

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

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**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 naive 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.

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### 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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**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), coinfection 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  $1500/\text{mm}^3$ , the neutrophil count below  $750/\text{mm}^3$  or the platelet (Plt) count below  $8 \times 10^4/\text{mm}^3$ , and was discontinued when the WBC count was below  $1000/\text{mm}^3$ , the neutrophil count below  $500/\text{mm}^3$  or the Plt count below  $5 \times 10^4/\text{mm}^3$ . 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 (/mm <sup>3</sup> )	5608 ± 1668	4901 ± 1664	4888 ± 1488	5113 ± 1426	4883 ± 1511	<0.001	
Neutrophils (/mm <sup>3</sup> )	2923 ± 1214	2425 ± 1031	2559 ± 1155	2535 ± 1017	2599 ± 1149	<0.001	
Red blood cells (×10 <sup>6</sup> /mm <sup>3</sup> )	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 (×10 <sup>9</sup> /mm <sup>3</sup> )	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 (/mm <sup>3</sup> )	6011 ± 1965	4874 ± 1346	4982 ± 1210	5079 ± 1877	4414 ± 871	<0.001	
Neutrophils (/mm <sup>3</sup> )	3214 ± 1511	2468 ± 971	2576 ± 950	2492 ± 1119	2521 ± 683	0.001	
Red blood cells (×10 <sup>6</sup> /mm <sup>3</sup> )	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 (×10 <sup>9</sup> /mm <sup>3</sup> )	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 ( $1.1 \pm 0.3$  µg/kg/week) for patients over 70 y.o. than for those under 70 y.o. ( $1.3 \pm 0.3$  µg/kg/week) and that of ribavirin decreased with age (<55 y.o.,  $10.3 \pm 1.9$  mg/kg/day; 55–59 y.o.,  $9.8 \pm 1.9$  mg/kg/day; 60–64 y.o.,  $9.3 \pm 2.3$  mg/kg/day; 65–69 y.o.,  $9.2 \pm 2.3$  mg/kg/day; ≥70 y.o.,  $8.5 \pm 2.5$  mg/kg/day). The same tendency was observed with genotype 2.

#### Sustained virologic response

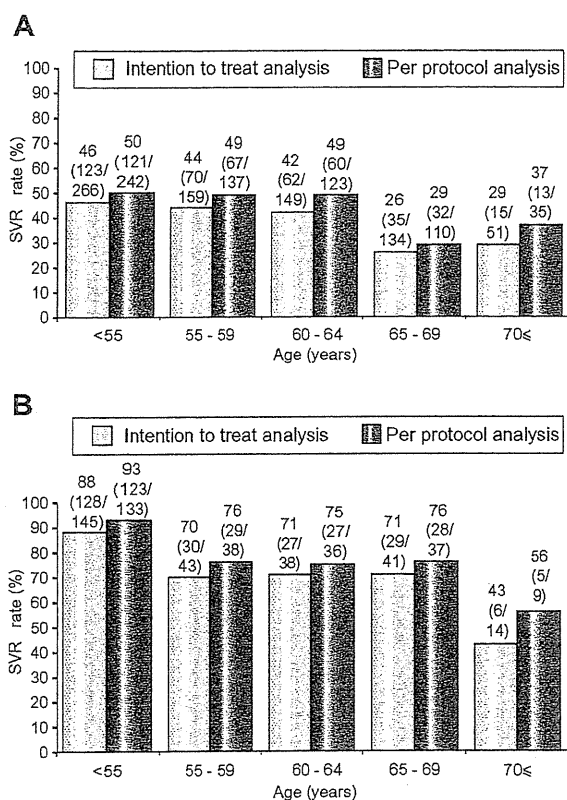
In genotype 1 patients, the overall SVR rate was 40% (305/759), being 46% (123/266) for group 1A, 44% (70/159) for group 1B, 42% (62/149) for group 1C, 26% (35/134) for group 1D, and 29% (15/51) for group 1E, following ITT analysis. The same tendency was observed using the PP analysis ( $n = 647$ ). 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.**

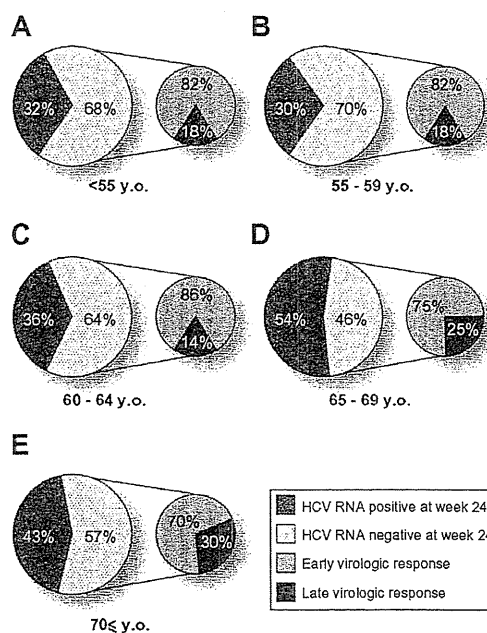
Factor	<55 y.o. (n = 363)	55 - 59 y.o. (n = 182)	60 - 64 y.o. (n = 169)	65 - 69 y.o. (n = 147)	≥70 y.o. (n = 58)	Total (n = 919)
Neutropenia	2	3	0	0	0	5
Thrombopenia	1	0	1	1	0	3
Anemia	0	4	3	2	9	18
Fatigue	1	1	3	3	1	9
Gastrointestinal disorder	2	1	0	0	1	4
Cough, Dyspnea	1	0	3	0	0	4
Vertigo	1	0	0	0	3	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
<b>Total</b>	<b>36 (10%)</b>	<b>27 (15%)</b>	<b>28 (17%)</b>	<b>28 (19%)</b>	<b>21 (36%)</b>	<b>140 (15%)</b>



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

*Timing of HCV RNA negatvation 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



**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.**

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 negatvation*

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.
<b>Genotype 1</b>					
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)
<b>Genotype 2</b>					
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 ( $\times 10^4/\text{mm}^3$ )	<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  $\geq 1.2$   $\mu\text{g}/\text{kg}/\text{week}$  on average during the first 12 weeks, and declined to 36% (8/22) in patients given 0.9–1.2  $\mu\text{g}/\text{kg}/\text{week}$  of Peg-IFN, and

to 14% (3/22) in patients administered with  $< 0.9$   $\mu\text{g}/\text{kg}/\text{week}$  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 (/mm <sup>3</sup> )		5063 ± 1474	5001 ± 1422	0.76
Neutrophils (/mm <sup>3</sup> )		2566 ± 1110	2551 ± 1071	0.87
Red blood cells (×10 <sup>4</sup> /mm <sup>3</sup> )		426 ± 36	421 ± 38	0.64
Hemoglobin (g/dl)		13.7 ± 1.2	13.5 ± 1.2	0.21
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> )		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 (×10 <sup>4</sup> /mm <sup>3</sup> )	<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 (/mm <sup>3</sup> )		5006 ± 1516	5030 ± 1409	0.81
Neutrophils (/mm <sup>3</sup> )		2575 ± 1130	2548 ± 1063	0.96
Red blood cells (×10 <sup>4</sup> /mm <sup>3</sup> )		427 ± 40	421 ± 36	0.53
Hemoglobin (g/dl)		13.8 ± 1.3	13.5 ± 1.2	0.14
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> )		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.

## Research Article

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.  $\geq 70$  y.o., 21% (6/28),  $p < 0.0001$ ; F3-4: <70 y.o., 0% (0/83) vs.  $\geq 70$  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  $\geq 1.2$   $\mu\text{g}/\text{kg}/\text{week}$ : <65 y.o., 55% (143/261) vs.  $\geq 65$  y.o., 33% (15/46),  $p < 0.0001$ ; F0-2, Peg-IFN during the first 12 weeks <1.2  $\mu\text{g}/\text{kg}/\text{week}$ : <65 y.o., 43% (26/60) vs.  $\geq 65$  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  $\mu\text{g}/\text{kg}/\text{week}$  of Peg-IFN leading to a low EVR rate for aged patients. If patients are difficult to treat with more than 1.2  $\mu\text{g}/\text{kg}/\text{week}$  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

# Hepatitis C virus-specific CD8+ T cell frequencies are associated with the responses of pegylated interferon- $\alpha$ and ribavirin combination therapy in patients with chronic hepatitis C virus infection

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**Aim:** Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTLs) play critical roles in elimination of the HCV-infected hepatocytes. However, the mechanism of HCV elimination by pegylated interferon- $\alpha$  (peg-IFN $\alpha$ ) plus ribavirin is not fully understood. We examined HCV-specific CTL responses during this combination therapy.

**Methods:** CD8+ T cells were isolated from 16 HCV infected patients treated by this combination therapy and were subjected to IFN- $\gamma$  enzyme-linked immunospot (ELISPOT) assay.

**Results:** The numbers of IFN- $\gamma$  spots against HCV Core or NS3 protein-derived peptides in HCV patients before treatment were similar to those in healthy donors, and those in HCV patients significantly increased 4 weeks after the initiation of combination therapy. All HCV Core or NS3 proteins-derived peptides specific CD8+ T cells responses in pre-treated patients were not associated with ALT levels and HCV viral loads of HCV patients before treatment. And those

in pre-treated patients were similar between sustained virologic responder (SVR) patients and non-SVR patients. Significant increase of HCV Core or NS3 proteins-derived peptides specific CD8+ T cells responses between before and 4 weeks after this combination therapy were observed in SVR patients, but not in non-SVR patients.

**Conclusions:** These results demonstrated that significant increase of HCV-specific CD8+ T cells at 4 weeks after the initiation of IFN treatment might be associated with the elimination of HCV. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of the combination therapy of peg-IFN $\alpha$  and ribavirin.

**Key words:** chronic hepatitis C, HCV-specific CTL, IFN- $\gamma$ , ELISPOT, peg-IFN $\alpha$ , ribavirin

## INTRODUCTION

CHRONIC INFECTION OF Hepatitis C virus (HCV) often leads to cirrhosis and hepatocellular carcinoma (HCC), which causes the poor prognosis of HCV-infected patients.<sup>1,2</sup> Combination therapy of pegylated interferon- $\alpha$  (Peg-IFN $\alpha$ ) plus ribavirin is standard treat-

ment for patients with chronic hepatitis C (CH-C), and sustained virologic response (SVR) in this combination therapy occurs in about 40–60% of genotype 1 patients,<sup>1,2</sup> which can improve the prognosis of HCV-infected patients. HCV-specific cytotoxic T lymphocytes (CTLs) is believed to play essential roles in determining the course of chronic infection,<sup>3</sup> and the insufficient activation, dysfunction, suppression of CTLs may cause persistent infection of HCV.<sup>4–6</sup> The elimination of HCV by HCV-specific CTLs is believed to consist of second slope of decay after viral decay during the first 24–48 h of IFN therapy.<sup>7</sup> However, the detail immune mechanism of HCV elimination by this combination therapy is not fully understood. In addition to direct antiviral

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property of Peg-IFN $\alpha$  and ribavirin against HCV infection, this combination therapy might have immunomodulatory activity. IFN- $\alpha$  enhances the maturation of antigen-presenting cells and CD4+ T cell function, but with little effect on CTLs. In contrast, ribavirin could induce a switch from Th2 to Th1 profile.<sup>8</sup> Although the base line immune responses of CTLs have been reported to be associated with the achievement of SVR in a few reports<sup>7,8</sup>, even now there are relatively little reports examining the detail of HCV-specific CTL responses during this combination therapy.

IFN- $\gamma$  enzyme-linked immunospot (ELISPOT) assay allows detection of finally differentiated effector CTLs, which means the ELISPOT data reflect the *in vivo* situation.<sup>9–11</sup> In the current study, we evaluated the HCV Core and NS3 proteins-derived peptides specific CD8+ T cells responses of the HCV infected patients by IFN- $\gamma$  ELISPOT assay and examined the relationship between CTL activity and the clinical outcome of the combination therapy of Peg-IFN $\alpha$  plus ribavirin. The frequencies of HCV-specific CD8+ T cells in pre-treated HCV patients were not associated with antiviral activity of this combination therapy in SVR. However, the significant increase of HCV-specific CD8+ T cells at 4 weeks after the starting of IFN treatment could be observed in SVR patients, but not in non-SVR patients. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

## MATERIALS AND METHODS

### Patients

SIXTEEN PATIENTS CHRONICALLY infected with HCV were examined for HCV specific CTL responses during the combination therapy of Peg-IFN $\alpha$  plus ribavirin. All patients enrolled in this study were infected with HCV genotype 1b with a high viral load and were HLA-A2 positive. The patients who were infected with other viruses (Hepatitis B virus, Human immunodeficiency virus) or had other forms of liver disease (alcohol liver disease, autoimmune hepatitis) were excluded from this study. Informed consent, under an Institutional Review Board-approved protocol, was obtained from each patient. All patients received Peg-IFN $\alpha$ -2b (PEGINTRON, Schering-Plough, Kenilworth, NJ) plus ribavirin (REBETOL, Schering-Plough) for the duration of the study of 48–72 weeks. In only one patient (Patients#11), treatment was stopped at 24 weeks because this patient remained HCV-RNA positive after

24 weeks and developed significant side effect. To evaluate the antiviral activity, serum HCV RNA levels were quantified during the combination treatment. Serum HCV RNA level was quantified using the COBAS AMPLI-CORE HCV MONITOR test (version 2.0; Roche Diagnostics, Branchburg, NJ). SVR was defined as the absence of detectable serum HCV RNA at 24 weeks after the end of the combination therapy. All treated patients were assessed the antiviral responses (SVR or non-SVR) as previously described.<sup>12</sup> The characteristics of patients with chronic HCV infection were summarized in Table 1.

### CD8+ T cells isolation from peripheral blood mononuclear cells (PBMC)

PBMC was obtained from 16 treated HCV infected patients before IFN treatment (pre-IFN) and 4 weeks after starting of this combination therapy (IFN-4week) and six healthy donors. CD8+ T cells were isolated from PBMC by magnetic cell sorting using CD8 MicroBeads according to the manufacturer's instructions (Miltenyl Biotech, Auburn, CA). More than 95% of the cells were CD8+ lymphocytes.

### IFN- $\gamma$ ELISPOT assays for HCV Core and NS3 protein-derived peptide-specific CD8+ T cells responses

To evaluate the frequencies of CD8+ T cells recognizing peptide epitopes, IFN- $\gamma$  ELISPOT assay were performed as previously described.<sup>11</sup> Briefly, 96-well multiscreen hemagglutinin antigen plates (Millipore, Billerica, MA) were coated with 10  $\mu$ g/mL of anti-human IFN- $\gamma$ mAb (1-D1K; Mabtech, Stockholm) in phosphate-buffered saline (PBS) overnight at 4 °C. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI 1640/10% human serum (1 h, 37 °C),  $1 \times 10^5$  CD8+ T cells were co-cultured with  $2 \times 10^4$  T2.DR4 cells (HLA-A2 positive peptide-presenting cells generously provided from Dr Walter J. Storkus, University of Pittsburgh, School of Medicine, Pittsburgh, PA) pulsed with HCV Core and NS3 derived peptides (a final concentration of 10  $\mu$ g/mL). HLA-A2-restricted HCV Core protein derived peptides (Core<sub>35–44</sub>, YLLPRPGPRL, Core<sub>131–140</sub>, ADLMGYIPLV) or NS3 protein derived peptides (NS3<sub>1073–1081</sub>, CINGVCWTV, NS3<sub>1406–1415</sub>, KLVALGINAV) were synthesized as previously described.<sup>13</sup> Negative control wells contained CD8+ T cells with T2.DR4 cells pulsed with HIV-nef<sub>190–198</sub> peptide (AFHHVAREL). After 24 h incubation of the plates, cells were removed from the ELISPOT well by washing and captured cytokine was detected at sites of their secretion

Table 1 Characteristics of patients with chronic hepatitis C virus (HCV) infection

Subject	Age	Sex	HCV-RNA (KIU)	ALT (U/l)	Treatment duration	SVR
1	43	F	440	17	48 week	SVR
2	56	M	2000	146	48 week	non
3	49	F	1200	31	72 week	SVR
4	49	M	340	106	48 week	SVR
5	65	F	3800	24	72 week	SVR
6	58	M	320	25	48 week	SVR
7	56	M	2551	24	48 week	non
8	55	M	939	43	48 week	SVR
9	46	M	1200	64	48 week	SVR
10	46	M	1059	42	48 week	SVR
11	43	M	407	91	24 week	non
12	63	F	1621	61	48 week	non
13	63	F	1841	63	48 week	non
14	47	M	458	41	48 week	SVR
15	36	M	1024	79	48 week	non
16	61	F	677	148	48 week	non

ALT, alanine aminotransferase; F, female; M, male; non, non-SVR; SVR, sustained virologic response.

by incubation for 2 h with biotinylated mAb anti-human IFN- $\gamma$  (7-6B-1, Mabtech) at 2  $\mu$ g/mL. Plates were washed six times and avidin-peroxidase complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) were added for 1 h. Unbound complex was removed by washing and 3-Amino-9-ethylcarbazole substrate (Sigma, St Louis, MO) was added for 5 min. The data are represented as mean IFN- $\gamma$  spots per 100 000 T cells analyzed.

### Statistics

All values were expressed as the mean and standard deviation (SD). The statistical significance of differences between the groups was determined by applying Mann-Whitney *U*-test. We defined statistical significance as  $P < 0.05$ .

## RESULTS

### Analysis of HCV derived peptide-specific IFN- $\gamma$ release of peripheral blood CD8+ T cells in ELISPOT assay

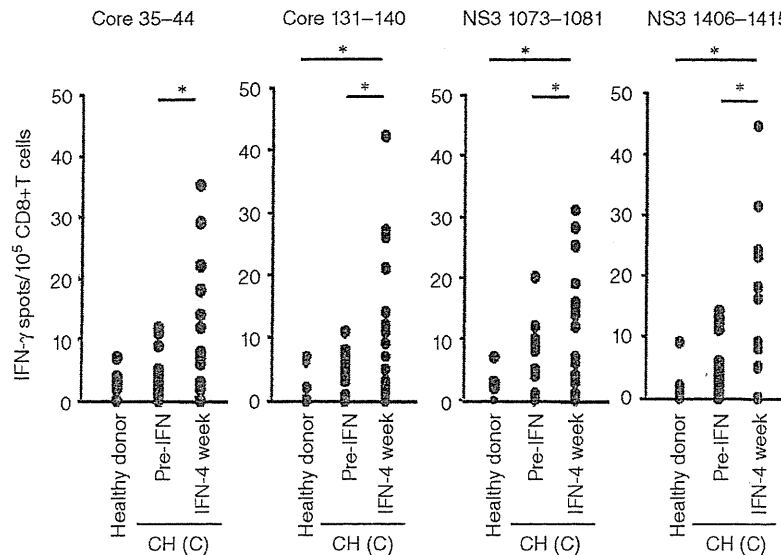
WE ASSESSED PERIPHERAL blood CD8+ T cell responses against HCV derived peptides (Core<sub>35-44</sub>, Core<sub>131-140</sub>, NS3<sub>1073-1081</sub>, NS3<sub>1406-1415</sub>) in 16 HLA-A2+ HCV patients and 6 healthy donors. As shown in Figure 1, the numbers of IFN- $\gamma$  spots (per 100 000 CD8+ T cells) observed for T cell responses against HCV peptides in pre-IFN patients were as low as those observed in healthy HLA-A2+ donors. In contrast, significant eleva-

tions of ELISPOT reactivity to three peptides (Core<sub>131-140</sub>, NS3<sub>1073-1081</sub>, NS3<sub>1406-1415</sub>) were observed in IFN-4week patients compared with healthy donors. The number of IFN- $\gamma$  spots against Core<sub>35-44</sub> peptides in IFN-4week patients also tended to be higher than those in healthy donors. In treated HCV patients, the numbers of IFN- $\gamma$  spots against all four HCV derived peptides in IFN-4week patients were significantly higher than those in pre-IFN patients (Fig. 1). We also examined whether the frequencies of HCV-specific CD8+ T cell responses were associated with sex difference. The frequencies of CTLs against all four peptides were similar between males and females before and 4 weeks after starting treatment (data not shown).

### HCV-specific CD8+ T cell responses in pre-IFN patients were not associated with the antiviral activity of the combination therapy of Peg-IFN $\alpha$ -2b plus ribavirin

We examined the association between HCV-specific CD8+ T cell responses in pre-IFN patients and ALT levels or HCV viral load before treatment. No association was observed between the frequencies of HCV-specific CD8+ T cells in pre-IFN patients and ALT levels or HCV viral load of pre-treated patients (Fig. 2).

We next examined whether HCV-specific CD8+ T cell responses in pre-IFN patients were associated with the antiviral activity of this combination therapy. As shown in Figure 3, the frequencies of CD8+ T cell responses against all four HCV proteins-derived peptides in



**Figure 1** Interferon (IFN)- $\gamma$  enzyme-linked immunospot (ELISPOT) analysis of hepatitis C virus (HCV)-specific CD8+ T cell responses in HCV patients treated with the combination therapy of peg-IFN $\alpha$  plus ribavirin. Peripheral blood CD8+ T cells were isolated from HLA-A2+ healthy donors and chronic hepatitis C (CH-C) patients. The CH-C patients were treated with the combination therapy of peg-IFN $\alpha$  plus ribavirin and PBMC were isolated from pre-treated patients (Pre-IFN) and treated patients 4 weeks after starting treatment (IFN-4week). HCV-specific CD8+ T cell responses were evaluated by IFN- $\gamma$  ELISPOT as outlined in "Materials and Methods". Data are reported as IFN- $\gamma$  spots/100 000 CD8+ T cells and represent the mean of triplicate determinations. T cell reactivity against T2.DR4 cells pulsed with HLA-A2-presenting HIV-nef<sub>190–196</sub> epitope served as the negative control in all cases, and this value was subtracted from all experimental determinations to determine HCV specific spots numbers. Each symbol within a panel represents the response of an individual donor to the indicated HLA-A2-presenting HCV Core- or NS3-peptides. \* $P < 0.05$ .

pre-IFN patients were not significantly different between SVR, the group of the patients who were observed SVR, and non-SVR, the group of the patients who were not observed SVR. These results suggested that the baseline HCV-specific CD8+ T cell responses in HCV patients were not associated with the antiviral activity of this combination therapy.

#### Significant early elevation of HCV-specific CD8+ T cell responses were associated with the antiviral activity of the combination therapy of Peg-IFN $\alpha$ plus ribavirin

We examined the association between early elevation of HCV-specific CD8+ T cell responses and the antiviral activity of this combination therapy. We evaluated the frequencies of CD8+ T cell responses against HCV proteins-derived peptides before and 4 weeks after starting treatment. As shown in Figure 4, in SVR patients, the frequencies of CD8+ T cell responses against all four HCV peptides (Core<sub>35–44</sub>, Core<sub>131–140</sub>, NS3<sub>1073–1081</sub>, NS3<sub>1406–1415</sub>) increased significantly 4 weeks

after starting treatment. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-SVR patients. These results demonstrated that significant early elevation of HCV-specific CD8+ T cell responses were associated with the antiviral activity of this combination therapy.

#### DISCUSSION

HCV-SPECIFIC CD8+ CTLs have been reported to play a significant role in the elimination of HCV in acute hepatitis of HCV.<sup>4,9</sup> In contrast, in chronic infection of HCV, HCV-specific CD8+ T cell responses were weak and were directed against a limited series of epitopes compared with acute hepatitis.<sup>9</sup> These might cause persistent infection of HCV in the HCV infected host. However, conflicting results have been reported with respect to HCV-specific CD8+ T cell responses on the antiviral activity of IFN therapy. IFN $\alpha$  monotherapy may promote viral clearance by enhancing the host CTL responses.<sup>14,15</sup> But Rehermann et al. reported that CTL

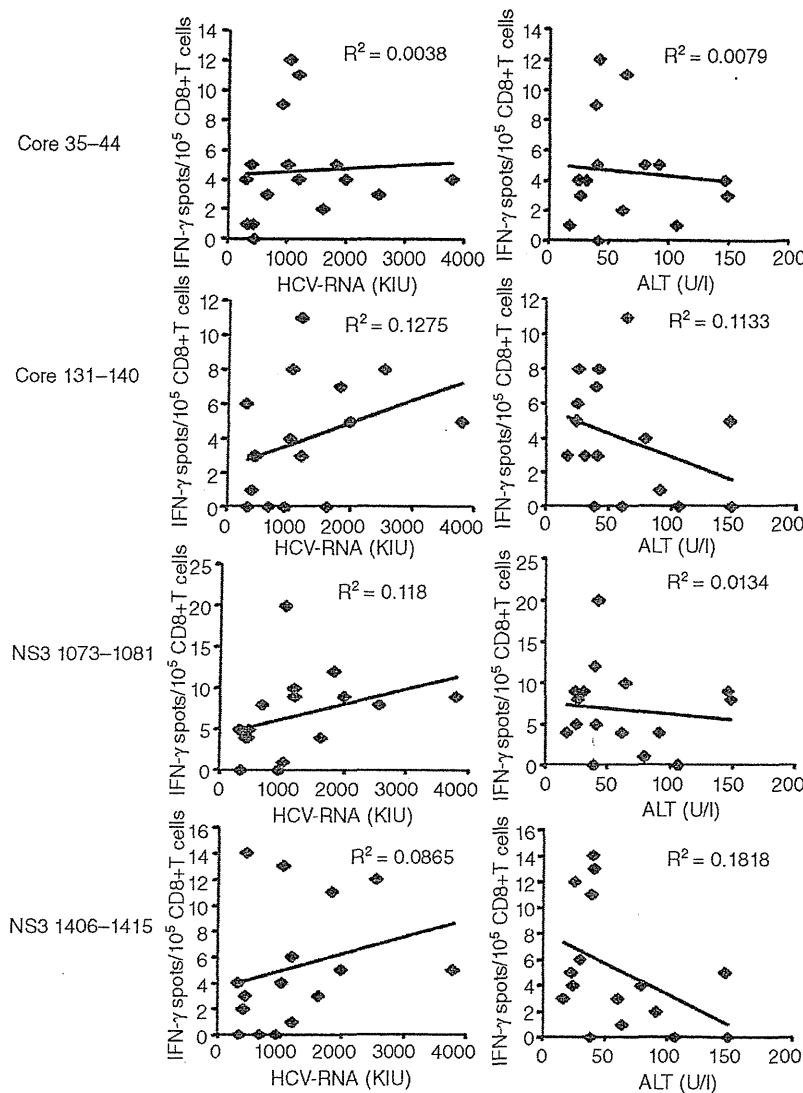


Figure 2 The association between the hepatitis C virus (HCV)-specific CD8+ T cell responses of pre-IFN patients and the serum alanine aminotransferase (ALT) levels or the HCV viral load of patients before treatment. The frequencies of HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-IFN HCV patients were evaluated by interferon (IFN)- $\gamma$  enzyme-linked immunospot (ELISPOT). We examined the association between the frequencies of HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-IFN HCV patient and the serum ALT levels or HCV viral loads of patients before treatment.

precursor frequencies against a range of HCV epitopes did not change during or after the course of IFN $\alpha$  monotherapy.<sup>13</sup> Recently, the combination therapy of PegIFN $\alpha$  plus ribavirin is standard treatment in the treatment of HCV infected patients with the better results of viral clearance compared with IFN $\alpha$  monotherapy. This suggested that this combination therapy might modify the HCV specific CD8+ T cell responses. We evaluated HCV-specific CD8+ T cell responses by IFN- $\gamma$  ELISPOT assay, a functional assay of T cells. Significant increase of the frequencies of HCV-specific CD8+ T cells between pre-IFN and IFN-4week could be

observed in SVR patients, but not in non SVR patients. This is consistent with the previous report of evaluating the frequencies of HCV-specific CTLs by direct ex vivo staining with HCV-specific pentamers.<sup>16</sup> Thus the evaluation of reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

It has been reported that complete early virologic response (cEVR), which means HCV RNA negativity at week 12, is strongly related to SVR in the combination therapy of Peg-IFN $\alpha$  plus ribavirin.<sup>12,17</sup> cEVR itself has been reported to be an independent predictive factor of

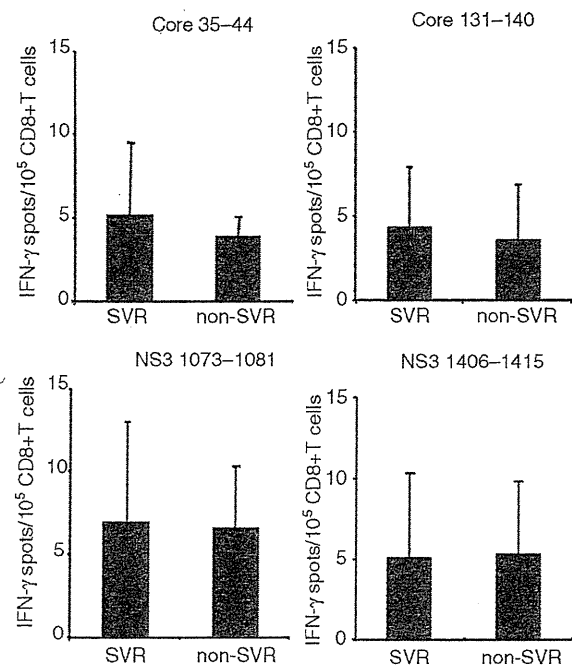


Figure 3 Comparison of the frequencies of hepatitis C virus (HCV)-specific CD8+ T cells in pre-treated HCV patients between sustained virologic response (SVR) and non-SVR. HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-treated HCV patients were evaluated by interferon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

SVR.<sup>1,12</sup> We also examined the association between cEVR and early elevation of HCV-specific CD8+ T cell responses. The frequencies of CD8+ T cell responses against all four HCV derived peptides in pre-IFN patients were not significantly different between cEVR and non-cEVR (Tatsumi T, unpublished data). In cEVR patients, the frequencies of CD8+ T cell responses against three HCV peptides Core<sub>35–44</sub>, Core<sub>131–140</sub>, NS3<sub>1406–1415</sub>) increased significantly 4 weeks after the starting treatment and those against NS3<sub>1073–1081</sub> peptide tended to increase although these were not significant. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-cEVR patients (Tatsumi T, unpublished data). The cEVR results were almost similar to those of the SVR results. Although we could not evaluate the HCV RNA levels at 4 week after starting treatment, the cEVR results sug-

gested that early elevation of the frequencies of HCV-specific CD8+ T cell responses might reflect the decrease of viral load of HCV.

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

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

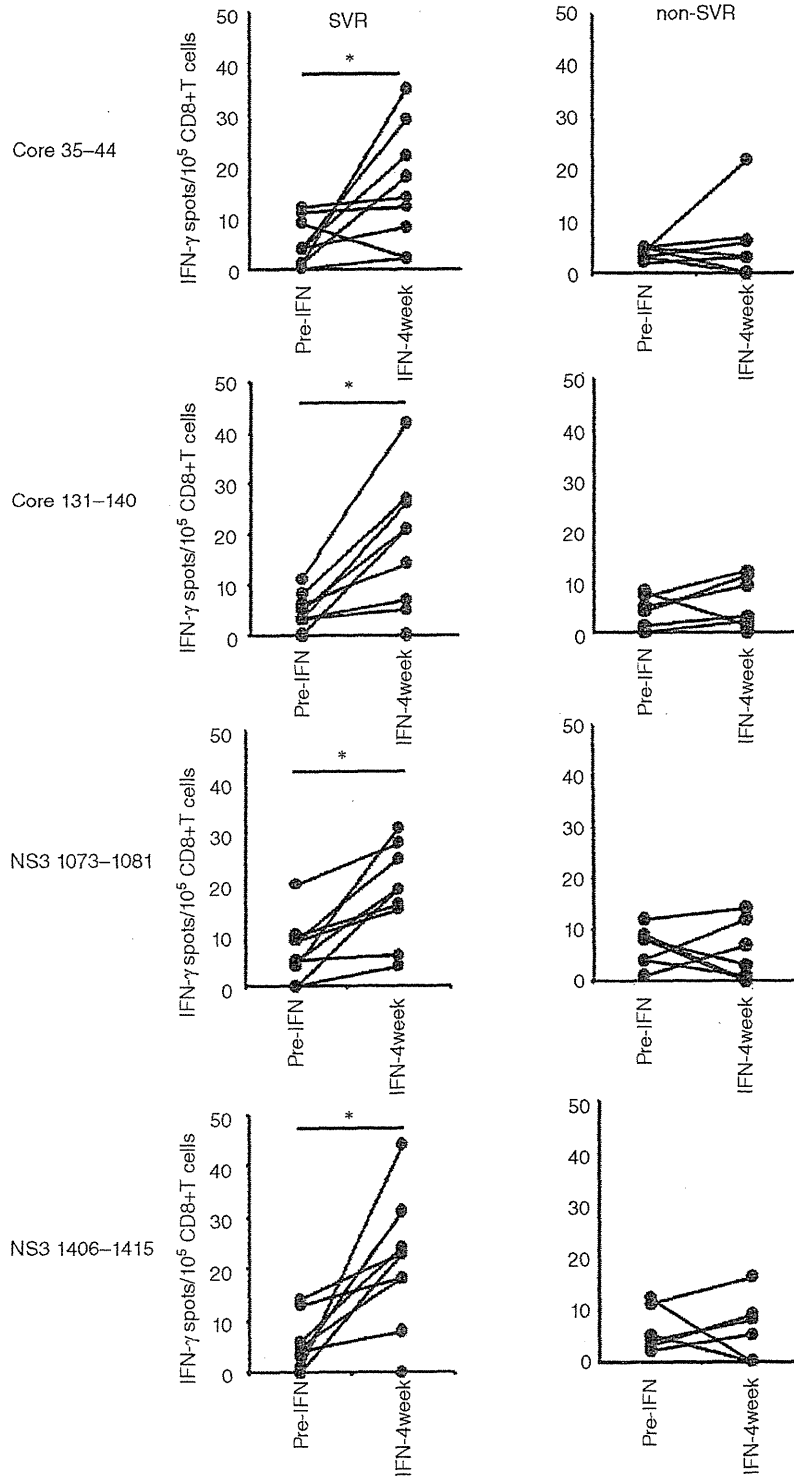


Figure 4 Analysis of the association of the change of hepatitis C virus (HCV)-specific CD8+ T cell responses between pre-IFN and IFN-4week chronic hepatitis C (CH-C) patients with the achieving sustained virologic response (SVR). Peripheral blood CD8+ T cells were isolated from pre-IFN and IFN-4week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

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

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

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

In spite of recent progress for HCV treatment, there remains significant room for improvement. To date, a variety of viral factors and host factors that correlate with SVR in the combination therapy have been noted. Recently, in addition to viral factors and host factors, response and adherence to treatment have been noted.<sup>2</sup> To establish the better treatment, the detail mechanism of HCV elimination should be elucidated. In the present study, we demonstrated that early enhancement of HCV-specific CD8+ T cell responses was associated with the achieving SVR in this combination therapy. These

suggest that activation of antiviral CTLs might be involved in the elimination of HCV. The early elevation of HCV-specific CTL responses in treated HCV patients may be a candidate for predicting SVR in this combination therapy.

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## Original Article

# $\alpha$ -Galactosylceramide activates antitumor immunity against liver tumor

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**Aim:**  $\alpha$ -Galactosylceramide ( $\alpha$ -GalCer) has been attracting attention as a novel approach to treat metastatic liver cancer. We investigated the detailed process of activating liver dendritic cells (DC) and immune cells after  $\alpha$ -GalCer treatment in the mouse liver tumor model.

**Methods:** BALB/c mice bearing CMS4 liver tumor (p53 peptide-expressing tumor) were treated by  $\alpha$ -GalCer. We evaluated the activation of liver DC and immune cells after  $\alpha$ -GalCer treatment. Interferon (IFN)- $\gamma$  enzyme-linked immunosorbent spot (ELISPOT) assay was performed to detect p53 peptide-specific cytotoxic T lymphocytes (CTL). To assess the impact of systemic acquired immunity by  $\alpha$ -GalCer treatment, 28 days after liver tumor treatment, CMS4 cells or Colon26 cells were re-challenged s.c.

**Results:** The liver weights of  $\alpha$ -GalCer-treated mice were significantly lighter than those of vehicle-treated mice. Depletion experiments revealed that natural killer (NK) cells were essential for the antitumor effect of  $\alpha$ -GalCer.  $\alpha$ -GalCer treatment

significantly increased the population of DC and NK cells in the liver. The expressions of co-stimulatory molecules on liver DC significantly increased with the peak at 1 day after  $\alpha$ -GalCer administration. IFN- $\gamma$  ELISPOT assay demonstrated that p53 peptide-specific CTL was generated efficiently in  $\alpha$ -GalCer-treated mice. <sup>51</sup>Cr-release assay revealed that CD8<sup>+</sup>, not CD4<sup>+</sup>, CTL against CMS4 cells were generated in  $\alpha$ -GalCer-treated mice. The mice that had been protected from CMS4 liver tumor by  $\alpha$ -GalCer injection became resistant against s.c. CMS4 re-challenge, but not against Colon26 re-challenge.

**Conclusion:** These results demonstrated the therapeutic potential of  $\alpha$ -GalCer against liver cancer through activating liver DC and immune cells in the liver.

**Key words:**  $\alpha$ -galactosylceramide, cytotoxic T lymphocytes, dendritic cells, liver, natural killer cells.

## INTRODUCTION

THE GLYCOLIPID ANTIGEN  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) induced the activation of natural killer (NK) T cells in a CD1d-dependent manner.<sup>1,2</sup> Recently,  $\alpha$ -GalCer has been attracting attention as a novel antitumor therapy. In *in vivo* animal studies, systemic administration of  $\alpha$ -GalCer showed antitumor effects against various tumors (including melanoma, sarcoma, colon carcinoma and lymphoma) in hepatic and lung

metastasis models.<sup>3,4</sup> Based on the promising results of preclinical studies demonstrating the antitumor potential of  $\alpha$ -GalCer, several phase 1 clinical studies on cancer immunotherapy by the i.v. administration of  $\alpha$ -GalCer has been carried out, but clinical responses of  $\alpha$ -GalCer has been limited.<sup>5</sup> No clinical trial against liver cancer has been reported to date. In view of future  $\alpha$ -GalCer treatment of liver cancer, the precise mechanism of activation of innate and acquired immunity in the liver by  $\alpha$ -GalCer should be examined. However, these are still not fully understood.

The liver contains both a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells (T cells).<sup>6,7</sup> Dendritic cells (DC) can induce the generation of both antigen-specific cytotoxic T lymphocytes (CTL) and T-helper (Th) cells.<sup>8,9</sup> Recent research of DC biology revealed that DC also contribute

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to innate immune responses by activating NK cells<sup>10–14</sup> and NKT cells<sup>1,15,16</sup> through interleukin (IL)-12 secretion and direct cellular interaction. Thus, DC can be expected to play critical roles in activating abundant T cells, NK cells and NKT cells in the liver after  $\alpha$ -GalCer administration. We previously reported that administration of  $\alpha$ -GalCer stimulated hepatic NKT cells and led to activation of hepatic NK cells,<sup>4</sup> and that  $\alpha$ -GalCer activated liver DC have higher ability to generate acquired immunity.<sup>17</sup> However, the detailed process of activating liver DC and immune cells after  $\alpha$ -GalCer treatment should be elucidated.

In this study, we evaluated the liver DC activation and antitumor effect mediated by both innate and acquired immunity against mouse liver tumor after administration of  $\alpha$ -GalCer. Administration of  $\alpha$ -GalCer induced early activation of liver DC with upregulation of antigen presenting-related molecules and resulted in complete rejection of local liver tumor by NK cells. Followed by early rejection of liver tumor, tumor antigen-specific CTL were generated and complete rejection in s.c. re-challenge of tumor cells was observed. Sequential activation of liver DC, innate and acquired immune cells in the liver may be an attractive strategy for treatment of local and distant tumor of liver cancer.

## METHODS

### Mice

SIX-TO-EIGHT-WEEK-OLD female BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan). All mice were maintained in micro-isolator cages. Procedures were performed according to approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

### Cell lines and culture

CMS4 sarcomas (H-2<sup>d</sup>) express mutated p53 and present the wild-type p53<sup>232–240</sup> epitope recognized by H-2K<sup>d</sup>-restricted CTL.<sup>18</sup> Colon26, a mouse colon adenocarcinoma cell line, was kindly provided by Dr Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan). These cell lines were maintained as previously described.<sup>19,20</sup>

### $\alpha$ -GalCer

$\alpha$ -Galactosylceramide was purchased from Funakoshi (Tokyo, Japan) and prepared as previously described by Kawano *et al.*<sup>1</sup>

## Animal experiments

BALB/c mice were injected in the liver with  $5 \times 10^5$  CMS4 cells on day 0. On day 1, BALB/c mice were injected i.p. with  $\alpha$ -GalCer (2  $\mu$ g/100  $\mu$ L) or 100  $\mu$ L of vehicle. Two weeks after the tumor injection, the livers of treated mice were removed, and the weight was measured to examine intrahepatic tumor growth. To assess the impact of systemic immunity from i.p. injection of  $\alpha$ -GalCer, mice were injected intrahepatically with  $5 \times 10^5$  CMS4 cells on day 0 and i.p. treated with  $\alpha$ -GalCer on day 1. On day 28 after  $\alpha$ -GalCer treatment,  $1 \times 10^6$  CMS4 cells or Colon26 cells were injected in the right flank of treated mice. To confirm the involvement of CD8<sup>+</sup> T cells in this antitumor effect, we depleted CD8<sup>+</sup> T cells before re-challenge of CMS4 cells in  $\alpha$ -GalCer-treated mice. On day 1 and day 3 of re-challenge of CMS4 cells, anti-CD8 antibody (53–6.72 hybridoma, ATCC) was injected i.p. as previously described.<sup>19</sup> Tumor size was assessed every 3 or 4 days and recorded in mm<sup>2</sup> by determining the product of the largest perpendicular diameters measured by vernier calipers. Data are reported as the average tumor area  $\pm$  standard deviation (SD).

## NK cell depletion experiments

For NK cell depletion, mice were injected with anti-asialo GM-1 (ASGM1) antibody (Ab) (Wako, Osaka, Japan) on day 1, 5, 10, 15 and 20 after tumor inoculation. The efficiency of NK cell depletion was validated by flow cytometry analysis of splenocytes using phycoerythrin (PE)-conjugated anti-DX5 monoclonal antibody (BD-Pharmingen, San Diego, CA, USA). In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

## Preparation of hepatic mononuclear cells and liver DC

Hepatic mononuclear cells (MNC) were prepared as previously described.<sup>4</sup> CD11c<sup>+</sup> dendritic cells were isolated from hepatic MNC by magnetic cell sorting using MACS (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's protocol.

## Flow cytometry

For phenotypic analysis of liver DC, NK cells, NKT cells and CD4<sup>+</sup> and CD8<sup>+</sup> T cells, PE- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against mouse cell surface molecules (CD11c, CD40, CD80, CD86, T-cell receptor [TCR]- $\beta$ , CD49b [DX5], CD4, CD8, CD69 [all from BD-Pharmingen], major his-

to compatibility complex [MHC] class II [Miltenyi Biotec]), and appropriate isotype controls were used, and flow cytometric analysis was performed using a FACscan (Becton Dickinson, San Jose, CA, USA) flow cytometer. The results of flow cytometric analysis are reported in positive cell rates (%) determined by using isotype controls. DC were identified as CD11c<sup>+</sup>/MHC class II<sup>+</sup> cells. NK cells were identified as DX5<sup>+</sup>/TCR-β<sup>-</sup> cells, NKT cells as DX5<sup>+</sup>/TCR-β<sup>-</sup> cells, as previously described.<sup>20</sup>

### IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays for p53<sub>232–240</sub> peptide-reactive CD8<sup>+</sup> T-cell responses

Bone marrow derived DC (BMDC) were generated from normal mice as previously described<sup>21</sup> and were used as peptide-presenting cells. On day 14 after treatment of α-GalCer or vehicle, CD8<sup>+</sup> T cells were isolated from the spleen cells of treated mice by using magnetic beads (Miltenyi Biotec). We used a mouse IFN-γ ELISPOT kit (R&D systems, Minneapolis, MN, USA) to detect the p53<sub>232–240</sub> peptide-specific CD8<sup>+</sup> T-cell responses. To evaluate the p53<sub>232–240</sub> peptide, strongly expressing on CMS4 cells,<sup>18</sup> specific CTL induction, isolated CD8<sup>+</sup> T cells (1 × 10<sup>5</sup> cells/well) were co-cultured with syngeneic BMDC (2 × 10<sup>4</sup> cells/well) pulsed with p53<sub>232–240</sub> peptide in an ELISPOT culture plate. BMDC cells without p53<sub>232–240</sub> peptide served as the negative control, and this value was subtracted from all experimental determinations to establish p53<sub>232–240</sub> peptide-specific spot numbers. The data are represented as mean IFN-γ spots ± SD per 100 000 CD8<sup>+</sup> T cells analyzed.

### Cytolytic assays

Splenocytes from α-GalCer or vehicle-treated mice were harvested 14 days after tumor inoculation. After 5 days of *in vitro* stimulation with mitomycin-C (Kyowa Hakko, Tokyo, Japan)-treated CMS4 cells, lymphocytes were analyzed for their ability to kill CMS4 cells in 4-h <sup>51</sup>Cr-release assays (effector cells/target cells ratio, 60:1), as previously described.<sup>21</sup> CD4<sup>+</sup> or CD8<sup>+</sup> T cells were depleted by magnetic sorting using CD4 or CD8 microbeads (Miltenyi Biotec), respectively.

### Statistical analyses

All experiments with three or more groups in which treatment was applied with a completely random design were first analyzed by a one-way factorial ANOVA. If the resulting *P*-value was less than 0.05, specific pairwise

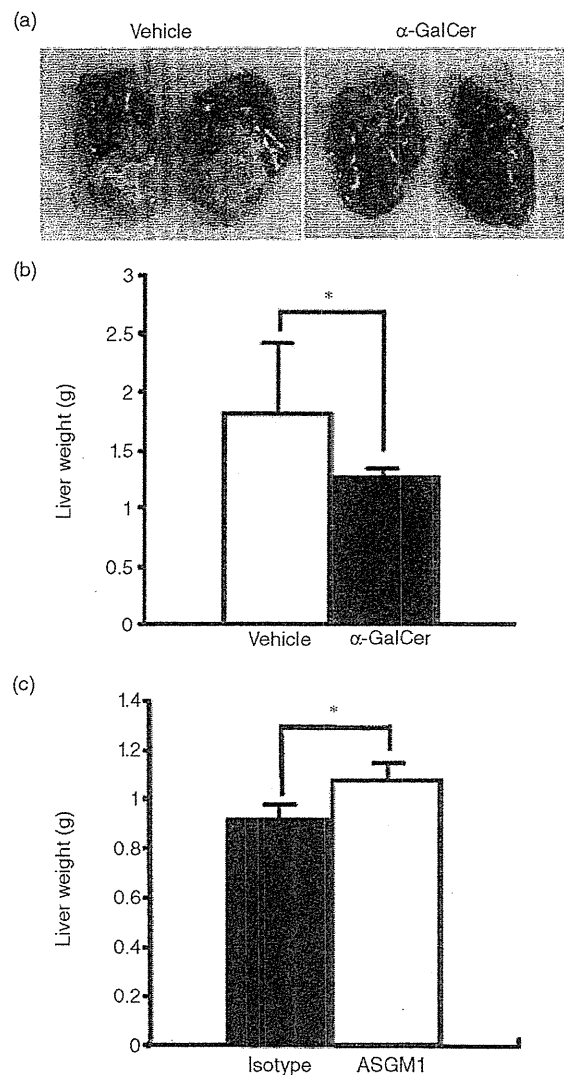


Figure 1 Therapeutic effectiveness of i.p. injection of α-galactosylceramide (α-GalCer) in CMS4 liver tumor model. BALB/c mice were injected intro-hepatically with 5 × 10<sup>5</sup> CMS4 cells. One day later, BALB/c mice were treated with i.p. injection of α-GalCer or vehicle (all treatment groups, n = 7). Two weeks after the CMS4 tumor injection, the livers of treated mice were removed. (a) Representative liver macroscopic view of each group. (b) Comparison of liver weight of each group. \**P* < 0.05. (c) To prove that the therapeutic benefit of α-GalCer treatment in the CMS4 liver tumor model is natural killer (NK)-cell dependent, *in vivo* depletion of NK cells was performed (as described in Methods, ASGM1). In control mice, isotype antibody (Ab) was injected i.p. (isotype). Both mice were treated by α-GalCer. Ab-mediated *in situ* depletion of NK cells markedly reduces the therapeutic efficacy of α-GalCer treatment (all treatment groups n = 5). \**P* < 0.05.