treatment. **b** HCV RNA negative rate on re-treatment according to partial early virologic response (p-EVR) or non-p-EVR in previous treatment. *White bars* patients with relapse in previous treatment. *Dark gray bars* patients with non-response in previous treatment. *Light gray bars* patients with p-EVR in previous treatment. *Black bars* patients with non-p-EVR in previous treatment. *$p < 0.0001$; **$p < 0.01$; compared to non-response. †$p < 0.001$; compared to patients without p-EVR