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, $p = 0.01$), while those without an SVR on re-treatment could be treated for almost the same period as that in the previous treatment (re-treatment, 58.8 ± 12.8 weeks vs. previous treatment, 54.2 ± 11.3 weeks, $p = 0.38$).

Comparison of the timing to the first undetectable HCV RNA level in the previous treatment and re-treatment could be carried out in 50 patients; most patients attained HCV RNA negativity on re-treatment earlier or with the same timing as in the previous treatment, and only one patient showed a later timing for re-treatment. The SVR rate on re-treatment was low, at 13% (3/24) among the patients with detectable HCV RNA at week 24 in the previous treatment. Among the 10 patients with HCV RNA negativity on re-treatment with the same timing as that in the previous treatment, an SVR was attained only by the patients who were re-treated for 72 weeks. Among the 23 patients with earlier HCV RNA negativity on re-treatment, an SVR of 61% was attained (14/23). The patients with an RVR on re-treatment attained a high SVR rate (88%, 7/8) regardless of the virologic response in the previous treatment (Fig. 2).

In genotype 2 patients, the HCV RNA negative rate on re-treatment was 56% (10/18) at week 4, 83% (15/18) at week 12, and 89% (16/18) at week 24, and the SVR rate was 56% (10/18). The two patients without a c-EVR in the previous treatment did not attain an SVR on re-treatment. Among the patients with an RVR on re-treatment, the SVR rates were 60% (3/5) in those with 24-week treatment and 100% (5/5) in those with 48-week treatment.

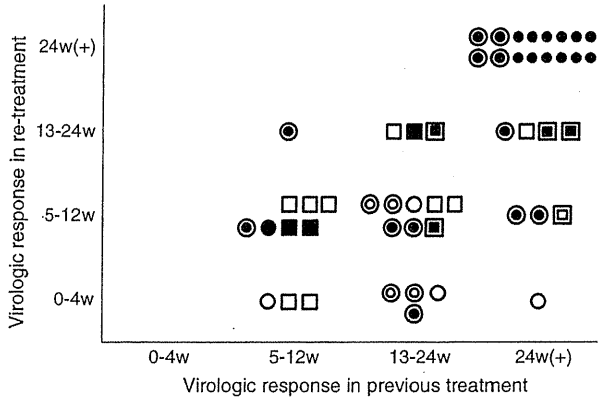

Fig. 2 Virologic response on re-treatment according to the timing of HCV RNA negativity in previous treatment and re-treatment. *Open double circles/open circles* sustained virologic response (SVR) with 48 weeks of re-treatment (*open double circles*, pegylated interferon [Peg-IFN] α-2a plus ribavirin; *open circles* Peg-IFN α-2b plus ribavirin). *Open double squares/open squares*, SVR with 72 weeks of re-treatment (*open double squares* Peg-IFN α-2a plus ribavirin; *open squares*, Peg-IFN α-2b plus ribavirin). *Closed double circles/closed circles*, non-SVR with 48 weeks of re-treatment or non-response (NR) with 24 weeks of re-treatment (*closed double circles*, Peg-IFN α-2a plus ribavirin; *closed circles* Peg-IFN α-2b plus ribavirin). *Closed double squares/closed squares*, non-SVR with 72 weeks of re-treatment (*closed double squares* Peg-IFN α-2a plus ribavirin; *closed squares*, Peg-IFN α-2b plus ribavirin)

Discussion

In the present study of the re-treatment of chronic hepatitis C patients who failed to show an SVR to Peg-IFN plus ribavirin therapy, the patients with relapse in the previous treatment showed a significant response on re-treatment compared with those with NR. This result showed similar findings to the evaluation of peg intron in control of hepatitis C chirrosis (EPIC) study of relapse and NR [10]. In addition, in the present study, p-EVR in the previous treatment was a good indicator of negative prediction for SVR on re-treatment; no patient without p-EVR in the previous treatment attained SVR on re-treatment; that is, the negative predictive value for SVR on re-treatment was 100%. 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 [12–15]. Among Japanese genotype 1 patients, it has been reported that those with the major single-nucleotide polymorphism (SNP) allele of IL28B (rs8099917) show an SVR rate of 39%, while those with the minor allele show an SVR rate of only 11%. Hence, in re-treatment for patients who failed to show a SVR to Peg-IFN plus ribavirin therapy, pretreatment prediction should be done by taking IL28B SNPs and the previous treatment response into account. Patients with the minor SNP allele of IL28B s who did not attain a p-EVR in the previous treatment should wait until new drugs become commercially available.

The next question is how the patients should be re-treated in order to attain an SVR on re-treatment. In the present study, the patients with a low serum HCV RNA level (less than 5 $\log_{10}$ IU/ml) at the start of re-treatment showed a significant rate of cure on re-treatment, and this is almost the same result as that previously reported [9, 10]. In the present study, one patient with NR in the previous treatment started re-treatment with HCV RNA of 52 KIU/ml and attained an RVR and SVR. HCV RNA levels declined on re-treatment among 61% (34/56) of the patients compared to the start of the previous treatment, and it is important not to miss the timing of when the HCV RNA level is low.

With respect to treatment duration among patients with HCV RNA negativity during re-treatment, 72 weeks of treatment tended to increase the SVR rate compared to

48 weeks of treatment (72 weeks, 68%, 15/22, vs. 48 weeks, 44%, 7/16, $p = 0.13$). This result was almost the same as that of the re-treatment of patients with chronic hepatitis C who do not respond to peginterferon-alpha 2b. A randomized trial (REPEAT) study [9]. Furthermore, in the present study, among the patients with relapse in the previous treatment, those who attained an SVR on re-treatment required a longer re-treatment duration than the duration of the previous treatment. In fact, the longer treatment brought about an SVR in some patients whose timing of HCV RNA negativity on re-treatment was the same as that in the previous treatment, as shown in Fig. 2. Thus, especially to be noted is that the relapsers in the previous treatment should be re-treated for a longer period than that of the previous treatment.

It has been reported that splenectomy and partial splenic embolization (PSE) are considered to make it possible for patients with cirrhosis and thrombocytopenia to initiate and continue anti-viral therapy safely, by increasing the platelet counts [16–19]. If poor adherence and inappropriate duration have contributed to a poor response in previous treatment due to thrombocytopenia, there is a possibility that increasing the platelet counts by splenectomy or PSE contributes to improving the tolerability of and adherence to re-treatment, and to increasing the SVR rate in re-treatment. In the present study, one patient with cirrhosis and thrombocytopenia who showed NR in the previous treatment owing to poor adherence to the Peg-IFN α-2b (0.78 μg/kg) regimen underwent splenectomy before re-treatment. As a result, the patient could continue with a sufficient dose of Peg-IFN (1.53 μg/kg) in the re-treatment and attained HCV negativity at re-treatment week 24 and an SVR by extended treatment. Further study is needed on the issue of the effect of splenectomy or PSE in re-treatment on the efficacy of re-treatment with Peg-IFN plus ribavirin therapy.

In the present study, the SVR rate was relatively high (56%) in patients with genotype 2. The patients who could not attain SVR on re-treatment (2 patients) had not attained a c-EVR in the previous treatment. And, among the patients with an RVR on re-treatment, all patients treated for 48 weeks attained an SVR (5 patients), while 40% (2/5) of patients treated for 24 weeks could not attain an SVR. Thus, in patients with genotype 2, as well as in those with genotype 1, the previous treatment response and response-guided therapy can be useful in decisions on the indication for re-treatment or the treatment duration on re-treatment. However, in this study, detailed analysis was not possible because of the small number of genotype 2 patients. Further investigation is needed to clarify this.

The limitation of the present study was that two types of Peg-IFN were used. As for the type of Peg-IFN, some reports have suggested that Peg-IFN α-2a has a stronger anti-viral effect than Peg-IFN α-2b [20, 21], and others have suggested that the two types of Peg-IFN have an almost equal anti-viral effect [22]. In this study, the HCV RNA negative rate at re-treatment week 12 was similar (α-2a, 59%, 13/22, vs. α-2b, 50%, 16/32, $p = 0.51$) between the patients with Peg-IFN α-2a and those with Peg-IFN α-2b. Furthermore, among 24 patients treated with Peg-IFN α-2a on re-treatment, an SVR rate of 38% was attained with 48-week treatment and an SVR rate of 60% was attained with 72-week treatment among patients with a p-EVR in the previous treatment, but no patient without a p-EVR in the previous treatment attained an SVR on re-treatment. Similarly, among 32 patients treated with Peg-IFN α-2b in re-treatment, an SVR rate of 56% was attained with 48-week treatment and an SVR rate of 79% was attained with 72-week treatment among patients with a p-EVR in the previous treatment, but no patient without a p-EVR in the previous treatment attained an SVR on re-treatment. As noted above, since the virologic responses to both Peg-IFNs among re-treated patients were similar, in this study we analyzed the effect of re-treatment without distinction of the type of Peg-IFN.

In conclusion, our results have demonstrated that the efficacy of re-treatment for genotype 1 patients who failed to show an SVR to previous treatment with Peg-IFN plus ribavirin could be predicted by the previous treatment response, especially in terms of p-EVR and a low HCV RNA level at the start of re-treatment. Re-treatment for 72 weeks led to clinical improvement for genotype 1 patients who attained HCV RNA negativity on re-treatment.

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Research Article

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Indications and limitations for aged patients with chronic hepatitis C in pegylated interferon alfa-2b plus ribavirin combination therapy

Tsugiko Oze[1], Naoki Hiramatsu[1,*], Takayuki Yakushijin[1], Kiyoshi Mochizuki[1], Masahide Oshita[2], Hideki Hagiwara[3], Eiji Mita[4], Toshifumi Ito[5], Hiroyuki Fukui[6], Yoshiaki Inui[7], Taizo Hijioka[8], Masami Inada[9], Kazuhiro Kaytayama[10], Shinji Tamura[11], Harumasa Yoshihara[12], Atsuo Inoue[13], Yasuharu Imai[14], Michio Kato[15], Takuya Miyagi[1], Yuichi Yoshida[1], Tomohide Tatsumi[1], Shinichi Kiso[1], Tatsuya Kanto[1], Akinori Kasahara[1], Tetsuo Takehara[1], Norio Hayashi[1]

[1]*Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Japan;* [2]*Osaka Police Hospital, Osaka, Japan;* [3]*Kansai Rousai Hospital, Amagasaki, Japan;* [4]*National Hospital Organization Osaka National Hospital, Osaka, Japan;* [5]*Osaka Koseinenkin Hospital, Osaka, Japan;* [6]*Yao Municipal Hospital, Yao, Japan;* [7]*Hyogo Prefectural Nishinomiya Hospital, Nishinomiya, Japan;* [8]*National Hospital Organization Osaka Minami Medical Center, Kawachinagano, Japan;* [9]*Toyonaka Municipal Hospital, Toyonaka, Japan;* [10]*Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan;* [11]*Minoh City Hospital, Minoh, Japan;* [12]*Osaka Rosai Hospital, Sakai, Japan;* [13]*Osaka General Medical Center, Osaka, Japan;* [14]*Ikeda Municipal Hospital, Ikeda, Japan;* [15]*National Hospital Organization Minami Wakayama Medical Center, Tanabe, Japan*

Background & Aims: This study investigated the efficacy and adverse effects of pegylated interferon (Peg-IFN) plus ribavirin therapy in aged patients with chronic hepatitis C (CH-C).

Methods: A total of 1040 naïve patients with CH-C (genotype 1, $n = 759$; genotype 2, $n = 281$), of whom 240 (23%) over 65 years old (y.o.), were treated with Peg-IFN alfa-2b plus ribavirin and assessed after being classified into five categories, according to age.

Results: The discontinuance rate was higher for patients over 70 y.o. (36%), the most common reason being anemia. In the presence of genotype 1, the SVR rate was similar (42–46%) among patients under 65 y.o. and declined (26–29%) among patients over 65 y.o. For patients over 65 y.o., being male (Odds ratio, OR, 3.5, $p = 0.035$) and EVR (OR, 83.3, $p < 0.001$) were significant factors for SVR, in multivariate analysis. The Peg-IFN dose was related to EVR, and when EVR was attained, 76–86% of patients over 65 y.o. achieved SVR. SVR was not achieved (0/35, 0/38, respectively) if a 1-log decrease and a 2-log decrease were not attained at week 4 and week 8, respectively. In the presence of genotype 2, the SVR rate was similar (70–71%) among patients under 70 y.o. and declined among patients over 70 y.o. (43%).

Conclusions: Aged patients up to 65 y.o. with genotype 1 and 70 y.o. with genotype 2 can be candidates for Peg-IFN plus ribavirin therapy. The response-guided therapy can be applied for aged patients with genotype 1.

Introduction

Pegylated interferon (Peg-IFN) plus ribavirin combination therapy has led to a marked progress in the treatment of chronic hepatitis C (CH-C) [1–4]. However, in aged patients, problems remain with respect to its anti-viral effect and tolerability [5–9]. Recently, the addition of a protease inhibitor to Peg-IFN plus ribavirin combination therapy has been reported, on the one hand, to improve the anti-viral effect, and, on the other hand, to increase side effects, especially severe anemia [10–11].

Therefore, this new therapy does not solve the problems encountered when treating aged patients.

With aging, the progression of liver fibrosis and the occurrence of hepatocellular carcinoma (HCC) have been shown to be accelerated, especially in patients over 60 y.o. [12–14]. In general, the anti-viral therapy can lead to an improvement in liver fibrosis and thus diminish the risk of HCC and ameliorate the prognosis in patients with CH-C [15–21]. Among aged patients, those results are mainly achievable upon eradication of the hepatitis C virus (HCV) [18,21]. Accordingly, the first goal of treatment of aged patients with a high-risk of HCC should be HCV elimination.

Thus, a treatment strategy, aiming at the improvement of the anti-viral efficacy in aged patients, should be established based on detailed large-scale studies.

Some points need to be further elucidated when using the Peg-IFN plus ribavirin combination therapy for the treatment of aged patients with CH-C: (i) the characteristics before treatment

Keywords: Pegylated interferon plus ribavirin therapy; Chronic hepatitis C; Aged patients.
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*Corresponding author. Address: Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita City, Osaka 565-0871, Japan. Tel.: +81 6 6879 3621; fax: +81 6 6879 3629.
E-mail address: hiramatsu@gh.med.osaka-u.ac.jp (N. Hiramatsu).

Abbreviations: HCV, hepatitis C virus; CH-C, chronic hepatitis C; HCC, hepatocellular carcinoma; Peg-IFN, pegylated interferon; SVR, sustained virologic response; RVR, rapid virologic response; EVR, early virologic response; LVR, late virologic response; NR, non-response; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; Plt, platelet; G-CSF, granulocyte-macrophage colony stimulating factor.

that would lead to the successful elimination of HCV, (ii) the prediction factors of treatment efficacy after the initiation of the therapy, and (iii) the utility of a response-guided therapy established in the treatment.

In the present study, using a large cohort, we aimed at clarifying these points taking into account the patients' age.

Patients and methods

Patients

This study was a retrospective, multicenter trial conducted by the Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 1040 naïve patients with CH-C were enrolled between December 2004 and June 2007. All patients were Japanese, infected with a viral load of more than 10^5 IU/ml, and treated with a combination of Peg-IFN alfa-2b plus ribavirin. Patients were excluded from the study if they had decompensated cirrhosis or other forms of liver disease (alcohol liver disease, autoimmune hepatitis), co-infection with hepatitis B or anti-human immunodeficiency virus. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki 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). Treatment duration was 48 weeks for patients with genotype 1 and 24 weeks for those with genotype 2. As a starting dose, Peg-IFN alfa-2b was given once weekly, at a dosage of 1.5 μg/kg, and ribavirin was given 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 and discontinuance

Dose modification followed, as a rule, the manufacturer's drug information on the intensity of the hematologic adverse effects. The Peg-IFN alfa-2b dose was reduced to 50% of the assigned dose when the white blood cell (WBC) count was below 1500/mm^3, the neutrophil count below 750/mm^3 or the platelet (Plt) count below 8×10^4/mm^3, and was discontinued when the WBC count was below 1000/mm^3, the neutrophil count below 500/mm^3 or the Plt count below 5×10^4/mm^3. Ribavirin was also reduced from 1000 to 600 mg, 800 to 600 mg, 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. Peg-IFN alfa-2b and ribavirin had to be both discontinued if there was a need to discontinue either of them. No ferric medicine or hematopoietic growth factors, such as epoetin alpha, or granulocyte-macrophage colony stimulating factor (G-CSF), were administered.

Virologic assessment and definition of virologic response

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) and qualitatively analyzed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/ml; Roche Diagnostics). The rapid virologic response (RVR) was defined as undetectable serum HCV RNA at week 4; the early virologic response (EVR) as undetectable serum HCV RNA at week 12; and the late virologic response (LVR) as detectable serum HCV RNA at week 12 and undetectable serum HCV RNA at week 24. Moreover, the sustained virologic response (SVR) was defined as undetectable serum HCV RNA, 24 weeks after treatment.

According to the protocol, genotype 1 patients, with less than a 2-log decrease in HCV RNA level at week 12 compared to the baseline, or with detectable serum HCV RNA at week 24, had to stop the treatment and were regarded as non-response (NR). Treatment discontinuance was evaluated except for those patients who had discontinued the treatment at up to 24 weeks, due to absence of response. Anti-viral efficacy was evaluated, for all study patients, using the intention-to-treat analysis (ITT analysis) and the per protocol analysis (PP analysis) for patients without treatment discontinuation due to side effects, and was assessed considering the definition of EVR or LVR for genotype 1, and RVR or non-RVR for genotype 2, as previously reported [1].

Assessment of drug exposure

The amounts of Peg-IFN alfa-2b and ribavirin, taken by each patient during the full treatment period, were evaluated by reviewing the medical records. The mean doses of Peg-IFN alfa-2b and ribavirin were calculated individually as averages, on the basis of the body weight at baseline: Peg-IFN alfa-2b expressed as μg/kg/week, ribavirin expressed as mg/kg/day.

Statistical analysis

Patients' baseline data are expressed as means ± SD or median values. To analyze the difference between baseline data, ANOVA or Mantel–Haenszel Chi-square test were performed. Factors associated with the viral response were assessed by univariate analysis using the Mann–Whitney *U* test or Chi-square test and multivariate analysis using logistic regression analysis. A two-tailed p value <0.05 was considered significant. The analysis was conducted with SPSS version 15.0J (SPSS Inc., Chicago, IL).

Results

Patient's profile

Baseline characteristics of the patients categorized by age are shown in Table 1.

Genotype 1 patients (n = 759) were distributed into five categories: 266 patients were under 55 y.o. (group 1A), 159 were 55–59 y.o. (group 1B), 149 were 60–64 y.o. (group 1C), 134 were 65–69 y.o. (group 1D), and 51 were 70 y.o. or older (group 1E). With advancing age, the male-to-female ratio and peripheral blood cell count (WBC, neutrophil count, Red blood cell (RBC), Hb, Plt) decreased significantly. Patients with a progression of liver fibrosis (METAVIR fibrosis score 3 or 4) significantly increased with age (Table 1A).

Genotype 2 patients (n = 281) were also distributed into five categories: 145 patients were under 55 y.o. (group 2A), 43 were 55–59 y.o. (group 2B), 38 were 60–64 y.o. (group 2C), 41 were 65–69 y.o. (group 2D), and 14 were 70 y.o. or older (group 2E). As observed in genotype 1 patients, the peripheral blood cell count decreased and the ratio of advanced fibrosis (score 3–4) increased significantly with age (Table 1B). For both genotypes, the initial doses of Peg-IFN in patients over 70 y.o. were lower than in those under 70 y.o., this was not the case for the ribavirin doses.

Dose reduction and discontinuance for adverse event

The overall discontinuance rate of treatment was 15% (140/919); 18% (112/639) for genotype 1 and 10% (28/280) for genotype 2, respectively. Table 2 shows the reason for and the rate of treatment discontinuance according to age. The discontinuance rate increased with age, being 10% (36/363) for patients under 55 y.o., 15% (27/182) for patients with 55–59 y.o., 17% (28/169) for patients with 60–64 y.o., 19% (28/147) for patients with 65–70 y.o., and significantly higher, 36%, (21/58) for patients over 70 y.o. The discontinuance of treatment due to hemolytic anemia was significantly higher for patients over 70 y.o. as compared to those under 70 y.o. (<70 y.o., 1% (9/861) vs. ⩾70 y.o., 16% (9/58), p <0.0001).

The rate without dose reduction of both drugs decreased with age (<55 y.o., 41% (171/411); 55–59 y.o., 20% (40/202); 60–64 y.o., 26% (48/187); 65–69 y.o., 23% (41/175); ⩾70 y.o., 18% (12/65)). In the presence of genotype 1, the mean dose of Peg-IFN

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Table 1. Baseline characteristics of patients.

Patients with genotype 1							
Factor		<55 y.o.	55 - 59 y.o.	60 - 64 y.o.	65 - 69 y.o.	≥70 y.o.	*p* value
Number		266	159	149	134	51	
Age (y.o.)		44.4 ± 8.1	56.9 ± 1.4	62.0 ± 1.4	66.8 ± 1.4	71.4 ± 1.7	<0.001
Sex: male / female		160 / 106	64 / 95	57 / 92	54 / 80	23 / 28	<0.001
Body weight (kg)		64.6 ± 11.7	58.3 ± 9.4	58.1 ± 9.6	56.3 ± 9.3	56.3 ± 9.2	<0.001
White blood cells (/mm³)		5608 ± 1668	4901 ± 1664	4888 ± 1488	5113 ± 1426	4883 ± 1511	<0.001
Neutrophils (/mm³)		2923 ± 1214	2425 ± 1031	2559 ± 1155	2535 ± 1017	2599 ± 1149	<0.001
Red blood cells (×10⁴/mm³)		454 ± 47	432 ± 38	427 ± 40	424 ± 37	424 ± 46	<0.001
Hemoglobin (g/dl)		14.4 ± 1.5	13.8 ± 1.2	13.7 ± 1.3	13.6 ± 1.2	13.7 ± 1.4	<0.001
Platelets (×10⁴/mm³)		18.6 ± 6.2	16.3 ± 5.7	15.4 ± 5.3	15.1 ± 5.0	14.4 ± 4.2	<0.001
AST (IU/L)		62 ± 50	62 ± 45	64 ± 46	72 ± 45	64 ± 40	0.295
ALT (IU/L)		79 ± 68	76 ± 64	73 ± 63	77 ± 58	65 ± 41	0.657
Serum HCV RNA (KIU/ml)*		1800	1600	1700	1700	1700	0.691
Histology (METAVIR)†	Fibrosis, 0 - 2 / 3 - 4	177 / 19	99 / 20	90 / 19	76 / 28	21 / 9	0.001
	Activity, 0 - 1 / 2 - 3	117 / 79	63 / 56	59 / 50	47 / 57	13 / 16	0.146
Peg-IFN dose (μg/kg/week)¶		1.47 ± 0.14	1.47 ± 0.16	1.46 ± 0.18	1.44 ± 0.18	1.36 ± 0.24	<0.001
Ribavirin dose (mg/kg/day)¶		11.5 ± 1.1	11.5 ± 1.4	11.5 ± 1.4	11.5 ± 1.7	11.2 ± 2.2	0.65

Patients with genotype 2							
Factor		<55 y.o.	55 - 59 y.o.	60 - 64 y.o.	65 - 69 y.o.	≥70 y.o.	*p* value
Number		145	43	38	41	14	
Age (y.o.)		40.9 ± 8.9	56.7 ± 1.3	62.3 ± 1.4	66.7 ± 1.5	71.8 ± 1.8	<0.001
Sex: male / female		78 / 67	17 / 26	17 / 21	18 / 23	6 / 8	0.441
Body weight (kg)		63.4 ± 12.0	59.5 ± 11.5	58.6 ± 11.7	58.5 ± 9.8	55.9 ± 6.8	0.783
White blood cells (/mm³)		6011 ± 1965	4874 ± 1346	4982 ± 1210	5079 ± 1877	4414 ± 871	<0.001
Neutrophils (/mm³)		3214 ± 1511	2468 ± 971	2576 ± 950	2492 ± 1119	2521 ± 683	0.001
Red blood cells (×10⁴/mm³)		454 ± 48	430 ± 42	432 ± 50	430 ± 43	408 ± 48	<0.001
Hemoglobin (g/dl)		14.3 ± 1.6	13.5 ± 1.3	13.9 ± 1.4	13.9 ± 1.3	13.3 ± 1.2	0.001
Platelets (×10⁴/mm³)		21.3 ± 5.4	18.3 ± 6.1	17.0 ± 5.2	15.8 ± 5.4	13.9 ± 4.7	<0.001
AST (IU/L)		53 ± 59	57 ± 45	55 ± 38	83 ± 48	68 ± 29	0.029
ALT (IU/L)		65 ± 59	73 ± 70	68 ± 62	105 ± 62	78 ± 43	0.008
Serum HCV RNA (KIU/ml)*		1700	1100	900	1100	500	0.008
Histology (METAVIR)‡	Fibrosis, 0 - 2 / 3 - 4	102 / 0	25 / 3	29 / 2	21 / 9	7 / 1	<0.001
	Activity, 0 - 1 / 2 - 3	68 / 34	18 / 10	18 / 13	9 / 21	5 / 3	0.01
Peg-IFN dose (μg/kg/week)¶		1.48 ± 0.16	1.48 ± 0.14	1.45 ± 0.18	1.46 ± 0.15	1.28 ± 0.26	0.001
Ribavirin dose (mg/kg/day)¶		11.5 ± 1.1	11.4 ± 1.2	11.5 ± 1.4	11.3 ± 1.6	11.0 ± 1.4	0.55

*, Data shown are median values.
†, 201 Missing.
‡, 82 Missing.
¶, Initial doses.

during the whole treatment period was lower (1.1 ± 0.3 μg/kg/week) for patients over 70 y.o. than for those under 70 y.o. (1.3 ± 0.3 μg/kg/week) and that of ribavirin decreased with age (<55 y.o., 10.3 ± 1.9 mg/kg/day; 55–59 y.o., 9.8 ± 1.9 mg/kg/day; 60–64 y.o., 9.3 ± 2.3 mg/kg/day; 65–69 y.o., 9.2 ± 2.3 mg/kg/day; ⩾70 y.o., 8.5 ± 2.5 mg/kg/day). The same tendency was observed with genotype 2.

Sustained virologic response

In genotype 1 patients, the overall SVR rate was 40% (305/759), being 46% (123/266) for group 1A, 44% (70/159) for group 1B, 42% (62/149) for group 1C, 26% (35/134) for group 1D, and 29% (15/51) for group 1E, following ITT analysis. The same tendency was observed using the PP analysis ($n = 647$). The SVR rates for patients over 65 y.o. were significantly lower than those for patients under 65 y.o. (ITT analysis: ⩾65 y.o., 27% vs. <65 y.o., 44%, $p < 0.0001$; PP analysis: ⩾65 y.o., 31% vs. <65 y.o., 50%, $p < 0.0001$) (Fig. 1A). Among genotype 1 patients over 65 y.o., the SVR rate was significantly lower for female patients than for male patients (ITT analysis: male, 40% (31/77) vs. female, 18% (19/108), $p < 0.001$; PP analysis: male, 49% (27/55) vs. female, 20% (18/90), $p < 0.001$).

Moreover, for genotype 2 patients, the overall SVR rate was 78% (220/281), being 88% (128/145) for group 2A, 70% (30/43) for group 2B, 71% (27/38) for group 2C, 71% (29/41) for group 2D, and 43% (6/14) for group 2E, following ITT analysis. The same tendency was observed with the PP analysis ($n = 253$). The SVR rates for patients over 70 y.o. were significantly lower than those for patients under 70 y.o. (ITT analysis: ⩾70 y.o., 43% vs. <70 y.o., 80%, $p < 0.0001$; PP analysis: ⩾70 y.o., 56% vs. <70 y.o., 85%, $p < 0.05$) (Fig. 1B). Among patients over 70 y.o. with genotype 2, the difference according to gender was not clear because of the small sample.

Table 2. Reasons for treatment discontinuation.

Factor	<55 y.o. (n = 363)	55 - 59 y.o. (n = 182)	60 - 64 y.o. (n = 169)	65 - 69 y.o. (n = 147)	≥70 y.o. (n = 58)	Total (n = 919)
Neutropenia	2	3	0	0	0	5
Thrombopenia	1	0	1	1	0	3
Anemia	0	4	3	2	9	18
Fatigue	1	1	3	3	1	9
Gastrointestinal disorder	2	1	0	0	1	4
Cough, Dyspnea	1	0	3	0	0	4
Vertigo	1	0	0	0	3	4
Psychosis (depression)	7 (3)	7 (3)	4 (4)	3 (3)	2 (2)	23
Rash	5	2	5	7	1	20
Thyroid dysfunction	2	0	2	0	0	4
Fundal hemorrhage	0	2	0	2	0	4
Drug-induced hepatitis	3	1	0	0	0	4
Interstitial pneumonia	0	1	0	1	1	3
Cerebral hemorrhage, infarction	2	0	0	1	0	3
Others	9	5	7	8	3	32
Total	36 (10%)	27 (15%)	28 (17%)	28 (19%)	21 (36%)	140 (15%)

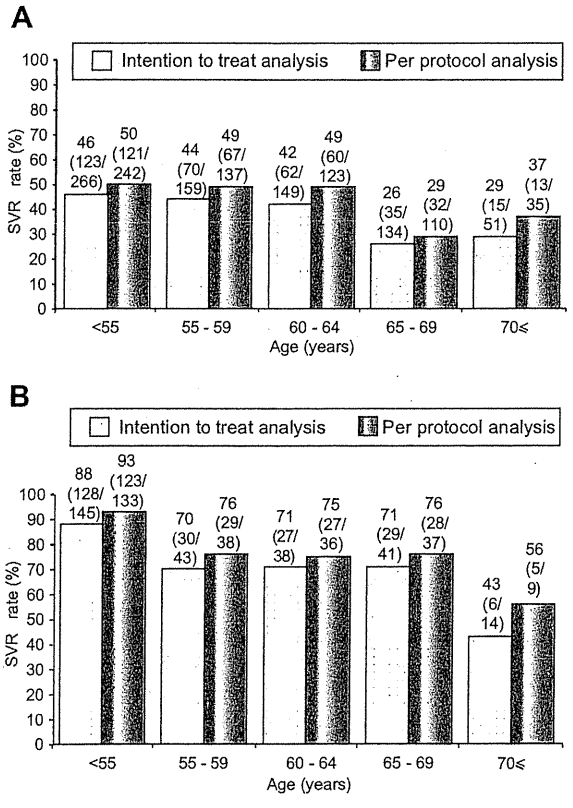

Fig. 1. SVR rate according to age. (A) Genotype 1. (B) Genotype 2.

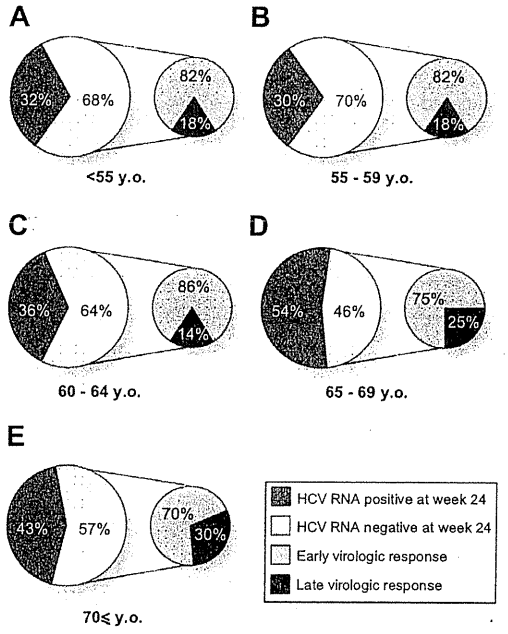

Fig. 2. Antiviral effect during treatment according to age. (A) <55 y.o. (B) 55–59 y.o. (C) 60–64 y.o. (D) 65–69 y.o. (E) ≥70 y.o.

Timing of HCV RNA negativation for genotype 1, according to age

Treatment responses distributing EVR, LVR, and NR according to age are shown in Fig. 2. The rates of NR were similar in patient groups under 65 y.o. (30–36%), but increased in almost half of the patients over 65 y.o. (p <0.0001). Moreover, among the virologic responders, the proportion of LVR tended to increase in patients over 65 y.o. (25–30%) compared to patients under 65 y.o. (14–18%) (p = 0.06).

SVR rate according to the timing of HCV RNA negativation

SVR rates according to EVR or LVR in genotype 1, and RVR or non-RVR in genotype 2 are summarized in Table 3. Genotype 1 patients with EVR achieved high SVR rates regardless of age; in particular, if EVR had been attained, 76% of patients with 65–69

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Table 3. SVR rate according to genotype and viral response in patients responding to PEG-IFN plus ribavirin combination therapy.

Factor	<55 y.o.	55 - 59 y.o.	60 - 64 y.o.	65 - 69 y.o.	≥70 y.o.
Genotype 1					
with EVR, % (n)	85 (114/134)	79 (62/79)	81 (55/68)	76 (29/38)	86 (12/14)
with LVR, % (n)	23 (7/30)	29 (5/17)	46 (5/11)	23 (3/13)	17 (1/6)
Genotype 2					
with RVR, % (n)	93 (57/61)	82 (14/17)	85 (17/20)	92 (11/12)	100 (4/4)
without RVR*, % (n)	96 (22/23)	60 (6/10)	57 (4/7)	50 (4/8)	0 (0/3)

RVR, rapid virologic response.
EVR, early virologic response.
LVR, late virologic response.
*, Serum HCV RNA was detectable at week 4, but undetectable at week 24.

Table 4. Multivariate analysis for the factors associated with SVR among all patients.

Factor	Category	Odds ratio	95% CI	*p*
Age (y.o.)	<65 / ≥65	0.485	0.295 - 0.799	0.005
Sex	male / female	0.524	0.353 - 0.777	0.001
Platelets ($\times 10^4/mm^3$)	<12 / ≥12	1.780	1.039 - 3.049	0.040
Serum HCV RNA (KIU/ml)	<2000 / ≥2000	0.599	0.401 - 0.896	0.010
Histology (METAVIR): Fibrosis	0 - 2 / 3 - 4	0.599	0.333 - 1.076	0.090

y.o. and 86% of patients over 70 y.o. achieved SVR, and these SVR rates compared favorably with those of younger patients. On the other hand, the SVR rates for patients with LVR ranged from 17% to 46%, which were lower than those for EVR patients in each age group, and no significant differences of SVR rates were found among LVR patients by age.

With genotype 2, patients with RVR achieved high SVR rates ranging from 82% to 100% regardless of age. Even for patients without RVR, 96% of those under 55 y.o. attained SVR, a rate that was significantly higher than that for patients over 55 y.o. (50%, 14/28) (p <0.001).

Factors associated with SVR for genotype 1

The factors associated with SVR were assessed for the variables shown in Table 1. The factors selected as significant by the univariate analysis: age, gender, WBC, neutrophils, RBC, Hb, Plt, aspartate aminotransferase, serum HCV RNA level, the degree of liver fibrosis, and the initial dose of Peg-IFN, were evaluated by multivariate logistic regression analysis. The factor of age over 65 y.o. was the independent factor for SVR (p = 0.005), apart from the gender (p = 0.001), Plt value (p <0.05), and serum HCV RNA level (p = 0.01) (Table 4).

Factors associated with EVR and SVR for patients over 65 y.o. with genotype 1

The results of univariate analysis for EVR among patients over 65 y.o. are shown in Table 5A. Gender, Plt value, and mean dose of Peg-IFN during the first 12 weeks were factors significantly associated with EVR. In multivariate analysis, the mean dose of Peg-IFN during the first 12 weeks was the independent factor for EVR (p = 0.03), apart from gender (p = 0.002) (Table 5B). The EVR rates were 41% (41/101) in patients who received ⩾1.2 μg/kg/week on average during the first 12 weeks, and declined to 36% (8/22) in patients given 0.9–1.2 μg/kg/week of Peg-IFN, and to 14% (3/22) in patients administered with <0.9 μg/kg/week of Peg-IFN.

The baseline and on-treatment factors, which are correlated with the SVR among the patients over 65 y.o., were assessed by univariate and multivariate analyses. Univariate analysis showed that factors significantly associated with SVR were gender and virologic response (Table 6A), and they were also selected as significant independent factors in multivariate analysis (p = 0.035, p <0.001) (Table 6B).

Negative prediction of SVR for patients over 65 y.o. with genotype 1

We tried positive and negative predictions of SVR for aged patients, focusing on the decrease of HCV RNA at treatment week 4 and 8. The SVR rate was 47% (29/62) for patients with more than a 1-log decrease in HCV RNA level at week 4, while no patients with less than a 1-log decrease at week 4 attained SVR (0/35) (p <0.0001). Similarly, 55% (35/64) of patients with more than a 2-log decrease at week 8 attained SVR, whereas no patients with less than a 2-log decrease at week 8 attained SVR (0/38) (p <0.0001).

Discussion

Peg-IFN plus ribavirin combination therapy can improve anti-viral efficacy and is presently recommended as first-line therapy [1–4]. However, with respect to aged patients with CH-C, there have been only a few small-scale cohort studies which reported poor anti-viral effect and poor tolerability in comparison with non-aged patients [5–9]. The problem in the treatment of aged patients with CH-C is most serious in Japan, because HCV carriers in Japan are 10–20 years older than those in the United States and European countries [22]. Therefore, in the present study, we examined the efficacy and prevalence of side effects with a focus on patient's age using a large-scale cohort.

Table 5. Factors associated with EVR among patients over 65 y.o.

Univariate analysis				
Factor		EVR	Non-EVR	*p* value
Number		52	93	
Age (y.o.)		67.9 ± 2.3	67.8 ± 2.5	0.66
Sex: male / female		28 / 24	27 / 66	0.003
White blood cells (/mm³)		5063 ± 1474	5001 ± 1422	0.76
Neutrophils (/mm³)		2566 ±1110	2551 ± 1071	0.87
Red blood cells (×10⁴/mm³)		426 ± 36	421 ± 38	0.64
Hemoglobin (g/dl)		13.7 ± 1.2	13.5 ± 1.2	0.21
Platelets (×10⁴/mm³)		16.5 ± 5.5	14.0 ± 4.6	0.009
AST (IU/L)		70 ± 51	70 ± 40	0.49
ALT (IU/L)		76 ± 58	70 ± 41	0.80
Serum HCV RNA (KIU/ml)*		1700	1900	0.62
Histology (METAVIR)†	Fibrosis, 0 - 2 / 3 - 4	25 / 10	47 / 20	0.54
	Activity, 0 - 1 / 2 - 3	16 / 19	29 / 37	0.52
Peg-IFN dose (μg/kg/week)‡		1.35 ± 0.24	1.25 ± 0.31	0.03
Ribavirin dose (mg/kg/day)‡		10.0 ± 2.2	9.6 ± 2.3	0.40

Multivariate analysis				
Factor	Category	Odds ratio	95% CI	*p* value
Sex	male / female	0.309	0.149 - 0.644	0.002
Platelets (×10⁴/mm³)	<12 / ≥12	-	-	N.S
Peg-IFN dose (μg/kg/week)‡	<1.2 / ≥1.2	2.481	1.079 - 5.705	0.03

*, Data shown are median values.
†, 43 Missing.
‡, Mean doses during 0 to 12 weeks.
N.S., not statistically significant.

Table 6. Factors associated with SVR among patients over 65 y.o.

Univariate analysis				
Factor		SVR	Non-SVR	*p* value
Number		45	100	
Age (y.o.)		68.0 ± 2.4	67.7 ± 2.5	0.45
Sex: male / female		27 / 18	28 / 72	<0.001
White blood cells (/mm³)		5006 ± 1516	5030 ± 1409	0.81
Neutrophils (/mm³)		2575 ±1130	2548 ± 1063	0.96
Red blood cells (×10⁴/mm³)		427 ± 40	421 ± 36	0.53
Hemoglobin (g/dl)		13.8 ± 1.3	13.5 ± 1.2	0.14
Platelets (×10⁴/mm³)		16.1 ± 5.6	14.3 ± 4.7	0.09
AST (IU/L)		71 ± 54	69 ± 40	0.47
ALT (IU/L)		76 ± 56	70 ± 43	0.77
Serum HCV RNA (KIU/ml)*		1700	2000	0.51
Histology (METAVIR)†	Fibrosis, 0 - 2 / 3 - 4	21 / 8	51 / 22	1.00
	Activity, 0 - 1 / 2 - 3	14 / 15	31 / 41	0.66
Peg-IFN dose (μg/kg/week)‡		1.27 ± 0.28	1.23 ± 0.33	0.31
Ribavirin dose (mg/kg/day)‡		8.8 ± 2.1	9.1 ± 2.5	0.38
Virologic response: EVR / non-EVR		41 / 4	11 / 89	<0.001

Multivariate analysis				
Factor	Category	Odds ratio	95% CI	*p* value
Sex	male / female	0.283	0.088 - 0.914	0.035
Virologic response	EVR / non-EVR	0.012	0.004 - 0.043	<0.001

*, Data shown are median values.
†, 43 Missing.
‡, Mean doses during treatment.

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With respect to the side effects and discontinuance rate of treatment in aged patients with CH-C, treated with Peg-IFN plus ribavirin combination therapy, Reddy et al. reported that there was no difference related to the incidence and reason for side effects between non-aged and aged patients [6]. Another paper reported that the incidence of side effects was more frequent in aged patients [5]. In our study, not only the continuance rate without reduction of both drug decreased with age, but also the discontinuance rate of treatment increased with age, with a third of the patients over 70 y.o. discontinuing the treatment. The discrepancy, existing between our results and those reported in the former study cited above, is due to the difference in the number of aged patients enrolled; Reddy' s study analyzed a small cohort including only a few cases of patients over 65 y.o. and classified all those over 50 y.o. as aged patients.

Discontinuance of treatment due to progression of anemia was significantly higher in patients over 70 y.o., accounting for 43% (9/21) of the discontinuance in this group. Although the ratio of advanced fibrosis (score 3–4) increased with age, the high discontinuance rate due to anemia among patients over 70 y.o. was similar regardless of the progression of fibrosis (F0-2: <70 y.o., 1% (6/559) vs. ⩾70 y.o., 21% (6/28), p <0.0001; F3-4: <70 y.o., 0% (0/83) vs. ⩾70 y.o., 22% (2/9), p <0.0001). It is possible that poor hematopoietic function and renal function led to the progression of anemia in aged patients. For patients who develop severe anemia, using epoetin alpha or taribavirin, which are ribavirin prodrugs, has been shown to result in a lower incidence of anemia, although no significant increase of SVR has been reported so far, even with the addition of taribavirin to Peg-IFN [23–24].

With genotype 1 patients, the SVR rates were almost equal up to 65 y.o. (49–50%), but decreased to 31% (45/145) among the patients that were over 65 y.o., and even for those who completed the entire treatment schedule in this study. Since the degree of liver fibrosis and drug exposure have been shown to be associated with anti-viral efficacy, the progression of liver fibrosis or decrease of drug exposure with age could account for the reduction of SVR rate among the aged patients. However, the stratified analysis, according to the progression of liver fibrosis and drug exposure, revealed that older patients still yielded low a SVR rate (F0-2, Peg-IFN during the first 12 weeks ⩾1.2 μg/kg/week:<65 y.o., 55% (143/261) vs. ⩾65 y.o., 33% (15/46), p <0.0001; F0-2, Peg-IFN during the first 12 weeks <1.2 μg/kg/week:<65 y.o., 43% (26/60) vs. ⩾65 y.o., 23% (6/26), p = 0.07), which means that older patients would be difficult to treat. From our results showing a low SVR rate and a high discontinuance rate for patients over 65 y.o., the genotype 1 patients under 65 y.o were those who benefited the most from Peg-IFN plus ribavirin combination therapy. The high prevalence of treatment failure (non-SVR) among the aged patients seems to be due to the high populations of NR and LVR (Fig. 2). A high population of LVR is considered to lead to a higher transient response rate among aged patients, since those over 65 y.o. with LVR showed a much higher relapse rate (79%, 15/19) than those with EVR (21%, 11/52) (p <0.0001), as can be seen from Table 3.

In this study, multivariate analysis for SVR, in patients over 65 y.o., showed that the factors associated with SVR were EVR and gender. This indicates that better SVR can be expected even with older patients if EVR is attained and response-guided therapy guidelines can be useful for aged patients. A low SVR rate among aged female patients was as previously reported [7], although the mechanism remains unclear. This finding suggests that female patients should be treated before 65 y.o.

The next question is how aged patients should be treated in order to attain EVR. We have examined the impact of drug exposure on treatment efficacy [25–26] and reported that Peg-IFN is dose-dependently correlated with EVR [25]. In this study, the dose-dependent efficacy of Peg-IFN for EVR was also revealed in aged patients over 65 y.o., with less than 0.9 μg/kg/week of Peg-IFN leading to a low EVR rate for aged patients. If patients are difficult to treat with more than 1.2 μg/kg/week of Peg-IFN, using as much Peg-IFN as possible is desirable, in order to attain higher EVR rates. Accordingly, a reduction of Peg-IFN to 80% may need to be considered, although the manufacturer's drug information recommends reducing the dose of Peg-IFN to 50% of the assigned one. Since reduction of Peg-IFN has been reported to not affect the SVR rate after HCV RNA disappearance [26], using G-CSF for aged patients who develop severe neutropenia can be beneficial, especially in the first 12 weeks.

We also examined the negative prediction of SVR, i.e. an HCV RNA decrease at an earlier point of treatment than the usual prediction at treatment week 12 of a 2-log decrease, among aged patients with CH-C treated by Peg-IFN plus ribavirin combination therapy. We found that none of the patients without a 1-log decrease at week 4 or a 2-log decrease at week 8 could attain SVR, even if the complete treatment duration was given, the negative predictive value (NPV) for SVR equaled 100%. This earlier prediction is applied just as well to aged patients as to non-aged patients in order to avoid additional adverse effects. Recently, a genetic polymorphism near the *IL28B* gene has been reported to be associated with non-response to Peg-IFN plus ribavirin combination therapy [27–29], which is beneficial to patients. Nevertheless, even in the presence of this genetic polymorphism, NPV for SVR remains at 57–87%; 100% accuracy is not guaranteed. Thus, in addition to the pretreatment prediction, an earlier negative prediction for SVR during treatment is also considered to be useful.

We have shown in this study that, in the presence of genotype 2, HCV was easily eliminated even among aged patients; the SVR rates were over 75% for patients who had completed the treatment, and these rates were similar up to 70 y.o. The SVR rate of genotype 2 patients over 70 y.o. was 43%, however, the age limitation of the treatment among patients over 70 y.o. remains unclear, because of the small number of patients enrolled in this study. We have reported that the reduction of treatment drugs had little effect on anti-viral efficacy for patients with genotype 2, meaning that SVR can be attained even with aged patients who are usually given lower drug doses than non-aged patients [30]. Patients under 70 y.o. with genotype 2 should, at least, benefit from this therapy. The SVR rate was maintained among genotype 2 patients being 65–69 y.o., compared to genotype 1 patients. The higher efficacy with shorter treatment duration in genotype 2 aged patients can account for it.

In conclusion, the strategy of a response-guided therapy and an earlier negative prediction for SVR may be beneficial for aged patients, especially those with genotype 1. At present, aged patients up to 65–70 y.o. with CH-C can be candidates for Peg-IFN plus ribavirin combination therapy, if its efficacy and adverse effects are fully taken into account. At the same time, there is an urgent need to establish new treatment procedures, such as combination therapy with protease inhibitor plus polymerase inhibitor without Peg-IFN or ribavirin, for non-responders or patients

with poor tolerability for Peg-IFN plus ribavirin combination therapy among aged patients.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this paper.

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ORIGINAL ARTICLE

Comprehensive immunological analyses of colorectal cancer patients in the phase I/II study of quickly matured dendritic cell vaccine pulsed with carcinoembryonic antigen peptide

Mitsuru Sakakibara · Tatsuya Kanto · Michiyo Hayakawa · Shoko Kuroda · Hideki Miyatake · Ichiyo Itose · Masanori Miyazaki · Naruyasu Kakita · Koyo Higashitani · Tokuhiro Matsubara · Naoki Hiramatsu · Akinori Kasahara · Tetsuo Takehara · Norio Hayashi

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Abstract Dendritic cell (DC) vaccine has been used to treat patients with advanced colorectal cancer (CRC). The results of vaccine-induced clinical responses have not always been satisfactory partially because of DC incompetence. In order to evaluate the feasibility of novel mature DCs for therapeutic adjuvants against CRC, we conducted clinical trials with carcinoembryonic antigen (CEA) peptide-loaded DC quickly generated with a combination of OK432 (Streptococcuspyogenes preparation), prostanoid, and interferon-α (OPA-DC). In the ten patients enrolled in this study, the OPA-DC vaccine was well tolerated and administered four times every 2 weeks except for two patients, who were switched to other treatments due to disease progression. Among the eight evaluable patients, one displayed stable disease (SD), while the remaining seven showed progressive disease (PD). In the SD patient, natural killer (NK) cell frequency and cytolytic activity were increased. In the same patient, the frequency of CEA-specific cytotoxic T cells (CTLs) increased stepwise with repetitive vaccinations; however, most of the CTLs exhibited central memory phenotype. In those with PD, NK cells proliferated well regardless of failure of response, whereas CTLs failed to do so. We concluded that the OPA-DC vaccine is well tolerated and has immune-stimulatory capacity in patients with CRC. Additional modulation is needed to attain significant clinical impact.

Keywords Dendritic cell · Vaccine · Cancer · Clinical study

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M. Sakakibara · T. Kanto (✉) · M. Hayakawa · M. Miyazaki · N. Kakita · K. Higashitani · T. Matsubara · N. Hiramatsu · T. Takehara
Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: kantot@gh.med.osaka-u.ac.jp

T. Kanto · S. Kuroda
Department of Dendritic Cell Biology and Clinical Applications, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

H. Miyatake
Osaka Police Hospital, Osaka, Japan

I. Itose · N. Hayashi
Kansai Rosai Hospital, Hyogo, Japan

A. Kasahara
Department of General Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Introduction

Colorectal cancer (CRC) is one of the most intractable malignant diseases causing the death of 600 thousand people annually around the world. Recently, the development of new chemotherapeutic regimens including molecular-targeted drugs has significantly improved the clinical outcomes of patients with CRC [1]. However, the severe adverse effects of chemotherapeutic agents often deteriorate their quality of life (QOL), thus limiting continuation of therapy at full dose. The development of better therapies against CRC is needed.

Dendritic cells (DCs) are the most potent antigen-presenting cells that enhance innate and adaptive immune reactions. Previous studies have demonstrated that antigen-loaded DC is one of the most promising candidates for a

therapeutic vaccine to induce tumor-specific immune reactions and preferable clinical responses in cancer patients with limited side effects. However, less than 10% of vaccinated patients showed favorable clinical responses [2, 3]. One of the primary reasons for such unsatisfactory results may be that the DCs have not been fully exploited to induce anti-tumor immunity.

Previous DC-vaccine studies have shown that mature DCs are better than immature ones for inducing anti-tumor responses in patients. The protocols of maturation stimuli are yet to be standardized. Although a combination stimulus using interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and prostaglandin (PG)-E2 is widely used as a monocyte-conditioned medium (MCM) mimic for monocyte-derived DCs (MoDCs), it lacks the ability to promote DCs to secrete IL-12p70 [4], a well-known enhancer of cytotoxic activity of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) [5, 6]. From the mechanistic point of view, DCs loaded with antigens migrate into draining lymph nodes, where they produce IL-12p70 and activate NK cells, prime type-1 helper-T (Th1) cells and CTLs [7, 8]. We have previously reported that novel mature DCs (OPA-DCs) more effectively exert such functions than MCM-mimic DCs, which can be generated even from monocytes of refractory cancer patients within 3 days by using a combination of OK432 (Streptococcus pyogenes preparation), low-dose prostanoid, and interferon (IFN)-α [9] (OPA). In order to evaluate the tolerability and the clinical or immunological responses of OPA-DC vaccine, we conducted a phase I/II clinical study of OPA-DC vaccine in patients with CRC, which shed light on the importance of NK cells and the limitations of CTL differentiation even with fully matured DC.

Table 1 Eligibility criteria for the enrollment of patients with colorectal cancer for receiving quickly matured dendritic cell vaccine

(1) Eligibility criteria	(a) Patients with colon cancer (stage IV)
	(b) Performance status: 0–2
	(c) Age: 20–75 years
	(d) Tolerability for apheresis
	(e) Stability in bone marrow, liver, and renal functions:
	2,000 < WBC (/μl) < 15,000, Plt (/μl) > 75,000, AST and ALT (IU/l) < 150, T-bil (mg/dl) < 2.0, Crn < 2.0
	(f) Acquired informed consent
	(g) Having metastatic lesions for the assessment of therapeutic efficacy
	(h) More than 4 weeks have passed from previous anti-cancer treatments
	(i) HLA-A*2402 positive
	(j) Increased serum CEA level: >5 ng/ml
	(k) Positivity for CEA in cancer tissues
(2) Exclusion criteria	(a) Pregnant or lactating woman
	(b) Severe bleeding tendency: PT < 50%, APTT > 60 s, fibrinogen < 100 mg/dl, FDP > 20 μg/ml
	(c) Patient with infectious diseases (HIV, HBV, HCV, HTLV, RPR)
	(e) Patient with autoimmune disorders
	(f) Patient who needs to take steroids or immunosuppressive drugs during treatment
	(g) Patient who has uncontrollable metastatic brain or intrathecal tumors
	(h) Patient whom the doctors define as inappropriate

Subjects and methods

Subjects

Patients with advanced CRC (stage IV) who had been followed in or referred to Osaka University Hospital were enrolled as candidates in this clinical trial. The eligibility and exclusion criteria are summarized in Table 1. Among them, ten patients were selected. Their clinical backgrounds are given in Table 2.

Study design

The current clinical trial was performed to evaluate tolerability as well as immune and clinical responses; it has been registered with the University-hospital Medical Information Network Clinical Trials Registry (UMIN-CTR), under the code of UMIN000000743. The protocol was approved by the ethical committee of the Osaka University Graduate School of Medicine and also reviewed by the Translational Research Review Board, Osaka University Hospital. Standard operating procedures for manufacturing OPA-DCs were reviewed by the Medical center for Translational Research (MTR). Every detailed procedure described in the Manufacturing Instructions and Records was programmed to automatic process-control software to avoid operational errors. All patients gave written informed consent prior to the treatment.

The patients were vaccinated four times every 2 weeks in conformity with the regulation in previous DC vaccine [10–12]. In order to collect peripheral blood mononuclear cells (PBMCs) as a DC source, they underwent leukocyte apheresis at the first day of each session. After OPA-DCs had been generated as described in the next paragraph, they were administered subcutaneously at bilateral groin sites. Blood samples were collected before and 2 weeks after DC injection for the purpose of immunological and biochemical tests. Serum carcinoembryonic antigen (CEA) levels were determined on the day of blood collection. Two

Table 2 Clinical characteristics and outcomes of ten patients enrolled in this study

Case	Gender	Age	Metastases	CEA (ng/ml)[a]	Total tumor volume (cm^3)[a]	Administrated DCs (mean) ($\times 10^6$/injection)	DC injection time	CEA decline	RECIST
1	Female	62	Lung	111	4.7	106.7	8	+	SD
2	Female	64	Lung, liver	647	590.8	11.2	4	–	PD
3	Female	63	Lung, liver	987	603.3	19.2	4	–	PD
4	Male	60	Lung, liver	631	694.4	121.1	4	–	PD
5	Female	48	Lung, liver, peritoneal dissemination	482	Difficult to measure[b]	Withdrawn	2	Withdrawn	
6	Male	65	Liver	925	64.7	51.6	4	+	PD
7	Female	59	Liver, pancreas	692	543.7	115.0	4	–	PD
8	Female	42	Lung, liver	12	110.0	36.6	4	–	PD
9	Female	32	Lung, liver, abdominal wall, LN	402	163.7	Withdrawn	2	Withdrawn	
10	Male	49	Lung, abdominal LN	612	19.1	35.225	4	–	PD

[a] Before vaccination

[b] Peritoneal disseminations are too small to measure their diameter

weeks after the last vaccination, the clinical response was evaluated by the changes in tumor size measured on computerized tomography (CT) scans, based on the Response-Evaluation Criteria In Solid Tumors (RECIST) [13]. Toxicity was scored occasionally according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) v. 3.0. If severe toxicities of grade 3 or 4 developed, the patient was withdrawn from the study and switched to other appropriate treatment. A successive series of vaccinations could be applied to the patients evaluated as achieving a partial response (PR) or a stable disease (SD) condition based on the RECIST criteria.

Preparation of quickly inducible mature dendritic cells (OPA-DCs)

The OPA-DCs were generated as reported previously with some modifications [9]. At least 5×10^8 PBMCs were recovered via leukapheresis (Amicus) (Baxter, Deerfield, IL) from the patients. The PBMCs were incubated at a concentration of at least 5×10^6 cells/ml with serum-free AIM-V in 225 cm^2 culture flasks (Iwaki, Japan) for 2–3 h at 37°C in 5% CO_2. Subsequently, these flasks were washed with saline in order to separate monocytes by means of their preferential adherence to plastic [9]. Briefly, monocytes were cultured with 50 ng/ml GM-CSF and 20 ng/ml IL-4 for 3 days at 37°C in 5% CO_2. During the final 24 h, the cells were matured with 0.1 KE/ml OK432, 500 IU/ml IFN-α, and 50 ng/ml PG-E1. Concomitantly, 20 μg/ml CEA.652(9) was loaded to the cells. On day 3 of culture, non-adherent cells (OPA-DCs) were harvested. These procedures were performed in the cell-processing center affiliated with MTR under a clean condition according to the guidelines for translational research using human stem cells by Japanese Ministry of Health, Welfare and Labor.

Quality assessment of DC injections

During the DC-preparation process, samples of the culture supernatant were collected. No bacterial contamination was confirmed by means of their endotoxin measurement. Before handling the OPA-DC injections, we examined their viability and cell number using 0.3% trypan-blue staining. Viability was assessed as the percentage of viable cells among all countable cells. All the final products were confirmed as having at least 70% viability and containing at least 3.0×10^6 viable cells in conformity with the regulation in previous DC vaccine [10–12]. The purity of DCs was also examined. Briefly, the cells sampled from each final product were stained with fluorescent-material-conjugated anti-CD14 monoclonal antibody (mAb), anti-CD11c mAb, and anti-HLA-DR mAb. They were analyzed on a FACS Caliber (BD, Franklin Lakes, NJ). The live cells, except for lymphoid cells, were gated at forward scatter versus side scatter plot. Subsequently, the percentage of the CD14-/CD11c+/HLA-DR+ cells among the gated cells was analyzed as the purity. All final products were confirmed as having at least 70% purity.

Reagents

Recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 were purchased from Primmune Inc., Japan. OK432 (Picibanil®) was purchased from Chugai, Japan. The amount of OK432 is expressed in units designated as KE (Klinische Einheit

[clinical unit]). One KE OK432 is equivalent to 0.1 mg dry streptococci. Natural human IFN-α (OIF®) was purchased from Otsuka, Japan. PG-E1 (Prostandin®) was purchased from Ono, Japan. The 9-mer peptide (CEA.652(9): TYACFVSNL), reported to be a human leukocyte antigen (HLA)-A*2402 restricted CTL epitope in CEA [9], was purchased from TaKaRa Bio (Japan) or Mimotope-Genzyme Pharmaceuticals (Switzerland). Therapeutic grade medium (AIM-V) was purchased from Invitrogen (Carlsbad, CA).

Fluorescent antibodies and HLA/peptide-pentamer

Fluorescein-isothiocyanate (FITC)-conjugated Lineage-Cocktail 1, anti-CD14 mAb (M5E2), anti-CD3 mAb (UCHT1), anti-CD4 mAb (RPA-T4), anti-CD27 mAb (M-T271), and anti-CD45RO mAb (UCHL1) were purchased from Becton–Dickinson (BD), Franklin Lakes, NJ. FITC-conjugated anti-CCR7 mAb (150503) was purchased from R&D Systems, McKinley Place, NE. Phycoerythrin (PE)-conjugated anti-CCR4 mAb (1G1), anti-CD4 mAb (RPA-T4), anti-CD45RA mAb (HI100), and anti-CD69 mAb (FN50) were obtained from BD. PE-conjugated anti-FoxP3 mAb (PCH101) was obtained from eBiosciences, San Diego, CA. PE-conjugated anti-CXCR3 mAb (49801) was purchased from R&D Systems. Peridinin-chlorophyll-protein-complex (PerCP)-conjugated anti-CD4 mAb (SK3), anti-CD8 mAb (SK1), and anti-HLA-DR mAb were purchased from BD. Allophycocyanin (APC)-conjugated anti-CD11c mAb (B-ly6) and anti-CD56 mAb (B159) were obtained from BD. APC-conjugated anti-CD4 mAb (13B8.2) was purchased from Beckman Coulter Inc., Fullerton, CA. PE-conjugated HLA-A*2402/CEA.652(9)-pentamer (CEA(24)-pentamer) and HLA-A*2402/EBV peptide (HLA-A*2402 restricted peptide derived from Epstein-Bar virus (EBV) LMP2: TYGPVFMSL)-pentamer were purchased from ProImmune Ltd., Bradenton, FL.

Cell lines

The NK-cell-sensitive cell line (K562) was obtained from ATCC (Manassas, VA). T2-A24 is a transporter associated with an antigen processing (TAP) deficient cell line (T2) transfected with HLA-A*2402 gene. This cell line expresses a high level of HLA-A24 protein and is used for targets in cytotoxicity assay. K562 and T2-A24 were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2.

Calculation of total tumor volume

We calculated the total tumor volume of each patient before vaccination. Maximum diameters (D_1, D_2, ...) were measured for all visible tumors in CT images from the neck to the perineum, and total tumor volume was calculated as follows:

$$\text{Total Tumor Volume} = 3.14 \times \left(D_1^3 + D_2^3 + \ldots\right)/6.$$

Assessment of immune responses

(1) Frequency analyses of immune cells

PBMCs derived from blood samples were stained with fluorescent-material conjugated antibodies or a CEA(24)-pentamer. We analyzed the frequency of NK, Th1, Th2, FoxP3+ regulatory CD4+ T cells (Treg), and CEA.652(9)-specific CTLs on a FACS canto II (BD). For the analysis of Tregs, the cells were permeabilized with the Human FoxP3 Buffer Set (BD). Th1 and Th2 cells were detected as CD4+/CD45RO+/CXCR3+ and CD4+/CD45RO+/CCR4+ cells, respectively [14]. NK cells were characterized as CD3−/CD56+ cells and their active phenotypes were assessed by the expression of CD69. The ratios of CD69+ NK-cell frequency before and 2 weeks after every DC injection were calculated as the CD69+ NK variation rate (VR). We analyzed FoxP3+ Tregs as CD4+/CD45RO+/$CD25^{high}$/Foxp3+ cells. Highly avid CTLs for CEA.652(9) were identified as CD8+/CEA(24)-pentamer+ cells. Additionally, the CTLs were subdivided into CCR7+/CD45RA- central memory cells, CCR7−/CD4RA− effector memory cells, and CCR7−/CD45RA+ terminal differentiated effector memory cells [15].

(2) Analysis of NK cell activity

The cytotoxic ability of NK cells during vaccination was analyzed by flow-cytometric methods [16]. NK cells were separated from PBMCs using CD56 microbeads (Miltenyi Biotec). K562 cells were labeled with carboxyfluorescein succinimidyl ester (Invitrogen) and cultured with or without NK cells in 24-well culture plates (Falcon) for 12 h at 37°C in 5% CO_2 (E/T ratio: 24/1). At the end of the incubation, the samples were put on ice and 50 μg/ml propidium iodide (SIGMA) was added for DNA labeling of the dead cells. The samples were then incubated for 10 min on ice and analyzed within 60 min using FACS Canto II (BD). The percentage of specific-target-cell death (cytotoxicity) was calculated as:

$$\text{cytotoxicity}(\%) = \frac{\text{dead targets in the sample}(\%) - \text{spontaneously dead targets}(\%)}{100 - \text{spontaneously dead targets}(\%)} \times 100$$

The representative plots of flow-cytometric analyses are given in Online Resource 1.

Statistical analysis

For the vaccinated patients, a comparison was made between plasma cytokine level and total tumor volume

before vaccination using the Pearson's product-moment correlation coefficient (r), which was analyzed with Prism 5 software (Graph Pad Software, San Diego, CA). A P value is the probability that an r value is zero. Therefore, a P value of less than 0.05 indicates that the two variables are correlated.

Results

Quality of the OPA-DC vaccine

The mean DC number administered at each vaccination is shown in Table 2. The viability and purity of DC from all patients were 91.1 ± 2.6% (mean ± SD) and 88.8 ± 4.28%, respectively. All products met the quality assessment of the OPA-DC vaccine except for one form Case 3 due to its lesser number.

Clinical outcomes

Eight of the ten patients enrolled in this study were able to receive at least one series of vaccinations. Two patients (Case 5 and 9) were withdrawn from the study because of ileus, respiratory distress, or obstructive jaundice of grade 3 accompanied by cancer progression. One patient (Case 1) displayed a clinical response of SD lasting 2 months from the initiation of vaccination. Two weeks after the first session, the diameter of the patient's maximum lung metastasis increased by less than 20% and cavity formation was observed at the core of the lesion (Fig. 1a). Based on such clinical responses to the first session, this patient received the second series of vaccinations. Seven other patients had PD after the therapy.

Decline in the serum CEA level during the vaccination period was observed in two patients (Case 1 and 6) (Fig. 1b). They had clearly smaller total tumor volumes in the body before vaccination than the other patients (Fig. 2).

Immunological responses

We previously reported that OPA-DC has potent Th1 priming ability in vitro [9]. Therefore, we analyzed the frequency of peripheral Th1 and Th2 cells during the vaccination period (Fig. 3a). In Cases 1 and 8, the Th1/Th2 ratio remained high during vaccination compared with the pre-vaccination value. In Case 1, the patient with SD continued to show increment of the Th1/Th2 ratio after vaccination during the two series of sessions.

OPA-DCs have potent ability to activate NK cells and antigen-specific CTLs in vitro [9]. Therefore, we analyzed the frequency of NK cells before and during vaccination (Fig. 3b). In all except Cases 2 and 10, the mean frequency of NK cells during vaccination increased compared with the pre-vaccination value. Moreover, in Cases 1 and 6 demonstrating a CEA decline, the NK cells displayed strong lytic activity against K562 cells after vaccination (Fig. 3c).

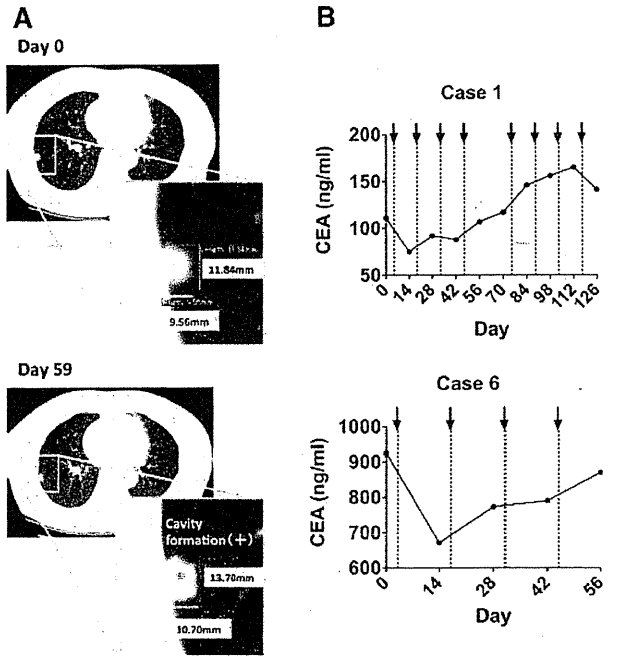


Fig. 1 Changes in CT images of metastatic lesion and serum CEA levels in patients after receiving the OPA-DC vaccines. **a** CT images of the patient with stable disease after OPA-DC vaccination (Case 1) are shown. The diameters of the maximum metastatic lesion in the lung are shown before (day 0) and after (day 59) the vaccination period. **b** The changes in serum CEA levels during vaccine treatment in the patients (Case 1 and 6) are shown. *Downward arrows* indicate the time points of OPA-DCs injections

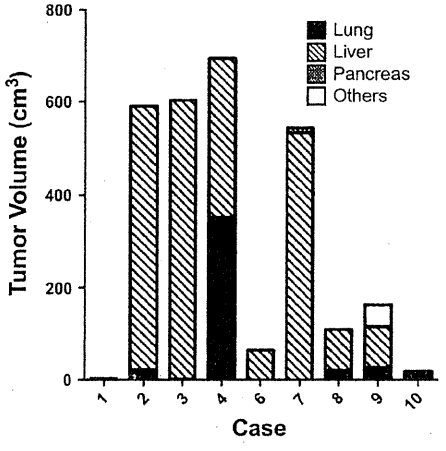
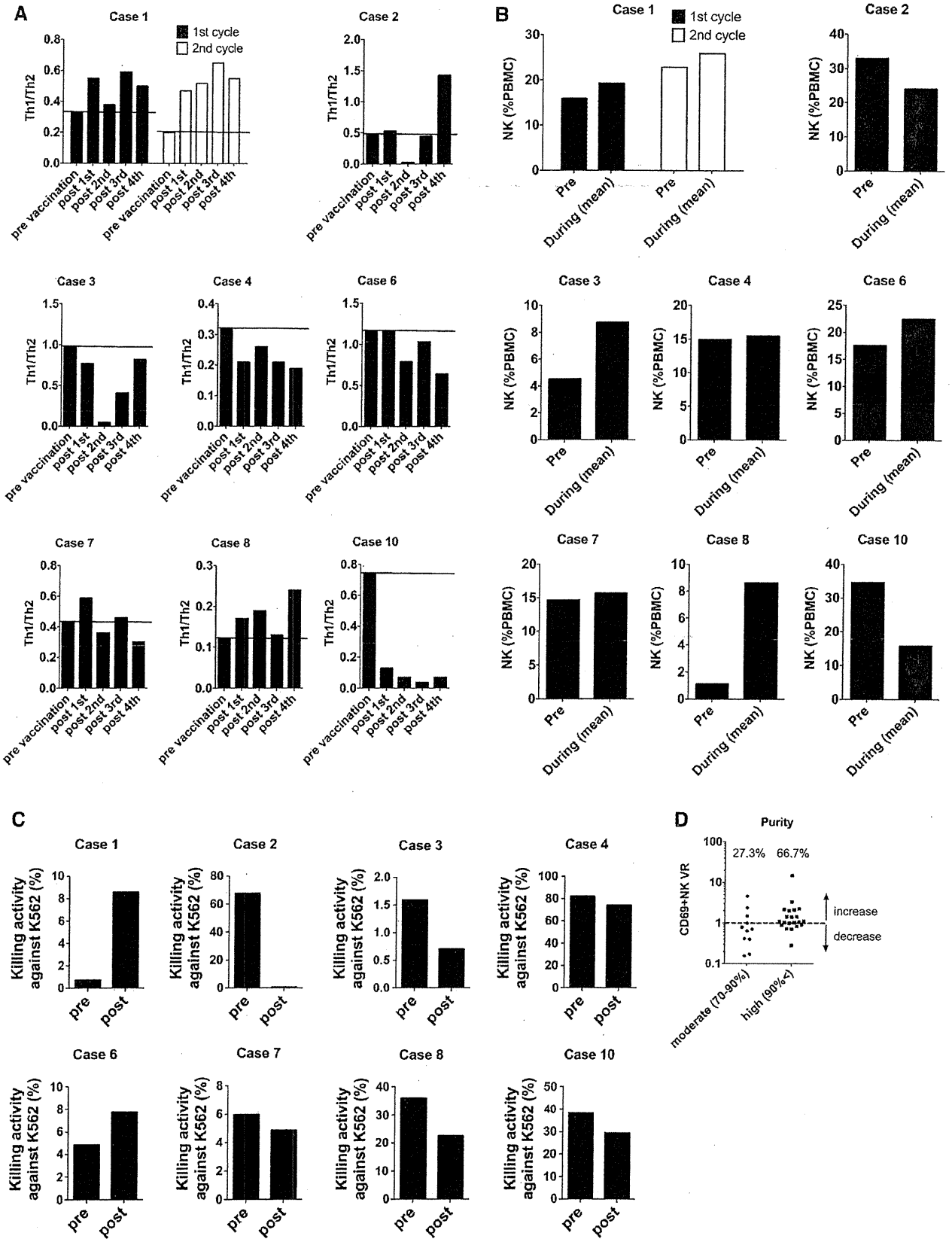


Fig. 2 Absence of correlation between total tumor volume and serum immunosuppressive cytokines in the vaccinated patients. Total tumor masses of each patient before vaccination were measured as described in "Subjects and methods". Localizations and volumes of metastatic tumors are shown


A
Case 1
1st cycle
2nd cycle
Th1/Th2
pre vaccination
post 1st
post 2nd
post 3rd
post 4th
Case 2
Case 3
Case 4
Case 6
Case 7
Case 8
Case 10
B
Case 1
1st cycle
2nd cycle
NK (%PBMC)
Pre
During (mean)
Case 2
Case 3
Case 4
Case 6
Case 7
Case 8
Case 10
C
Case 1
Killing activity against K562 (%)
pre
post
Case 2
Case 3
Case 4
Case 6
Case 7
Case 8
Case 10
D
Purity
CD69+NK VR
27.3%
66.7%
increase
decrease
moderate (70-90%)
high (90%<)

◀ **Fig. 3** Changes in Th1/Th2 ratio and NK cells before and during/after the OPA-DC vaccines. **a** Ratios between Th1 and Th2 frequency (Th1/Th2) were determined before and 2 weeks after every vaccination as described in "Subjects and methods". In Case 1, four additional injections of DCs were performed (*empty bars*). *Horizontal lines* in each graph indicate the Th1/Th2 value before vaccination. **b** NK cell frequency was examined before and during the vaccinations. The *values* are shown as the mean of the NK cell frequencies between the first and the last DC injections. **c** NK activities are evaluated by the killing of NK-sensitive K562 cells before and 2 weeks after the first DC injection. **d** CD69+ NK variation rates (VR) are shown for all DC injections of all patients in the first session. VR values above 1 mean that CD69+ NK cells increased with that single injection of DC. Percentages in the graphs depict the rate of CD69+ NK increment induced by DCs with quality

We and other investigators have found that DCs primed with OK432 can activate NK cells within 48 h [9, 17]. Such observations support the possibility of vaccine-dependent activation of NK cells with each DC injection. Therefore, we analyzed the increment of CD69 + NK cells after every DC injection with respect to its relationship with DC purity. In total, 32 injections were performed with eight patients in their first sessions. Among such vaccinations, 21 injections were done with highly pure DCs (purity: >90%) and the remaining 11 with moderately pure DCs (purity: 70–90%). As shown in Fig. 3d, regardless of the differences in clinical backgrounds of the patients, a post-vaccine increment of CD69 + NK cells was observed in 14 out of the 21 given highly pure DC injections (66.7%). In contrast, such an increment was detected only in 3 out of the 11 given moderately pure DC injections (27.3%). Therefore, highly pure DC injections resulted in a higher rate of NK activation compared with moderately pure DC injections.

Next, to assess the frequency of antigen-specific CTLs induced with OPA-DC vaccine, we analyzed PBMCs from vaccinated patients with CEA(24)-pentamer staining (Fig. 4a). In Case 1 with the SD response, the frequency of the pentamer-positive CTLs increased with vaccination. Of particular interest, during the second sessions, the frequency increased much more compared with those in the first sessions (Fig. 4a). In contrast, the frequency of specific CTLs against control peptide derived from Epstein-Bar virus (EBV) kept under 1% of all CD8+ T cells during all sessions (Online Resource 2). According to the differentiation stages, human CD8+ T cells have been subdivided into different populations based on their CD45RA and CCR7 expressions [15]. Therefore, we analyzed the frequencies of CEA.652(9)-specific central memory T (Tcm), effector memory T (Tem) and terminally differentiated Tem (Tem/td) cells during vaccination in Case 1 (Fig. 4b). The CEA.652(9)-specific Tcm cells increased gradually with OPA-DC vaccination; however, antigen-specific Tem or Tem/td cells were not induced. In other cases, including Case 6 with CEA decline, we did not observe such increment of antigen-specific CTLs after the vaccinations.

We performed ELISPOT assay in order to enumerate IFN-γ-producing CD8 cells reacting to the CEA peptide. Also, we had intended to measure the antigen-specific lytic activity of CTLs against CEA.652(9) pulsed T2-A24 cells by ^{51}Cr-releasing assay, but in all cases, no such responses were observed (data not shown).

Regulatory T cells (Treg) play an active role in the suppression of anti-tumor immune responses. Many investigators reported that the accumulation of Tregs is observed in various types of cancers including CRC [18–21]. Therefore, we analyzed the frequency of Tregs during vaccination. In Cases 2, 3, 6, and 7, Tregs decreased after the vaccinations (Fig. 5). It is noteworthy that in Case 6, the reduction in Tregs was maintained throughout the vaccination period. Delayed-type hypersensitivity (DTH) against CEA.652(9) peptide was not observed in any of the cases (data not shown).

Toxicity

All patients experienced grade-1 fever after every vaccination, but this could be controlled with anti-inflammatory drugs. In addition, induration of the vaccinated groin sites was observed about 2 weeks after the first vaccination with all patients. They were followed without treatment, except for one patient (Case 8) who received temporal antibiotics for abscess formation of the induration. OPA-DC vaccine could be performed without severe toxicity for all patients.

Discussion

Dendritic cells pulsed with CEA peptide are one of the feasible vaccines to induce anti-tumor immunity in patients with CRC [22]. We have previously reported that novel mature DCs (OPA-DCs) can be generated from monocytes using OK-432, low-dose prostanoid, and IFN-α (OPA) by a short-term process. OPA-DCs possess potent migrating ability and stimulating activity for Th1, CTL, and NK cells, which are desirable for DC vaccines using peptide antigen against cancers [9]. In this phase I/II clinical study, we evaluated the safety and efficacy of vaccination with OPA-DCs pulsed with CEA.652(9) peptide in ten patients with metastatic CRC. We chose the peptide as a target antigen because it has been reported as a tumor-associated antigen achieving preferable responses in previous cancer vaccine studies [23, 24]. OPA-DCs offer several advantages in clinical settings [25]. First, even with serum-free media, OPA-DCs are likely to possess better functional abilities with large-scale yield. Second, they can avoid the possibility of contamination and can save costs with the

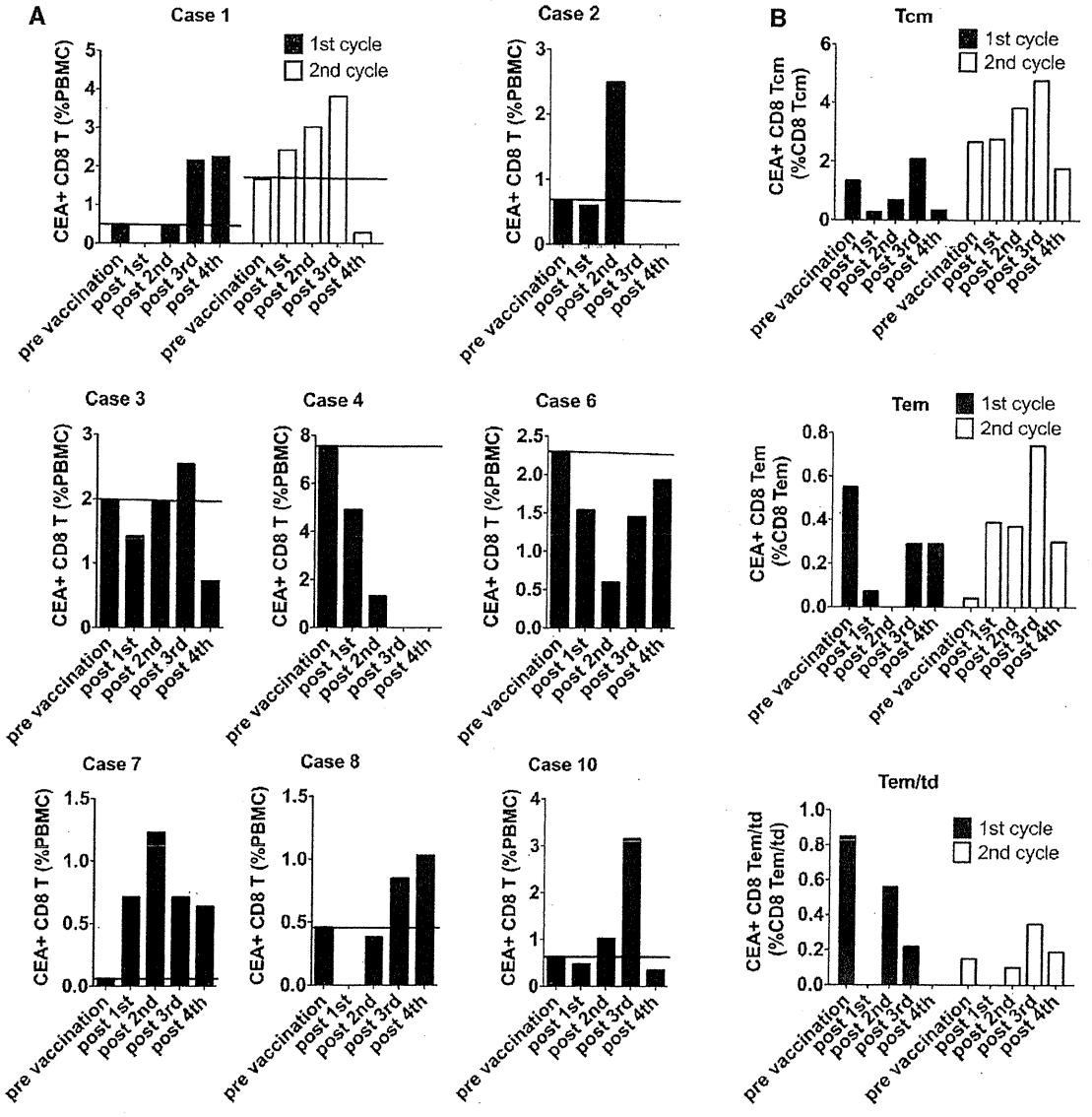


Fig. 4 Differentiation of CTLs was impaired regardless of the increased frequency of CEA-pentamer-positive CD8 T cells after DC vaccinations. **a** CEA-specific CTLs, as judged by CEA-pentamer-positive CD8+ T cells, were counted as described in "Subjects and methods". The *horizontal lines* in each graph indicate the frequency of CEA-pentamer-positive cells before vaccination. **b** In the patient who showed an SD response (Case 1), the frequency of CEA-pentamer-positive CD8+ central memory T cells (Tcm), effector memory T cells (Tem), and terminal differentiated effector memory T cells (Tem/td) was analyzed as described in "Subjects and methods"

generation of clinical-grade DCs. Alternate strategies using short-term cultured DCs have been reported elsewhere [26–28]; however, this is the first clinical study of anti-tumor vaccine using quickly generated DCs.

Regarding toxicity, the vaccination with OPA-DCs was well tolerated in patients with advanced CRC. The most common adverse events were grade-1 fever and indurations at the injection sites. Such toxicities were comparable to findings reported for previous DC-vaccine trials [29–32]. Two patients were withdrawn from the study because of grade-3 ileus, respiratory distress, or obstructive jaundice; however, these problems were caused by exacerbation of preexisting peritoneal disseminations, lymphangitic carcinomatosis, or liver metastases.

In this clinical study, we observed an SD response in one patient (Case 1). Interestingly, several tumor lesions in this patient formed a central cavity, which was probably attributed to immunological tumor necrosis triggered by the OPA-DC vaccine. Histological analysis could have offered support for this, but we could not perform biopsies of the lesions for ethical reasons. Instead, some immunological events that could have contributed to her clinical outcomes were observed in the peripheral blood during vaccination. First, Th1 cells were dominant over Th2 cells

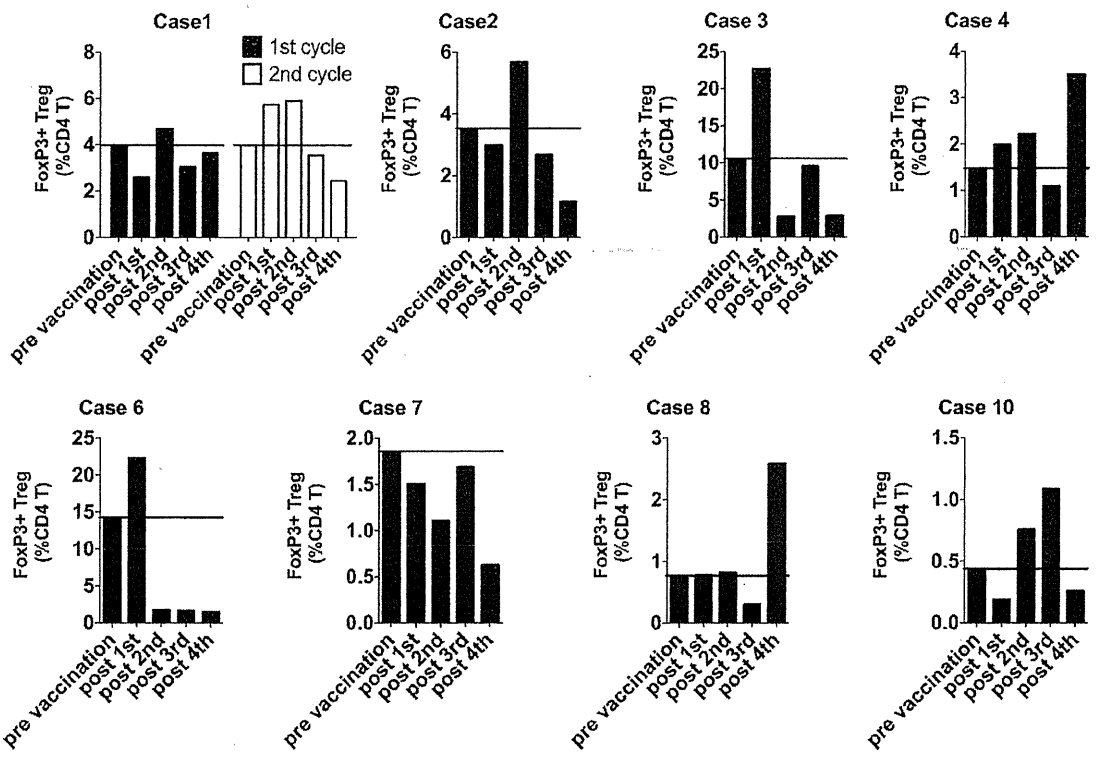


Fig. 5 Changes in FoxP3+ regulatory T cells varied during the OPA-DC vaccination period. Frequency of FoxP3+ CD4+ CD25+ T cells (depicted as Tregs) during the vaccination period is shown. *Horizontal lines* in each graph indicate the frequency of Tregs before vaccination

throughout her vaccination period. Second, NK cells had increased and were activated, and their in vitro lytic activity was enhanced after vaccination. In addition, specific CTLs that possess strong avidity to HLA-A*2402/CEA.652(9)-pentamer (CEA(24)-pentamer) were increased gradually with OPA-DC injections. These findings demonstrate that OPA-DCs can have significant immunological impact on patients even in refractory stages.

As for clinical outcomes, we observed decline in CEA in only 2 of 8 patients (Case 1 and 6) and stable disease in one (Case 1). Such results are comparable with those of previous reports regarding DC vaccine against CRC [31, 33, 34], which implies that additional modifications, other than highly active DC, are required to improve clinical responses with DC vaccine. In our study, the responders had lesser tumor volume than non-responders at the beginning of the vaccination period. In addition, regulatory T cells (Tregs) were reduced after OPA-DC vaccine in half of the treated patients. In a patient with CEA decline (Case 6), a sustained reduction in Tregs was observed throughout the vaccination period. It is not clear whether OPA-DCs directly reduce Tregs; however, Treg reduction may exert a favorable impact on the clinical outcome. Even if such inhibitory factors could be removed, a sizable number of cancer cells could not be completely eliminated by a limited number of effector cells educated by DC vaccine. Therefore, initiating vaccination at earlier stages of the disease could be a key to success for DC therapy.

Natural killer (NK) cells are potent anti-tumor effectors that reciprocally interact with DCs [35]. Many previous reports about DC vaccine have regarded CTLs as a principal effector providing anti-tumor immunity. However, there are few studies reporting NK cell activation in response to DC vaccine. Osada et al. [36] reported that NK number increased in 5 of 9 patients (55.6%) vaccinated with CEA-gene-transfected MoDCs, of which the clinical outcomes were correlated with NK activity rather than with T-cell responses. Our study showed that NK cell frequency was increased in 6 of 8 OPA-DC-vaccinated patients (75%). Such a high response rate shows that OPA-DCs possess potent ability to stimulate NK cells in vivo. In addition, our results showed that highly pure DC could activate NK cells compared with moderately pure one, which indicate that such NK activation is dependent on specific action of administrated DCs. Of interest, in two patients (Case 1 and 6), NK cells gained strong lytic activity after vaccination. Interestingly, only these two patients showed decline in serum CEA or a preferable clinical outcome as SD. In support of this possibility, Shimizu and Fujii [37] have reported that mice immunized with DC vaccine acquired "primed" NK cells, which could be quickly re-activated in response to tumor-cell challenge