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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

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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 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.



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.

## 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

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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 $8 \times 10^4$/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 $5 \times 10^4$/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%) |

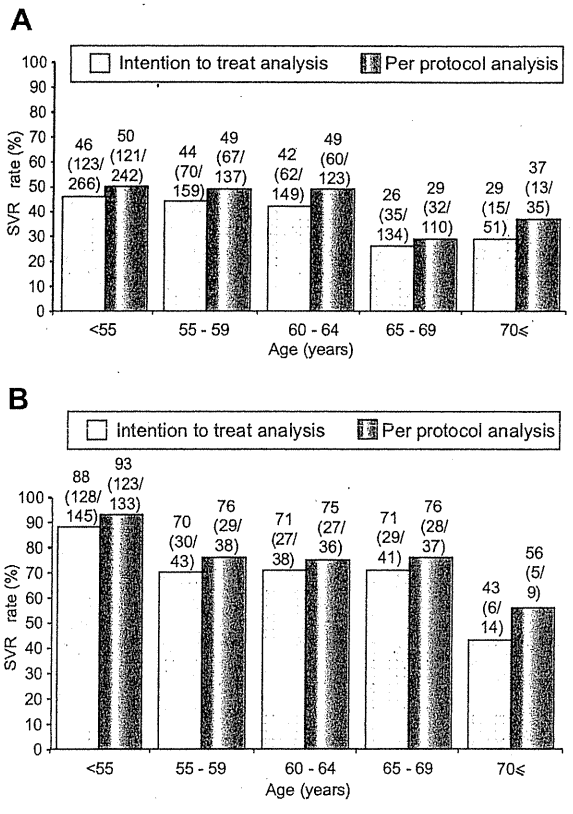


**Fig. 1. SVR rate according to age.** (A) Genotype 1. (B) Genotype 2.

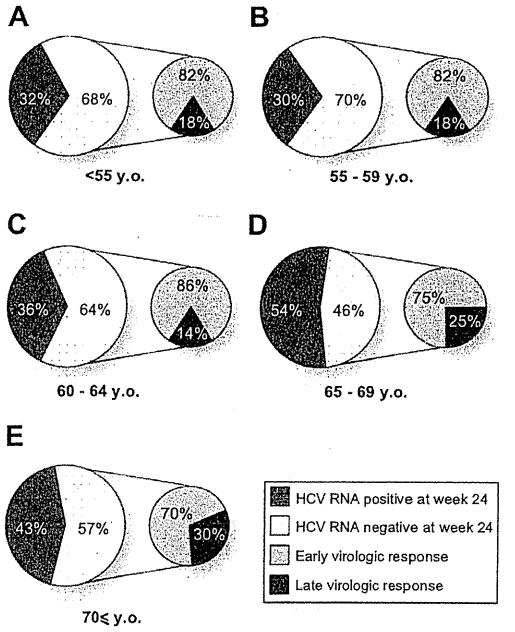


**Fig. 2. Antiviral effect during treatment according to age.** (A) <55 y.o. (B) 55–59 y.o. (C) 60–64 y.o. (D) 65–69 y.o. (E) ≥70 y.o.

*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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# Reduced risk of hepatocellular carcinoma after interferon therapy in aged patients with chronic hepatitis C is limited to sustained virological responders

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SUMMARY. This study was undertaken to investigate the effect of interferon (IFN) monotherapy on the risk of hepatocellular carcinoma (HCC) in aged-patients with chronic hepatitis C. Seven hundred and twenty-five patients with histologically proven chronic hepatitis C were enrolled in this retrospective cohort study; 531 received IFN monotherapy for 6 months between 1992 and 1995, and 157 were collected as a historical control. The effect of IFN therapy on the development of HCC was compared between the patients with chronic hepatitis C under 60 years old (non-aged group, $n = 531$) and those 60 and over (aged group, $n = 194$). A stepwise Cox proportional-hazards regression analysis in the non-aged group revealed that IFN therapy (risk ratio 0.52, 95% CI 0.33–0.81, $P = 0.004$), older age ($P = 0.001$), and higher histological stage ($P < 0.001$) were independent factors associated with the development of HCC. In the aged-group, only higher histological stage ($P = 0.002$) and male gender ($P = 0.011$), but not IFN therapy (risk ratio 0.77, 95% CI 0.42–1.40, $P = 0.386$), were identified as independent risk factors for HCC, although HCC was significantly reduced when sustained virological response (SVR) was obtained (risk ratio 0.23, 95% CI 0.08–0.64, $P = 0.005$). In conclusion, inhibitory effect of IFN on development of HCC in the patients with chronic hepatitis C aged 60 and over was limited to the patients achieving SVR when treated with 6 months-IFN monotherapy.

*Keywords*: aged patients, chronic hepatitis C, hepatocellular carcinoma, interferon, sustained virological response.

## INTRODUCTION

In Japan, based on the epidemiological surveillance as well as the study on molecular tracing of hepatitis C virus (HCV), HCV infection is considered to spread from the 1920s and to expand more after World War II [1–5], The data of first-time blood donor candidates in Osaka demonstrated that the prevalence of anti-HCV antibodies among the candidates born in 1925–1935 was 7–10%, which was much higher than the prevalence of anti-HCV antibodies among the younger population [6]. Accordingly, chronic hepatitis C patients have become aged in Japan and HCV-related hepatocellular carcinoma (HCC) patients have also been shown to be old with a peak around age 70 and tended to decrease [1,3,5]. More importantly, the main cause of death in the patients with chronic hepatitis C has been reported to be HCC [7–10].

In the 1990s, interferon (IFN) therapy was used for the treatment of the patients with chronic hepatitis C worldwide and it has been shown by many studies including our reports that IFN therapy reduced the risk of HCC in patients with chronic hepatitis C [7,11–17]. This inhibitory effect of IFN therapy on hepatocarcinogenesis is notable when sustained virological response (SVR) was obtained, although SVR rate of IFN monotherapy was not very high. It has been also

Abbreviations: IFN, interferon; HCC, hepatocellular carcinoma; SVR, sustained virological response; HCV, hepatitis C virus; non-SVR, nonsustained virological response.

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reported that HCC development was significantly reduced in the patient achieving SVR as compared with those without SVR in chronic hepatitis C patients treated with IFN and ribavirin [18].

For the treatment of the patients with chronic hepatitis C, a combination of peginterferon and ribavirin has become a standard therapy, which has a high SVR rate [19–21]. However, the combination treatment has several adverse effects such as haemolytic anaemia which may not be tolerable for aged patients with chronic hepatitis C. On the other hand, aging is a significant risk factor for HCC in chronic hepatitis C patients. Accordingly, it is an important issue whether IFN monotherapy could reduce incidence of HCC in aged patients with chronic hepatitis C. Recently, Arase *et al.* [22] reported that long-term IFN monotherapy using low-dose of natural IFN-α was effective in preventing hepatocarcinogenesis in aged patients with chronic hepatitis C. In contrast, the hepatitis C antiviral long-term treatment against cirrhosis (HALT-C) Trial has shown that maintenance peginterferon therapy for 3.5 years did not reduce the incidence of HCC and the rate of disease progression in chronic hepatitis C patients with bridging fibrosis or cirrhosis who failed to respond to the combination therapy of peginterfeon-α2a and ribavirin [23,24].

We conducted a long-term multicenter retrospective cohort study to clarify the effect of 6-month IFN monotherapy on the incidence of HCC in aged patients with chronic hepatitis C.

## MATERIAL AND METHODS

### *Patients*

This study was conducted at Osaka University Hospital and six university-affiliated hospitals. IFN-treated patients consisted of 568 consecutive patients with chronic hepatitis C who had undergone liver biopsy 1 week to 2 months before IFN therapy and received either human lymphoblastoid IFN, recombinant IFN-α2a or recombinant IFN-α2b for 6 months between 1992 and 1995. The control group consisted of 158 consecutive patients with chronic hepatitis or cirrhosis who had undergone liver biopsy between January 1986 and December 1989, when IFN therapy had not been available in Japan. All the patients were positive for anti-HCV. The inclusion criteria in this study were as follows: (1) histological diagnosis of chronic hepatitis or cirrhosis; (2) no history of clinical signs at entry into the study of complications of cirrhosis, i.e. ascites, jaundice, encephalopathy, or variceal bleeding; (3) no previous IFN therapy; (4) no evidence of HCC at entry into the study as assessed by ultrasonography and/or computed tomography; (5) absence of serum hepatitis B surface antigen; (6) absence of co-existing liver diseases such as autoimmune hepatitis or primary biliary cirrhosis and (7) absence of excessive alcohol consumption (>80 g/day).

Sustained virological response was defined as persistent HCV RNA negativity during IFN therapy and follow-up. Patients showing positive HCV RNA after IFN therapy were classified as nonsustained virological response (non-SVR). In the patients with non-SVR, patients whose ALT levels decreased to the normal range and remained normal during IFN therapy were classified as transient biochemical response and patients without a decrease of ALT levels of the normal range during the therapy were classified as zbiochemical nonresponse.

Hepatitis C virus antibody was measured by first-, second-, or third-generation enzyme-linked immunosorbent assays (Ortho Diagnostics, Tokyo, Japan). Serum HCV RNA was measured by reverse transcription polymerase chain reaction or complementary DNA assay [25].

### *Follow-up*

The starting date of follow-up of the patients was defined as the date of liver biopsy. Abdominal ultrasonography or computed tomography and biochemical examinations including α-fetoprotein were performed every 3–6 months during follow-up equally in the IFN-treated and control patients. The diagnosis of HCC was confirmed by needle biopsy, by surgically resected tumour specimens, or by typical radiological findings on hepatic angiography or dynamic computed tomography. In the patients residing in Osaka whose follow-up data were not obtained, the Osaka Cancer Registry was used to determine whether HCC had occurred and the data were available until the end of 2002 in this study [13,26]. Accordingly, we decided to use the date of the development of HCC or the end of 2002 as the end of follow-up. As the longest observation period of the patients in the IFN group was 11 years, only the follow-up data for the first 11 years were considered in the control group. The study protocol was in accordance with the Helsinki Declaration of 1975 (revised in 1983) and approved by the Ethical Committee of the Ikeda Municipal Hospital.

### *Histological evaluation*

The sections were stained with haematoxylin–eosin and Azan-Mallory and histology of liver biopsy specimens was scored by two authors in a blinded manner using two scoring methods as described before [13]. Briefly, fibrosis score of Desmet *et al.* was used for the assessment of histological staging and a total score of histological activity (components 1–3) using the Knodell histological activity index was used for the assessment of histological grading [13,27,28].

### *Statistical analysis*

Patients who did not complete the treatment protocol were included for the analysis on an intention-to-treat basis. The chi-square test and Student's *t*-test were used to compare the

baseline characteristics. The Kaplan–Meier method was used to calculate the cumulative incidence of HCC, and the log-rank test was used to compare the cumulative incidence of HCC between the groups. To estimate independent risk factors for the development of HCC, a stepwise Cox proportional-hazards regression analysis was used. For the analysis, IFN therapy, age, gender, and histological staging and activity scores were used as variables. A $P$ value <0.05 was considered statistically significant. Data are presented as the mean ± SD and were analysed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

## RESULTS

Table 1 shows the baseline characteristics of the aged (60 years old and over) and non-aged (under 60 years old) groups. Both the histological stage and activity were significantly higher in the aged group than in the non-aged group. The proportion of male patients of the non-aged group was significantly higher than that of the aged group. In Table 2, baseline characteristics of controls and IFN-treated patients in the aged and non-age groups were compared. In the non-aged group, age at entry, proportion of male gender, histological activity score, serum ALT level and platelet count did not differ between the control and IFN-treated patients. However, histological stage of IFN-treated patients was less advanced as compared with that of the control patients. In the age-group, age at entry, proportion of male gender, histological stage and activity, serum ALT level and platelet count did not differ between the control and IFN-treated patients.

During the follow-up period, HCC was found in 35 controls and 44 IFN-treated patients among the non-aged group and in 14 controls and 48 IFN-treated patients among the aged group. The median tumour sizes of HCC in controls and IFN-treated patients at the time of discovery on ultrasonography or computed tomography were 22 mm (range, 10–55 mm) and 19 mm (range, 8–52 mm) respectively ($P \geq 0.2$). In the non-aged group, the cumulative incidence of HCC estimated by the Kaplan–Meier Method of IFN-treated patients was significantly lower than that of control patients (log-rank test, $P < 0.001$, Fig. 1a), whereas there was no difference in the cumulative incidence of HCC between controls and IFN-treated patients in the aged group (log-rank test, $P = 0.498$, Fig. 1b). The cumulative incidence of HCC of SVR and non-SVR patients and controls of the aged and non-aged groups are shown in Fig. 2. The 10-year incidences of HCC for controls, non-SVR and SVR patients in the non-aged group were 30.1%, 15.8%, 4.5% respectively (log-rank test, $P < 0.001$, Fig. 2a). Also, the 10-year incidences of HCC for controls, non-SVR and SVR patients in the aged group were 39.1%, 38.9%, 12.7% respectively (log-rank test, $P = 0.015$, Fig. 2b).

In Table 3, risk ratios for the development of HCC calculated by a stepwise Cox regression analysis in the aged and non-aged patients with chronic hepatitis C according to virological and biochemical responses to IFN are summarized. In the 410 IFN-treated patients of non-aged group, 134 patients (32.7%) achieved SVR and the remaining 276 showed non-SVR (Table 3). Of this 276 patients showing non-SVR, 163 showed transient biochemical response and 113 showed biochemical nonresponse during the IFN treatment. On the other hand, 41 (25.9%) of 158 IFN-treated patients of the aged group obtained SVR and the other 117 did not obtain SVR (Table 3). Of the 117 non-SVR patients, 57 showed transient biochemical response and 60

Table 1 Baseline characteristics of aged and non-aged patients with chronic hepatitis C

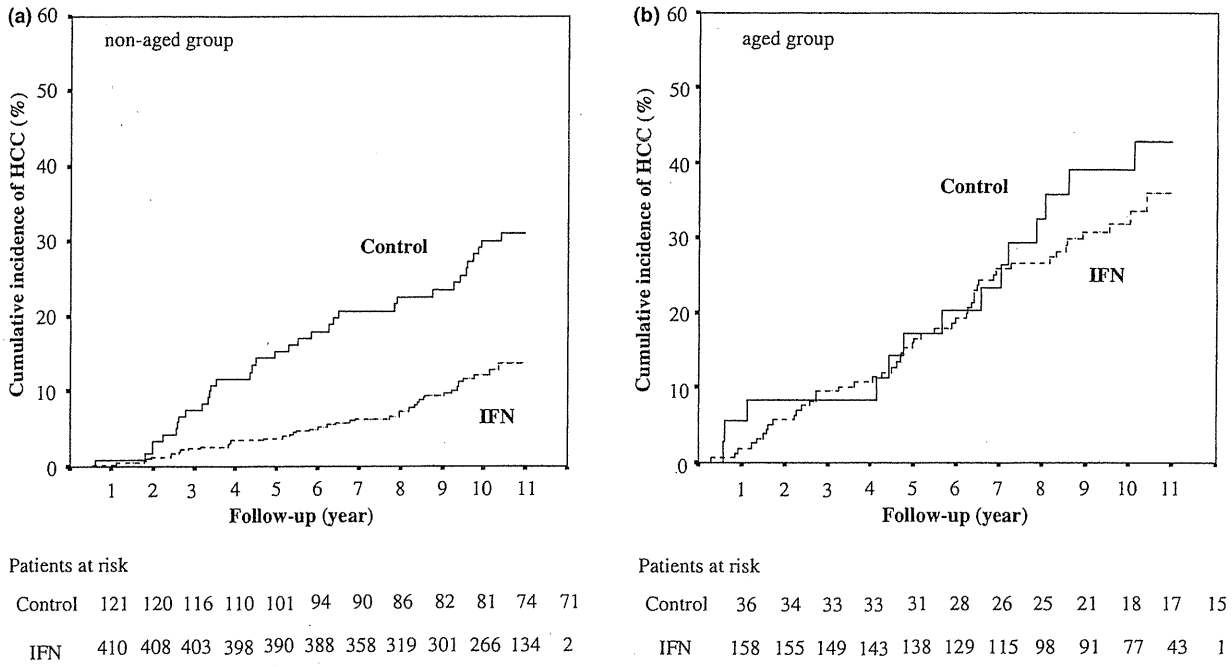
| | Non-aged group ($n$ = 531) | Aged group ($n$ = 194) | $P$ value |
|---|---|---|---|
| Control group ($n$)/IFN group ($n$) | 121/410 | 36/158 | 0.262 |
| Age | 48.1 ± 9.7 | 63.7 ± 3.3 | <0.001 |
| Gender | | | |
| Male | 353 | 108 | 0.009 |
| Female | 178 | 86 | |
| Histological stage* | | | |
| F0, 1 | 186 | 37 | 0.001 |
| F2 | 157 | 69 | |
| F3 | 141 | 69 | |
| F4 | 47 | 19 | |
| Histological activity† | | | |
| <10 | 329 | 104 | 0.049 |
| ≧ 10 | 202 | 90 | |
| ALT (IU/L) | 117 ± 86 | 104 ± 60 | 0.053 |
| Platelete count ($10^4/\mu$L) | 15.4 ± 5.6 | 14.4 ± 5.6 | 0.040 |

*According to Desmet *et al.*[27] †Based on components 1–3 of the Knodell histological activity.

**Table 2** Baseline characteristics of controls and IFN-treated patients in aged and non-aged groups

| | Non-aged group | | | Aged group | | |
|---|---|---|---|---|---|---|
| | Controls | IFN-treated | *P* value | Controls | IFN-treated | *P* value |
| *n* | 121 | 410 | | 36 | 158 | |
| Age | 48.4 ± 10.5 | 48.0 ± 9.4 | 0.736 | 64.6 ± 3.6 | 63.5 ± 3.2 | 0.059 |
| Gender | | | | | | |
| Male | 75 | 278 | 0.273 | 22 | 86 | 0.579 |
| Female | 46 | 86 | | 14 | 72 | |
| Histologic stage* | | | | | | |
| F0,1 | 27 | 159 | <0.001 | 8 | 29 | 0.933 |
| F2 | 28 | 129 | | 12 | 57 | |
| F3 | 47 | 94 | | 12 | 57 | |
| F4 | 19 | 28 | | 4 | 15 | |
| Histologic activity† | | | | | | |
| <10 | 72 | 257 | 0.525 | 20 | 84 | 0.854 |
| ≧ 10 | 49 | 153 | | 16 | 74 | |
| ALT (IU/L) | 127 ± 80 | 114 ± 88 | 0.132 | 110 ± 85 | 103 ± 53 | 0.523 |
| Platelete count ($10^4/\mu L$) | 15.2 ± 6.1 | 15.4 ± 5.4 | 0.766 | 15.0 ± 5.4 | 14.3 ± 5.7 | 0.486 |
| HCV RNA load | | | | | | |
| High | ND‡ | 166 | | ND‡ | 54 | |
| Low | ND‡ | 116 | | ND‡ | 30 | |
| HCV RNA serotype | | | | | | |
| 1 | ND‡ | 231 | | ND‡ | 90 | |
| 2 | ND‡ | 102 | | ND‡ | 32 | |

*According to Desmet *et al.* [27] †Based on components 1–3 of the Knodell histologic activity. ‡Not done.



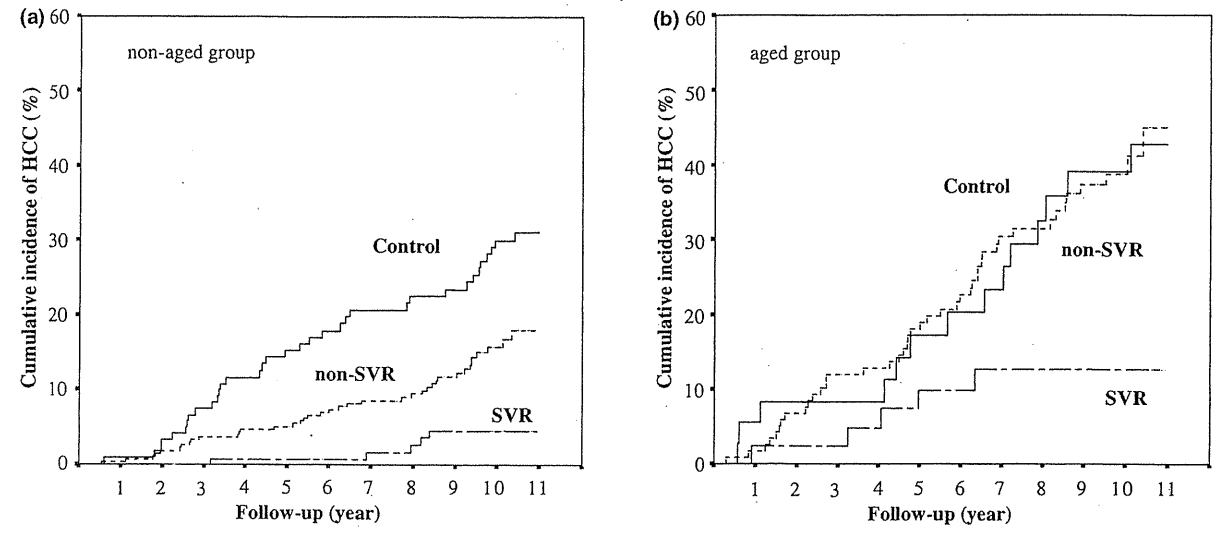

Patients at risk

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 121 | 120 | 116 | 110 | 101 | 94 | 90 | 86 | 82 | 81 | 74 | 71 |
| IFN | 410 | 408 | 403 | 398 | 390 | 388 | 358 | 319 | 301 | 266 | 134 | 2 |

Patients at risk

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 36 | 34 | 33 | 33 | 31 | 28 | 26 | 25 | 21 | 18 | 17 | 15 |
| IFN | 158 | 155 | 149 | 143 | 138 | 129 | 115 | 98 | 91 | 77 | 43 | 1 |

**Fig. 1** Cumulative incidence of hepatocellular carcinoma in IFN-treated (dotted line) and control (solid line) patients of the non-aged group (a) and the aged group (b). A log-rank test of the two curves showed a significant difference in the non-aged group ($P < 0.001$), whereas no significant difference was observed in the aged group ($P = 0.498$).




Patients at risk

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 121 | 120 | 116 | 110 | 101 | 94 | 90 | 86 | 82 | 81 | 74 | 71 |
| Non-SVR | 276 | 274 | 269 | 264 | 259 | 257 | 239 | 212 | 199 | 179 | 85 | 2 |
| SVR | 134 | 134 | 134 | 134 | 131 | 131 | 119 | 107 | 102 | 87 | 49 | 0 |

Patients at risk

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 36 | 34 | 33 | 33 | 31 | 28 | 26 | 25 | 21 | 18 | 17 | 15 |
| Non-SVR | 117 | 115 | 115 | 103 | 100 | 93 | 82 | 67 | 60 | 50 | 26 | 1 |
| SVR | 41 | 40 | 40 | 40 | 38 | 36 | 33 | 31 | 31 | 27 | 17 | 0 |

Fig. 2 (a) Cumulative incidence of hepatocellular carcinoma categorized by sustained virological response (dashed line), nonsustained virological response (dotted line), and controls (solid line) of the non-aged group (a) and the aged group (b). A log-rank test of the three curves showed a significant difference between these groups (non-aged group, $P < 0.001$; aged group, $P = 0.015$).

showed biochemical nonresponse. In the non-aged group, stepwise Cox regression analysis identified IFN therapy (risk ratio 0.52, 95% CI 0.33–0.81, $P = 0.004$), older age (risk ratio 1.07, 95% CI 1.03–1.10, $P = 0.001$), and higher histological stage (score 3 or 4) (risk ratio 4.03, 95% CI 2.41–6.76, $P < 0.001$) as independent risk factors associated with the development of HCC. In the non-aged group, the development of HCC was strongly suppressed when SVR was achieved (risk ratio 0.20, 95% CI 0.08–0.50, $P < 0.001$) (Table 3). In the patients with transient biochemical response of the non-SVR group among the non-aged group, HCC development was also significantly reduced (risk ratio 0.47, 95% CI 0.26–0.86, $P = 0.015$). In the aged group, stepwise Cox regression analysis revealed that only higher histological stage (score 3 or 4) (risk ratio 2.27, 95% CI 1.36–3.78, $P = 0.002$) and male gender (risk ratio 2.00, 95% CI 1.17–3.41, $P = 0.011$) were independent factors responsible for the development of HCC (Table 3). Although IFN therapy was not identified as an independent variable for HCC, the risk of HCC was significantly decreased in the patients with SVR in the aged group as shown in the Table 3 (risk ratio 0.23, 95% CI 0.08–0.64, $P = 0.005$). In the

**Table 3** Risk ratios for hepatocellular carcinoma in aged and non-aged patients with chronic hepatitis C according to virological and biochemical responses to interferon*

| | Non-aged group ($n$ = 531) | | | | Aged group ($n$ = 194) | | | |
|---|---|---|---|---|---|---|---|---|
| | $n$ | Risk ratio | 95% CI | $P$ value | $n$ | Risk ratio | 95% CI | $P$ value |
| Control group | 121 | 1.00 | | | 36 | 1.00 | | |
| IFN group | 410 | 0.52 | 0.33–0.81 | 0.004 | 158 | 0.77 | 0.42–1.40 | 0.388 |
| Sustained virological response | 134 | 0.20 | 0.08–0.50 | 0.001 | 41 | 0.23 | 0.08–0.64 | 0.005 |
| Nonsustained virological response | 276 | 0.65 | 0.41–1.03 | 0.068 | 117 | 1.07 | 0.58–1.97 | 0.821 |
| Transient biochemical response† | 163 | 0.47 | 0.26–0.86 | 0.015 | 57 | 0.67 | 0.32–1.43 | 0.303 |
| Biochemical nonresponse† | 113 | 0.86 | 0.51–1.47 | 0.584 | 60 | 1.46 | 0.77–2.78 | 0.245 |

*A stepwise Cox regression analysis was carried out by using interferon therapy, age, gender, and histologic stage and histologic activity scores as variables. †Nonsustained virological response was classified into transient biochemical response and biochemical nonresponse according to the ALT response during the interferon treatment.

patients with transient biochemical response of the non-SVR group of aged patients, HCC development was not reduced (risk ratio 0.67, 95% CI 0.32–1.43, $P = 0.303$, Table 3) in contrast to the patients showing transient biochemical response in the non-aged group.

As the cumulative incidence of HCC calculated by the Kaplan–Meier Method of the patients with SVR in the aged group was much higher than that in the non-aged group, we also carried out Cox proportional-hazards regression analysis to estimate risk factors responsible for HCC development in the 175 patients achieving SVR. As a result, older age (risk ratio 1.09, 95% CI 1.01–1.18, $P = 0.025$) and higher histological activity before IFN therapy started (10 or more of the total score of components 1–3 in Knodell's histological activity index) (risk ratio 4.16, 95% CI 1.07–16.25, $P = 0.040$) were identified as risk factors associated with HCC among the patients with SVR.

## DISCUSSION

In this long-term retrospective cohort study, an inhibitory effect of 6 months-IFN monotherapy in early 1990s on the cumulative incidence of HCC were compared between the patients with histologically proven chronic hepatitis C under 60 years old (non-aged group) and those 60 years old and over (aged group). Because of retrospective analysis, there were some differences in baseline characteristics between the two groups. In the aged group, the histological stage and activity as well as the proportion of male patients were significantly higher than in the non-aged group. Also, SVR rate in the aged group was lower than that in the non-aged group. To avoid the influence of these biases, we performed Cox proportional-hazards regression analysis to see whether IFN monotherapy reduced the risk of HCC in the aged and non-aged groups. Then, we found that IFN therapy for 6 months significantly reduced the risk of HCC (risk ratio 0.52) in the non-aged group, whereas this inhibitory effect of IFN monotherapy on HCC development was recognized only in the patients achieving SVR among the aged-patients.

It is difficult to explain why IFN had no inhibitory effect on HCC development in the aged patients, whereas IFN had significant inhibitory effect in the non-aged patients of this study. Many clinical studies have demonstrated that aging was an independent risk factor associated with HCV-related HCC other than advanced histological staging and male gender [7,11–17,29]. However, molecular mechanism of the impact of aging on haepetocarcinogenesis has not been elucidated. Moriya *et al.* reported that lipid hydroperoxide products accumulated in the liver without inflammation and may play a role in the development of HCC in HCV core gene transgenic mice [30,31]. A long-term infection of HCV may lead to HCC through some molecular alterations.

Recently, there have been two controversial reports from the United States and Japan as to the long-term effect of low-dose IFN therapy on the incidence of HCC in chronic hepatitis C [22,24]. The report from Japan was a non-randomized retrospective study and observed beneficial effect of long-term natural IFN-α therapy on hepatocarcinogenesis in aged chronic hepatitis C patients [22]. The HALT-C Trial from the United States, a large prospective randomized study, reported that treatment with peginterfeon-α2a at a dose of 90 μg weekly for 3.5 years did not prevent HCC development in the patients with bridging fibrosis or cirrhosis who did not obtain SVR by combination therapy of peginterferon and ribavirin [24]. The result was consistent with our data in the aged patients. However, the annual incidence of HCC of the HALT-C Trial, about 1%, was much lower than that in the aged group in this study, about 4%. Accordingly, a randomized prospective study to determine the effect of long-term IFN or peginterferon therapy on the incidence of HCC in chronic hepatitis C, especially in the aged patients, may be needed in Japan.

This study has a limitation, because we used historical controls as control patients. A lead-time bias may have occurred. Detection of HCC by the screening program could be less effective in controls than IFN-treated patients. In that case, we might underestimate the effect of IFN on the cumulative incidence of HCC. However, such underestimation may be unlikely as the tumour sizes at the time of detection were not different between the control and IFN-treated patients.

The 10-year incidence of HCC for SVR patients of the aged group (12.7%) was much higher than that of non-aged group (4.5%) in our study. Makiyama *et al.* [32] studied the risk factors for developing HCC after obtaining sustained biochemical response to IFN therapy in chronic hepatitis C and reported that older age, male gender and advanced fibrosis were associated with HCC. Consistent with their results, we found that older age was an independent risk factor for HCC in the patients with SVR, suggesting a high potential of developing HCC even after eradication of HCV RNA in the aged patients. Another possibility is that malignant foci, which could not be detected by imaging modalities, had already existed before IFN therapy. Our finding indicates that even in the patients showing SVR, a follow-up examination to investigate HCC should be carried out for at least 10 years, particularly in the aged patients.

In conclusion, IFN monotherapy reduced the risk of HCC in the patients with chronic hepatitis C under 60 years old. In contrast, this inhibitory effect of IFN on hepatocacinogenesis was limited to patients showing SVR in the aged-patients when treated with 6 months-IFN monotherapy. These results suggest that combination therapy of peginterferon and ribavirin is recommended even in the aged patients with chronic hepatitis C to obtain better preventive effect of IFN on HCC development. For reasons of relatively high cumulative incidence of HCC in the aged chronic hepatitis C patients with SVR to IFN therapy, they should be followed carefully even after eradication of HCV by IFN therapy.

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Research Article

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# Altered interferon-α-signaling in natural killer cells from patients with chronic hepatitis C virus infection☆

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**Background & Aims**: Natural killer (NK) cells play an important role in the immune response against virus infection. Interferon (IFN)-α, an essential component in therapy against hepatitis C virus (HCV) infection, regulates NK cell function. However, it remains obscure how chronic HCV infection (CHC) modifies intracellular IFN-α signaling in NK cells. We investigated IFN-α signaling in NK cells in patients with CHC.

**Methods**: Peripheral blood mononuclear cells were obtained from patients with CHC and healthy subjects (HS) as controls.

**Results**: The expression level of signal transducer and activator of transcription (STAT) 1, a key molecule of IFN-α signaling, was clearly higher in NK cells from the CHC patients than in those from HS. The phosphorylation level of STAT1 with IFN-α stimulation was significantly greater in NK cells from the CHC patients than in those from the HS, while that of STAT4 was significantly less. These phosphorylation levels of STAT1 and STAT4 positively and negatively correlated with the STAT1 level in NK cells, respectively. The IFN-α induced messenger RNA level of the suppressor of cytokine signaling 1, which is a downstream gene of phosphorylated-STAT1, was clearly greater in NK cells from the CHC patients than in those from the HS, while that of IFN-γ, which is a downstream gene of phosphorylated-STAT4, was clearly lower.

**Conclusions**: These results indicate altered IFN-α signaling in NK cells in CHC patients, suggesting that this alteration is associated with the persistence of HCV infection and resistance to IFN-α therapy.



Keywords: Natural killer cells; Interferon; Hepatitis C virus; Signal transducer and activator of transcription.

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*Abbreviations:* NK, natural killer; IFN, interferon; STAT, signal transducer and activator of transcription; HCV, hepatitis C virus; CHC, chronic hepatitis C virus infection; HS, healthy subject; SOCS, suppressor of cytokine signaling; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cell; IL, interleukin; pSTAT, phosphorylated-signal transducer and activator of transcription; RT-PCR, reverse transcription polymerase chain reaction; NKT, natural killer T; ISG, interferon stimulated gene.

## Introduction

Natural killer (NK) cells play an important role in innate immune responses against a variety of viral infections by directly killing infected cells with cytotoxic molecules such as perforin and granzyme [1]. The cells also have a great ability to secrete a key cytokine, interferon (IFN)-γ, which activates subsequent adaptive immune responses as well as inhibits viral replication [1,2]. Another major component in innate immune responses during viral infections is IFN-α, which is the most abundant cytokine released during viral infections [3]. In addition to its anti-viral effects, IFN-α activates NK cells to induce IFN-γ production via activation of the signal transducer and activator of transcription (STAT) 4, as well as its cytotoxic ability via activation of STAT1 [4–7].

Hepatitis C virus (HCV) causes persistent infection in more than 70% of infected patients. Whereas some of the patients show a carrier-like state, most develop chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, which is why HCV infection is a worldwide health problem [8]. The administration of IFN-α is a well-established anti-viral therapy for HCV infection. More than 90% of patients with acute HCV infection respond to IFN-α based therapy, while only around 50% of patients with chronic HCV infection (CHC) do [9–12], suggesting a mechanism by which persistent HCV infection leads to resistance to IFN-α based therapy. NK cell number has been demonstrated to decrease in patients with CHC, while it is controversial whether NK cell functions are impaired in patients with CHC [13–15]. It thus remains unclear whether perturbation of NK cells is involved in the persistence of CHC as well as resistance to the therapy [13–15].

In the present study, we investigated how chronic HCV infection modifies intracellular IFN-α signaling in NK cells. The expression level of total STAT1, a key molecule of IFN-α signaling, was clearly higher in NK cells from patients with CHC than in those from healthy subjects (HS). Phosphorylation of STAT1, resulting in induction of the suppressor of cytokine signaling (SOCS) 1 messenger RNA (mRNA) expression in response to IFN-α was clearly greater in NK cells from the CHC patients than

in those from the HS. On the other hand, the phosphorylation of STAT4, resulting in induction of IFN-γ mRNA expression in response to IFN-α, was clearly less in NK cells from the CHC patients than in those from the HS. NK cell degranulation was significantly enhanced in response to IFN-α in the HS, but not in the CHC patients. These findings suggest altered IFN-α signaling in NK cells in patients with CHC. The alteration of IFN-α signaling might be associated with the persistence of chronic HCV infection and resistance to IFN-α therapy.

## Materials and methods

*Subjects*

Twenty-six patients with CHC (HCV RNA genotype 1) and 26 healthy volunteers were enrolled in this study. The profile of these subjects is shown in the Supplementary data and Supplementary Table 1. The study was approved by the ethical committee of Osaka University Hospital.

*Isolation of peripheral blood mononuclear cell (PBMC) populations*

PBMCs were isolated from fresh heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation as described [16].

*In vitro stimulation of cells*

Prepared cells were unstimulated or stimulated with either natural human IFN-α, recombinant human IFN-γ or recombinant human interleukin (IL)-12. The details are provided in the Supplementary data.

*Cell lysates and Western blot analysis*

Prepared cells were lysed as described [17]. A 25 μg sample of protein was separated on 10% SDS polyacrylamide gels and transferred onto PVDF membrane. Monoclonal anti-STAT1 antibody (1/Stat1) was purchased from BD Biosciences (San Jose, CA, USA). Polyclonal anti-β-actin antibody from Abcam (Cambridge, MA, USA) was used as the loading control. Detection of immunolabeled proteins was performed as described [17].

*Flow cytometric analysis*

The staining of prepared cells was performed as described [16,18]. Briefly, cells were stained with fluorescein isothiocyanate-conjugated anti-CD3 (UCHT1) and biotin-conjugated anti-CD56 antibody (B159), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and cold pure methanol, and then stained with phycoerythrin-conjugated anti-phosphorylated-STAT (pSTAT) 4 (pY693) (38/p-Stat4) and Alexa Fluor® 647-conjugated anti-pSTAT1 (pY701) antibody (4a), or phycoerythrin-conjugated anti-STAT1 (1/Stat1) antibody alone, or the corresponding isotype control, followed by staining with peridinin chlorophyll protein-conjugated streptavidin (BD Biosciences). All antibodies were purchased from BD Biosciences. The stained cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA), and the data were processed using the FlowJo program (Tree Star Inc., Ashland, OR, USA).

*NK cell enrichment from PBMCs*

To obtain pure populations of $CD56^+$ CD3 NK cells from PBMCs, NK cells were negatively isolated by magnetic cell sorting with a human NK cell isolation kit (Miltenyi Biotec, Gladbach, Germany). The purity of the isolated population was confirmed using FACS analysis and was more than 90%.

*RNA isolation and analysis*

Total RNA isolation and the real-time reverse transcription polymerase chain reaction (RT-PCR) analysis are presented in detail in the Supplementary data, Supplementary Fig. 1, and Supplementary Table 2.

*NK cell degranulation assay*

NK cell degranulation was assessed as described [19], with minor modifications. Details and representative data are provided in the Supplementary data and Supplementary Fig. 2.

*Statistical analysis*

The statistical significance of differences between the patient and control groups or that of changes due to IFN-α stimulation in the NK cell degranulation assay was determined by applying unpaired or paired Student's *t*-test, respectively. Correlations were assessed using the Pearson product–moment correlation coefficient. The statistical significance was defined as $p < 0.05$.

## Results

*NK cells from CHC patients showed a higher level of STAT1 expression than those from HS*

Murine NK cells have been reported to exhibit a lower level of STAT1 expression than non-NK cells [17,18]. Also, the increase of STAT1 expression level in murine NK cells has been observed in viral infection [18]. We examined whether similar findings could be observed for human NK cells. Western blot analyses revealed that human NK cells from representative HS have a clearly lower level of STAT1 expression than non-NK cells (Fig. 1A). We then examined whether chronic HCV infection affected the STAT1 expression level in NK cells. It was clearly higher in NK cells from the CHC patients than in those from the HS (Fig. 1B).

We next examined STAT1 expression level within individual cells by flow cytometry. Consistent with the results of Western blot analyses, flow cytometric analyses demonstrated that NK cells from a representative healthy subject had a lower level of STAT1 expression than non-NK cells such as T cells or natural killer T (NKT) cells (Fig. 2A). NK cells from a representative CHC patient displayed a clearly higher level of STAT1 expression than

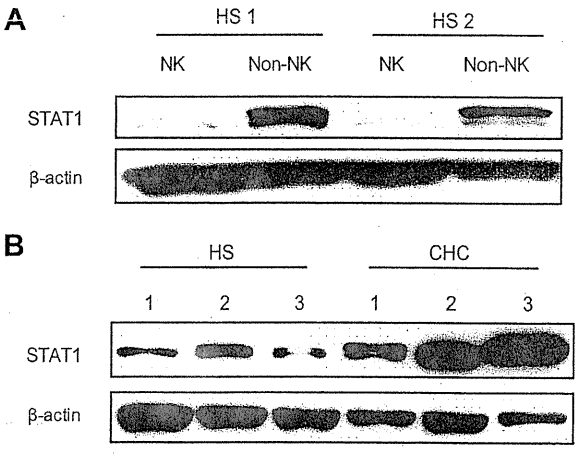


**Fig. 1. STAT1 expression in NK cells from patients with chronic HCV infection.** STAT1 protein levels were evaluated by Western blot analyses with β-actin measurement as a loading control. PBMCs were obtained from patients with chronic HCV infection (CHC) and healthy subjects (HS). NK cells and non-NK cells were purified from those cells. (A) STAT1 protein levels in NK cells and non-NK cells from two representative HS are shown. (B) STAT1 protein levels in NK cells from three other representative HS and three representative patients are shown.

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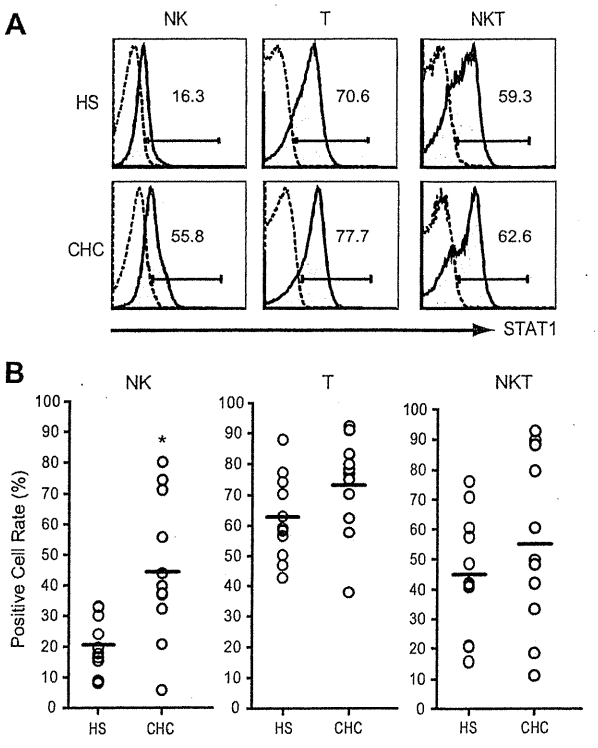


**Fig. 2. STAT1 expression level in NK, T or NKT cells from patients with chronic HCV infection.** STAT1 protein level was evaluated by flow cytometry, electronically gating on $CD56^+$ CD3 NK cells, CD56 $CD3^+$ T cells, and $CD56^+$ $CD3^+$ NKT cells. PBMCs were derived from patients with chronic HCV infection (CHC) and healthy subjects (HS). (A) Representative histograms from a patient and an HS are shown. Dotted lines show staining with the isotype control. Solid lines with shaded areas show staining with the antibody. Numbers are percentages of positive cells (positive cell rate) determined based on the isotype control staining. (B) Comparison of STAT1 expression level between the patients with CHC ($n = 11$) and HS ($n = 11$) are shown as positive cell rates. Each circle represents data for an individual. Horizontal bars represent means. *$p$ <0.005 vs. HS.

those from the healthy subject. Fig. 2B summarizes the profile of the intracellular STAT1 expression level of NK, T, and NKT cells. The intracellular STAT1 expression level of NK cells from the CHC patients was significantly higher than that from the HS, while that of T cells or NKT cells did not show any significant difference.

*Altered activation of STAT1/4 occurred in response to IFN-α in NK cells from CHC patients*

Activation of STAT1/4 in response to IFN-α in murine NK cells has been reported to shift from pSTAT4 dominant to pSTAT1 dominant as the intracellular STAT1 expression level increases [18]. This led us to examine the activation of STAT1/4 in response to IFN-α in human NK cells using samples from CHC patients and HS. Although IFN-α can phosphorylate both STAT1 and STAT4, IFN-γ can phosphorylate STAT1 and IL-12 can phosphorylate STAT4 in NK cells [1,7]. We examined the phosphorylation level of STAT1/4 in response to IFN-α, compared with IFN-γ or IL-12, in NK cells from the subjects by flow cytometry. It was found that IFN-α phosphorylated STAT1 more strongly in NK cells from the representative CHC patient than in those from the representative HS, while IFN-γ did not (Fig. 3A). On the other hand, IFN-α phosphorylated STAT4 more weakly in NK cells from the CHC patient than in those from the HS, while IL-12 did not. Fig. 3B summarizes the profile of STAT1/4 phosphorylation level in NK cells in response to IFN-α, IFN-γ or IL-12. IFN-α, but not IFN-γ, phosphorylated STAT1 significantly more strongly in NK cells from the CHC patients than in those from the HS. IFN-α, but not IL-12, phosphorylated STAT4 significantly more weakly in NK cells from the CHC patients than in those from the HS. These results suggested altered signaling of IFN-α, but not of IFN-γ or of IL-12, in NK cells of patients with CHC.

We then examined the relationship between STAT1 expression level and STAT1/4 phosphorylation level in response to IFN-α in NK cells from the CHC patients. The phosphorylation level of STAT1 in response to IFN-α correlated significantly and positively with the STAT1 expression level in NK cells ($R^2 = 0.67$, $p < 0.003$), while that of STAT4 correlated significantly and negatively ($R^2 = 0.49$, $p < 0.02$) (Fig. 4). These results suggested that, as in murine NK cells, the activation of STAT1/4 in response to IFN-α in human NK cells shifts from a preference for pSTAT4 to one for pSTAT1 as intracellular STAT1 expression level increases.

*Altered induction of interferon stimulated gene (ISG) expression occurred in response to IFN-α in NK cells*

We examined how the altered activation of STAT1/4 in response to IFN-α in NK cells affected the induction of downstream gene expression. Real-time RT-PCR analyses revealed that the induction level of SOCS1 mRNA expression with IFN-α stimulation, which is a downstream gene of pSTAT1, was clearly greater in NK cells isolated from the CHC patients than in those from the HS, and that the induction level of IFN-γ mRNA expression with IFN-α stimulation, which is a downstream gene of pSTAT4, was clearly lower (Fig. 5). On the other hand, the mRNA induction level of perforin or granzyme B, which is a cytotoxic molecule induced by IFN-α via pSTAT1, was not greater but modestly lower, suggesting negative regulation by the large induction of SOCS1, which is a negative regulator of the pSTAT1 pathway [20].

*Altered activation of NK cells occurred in response to IFN-α*

To examine how the altered IFN-α signaling in NK cells affected the activation of NK cells in response to IFN-α, we evaluated the NK cell degranulation ability in response to IFN-α. NK cell degranulation assay showed that CD107a expression, as a marker of degranulation, in the presence of K562 cells was significantly up-regulated in response to IFN-α in NK cells from HS, but not in those from CHC patients (Fig. 6), suggesting altered NK cell activation in response to IFN-α in CHC patients.

## Discussion

Both IFN-α and IFN-γ have been observed in sera from patients with CHC [21,22]. Their production may be induced by a host response to HCV within the liver, which would make these IFNs detectable in the systemic circulation of patients with CHC. The present study has shown that NK cells from patients with CHC display higher levels of STAT1 expression compared to those from HS (Fig. 1B and Fig. 2). STAT1 itself is one of the ISGs, whose

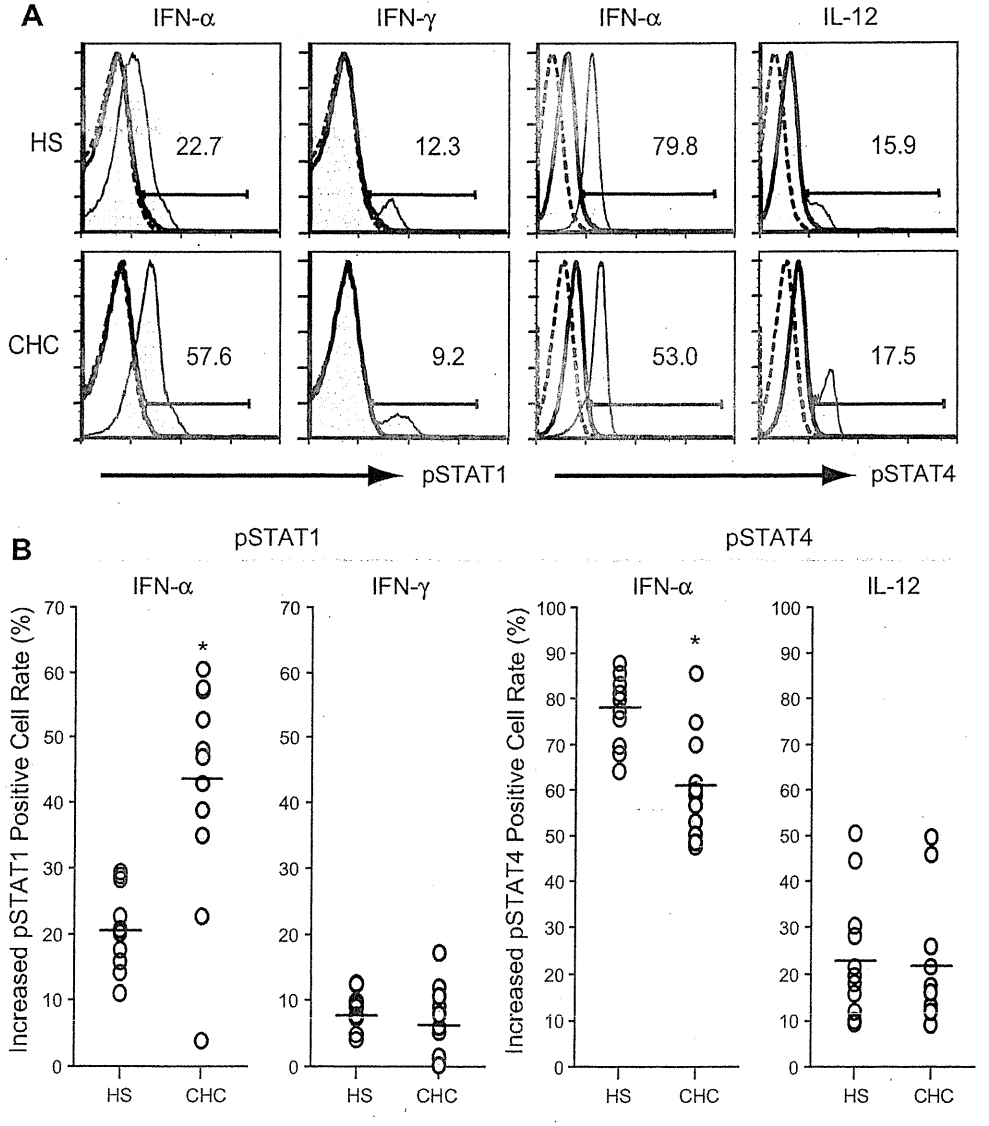


**Fig. 3. Altered activation of STAT1/4 in response to IFN-α, but not to IFN-γ or IL-12 in NK cells from patients with chronic HCV infection.** pSTAT1 and pSTAT4 protein levels were evaluated by flow cytometry with isotype control staining. PBMCs were derived from patients with chronic HCV infection (CHC) and healthy subjects (HS). (A) Prepared PBMCs were unstimulated or stimulated with natural IFN-α, IFN-γ or IL-12 for 90 min *in vitro*, and then collected. pSTAT1 and pSTAT4 protein levels were evaluated by flow cytometry, electronically gating on $CD56^+$ CD3 NK cells. Representative histograms of a patient and an HS are shown. Dotted lines show staining of stimulated cells with isotype control. Thick lines show staining of unstimulated cells with the antibody. Thin lines with shaded areas show staining of stimulated cells with the antibody. Positive cell rates were determined based on the staining with isotype controls. Numbers are increased positive cell rates which were determined by subtracting the positive cell rate of unstimulated cells from those of stimulated cells. (B) Comparison of pSTAT1/4 level in response to IFN-α, IFN-γ or IL-12 between the patients with CHC ($n = 11$) and the HS ($n = 11$) are shown as increased pSTAT1/4 positive cell rate. Each circle represents individual data. Horizontal bars represent means. *$p < 0.001$ vs. HS.

expression is up-regulated by IFN-α or IFN-γ [23,24]. It is thus possible that the higher level of STAT1 in NK cells from the CHC patients was up-regulated by IFN-α and/or IFN-γ induced by a chronic host response to HCV.

Our real-time RT-PCR analyses showed that the induction level of SOCS1 mRNA in response to IFN-α was significantly greater in NK cells from the CHC patients than in those from the HS (Fig. 5). This finding is consistent with the observation that the phosphorylation level of STAT1 in response to IFN-α was significantly stronger in NK cells from the CHC patients than in those from the HS (Fig. 3), because SOCS1 is a downstream gene of pSTAT1 [20]. On the other hand, SOCS1 is an inducible negative regulator which inhibits further activation of the pSTAT1 pathway [20]. It is therefore possible that this greater up-regulation of SOCS1 owing to the greater level of STAT1 phosphorylation in response to IFN-α finally results in a weaker response to IFN-α, that is, a weaker induction of ISGs via the pSTAT1 pathway. Indeed, an increase of the STAT1 level,