Table 3 Cox proportional hazards regression for recurrence

Variant	Univariate HR (95 % CI)	*p* value	Multivariable HR (95 % CI)	*p* value
Sex: female	0.763 (0.446–1.308)	0.326		
Age: ≥70 years	1.111 (0.671–1.840)	0.683		
Pre-MDSC ratio: ≥22 %	1.210 (0.698–2.096)	0.497		
Pre-neutrophil	0.990 (0.969–1.012)	0.385		
Pre-lymphocyte	1.014 (0.987–1.043)	0.311		
Pre-neutrophil/lymphocyte	0.978 (0.787–1.216)	0.844		
Pre-ALT	1.001 (0993–1.008)	0.882		
Pre-serum albumin: <3.5 mg/dl	1.143 (0.665–1.982)	0.647		
Pre-prothrombin time: <70 %	1.662 (0.961–2.903)	0.073	1.881 (0.522–6.777)	0.101
Post-MDSC ratio: ≥22 %	2.795 (1.150–6.792)	0.023	3.906 (1.313–11.616)	0.014
Post-neutrophil	1.005 (0.975–1.035)	0.762		
Post-lymphocyte	0.993 (0.960–1.027)	0.678		
Post-neutrophil/lymphocyte	1.003 (0.810–1.242)	0.980		
Post-ALT	0.995 (0.981–1.010)	0.501		
Type IV collagen 7S	1.122 (0.992–1.268)	0.067	1.192 (0.907–1.566)	0.207
AFP: ≥100 ng/ml	1.357 (0.743–2.480)	0.321		
Tumor size: ≥20 mm	1.29 (0.78–2.12)	0.328		
Tumor multiplicity: multiple	2.00 (1.18–3.40)	0.010	1.851 (0.721–4.753)	0.201

HR hazard ratio, *CI* confidence interval, *ALT* alanine aminotransferase, *AFP* alpha-fetoprotein

Another important finding of our study is that the frequency of MDSCs showed various changes after curative RFA and this frequency is an independent risk factor of HCC recurrence. In most of the patients, the frequency of MDSCs decreased after RFA. A similar phenomenon has also been reported in other cancer treatments [19, 21, 36]. Liu et al. [21] reported that MDSCs were decreased in non-small cell lung cancer patients who had clinical benefit from chemotherapy or who received curative surgery. These results suggest that a decrease in the frequency of MDSCs is due to tumor eradication.

It is well known that tumor factors including multiplicity, tumor diameter, serum levels of tumor marker, and hepatic reserve are risk factors of HCC recurrence after RFA [43, 44], but it has not been reported that the frequency of circulating MDSCs is also a risk factor. From our findings, there was a clear inverse correlation between the frequency of MDSCs after RFA and recurrence-free survival. Consistent with our results, in the patients with pancreatic, esophageal, and gastric cancer, Gabitass et al. [23] reported that an increase in MDSCs was associated with an increased risk of death and that the frequency of MDSCs was an independent prognostic factor for patient survival. Taken together with these findings, our results suggest that the frequency of MDSCs might be one of the prognostic factors of patients after cancer treatments.

As we showed, the frequency of MDSCs is primarily correlated with tumor progression. However, between the patients with high and low frequency of MDSCs after RFA, there was no significant difference in hepatic reserve and tumor factors before treatment. Although an incomplete HCC eradication at a microscopic level may allow a high frequency of MDSCs after RFA, there may be other mechanisms such as subsequently tumor-specific immune responses after RFA. In addition, there is a limitation of the present study because we used cryopreserved PBMCs for phenotypic analysis of MDSCs. Further studies using fresh PBMCs are needed for precise phenotypic analysis of MDSCs and elucidation of the mechanism to regulate the frequency of MDSCs after HCC treatment.

In conclusion, the frequency of MDSCs in HCC patients is correlated with tumor progression, and the frequency after RFA is inversely correlated with the prognosis of HCC patients. HCC patients who show a high frequency of MDSCs after RFA should be closely followed, and the inhibition or elimination of MDSCs after HCC treatments may improve the prognosis of HCC patients.

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Conflict of interest The authors declare no conflict of interest.

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ORIGINAL ARTICLE

In vivo immunological antitumor effect of OK-432-stimulated dendritic cell transfer after radiofrequency ablation

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Abstract Radiofrequency ablation therapy (RFA) is a radical treatment for liver cancers and induces tumor antigen-specific immune responses. In the present study, we examined the antitumor effects of focal OK-432-stimulated dendritic cell (DC) transfer combined with RFA and analyzed the functional mechanisms involved using a murine model. C57BL/6 mice were injected subcutaneously with colon cancer cells (MC38) in their bilateral flanks. After the establishment of tumors, the subcutaneous tumor on one flank was treated using RFA, and then OK-432-stimulated DCs were injected locally. The antitumor effect of the treatment was evaluated by measuring the size of the tumor on the opposite flank, and the immunological responses were assessed using tumor-infiltrating lymphocytes, splenocytes and draining lymph nodes. Tumor growth was strongly inhibited in mice that exhibited efficient DC migration after RFA and OK-432-stimulated DC transfer, as compared to mice treated with RFA alone or treatment involving immature DC transfer. We also demonstrated that the antitumor effect of this treatment depended on both CD8-positive and CD4-positive cells. On the basis of our findings, we believe that combination therapy for metastatic liver cancer consisting of OK-432-stimulated DCs in combination with RFA can proceed to clinical trials, and it is anticipated to be markedly superior to RFA single therapy.

Keywords Metastatic liver cancer · MC38 · Immunotherapy · Intratumoral injection · Tumor-infiltrating lymphocyte

Abbreviations

RFA	Radiofrequency ablation
DC	Dendritic cell
HCC	Hepatocellular carcinoma
TAE	Transcatheter hepatic arterial embolization
TLR	Toll-like receptor
GFP	Green fluorescent protein
ELISPOT	Enzyme-linked immunospot
Treg	Regulatory T cell
MDSC	Myeloid-derived suppressor cell
IFN-γ	Interferon-γ

Introduction

Liver is one of the most common organs to which various cancers spread from their site of origin. In some types of cancer, the liver metastasis lesion is a target of surgical treatment. For instance, surgical resection of hepatic metastasis achieves longer median survival in colorectal and breast cancer patients [1, 2]. However, even if the hepatic lesions are surgically treated, the prognosis of the

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patients is not satisfactory. As for colorectal cancers, the recurrence rate is over 50 % after radical resection of metastatic lesions [3]. Moreover, at the time of initial diagnosis, only a few patients meet the criteria for hepatic resection because of unresectability, low hepatic functional reserve or poor performance status [4].

Radiofrequency ablation therapy (RFA) has been developed as a radical and minimally invasive treatment method for metastatic liver cancers. Recently, RFA has been used as an adjunct to hepatic resection or as an alternative method to resection when surgical treatment is not feasible [5]. Additionally, it has been revealed that RFA for metastatic liver cancers generates tumor antigen-specific T-cell responses in man [6, 7]. We have previously reported that RFA could also control distant tumor growth in a murine hepatocellular carcinoma (HCC) model [8].

Dendritic cells (DCs) are potent antigen-presenting cells [9]. Recently, we have established new treatments using local DC injection with transcatheter hepatic arterial embolization (TAE) and have shown that this combination therapy could induce tumor antigen-specific T-cell responses in HCC patients [10].

OK-432 is derived from the Su strain of Group A *Streptococcus pyogenes* by means of treatment with benzylpenicillin and heat [11]. OK-432 can stimulate DCs via Toll-like receptor (TLR) 3, TLR4 and β2 integrin and subsequently induce antigen-specific cytotoxic lymphocytes [12–14].

On the basis of these results, we hypothesized that OK-432-stimulated DC transfer is a promising candidate for an enhancer that can strongly increase the antitumor effect of RFA. We have previously demonstrated in a clinical trial that the local infusion of OK-432-stimulated DC after TAE could prolong recurrence-free survival in HCC patients [15]. However, it remains unknown as to how the transferred DCs work in combination with RFA. In the present study, we examined the antitumor effects of OK-432-stimulated DCs when combined with RFA and analyzed the functional mechanisms involved using a murine subcutaneous colon cancer model.

Materials and methods

Animals

Wild-type 8–12-week-old female C57BL/6 J mice were obtained from Charles River Japan (Yokohama, Japan). Female C57BL/6-Tg (UBC-GFP) 30Scha/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were approved and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University, which strictly conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Cell lines and bone marrow-derived dendritic cells

A murine colorectal cancer cell line, MC38 and hybridomas, clone GK1.5 and clone 2.43 were cultured in RPMI-1640 containing 10 % fetal bovine serum (Life Technologies, Co., Carlsbad, CA, USA) supplemented with 100 μg/ml streptomycin and 100 units/ml penicillin (Wako Pure Chemical Industries Ltd., Osaka, Japan). Bone marrow-derived dendritic cells (BMDCs) were generated using 20 ng/ml of recombinant granulocyte macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, USA) as previously described [16]. OK-432 (Picibanil; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) was loaded into the supernatant from days 6–7 of the BMDC generation period at a concentration of 5 μg/ml.

In vitro evaluation of phagocytic activity by dendritic cells

MC38 cells were labeled with DiD dye (Life Technologies) according to the manufacturer's instructions followed by heat treatment at 80 °C for 90 s. OK-432-stimulated or immature DCs were co-incubated with the treated MC38 cells for 3 h at a ratio of 1:1. After incubation, the cell suspensions were observed using a fluorescence microscope (BZ9000: Keyence, Osaka, Japan) and analyzed by means of FACSCalibur (BD Immuno-Cytometry System, San Jose, CA, USA).

Animal model

Bilateral flanks of C57BL/6 mice were each injected subcutaneously with 1×10^6 MC38 cells. Seven days after injection, after they had grown to 5–6 mm in diameter, the subcutaneous tumors on one flank were treated using RFA, and 1×10^7 immature DCs or 1×10^7 OK-432-stimulated DCs were injected into the treated tumors at 24 h after RFA. After this, the volume of the untreated tumor on the contralateral flank was evaluated over a period of 10 days. Tumor volumes were calculated using the following formula: tumor volume (mm^3) = (longest diameter) × (shortest diameter)^2/2.

Radiofrequency ablation

Mice bearing tumors were anesthetized with an intraperitoneal injection of pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan), and the skin on the tumor was cut. Subsequently, an expandable RFA needle was inserted into the tumor, which was treated using a radiofrequency generator (RITA

500PA; RITA Medical Systems, Inc., Fremont, CA, USA). During the use of this system, the intratumor temperature was maintained at 70–90 °C, and the current was turned off when the tumor exhibited heat denaturation.

Flow cytometry

The DCs were detected by means of staining with anti-CD11c antibodies (Life technologies). The lymphocytes in the draining lymph node were stained with anti-CD4 antibodies, anti-CD8 antibodies, anti-CD11c antibodies and anti-CD69 antibodies (BD Bioscience, San Diego, CA, USA). The splenocytes were stained with anti-CD4 antibodies, anti-CD8 antibodies, CD11c antibodies, anti-NK1.1 antibodies, CD45 antibodies (BD Bioscience), anti-Gr-1 antibodies, and anti-CD11b antibodies and mouse regulatory T-cell staining solution (BioLegend, San Diego, CA, USA). The stained samples were analyzed using FACSAria II (BD Immuno-Cytometry System).

Immunohistochemical assay

The draining lymph nodes and the observed tumors were embedded in Sakura Tissue-Tek optimum cutting temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) for frozen sectioning. Tissue sections were fixed at −20 °C in methanol for 10 min. The draining lymph nodes were stained using rabbit anti-GFP antibody (Abcam, Cambridge, UK) that were detected using an EnVision+/HRP kit (Dako, Glostrup, Denmark). The observed tumors were stained with anti-CD4 and anti-CD8a (BD Bioscience), which were detected using the Nichirei Histofine Simple Stain Mouse Max PO (Rat) system (Nichirei Co., Tokyo, Japan) or the Vectastain ABC kit (Vector Laboratory, Inc., Burlingame, CA, USA).

Interferon gamma enzyme-linked immunospot assay

The splenocytes, the tumor-infiltrating lymphocytes (TILs) in the untreated tumors that were isolated by mechanical homogenizations and density gradient centrifugations, and the lymphocytes in the draining lymph nodes were loaded into the interferon gamma enzyme-linked immunospot assay to estimate the tumor-specific immune reactions, as previously described [8, 17]. Briefly, 3×10^5 lymphocytes or 1×10^5 TILs were incubated for 24 h with or without 6×10^5 MC38 lysates, which were prepared through five cycles of rapid freezing in liquid nitrogen, thawing at 55 °C and centrifugation. The number of MC38-specific IFN-γ spots was determined by subtracting the number of spots incubated without MC38 lysates from the number of spots incubated with MC38 lysates. For CD4 or CD8 depletion, we used magnetic CD4 beads or CD8 beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

In vivo CD4/CD8 depletion

For in vivo CD4 or CD8 depletion, B6 mice were injected intraperitoneally with 200 μg of purified monoclonal antibodies specific to CD4 or CD8 at 1 day before and 3 days after RFA treatment; the monoclonal antibodies were prepared from GK1.5 hybridoma and 2.43 hybridoma, respectively [18]. The depletion was confirmed by flow cytometry using peripheral blood lymphocytes stained with anti-CD4 and anti-CD8 antibodies.

Statistical analysis

The data obtained were analyzed statistically using the t test or one-way analysis of variance followed by Tukey's multiple-comparison test. A P value <0.05 was considered as being statistically significant.

Results

Migration efficacy and phagocytic ability of OK-432-stimulated DCs

We employed OK-432 as a modifying agent for DCs, because we have previously shown in clinical studies that OK-432 prolonged recurrence-free survival after combination therapy involving DC injection with TAE for HCC patients [10, 15]. We first confirmed that the OK-432-stimulated murine DCs showed higher expression of maturation markers such as CD40, CD80, CD86, MHC class II and CCR7 (Supplementary Fig. 1), as previously reported [19, 20].

To evaluate their phagocytic abilities, we incubated the immature DCs and the OK-432-stimulated DCs with MC38 tumor cells. Heat-treated MC38 cells were taken up well by both immature DCs and OK-432-stimulated DCs, as compared to nontreated MC38 cells (Fig. 1a–c). In addition, the phagocytic ability of OK-432-stimulated DCs was not inferior to that of immature DCs. These results were consistent with the dextran uptake assay (Supplementary Fig. 2) and our previous data on human monocyte-derived DCs [15]. Since heat-treated MC38 cells were thought to be in a similar condition to those in the MC38 tumor in mice treated with RFA, OK-432-stimulated DCs were expected to effectively phagocytose RFA-treated MC38 tumor cells in vivo.

We next estimated the kinetics of the transferred DCs in mice bearing subcutaneous MC38 tumors treated with RFA. Immature DCs or OK-432-stimulated DCs that were derived from GFP-Tg mice were injected intratumorally

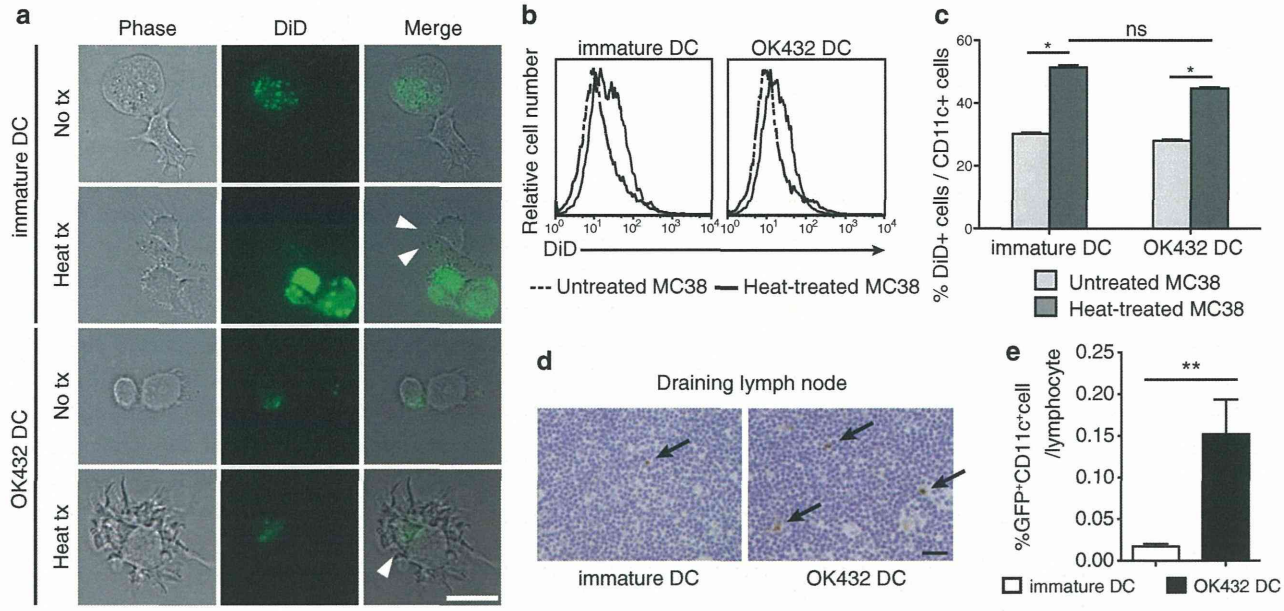

Fig. 1 Effects of OK-432 on murine bone marrow-derived DCs. **a** OK-432-stimulated DCs or immature DCs were co-incubated for 3 h with MC38 cells untreated or treated at 80 °C for 90 s after staining with DiD dye. After incubation, DC and MC38 cells were observed using a fluorescence microscope. *Arrowheads* indicate MC38 derivatives being phagocytosed by DCs. No tx, untreated MC38 cells; heat tx, heat-treated MC38 cells; *bar*, 20 μm. **b**, **c** Co-incubated MC38 cells and DCs were stained with anti-CD11c antibodies and analyzed using flow cytometry. The *histograms* show the DiD fluorescent intensity of the CD11c-positive fractions. The percentages of DiD^+ $CD11c^+$ cells in the $CD11c^+$ cell population are also shown in a *column graph*. The experiments were performed five times, and representative results are shown. Data are presented as the mean ± SE. $*P < 0.05$. **d** The migration abilities of the DCs after intratumoral transfer were evaluated. The draining lymph nodes were harvested at 3 days after RFA followed by the DC transfer. Frozen sections were prepared and stained with anti-GFP antibodies. *Arrows* indicate the GFP-positive cells in the lymph nodes. *Bar* 20 μm. **e** The draining lymph nodes were also analyzed using flow cytometry after staining with anti-CD11c antibodies. Data were obtained from six mice in each group. Percentages of GFP^+ $CD11c^+$ cell are presented as the mean ± SE. $**P < 0.01$

at 24 h after RFA treatment, and the subcutaneous tumors and the lymph nodes were harvested at 3 days after RFA. According to the immunohistochemical study involving the detection of GFP, the inguinal lymph node on the RFA-treated flank was thought to be the draining lymph node (Supplementary Fig. 3). Additionally, the number of transferred DCs in the draining lymph nodes was significantly higher in the mice treated with the OK-432-stimulated DCs than in those treated with the immature DCs (Fig. 1d, e). Our experimental results attested to the fact that the OK-432-stimulated DCs had both sufficient phagocytic ability and higher migration efficacy.

Effect of RFA in combination with the injection of OK-432-stimulated DCs on tumor growth

OK-432-stimulated DCs were used in combination therapy with RFA in this murine model (Fig. 2a). Namely, BMDCs stimulated with OK-432 were injected into RFA-treated tumor at 24 h after RFA treatment. We compared four groups of tumor-bearing mice as follows: (1) no treatment; (2) RFA only; (3) RFA with the injection of immature DCs; and (4) RFA with the injection of OK-432-stimulated DCs. Tumor volumes were measured for 10 days after treatment/no treatment. On the day after RFA, the treated tumors were covered with scars, started to shrink and had disappeared macroscopically at 4 days after RFA in all of the groups. This indicated that RFA treatment was highly effective for focal lesions. The injected DCs were detected in the treated tumors (Supplementary Fig. 3). With regard to the untreated tumors, as we previously reported, the group treated with RFA only showed an antitumor effect against distant tumors. The injection of immature DCs combined with RFA did not show any additional enhancement of the antitumor effect. On the other hand, the volumes of the untreated tumors in the group that underwent RFA combined with the injection of OK-432-stimulated DCs were strongly suppressed ($P < 0.001$) relative to other groups (Fig. 2b).

Recruitment of antigen-specific lymphocyte fractions in both splenocytes and tumor by injected OK-432-stimulated DCs

Ten days after RFA, the tumors and the spleens were harvested and analyzed using immunohistochemical staining.

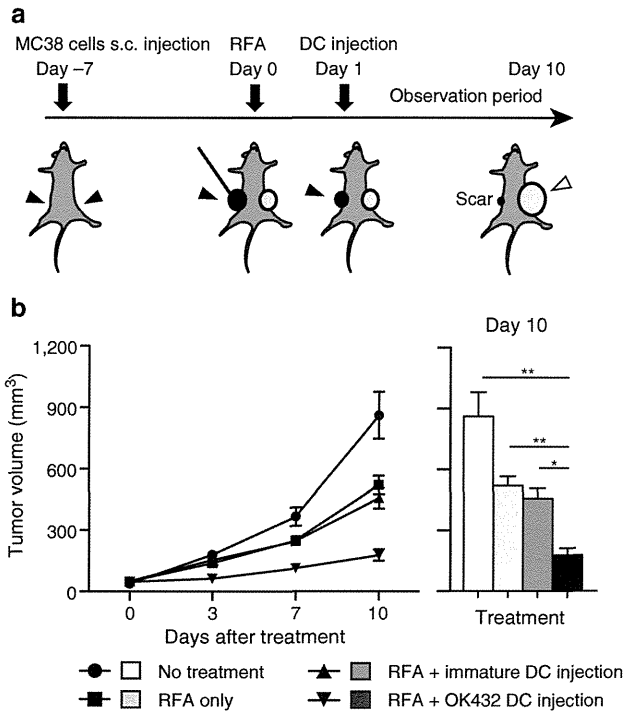

Fig. 2 Impact of injection of OK-432-stimulated DCs into murine MC38 subcutaneous tumors. **a** RFA was administered to a tumor on one flank followed by injection of 1×10^7 DCs into the treated tumor. The untreated tumor on the opposite flank was observed for 10 days. The *solid arrowheads* indicate the treatment intervention sites, and the *open arrowhead* indicates the observed untreated tumor. **b** The tumor volumes were compared among the four groups as follows: (1) no treatment; (2) RFA only; (3) RFA in combination with immature DC injection; and (4) RFA in combination with OK-432-stimulated DC injection. $n = 8$ mice per group. The data are presented as the mean ± SE. $^*P < 0.05$; $^{**}P < 0.001$

We examined the number of tumor-infiltrating CD4-positive or CD8-positive cells in the tumors by means of immunohistochemistry. The infiltration of these cells into the untreated tumors was found to be promoted by RFA. The injection of OK-432-stimulated DCs after RFA induced the additional recruitment of CD8-positive cells into the untreated tumors (Fig. 3a, b). CD11c-, CD11b- and NK1.1-positive cells were very marginal and showed no differences in number among the four groups (data not shown).

Systemically, in terms of analyzing splenocytes with flow cytometry, the number of CD4-positive and CD8-positive cells increased in the group treated with RFA in combination with OK-432-stimulated DCs. On the other hand, the CD11c and NK1.1 fractions, which were considered as DCs and NK cells, respectively, presented no difference among the four groups (Fig. 3c). In addition, we examined the effect of the injection of OK-432-stimulated DCs after RFA on inhibitory blood cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (Fig. 3c). Among CD4-positive cells, significantly fewer Tregs were detected in the group treated with RFA in combination with OK-432-stimulated DCs than in the group treated with RFA in combination with immature DCs. In the analysis of MDSCs, their rates of occurrence were not affected by treatment with either RFA alone or RFA in combination with DCs. Taking these results together, we concluded that treatment with RFA combined with OK-432-stimulated DCs enhanced the number of CD4- or CD8-positive T cells and reduced the Treg/CD4 ratio, but did not influence MDSC numbers.

Furthermore, we examined the number of tumor-specific IFN-γ-producing cells at 10 days after RFA using the ELISPOT assay. The number of IFN-γ-producing cells among splenocytes and TILs showed the same trend as the level of tumor growth control among the four groups (Fig. 3d); the group treated with RFA in combination with injected OK-432 DCs showed the most abundant specific spots. These results suggested that the augmented antitumor effects of RFA combined with OK-432-stimulated DCs depended in large part on tumor-specific immune responses by CD4 cells or CD8 cells.

Evaluation of tumor-specific immune responses in the draining lymph node after OK-432-stimulated DC transfer

CD4 T cells and CD8 T cells are now thought to have an important antitumor effect as a result of the OK-432-stimulated DC transfer. To elucidate the priming of the antigen-specific immune response, we analyzed the draining lymph nodes at 3 days after RFA focusing on CD4-positive or CD8-positive cells. CD69, the early activation marker, on CD4-positive and CD8-positive cells was examined and compared between the immature DC transfer group and the OK-432-stimulated DC transfer group. It was found that CD69 expression on both CD4-positive and CD8-positive cells was elevated in the OK-432-stimulated DC transfer group (Fig. 4a, b). The activations were also demonstrated to be tumor-specific using the IFN-γ ELISPOT assay in which each of CD4-negative and CD8-negative fractions was applied to the assay and both showed tumor-specific IFN-γ secretions (Fig. 4c).

Evaluation of the relationship between CD4-positive and CD8-positive cells and the antitumor effects of RFA and OK-432-stimulated DC transfer

We have demonstrated that combination therapy involving RFA and OK-432-stimulated DC transfer might generate enhanced antitumor effects via tumor-specific CD4-positive and CD8-positive cells. To obtain further evidence, we carried out in vivo CD4 or CD8 depletion studies in mice. Initially, we confirmed CD4 or CD8 depletion in the control in vivo study (Supplementary Fig. 4). The

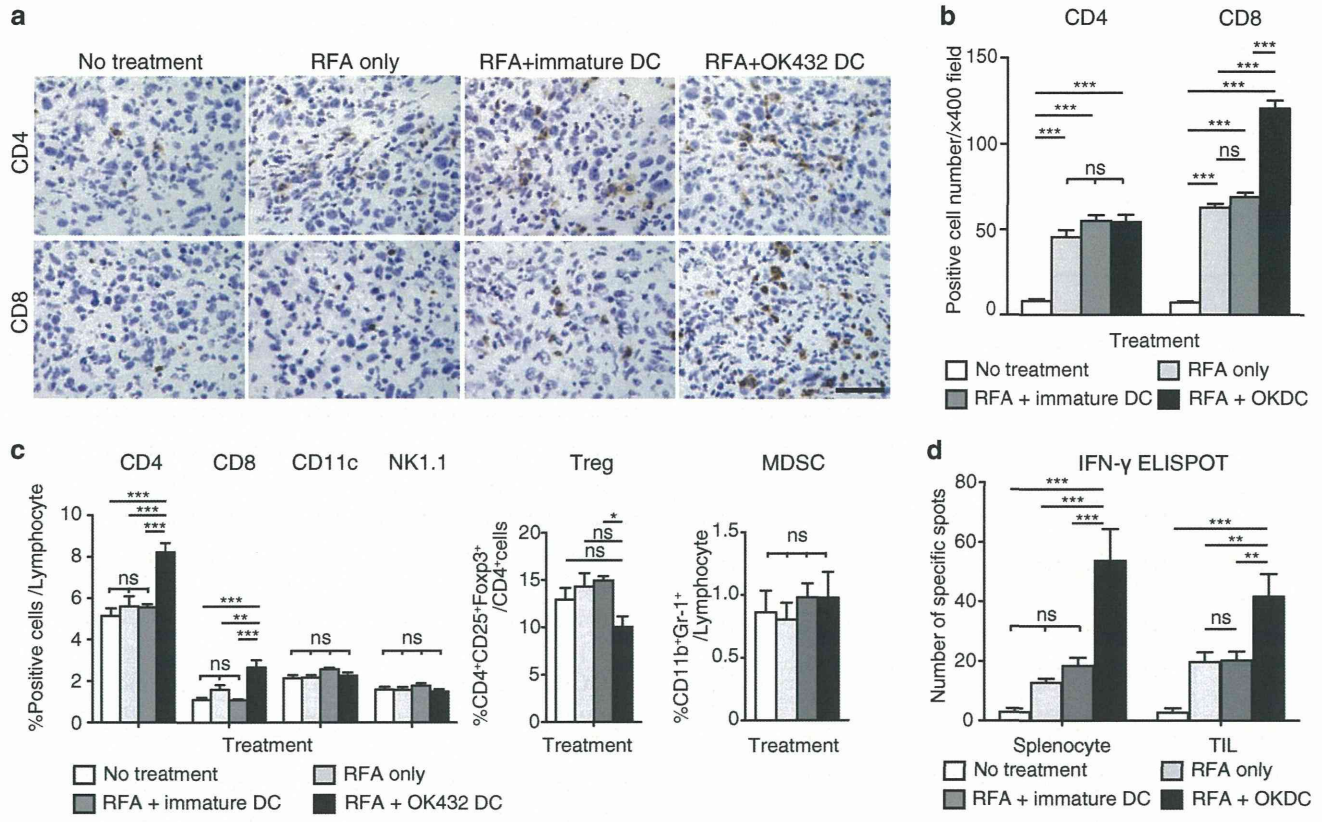

Fig. 3 Analysis of the tumor-infiltrating lymphocytes and the splenocytes after combination therapy with RFA and DC injection. **a** CD4-positive and CD8-positive cells in the observed untreated tumors were detected using immunohistochemistry at 10 days after RFA. The *black bar* represents 50 μm. **b** The number of positive cells was counted using a microscope. This was achieved by counting the number of cells in six randomly chosen tumor areas at 400-fold magnification. Three mice were used in each group. The data are presented as the mean ± SE. *** $P < 0.001$; *ns* not significant. **c** Ten days after RFA, splenocytes were stained with anti-CD4, anti-CD8, anti-NK1.1 and anti-CD11c antibodies and analyzed using flow cytometry. Regulatory T cells (Tregs) defined as $CD4^+CD25^+Foxp3^+$ cells and myeloid-derived suppressor cells (MDSCs) defined as $CD11b^+Gr\text{-}1^+$ cells were counted and compared among the four groups. Six mice were analyzed in each group. The data are presented as the mean ± SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *ns* not significant. **d** Immune responses by the splenocytes and the tumor-infiltrating lymphocytes (TILs) were examined by means of the IFN-γ enzyme-linked immunospot (ELISPOT) assay using MC38 lysate. In the assay for TILs, 1×10^5 TILs were mixed with 2×10^5 splenocytes from B6 mice and applied to the well. Six mice were analyzed in each group. The data are presented as the mean ± SE. **$P < 0.01$; ***$P < 0.001$; *ns* not significant

CD4-positive and CD8-positive fractions in the peripheral blood were greatly depleted at 7 days after injection of the antibodies. The experimental schedule was determined as follows. The depletion antibodies were injected at 1 day before and 3 days after RFA, and the tumors that were not treated with RFA were observed for 10 days. In addition, the draining lymph nodes were harvested at 3 days after RFA and analyzed (Supplementary Fig. 5). The antitumor effects of RFA treatment and the augmented effects from OK-432-stimulated DCs were cancelled out by depletion of both CD4 and CD8 cells (Fig. 5a). In the CD4 depletion study, there was no priming of the antitumor effect in the draining lymph nodes (Fig. 5b; Supplementary Fig. 6). On the other hand, in the CD8 depletion study CD4 cells were activated with tumor specificities in the draining lymph node in both groups, and the activation was stronger in the OK-432-stimulated DC transfer group (Fig. 5b; Supplementary Fig. 6). Tumor-specific reactions were also demonstrated in the splenocytes and the TILs at 10 days after RFA. There was a tendency for OK-432 DC transfer treatment to result in the recruitment of increased numbers of tumor-specific lymphocytes into the tumor on the opposite flank ($P = 0.184$; Fig. 5c). These results indicated that the tumor-specific activation of CD8 cells was necessary for the antitumor effect and was completely dependent on help from the CD4 cells.

Discussion

In the past decade, cytotoxic agents and molecular-targeted therapies have been developed, and the treatment outcomes

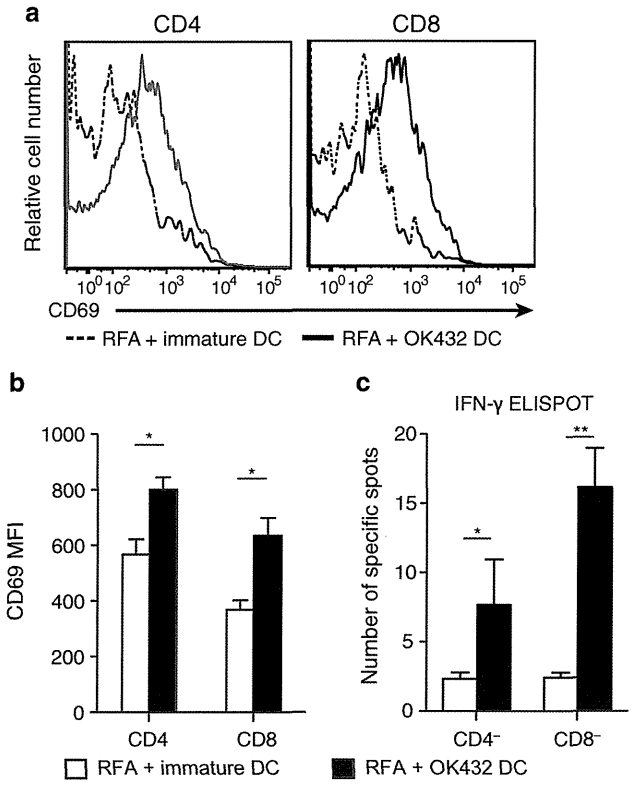

Fig. 4 Antigen-specific activation of both CD4-positive and CD8-positive cells in the draining lymph node. **a** Three days after RFA followed by DC transfer, the draining lymph node was harvested and analyzed by staining with anti-CD4 antibodies, anti-CD8 antibodies and anti-CD69 antibodies. The fluorescence intensities of CD69 in the CD4-positive and CD8-positive fractions are compared between the OK-432-stimulated DC transfer group and the immature DC transfer group. The data were obtained from six mice in each group. The *histograms* show the representative data. **b** The mean fluorescent intensities are also presented as the mean ± SE. $*P < 0.05$. **c** The antigen specificities of the T-cell activations were confirmed by means of the IFN-γ ELISPOT assay using MC38 lysate. After CD4 or CD8 depletion using CD4 and CD8 magnetic beads, the lymphocytes from the draining lymph nodes were submitted to IFN-γ ELISPOT assay. Data were obtained from six mice in each group. $*P < 0.05$; $**P < 0.01$

for various cancers have improved. However, few patients with advanced cancers have been completely cured, and thus, new strategies for anticancer therapy are required. Immunotherapy is considered to have the potential to effectively treat such advanced cancers, and many different approaches have been explored. For the utilization of the adoptive immune response in a cancer therapy, DCs are a key constituent of the immune system. This is because of their natural potential to present tumor-associated antigens to $CD4^+$ and $CD8^+$ lymphocytes and also to control both immune tolerance and immunity [21]. Thus, DCs are considered as an important target for cancer immunotherapy. Many trials and studies have been carried out regarding immunotherapy for cancer using DCs, some of which have been reported to have pronounced effects [22–25]. In recent studies, it has been revealed that RFA treatment induces tumor-specific T-cell responses, which is known as the abscopal effect; this has been mainly reported in radiotherapy studies and is augmented with combined immunotherapies [26, 27]. Brok et al. [28] have previously reported on the vaccination effects of combination therapy involving RFA and CTLA-4 antibody.

To our knowledge, this is the first study that has demonstrated using a murine metastatic cancer model that RFA in combination with focal DC injection could enhance the antitumor effects of RFA alone. Our results showed that immature DCs made no additional immunological contribution to RFA. In the analysis of draining lymph nodes, few transferred DCs were detected after the injection of immature DCs. It appeared that immature DCs did not act as sentinels in the adoptive immune system, partially because they exhibited low expression of CCR7 (the main molecule that promotes DC migration [29]), even though elevation of CCR7 expression using OK-432 was very modest in our study. There is another possibility immature DCs are easily lysed and excluded by the host immune system [30]. On the other hand, mature DCs can escape cell lysis [31].

Utilization of OK-432-stimulated DCs improved the number of migrating transferred DCs in the present study. These DCs, which could act as sentinels for immunity, induced expansion in the number of tumor-specific lymphocytes in the draining lymph nodes, in the splenocytes and in the distant nontreated tumors, without systemic expansion of inhibitory cells such as Tregs or MDSCs. We also demonstrated that these augmented antitumor effects after OK-432-stimulated DC transfer were primed in the draining lymph nodes with tumor-specific activations of CD4-positive and CD8-positive cells; it was proved that without CD4-positive or CD8-positive cells, both the antitumor effect by RFA and the additional effect of the injection of OK-432-stimulated DCs disappeared completely. In addition, the in vivo CD4 depletion study revealed that tumor-specific activations of CD8-positive cells were not seen in the draining lymph nodes in both groups after the injection of immature DCs and OK-432-stimulated DC injection; in other words, tumor-specific CD8 activation depended on CD4-positive cells entirely. In the CD8 depletion study, on the other hand, we found that tumor-specific CD4-positive cells appeared in the draining lymph nodes, the splenocyte population and the untreated tumor on the opposite flank, and these lymphocytes were considered to be CD4-positive cells. In the tumor-infiltrating lymphocytes, there was a tendency for more tumor-specific CD4-positive cells to be recruited after treatment involving OK-432-stimulated DC transfer. Many researchers have demonstrated the contribution of CD4 cells to cytotoxicity

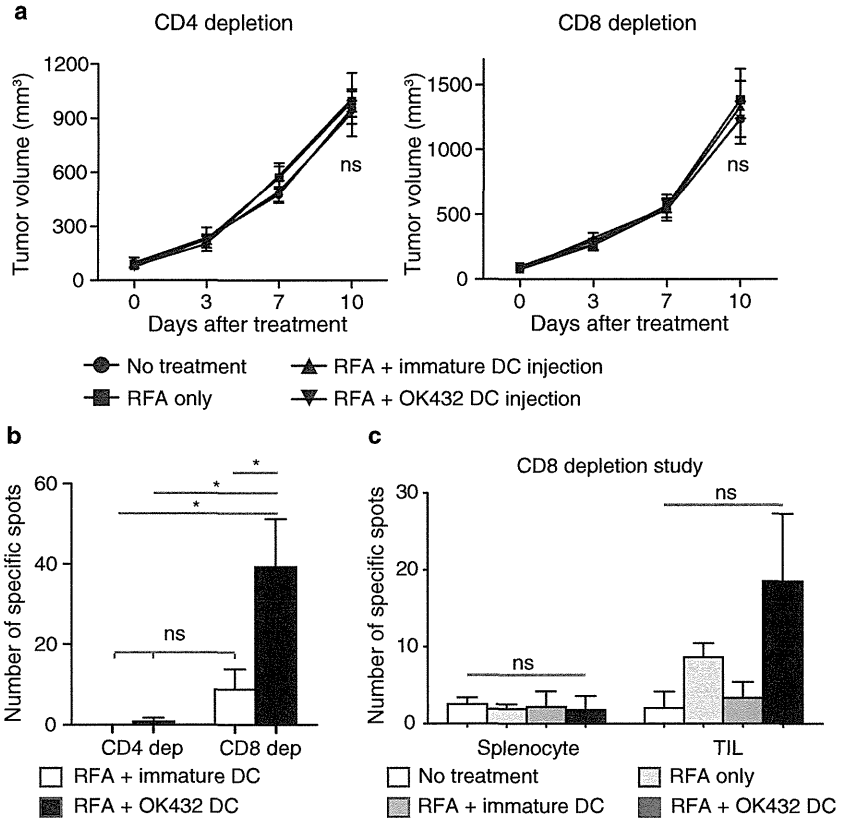

Fig. 5 The augmented antitumor effects depended on both CD4-positive and CD8-positive cells. **a** For in vivo CD4 or CD8 depletion, monoclonal antibodies specific to CD4 (GK1.5) or CD8 (2.43), respectively, were injected intraperitoneally at 1 day before and 3 days after RFA. Tumor volumes were compared among the four groups for 10 days after RFA. In each experiment, data were obtained from four mice per group and are presented as the mean ± SE. *ns* not significant. **b** The draining lymph nodes were harvested at 3 days after RFA and analyzed for their tumor specificities using the IFN-γ ELISPOT assay. Two mice were used in each group. Data are shown as the mean ± SE. $*P < 0.005$; *ns* not significant. **c** In the CD8 depletion study, splenocytes and tumor-infiltrating lymphocytes (TILs) were evaluated for their tumor specificities using the IFN-γ ELISPOT assay as described in Fig. 3. Four mice were used in each group. Data are shown as the mean ± SE. *ns* not significant

[32, 33]. However, in our experimental models, tumor-specific CD4-positive cells were not observed to contribute to the antitumor effect. Summarizing the above, in our study, the CD4-positive cells were required for the priming of the immune responses, and the CD8-positive cells acted as the effector cells after help from the CD4-positive cells.

In conclusion, we consider on the basis of our preclinical findings regarding combination therapy involving OK-432-stimulated DCs with RFA for the treatment of metastatic liver cancer that clinical trials can now proceed. It is anticipated that this combination therapy will be markedly superior to RFA single therapy.

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Conflict of interest The authors received financial support for this study from Chugai Pharmaceutical Co., Ltd.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Relevance of the Core 70 and IL-28B polymorphism and response-guided therapy of peginterferon alfa-2a ± ribavirin for chronic hepatitis C of Genotype 1b: a multicenter randomized trial, ReGIT-J study

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Abstract
Background We conducted a multicenter randomized clinical trial to determine the optimal treatment strategy against chronic hepatitis C virus (HCV) with genotype 1b and a high viral load (G1b/high).
Methods The study subjects included 153 patients with G1b/high. Patients were initially treated with PEG-IFNα-2a alone and then randomly assigned to receive different treatment regimens. Ribavirin (RBV) was administered to all patients with HCV RNA at week 4. Patients negative for HCV RNA at week 4 were randomly assigned to receive PEG-IFNα-2a (group A) or PEG-IFNα-2a/RBV (group B). Patients who showed HCV RNA at week 4 but were negative at week 12 were randomly assigned to receive weekly PEG-IFNα-2a (group C) or biweekly therapy (group D). Patients who showed HCV RNA at week 12 but were negative at week 24 were randomly assigned to receive PEG-IFNα-2a/RBV (group E) or PEG-IFNα-2a/RBV/fluvastatin (group F).
Results Overall, the rate of sustained virological response (SVR) was 46 % (70/153). The total SVR rate in the group (A, D, and F) of response-guided therapy was significantly higher than that in the group (B, C, and E) of conventional therapy [70 % (38/54) versus 52 % (32/61), $p = 0.049$]. Although IL28-B polymorphism and Core 70 mutation were significantly associated with efficacy, patients with rapid virological response (RVR) and complete early virological response (cEVR) achieved high SVR rates regardless of their status of IL-28B polymorphism and Core 70 mutation.
Conclusion In addition to knowing the IL-28B polymorphism and Core 70 mutation status, understanding the likelihood of virological response during treatment is critical in determining the appropriate treatment strategy.

Keywords Chronic hepatitis C · IL-28B · Peginterferon alfa-2a · Ribavirin · Response-guided therapy

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Introduction

The introduction of combined treatment with peginterferon (PEG-IFN) and ribavirin (RBV) has dramatically increased the rate of sustained virological response (SVR) in patients with genotype 1 high virus titer chronic hepatitis C (HCV RNA titer $\geq$ 5 Log IU/mL), a disease generally considered intractable, to approximately 50 % [1–4]. Currently, a protease inhibitor, telaprevir, can be used for the treatment of chronic hepatitis C, further increasing the SVR rate to approximately 70 % after initial treatment; however, adverse events such as severe anemia, dermatopathy, and renal dysfunction due to increased creatinine level have been reported [5, 6].

RBV is also associated with adverse events, such as anemia, dermatopathy and taste disturbance, and these events can be accentuated in elderly patients or patients with renal dysfunction or anemia. In Japan, there are many elderly patients with chronic hepatitis C and they often cannot tolerate a treatment combination involving RBV [7]. For such patients, PEG-IFN monotherapy could be a treatment option. It has been reported that patients with genotype 1 high virus titer chronic hepatitis C are more likely to achieve SVR if their HCV RNA becomes negative within 4 weeks after initiation of PEG-IFN monotherapy (Rapid Virological Response: RVR) [8].

Patients receiving the PEG-IFNα-2a/RBV combination therapy can also achieve an excellent SVR rate if their HCV RNA becomes negative within 12 weeks after initiation of treatment, whereas the rate is known to decrease with a delay in the timing of HCV RNA-negative conversion [3]. Based on these findings, we propose the use of "response-guided therapy", in which a treatment regimen is modified according to viral kinetics. For the treatment of genotype 1 chronic hepatitis C, proposed treatment strategies include shortening of treatment period in patients with RVR and extension of treatment period in patients with a delayed response to the initial treatment as judged at week 12 [9–17]. For the treatment of genotype 1 high virus titer chronic hepatitis C, shortening of the treatment period may not be recommended even if RVR is achieved because of a possible reduction in the SVR rate, whereas extension of the treatment period to 72 weeks has been reported to increase the SVR rate in patients showing a delayed response to the initial treatment [12, 14–18]. In addition, combined use of HMG-CoA reductase inhibitors and IFN has been shown to enhance the antiviral effects in a synergistic manner [19]. Addition of fluvastatin (FLV), an HMG-CoA reductase inhibitor reported to exhibit the highest antiproliferative activity against hepatitis C virus, to PEG-IFNα-2a/RBV combination therapy has improved the SVR rate [20–22].

Factors affecting the efficacy of PEG-IFN/RBV combination therapy can be divided into viral and host factors. The viral factors include virus titer, genotype, amino acid substitution at position 70 of the core protein (Core 70) and mutations in the interferon sensitivity-determining region (ISDR) in the HCV NS5A region [23–27]. The host factors include age, sex, the degree of liver fibrosis, and a single nucleotide polymorphism (SNP) close to the IL-28B gene [28–33].

We therefore conducted a randomized trial to explore the optimal treatment strategy for patients with genotype 1 high virus titer chronic hepatitis C by comparing several treatment regimens modified according to the concept of "response-guided therapy" in consideration of tolerability (PEG-IFNα-2a monotherapy, PEG-IFNα-2a weekly or biweekly/RBV combination, and PEG-IFNα-2a/RBV/FLV combination therapy). We also evaluated the relations of IL-28B polymorphism and Core 70 mutation to the rate of HCV-RNA-negative conversion and SVR.

Patients and methods

Patients

The study subjects included 153 patients with genotype 1b high virus titer chronic hepatitis C (HCV RNA $\geq$ 5 Log IU/mL) who visited 17 institutions from April 2007 to December 2010 and met the following inclusion criteria: laboratory data before study treatment of white blood cell count $\geq$ 3,000/mm^3, neutrophil count $\geq$ 1,500/mm^3, platelet count $\geq$ 90,000/mm^3, and hemoglobin $\geq$ 12 g/dL. Before the study treatments were carried out, all patients gave written informed consent after receiving a sufficient explanation of the therapy. All patients had genotype 1b chronic hepatitis C with a mean HCV RNA titer of 6.4 Log IU/mL. There were 63 male and 90 female patients with a mean age of 56.5 years. Sixty patients had received prior treatment with IFN, though it was ineffective in 30 of these patients (Table 1).

Treatment protocol

The study design is shown in Fig. 1. After a lead-in therapy with PEG-IFNα-2a 180 μg/week alone (for 4 weeks), RBV was added to the treatment for patients without HCV RNA-negative conversion (according to their weight; $\leq$ 60 kg, 600 mg/day; 60–80 kg, 800 mg/day; and >80 kg, 1,000 mg/day). Patients with negative HCV RNA (TaqMan < 1.2 Log IU/mL) at week 4 (rapid virological response, RVR) were randomly assigned to receive PEG-IFNα-2a alone (group A) or PEG-IFNα-2a/RBV combination (group B). Patients with negative HCV RNA

Table 1 Baseline characteristics of patients ($n = 153$)

Age (years)	56.5 ± 11.1
Gender (male/female)	63/90
HCV RNA (Log IU/mL)	6.4 ± 0.7
BMI (kg/m^2)	22.8 ± 3.3
ALT (IU/L)	60.5 ± 41.3
AST (IU/L)	51.7 ± 31.5
Previous IFN (no/yes)	93/60 (non-responder for 30)
Fibrosis (F0-2/F3-4)	72/32 (unknown for 49)
Activity (A0-1/A2-3)	49/56 (unknown for 48)
Core 70 (wild/mutant)	54/38 (unknown for 61)
IL-28B, rs8099917 (TT/non-TT)	43/26 (unknown for 84)

Values are mean ± standard deviation (SD)

BMI body mass index, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase

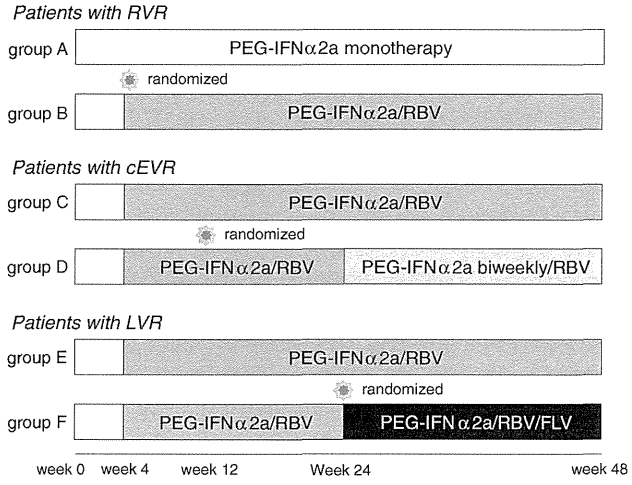

Fig. 1 Study design. After a lead-in therapy with PEG-IFNα-2a for 4 weeks, patients with negative HCV RNA at week 4 (RVR) were randomly assigned to receive PEG-IFNα-2a alone (group A) or PEG-IFNα-2a/RBV combination (group B). Patients with negative HCV RNA at week 12 (cEVR) were randomly assigned to receive weekly PEG-IFNα-2a/RBV combination (group C) or biweekly PEG-IFNα-2a/RBV combination (group D). Patients with negative HCV RNA at week 24 (LVR) were randomly assigned to receive PEG-IFNα-2a/RBV combination (group E) or PEG-IFNα-2a/RBV/fluvastatin (FLV) combination (group F)

at week 12 (complete early virological response, cEVR) were randomly assigned to receive weekly PEG-IFNα-2a/RBV combination (group C) or biweekly PEG-IFNα-2a/RBV combination (group D). Patients with negative HCV RNA at week 24 (late virological response, LVR) were randomly assigned to receive PEG-IFNα-2a/RBV combination (group E) or PEG-IFNα-2a/RBV/fluvastatin (FLV) combination (group F). For assignment, we used Microsoft Access to generate random numbers.

Cases with RVR: evaluation of necessity of RBV (PEG-IFNα-2a monotherapy versus PEG-IFNα-2a/RBV combination therapy)

Patients with negative HCV RNA at week 4 after the introduction of lead-in therapy with PEG-IFNα-2a alone (RVR) were randomly assigned to receive PEG-IFNα-2a alone (group A) or PEG-IFNα-2a/RBV combination (group B) to compare the efficacy and safety between the treatment groups and to evaluate the significance of addition of RBV in RVR cases.

Cases with cEVR: evaluation of dosage interval of PEG-IFNα-2a (weekly versus biweekly PEG-IFNα-2a in combination of RBV)

Patients positive for HCV RNA at week 4 but negative at week 12 (cEVR) were randomly assigned to receive weekly PEG-IFNα-2a/RBV combination (group C) or biweekly PEG-IFNα-2a/RBV combination (group D) after week 24, to compare the efficacy and safety between the treatment groups and to evaluate the dosage interval of PEG-IFNα-2a.

Cases with LVR: evaluation of clinical significance of addition of fluvastatin (PEG-IFNα-2a/RBV combination therapy versus PEG-IFNα-2a/RBV/FLV combination therapy)

Patients with positive HCV RNA at week 4 and 12 but negative HCV RNA at week 24 (LVR) were randomly assigned to a treatment group of PEG-IFNα-2a/RBV (group E) or PEG-IFNα-2a/RBV/FLV (group F) to compare the efficacy and safety between the treatment groups and to evaluate the significance of adding FLV. The dosage of FLV was set to 20 mg/day.

The primary efficacy endpoint was SVR. We also investigated correlations of IL-28B polymorphism (rs8099917) and Core 70 mutation with the rate of HCV RNA-negative conversion and SVR. The IL-28B polymorphism and Core 70 mutation were measured only in patients who wished to have this done. The genetic testing (IL-28B) was performed only in patients who gave written informed consent after obtaining the approval from the ethical committee. This study was a multicenter trial, and the numbers of patients with available HCV-RNA data were different for the week-4, -12, and -24 responses, because not all of the participating institutions completed all of these time points. Therefore, the numbers of patients with regard to IL28B and Core 70 mutation did not completely match at each time point.

If a decrease in the neutrophil count, platelet count, or Hb level reached a critical level or other adverse events

occurred, dose reduction or discontinuation of PEG-IFNα-2a or RBV was performed.

Statistical analysis

All statistical analyses were done using JMP version 9 (SAS). We used the *t* test, Chi-square test, and Fisher's exact test for univariate analysis. To identify factors affecting the SVR rate, we used the logistic regression test. A *p* value of less than 0.05 was considered statistically significant.

Results

Flowchart of the study

A flowchart of the study is shown in Fig. 2. PEG-IFNα-2a monotherapy was initiated in 153 patients, out of which 15 patients necessitated treatment discontinuation due to the patient's hope of recovery or adverse events. The timing of treatment discontinuation was within 4 weeks in three patients, between 5 and 12 weeks in nine patients, and between 13 and 24 weeks in three patients. RVR, cEVR, and LVR were achieved in 18, 70, and 27 patients, respectively, and these 115 patients were randomly assigned to treatment groups according to the response-guided therapy. However, 23 patients remained positive for HCV RNA (non-virological response, NVR) at week 24 and were finally judged as non-SVR.

Of 18 patients with RVR, 10 were assigned to group A (PEG-IFNα-2a monotherapy) and eight to group B (PEG-IFNα-2a/RBV combination); of 70 patients with cEVR, 39 were assigned to group C (weekly PEG-IFNα-2/RBV combination) and 31 to group D (biweekly PEG-IFNα-2/RBV combination); and of 27 patients with LVR, 14 were assigned to group E (PEG-IFNα-2a/RBV combination) and 13 to group F (PEG-IFNα-2a/RBV/FLV combination).

PEG-IFNα-2a monotherapy versus PEG-IFNα-2a/RBV combination therapy in cases with RVR (group A versus group B)

The SVR rate in 18 patients with negative HCV RNA at week 4 after initiation of PEG-IFNα-2a monotherapy (RVR) was 100 % (10/10) in group A (PEG-IFNα-2a monotherapy) and 87.5 % (7/8) in group B (PEG-IFNα-2a/RBV combination), showing no significant difference between the two groups ($p = 0.444$). The rate of treatment discontinuation was 0 % (0/10) in group A. However, treatment discontinuation was required in one patient (12.5 %) in group B due to hemolytic anemia caused by RBV, resulting in non-SVR. Although the rate of RVR by PEG-IFNα-2a monotherapy was only 12 % (18/153), once

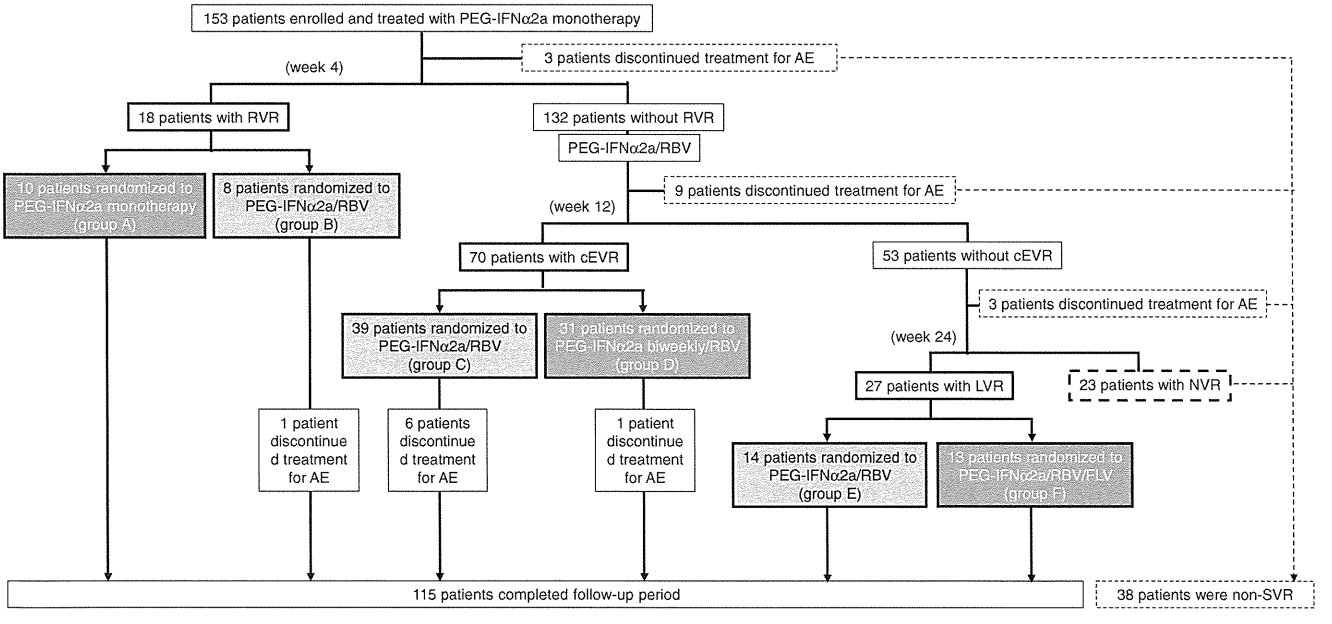

Fig. 2 Flowchart of the study. PEG-IFNα-2a monotherapy was initiated in 153 patients, of whom 15 patients necessitated treatment discontinuation. A total of 115 patients with RVR, cEVR, or LVR were randomly assigned to treatment groups, while 23 patients remained positive for HCV RNA (non-virological response, NVR) at week 24 and were finally judged as non-SVR. Of 18 patients with RVR, 10 were assigned to group A (PEG-IFNα-2a monotherapy) and eight to group B (PEG-IFNα-2a/RBV combination); of 70 patients with cEVR, 39 were assigned to group C (weekly PEG-IFNα-2/RBV combination) and 31 to group D (biweekly PEG-IFNα-2/RBV combination); and of 27 patients with LVR, 14 were assigned to group E (PEG-IFNα-2a/RBV combination) and 13 to group F (PEG-IFNα-2a/RBV/FLV combination)

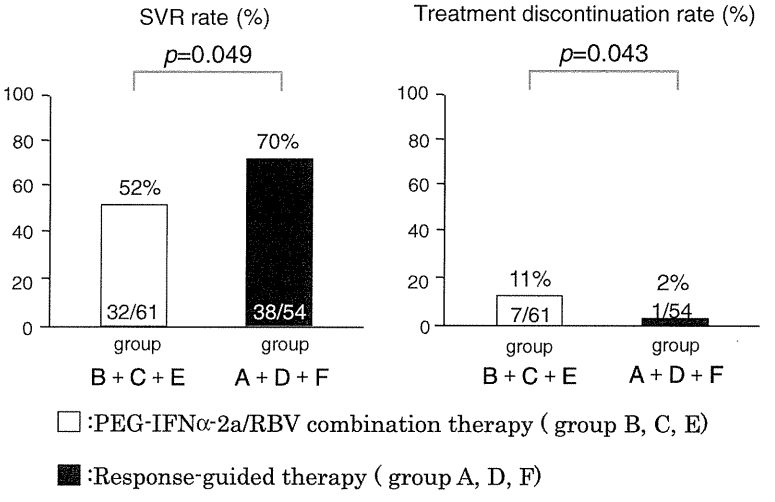

Fig. 3 The SVR and treatment discontinuation rate in the group (A + D + F) of treatment regimens modified according to response-guided therapy and in the group (B + C + E) of PEG-IFNα-2a/RBV combination therapy

RVR is achieved, PEG-IFNα-2a monotherapy without addition of RBV can induce SVR at a high rate with a high tolerability.

Weekly PEG-IFNα-2/RBV combination versus biweekly PEG-IFNα-2/RBV combination therapy in patients with cEVR (group C versus group D)

The SVR rate in 70 patients with cEVR was 54 % (21/39) in group C (weekly PEG-IFNα-2/RBV combination) and 65 % (20/31) in group D (biweekly PEG-IFNα-2/RBV combination). Adverse events leading to treatment discontinuation occurred in six patients (15 %) in group C (a decrease in Hb level, chest pain, fatigue, dizziness, a sense of feeling bad, and a suspicion of HCC) but in only one patient (3 %) in group D (depression), suggesting that the rate of treatment discontinuation tended to be higher in group C than in group D ($p = 0.123$). The difference in the SVR rates between groups C and D may reflect the difference in the rate of treatment discontinuation between the groups.

PEG-IFNα-2a/RBV combination versus PEG-IFNα-2a/RBV/FLV combination therapy in patients with LVR (group E versus group F)

The SVR rate in 27 patients with LVR was 29 % (4/14) in group E (PEG-IFNα-2a/RBV combination therapy) and 62 % (7/13) in group F (PEG-IFNα-2a/RBV/FLV combination therapy), suggesting that the rate tended to be higher in group F than in group E ($p = 0.085$). Thus, addition of an HMG-CoA inhibitor, FLV, increased the SVR rate even in patients with LVR showing delayed negative conversion of HCV RNA. There were no adverse events leading to treatment discontinuation in both groups, and FLV did not augment the adverse events in group F.

Group with PEG-IFNα-2a/RBV combination therapy versus group with response-guided therapy (groups B + C + E versus groups A + D + F)

We then divided all of these groups into two groups according to treatment regimens, a group (A + D + F) in which treatment regimen was modified according to response-guided therapy and a group (B + C + E) of PEG-IFNα-2a/RBV combination therapy. The SVR rate in the response-guided therapy group was significantly higher than in the PEG-IFNα-2a/RBV combination therapy group [70 % (38/54) versus 52 % (32/61), $p = 0.049$].

The rate of treatment discontinuation due to adverse events was significantly lower in the response-guided therapy group than in the PEG-IFNα-2a/RBV combination therapy group [11 % (7/61) versus 2 % (1/54), $p = 0.043$] (Fig. 3).

Factors influencing negative conversion of HCV RNA at week 4, 12, and 24

Factors influencing negative conversion of HCV RNA at week 4 were analyzed in 18 patients with negative HCV RNA and 132 patients with positive HCV RNA. Factors identified as significantly different between the negative and positive groups were age and HCV RNA titer before study treatment, but IL-28B polymorphism and Core 70 mutation were not associated with negative conversion at this time point. Comparison between 88 negative and 53 positive HCV RNA patients at week 12 and that between 115 negative and 23 positive HCV RNA patients at week 24 identified IL-28B polymorphism and Core 70 mutation as factors, showing differences with a statistical significance (Table 2).

Table 2 Characteristics of HCV RNA-negative or positive patients at week 4, 12, and 24

At week 4	Negative (n = 18)	Positive (n = 132)	p value
Age (years)	49.5 ± 14.6	57.6 ± 10.3	0.003
HCV RNA (Log IU/mL)	6.0 ± 0.7	6.4 ± 0.7	0.009
At week 12	Negative (n = 88)	Positive (n = 53)	p value
Core 70 substitution (wild/mutant)	39/14	13/22	<0.001
IL-28B, rs8099917 (TT/non-TT)	31/8	10/18	<0.001
At week 24	Negative (n = 115)	Positive (n = 23)	p value
Core 70 substitution (wild/mutant)	48/23	4/11	0.003
IL-28B, rs8099917 (TT/non-TT)	38/14	3/10	0.003

Value are mean ± standard deviation (SD)

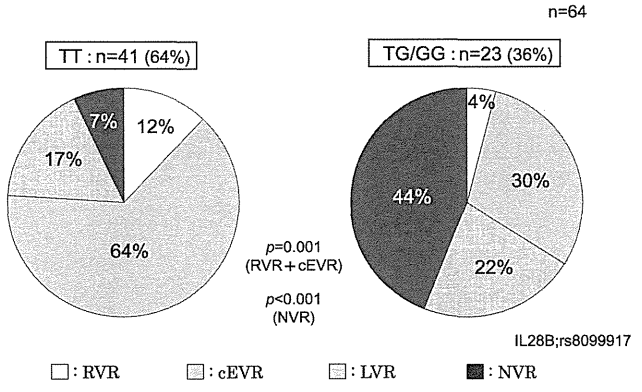

Fig. 4 Treatment response to PEG-IFNα-2a with or without RBV according to the IL-28B single nucleotide polymorphisms (TT versus TG/GG genotype)

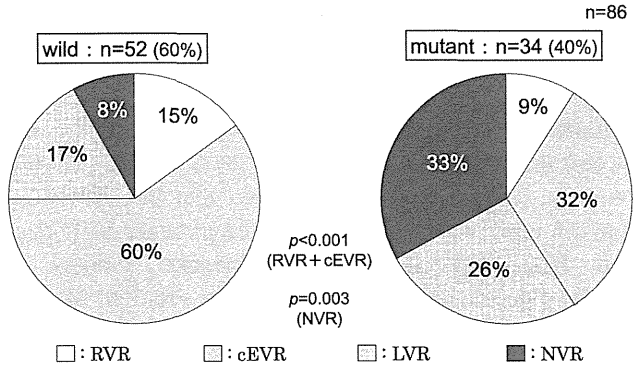

Fig. 5 Treatment response to PEG-IFNα-2a with or without RBV according to the Core 70 mutation (wild-type versus mutant Core 70)

We also investigated the correlation between IL-28B polymorphism and HCV RNA-negative conversion within 12 weeks (RVR + cEVR) in 64 patients in whom IL-28B polymorphism was examined. Negative HCV RNA was achieved within 12 weeks in 76 % of 41 patients with IL-28B TT genotype (major) and in 34 % of 23 patients with IL-28B TG or GG genotype (minor), showing a significant difference between them (p = 0.001). Especially in cases with NVR, negative HCV RNA was achieved in 7 % of patients with IL-28B major genotype and in 44 % of patients with IL-28B minor genotype (p < 0.001), suggesting that IL-28B polymorphism is strongly associated with treatment response (Fig. 4). Similarly, in 86 patients with determined Core 70 mutation status, negative HCV RNA was achieved within 12 weeks in 75 % of 52 patients with wild-type Core 70 and 41 % of 34 patients with mutant Core 70, showing a significant difference between them (p < 0.001). In patients with NVR, the rate of becoming HCV RNA-negative within 12 weeks was 8 % in patients with wild-type Core 70 and 33 % in those with mutant Core 70 (p = 0.003) (Fig. 5).

The SVR rates at different time points of HCV RNA-negative conversion by IL-28B polymorphism and Core 70 mutation

The SVR rates were investigated in patients with different time points of HCV RNA-negative conversion (RVR in six patients, cEVR in 33, LVR in 13, and NVR in 13) according to the IL-28B genotypes. The SVR rate was 100 % (5/5) in patients with RVR, 65 % (17/26) in patients with cEVR, 57 % (4/7) in patients with LVR, and 0 % (0/3) in patients with NVR with IL-28B major genotype; whereas the rate was 100 % (1/1) in patients with RVR, 43 % (3/7) in patients with cEVR, 83 % (5/6) in patients with LVR, and 0 % (0/10) in patients with NVR with IL-28B minor genotype. Similarly, the SVR rates were investigated in patients with different time points of HCV RNA-negative conversion (RVR in 11 patients, cEVR in 42, LVR in 18, and NVR in 15) according to the Core 70

Table 3 Characteristics of sustained virological response (SVR) and non-SVR patients

	SVR ($n = 70$)	Non-SVR ($n = 83$)	p value
Age (years)	53.1 ± 12.7	59.4 ± 8.7	<0.001
Gender (male/female)	29/41	34/49	0.954
HCV RNA (Log IU/mL)	6.4 ± 0.7	6.4 ± 0.7	0.782
BMI (kg/m^2)	22.7 ± 3.9	22.9 ± 2.8	0.815
Previous IFN (no/yes)	49/21	44/39	0.032
Fibrosis (F0-2/F3-4)	41/9	31/23	0.007
Activity (A0-1/A2-3)	24/27	25/29	0.938
NS5A mutation, *n* (0-1/2-)	31/10	47/3	0.013
Core 70 substitution (wild/mutant)	30/11	24/27	0.012
IL-28B, rs8099917 (TT/non-TT)	26/9	17/18	0.027
HCV RNA-negative at week 12 (yes/no)	58/12	30/41	<0.001
Treatment group (B,C,E/A,D,F)	32/38	29/16	0.049

Values are mean ± standard deviation (SD)

BMI body mass index

Table 4 Associated factors with sustained virological response (SVR) by multivariate logistic regression analysis

Factor	Odds ratio	95 % CI	p value
Age (per 1 year)	0.94	0.89–0.98	0.005
Previous IFN (no/yes)	1.62	0.62–4.27	0.323
Fibrosis (F0-2/F3-4)	3.38	1.15–10.8	0.026
NS5A mutation, *n* (2-/0-1)	7.18	1.32–61.0	0.021
Core 70 substitution (wild/mutant)	2.49	1.51–8.28	0.044
IL-28B, rs8099917 (TT/non-TT)	1.85	0.85-8.61	0.563
HCV RNA-negative at week 12 (yes/no)	7.89	2.92–24.0	<0.001

mutation status. The SVR rate was 100 % (RVR), 58 % (cEVR), 44 % (LVR), and 0 % (NVR) in patients with wild-type Core 70; whereas the rate was 67 % (RVR), 55 % (cEVR), 33 % (LVR), and 0 % (NVR) in patients with mutant Core 70. Thus, when the SVR rates were investigated according to the different time points of HCV RNA-negative conversion, there was no association of IL-28B polymorphism or Core 70 mutation with the SVR rates.

Factors affecting the SVR rate

An univariate analysis in 70 SVR patients and 83 non-SVR patients identified age, previous IFN treatment, fibrosis, NS5A mutation, Core 70 mutation, EVR, IL-28B, and treatment group as factors affecting the SVR rate (Table 3). In this analysis, we examined 83 non-SVR patients: 45 non-SVR patients are presented in Fig. 3, and 38 non-SVR patients (23 patients with NVR and 15 patients who discontinued the Peg-IFN-RBV treatment prior to the enrollment of the randomized trial) are presented in Fig. 2. Multivariate analysis using a logistic regression analysis revealed age (younger), fibrosis (mild), NS5A mutation (two or more mutations), Core 70 status (wild-type), and EVR (RVR + cEVR), to be independent factors affecting the SVR rate, and among them EVR was the most significant factor (odds ratio, 7.89; $p < 0.001$) (Table 4). Therefore, even in patients considered intractable based on the IL-28B genotype or Core 70 mutation status, SVR is expected to be achieved once RVR or cEVR is reached during treatment.

Discussion

The introduction of combined treatment with PEG-IFN and RBV has increased the SVR rate to approximately 40–50 % even in intractable cases with genotype 1b high virus titer chronic hepatitis C after a standard treatment course of 48 weeks [1–4]. In an attempt to further improve the SVR rate, we propose a concept of "response-guided therapy", in which the treatment regimen (such as an extension of a treatment period) is determined according to the viral response to the initial treatment [7–15]. In cases with positive HCV RNA at week 4 or 12, extension of the treatment period from 48 to 72 weeks has been reported to prevent the recurrence and improve the SVR rate [12–14]. Recently, Miyase et al. [34] showed that PEG-IFNα-2a/ribavirin combination therapy resulted in better SVR rates than PEG-IFNα-2b/ribavirin combination therapy in female, older or low-weight patients. In addition, Minami et al. [35] reported that the rate of severe adverse events was not negligible in PEG-IFN/ribavirin combination therapy, and the rate was affected by treatment regimens. Therefore, it is important to establish a treatment regimen of PEG-IFN/RBV combination therapy that has a high efficacy with minimal adverse events. We herein investigated the treatment regimens based on the concept of response-guided therapy to minimize the rate of treatment discontinuation, without changing the treatment period, in consideration of aged patients in Japan.