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# Predictive Value of Early Viral Dynamics During Peginterferon and Ribavirin Combination Therapy Based on Genetic Polymorphisms Near the *IL28B* Gene in Patients Infected With HCV Genotype 1b

**Hidenori Toyoda,[1]* Takashi Kumada,[1] Toshifumi Tada,[1] Kazuhiko Hayashi,[2] Takashi Honda,[2] Yoshiaki Katano,[2] Hidemi Goto,[2] Takahisa Kawaguchi,[3] Yoshiki Murakami,[3] and Fumihiko Matsuda[3]**

[1]*Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan*
[2]*Department of Gastroenterology, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[3]*Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan*

A study was carried out to determine whether early viral dynamics retain prediction of the outcome of peginterferon (PEG-IFN) and ribavirin combination therapy based on different genetic polymorphisms near the *IL28B* gene, the strongest baseline predictor of response to this therapy. A total of 272 patients infected with hepatitis C virus (HCV) genotype 1b were grouped according to genetic polymorphisms near the *IL28B* gene (rs8099917). The ability of reduced HCV RNA levels at 4 and 12 weeks after starting therapy to predict a sustained virologic response was evaluated based on these genotypes. Among patients with the TT genotype for rs8099917 (associated with a favorable response), the rates of sustained virologic response were higher in patients with a $\geq$3 $\log_{10}$ reduction in serum HCV RNA levels at 4 weeks after starting therapy ($P < 0.0001$). In contrast, among patients with the TG/GG genotype (associated with an unfavorable response), there were no differences in this rate based on the reduction in HCV RNA levels at 4 weeks. Early viral dynamics at 4 weeks after starting therapy retains its predictive value for sustained virologic response in patients with the TT genotype for rs8099917, but not in patients with the TG/GG genotype. Patients who are likely to achieve sustained virologic response despite unfavorable TG/GG genotype cannot be identified based on early viral dynamics during therapy. In contrast, lack of early virologic response at 12 weeks retains a strong predictive value for the failure of sustained virologic response regardless of *IL28B* polymorphisms, which remains useful as a factor to stop therapy. ***J. Med. Virol. 84:61–70, 2012.*** © 2011 Wiley Periodicals, Inc.

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**KEY WORDS:** chronic hepatitis C; early viral dynamics; genetic polymorphisms near the *IL28B* gene; peginterferon; response-guided therapy; ribavirin

## INTRODUCTION

The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although this treatment regimen has increased markedly the number of patients with a sustained virologic response, i.e., the eradication of hepatitis C virus (HCV), only 50% of patients infected with HCV genotype 1 achieved a sustained virologic response approximately.

Many investigators have examined factors that predict the treatment outcome of PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1. In addition to the baseline factors, the response of HCV during combination therapy, i.e., the changes in serum HCV RNA levels after starting therapy, has been shown to be an important predictor of the treatment outcome [Zeuzem et al., 2001; Buti

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*Correspondence to: Hidenori Toyoda, MD, PhD, Department of Gastroenterology, Ogaki Municipal Hospital 4-86, Minaminokawa, Ogaki, Gifu, 503-8502, Japan.
E-mail: hmtoyoda@spice.ocn.ne.jp

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et al., 2002; Berg et al., 2003], with the emphasis on "response-guided therapy" [Lee and Ferenci, 2008; Marcellin and Rizzetto, 2008]. Recent reports have emphasized the importance of evaluating the viral dynamics at 4 weeks after starting therapy to predict a sustained virologic response. A rapid virologic response, in which serum HCV RNA is undetectable at 4 weeks after starting therapy, has been the strongest predictive factor of a sustained virologic response reportedly [Martinez-Bauer et al., 2006; Poordad et al., 2008; de Segadas-Soares et al., 2009; Martinot-Peignoux et al., 2009]. In addition, the predictive value of reduced serum HCV RNA levels at 4 weeks after starting therapy has been clarified further, and a $\geq 3$ $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy has high predictive value that a patient will achieve a sustained virologic response as a final outcome, even in the absence of a rapid virologic response [Toyoda et al., 2011].

In contrast, the lack of an early virologic response, defined as either undetectable serum HCV RNA or HCV RNA levels decreased by $>2.0$ $\log_{10}$ from the pretreatment level at 12 weeks after starting therapy, has been the most important predictor for the failure of a sustained virologic response in patients infected with HCV genotype 1 reportedly [Fried et al., 2002; Davis et al., 2003]. Therefore, treatment may be discontinued in patients without an early virologic response at 12 weeks of treatment, according to the recommendation in the AASLD guidelines [Ghany et al., 2009].

More recently, several studies reported that genetic polymorphisms near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19 affect the virologic response to PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. Furthermore, genetic polymorphisms near the *IL28B* gene are the strongest baseline predictive factor of the final outcome of combination therapy. An additional report showed the effects of genetic polymorphisms near the *IL28B* gene on HCV viral dynamics during PEG-IFN and ribavirin combination therapy [Thompson et al., 2010].

Although early HCV viral dynamics during therapy was shown originally to have a high predictive value for a sustained virologic response in HCV genotype 1-infected patients before genetic polymorphisms near the *IL28B* gene were linked to a therapeutic response, it is not clear whether early viral dynamics retain their predictive value in light of this additional information. The purpose of the present study was to investigate whether response-guided therapy based on viral dynamics at 4 or 12 weeks after initiating therapy retains its ability to predict the final outcome of PEG-IFN and ribavirin combination therapy after accounting for genetic polymorphisms near the *IL28B* gene.

## MATERIALS AND METHODS

### Patients and Treatment

Between January 2007 and June 2008, a total of 402 patients with chronic hepatitis C received antiviral combination therapy with PEG-IFN and ribavirin for HCV infection at the Ogaki Municipal Hospital or the Nagoya University Hospital. Among these patients, 272 were infected with HCV genotype 1b and had pretreatment HCV RNA levels $>5.0$ $\log_{10}$ IU/ml based on a quantitative real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Molecular Systems, Pleasanton, CA; Lower limit of quantification, 1.7 $\log_{10}$ IU/ml: Lower limit of detection, 1.0 $\log_{10}$ IU/ml) [Colucci et al., 2007; Pittaluga et al., 2008]. This study did not include any patients infected with HCV genotype 1a because this genotype is not found in the general Japanese population.

All patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough, Kenilworth, NJ) daily. The PEG-IFN and ribavirin doses were adjusted based on the patient's body weight. Patients weighing $\leq 45$ kg were given 60 μg of PEG-IFN alpha-2b once a week, those weighing $>45$ and $\leq 60$ kg were given 80 μg, those weighing $>60$ and $\leq 75$ kg were given 100 μg, those weighing $>75$ and $\leq 90$ kg were given 120 μg, and those weighing $>90$ kg were given 150 μg. Patients weighing $\leq 60$ kg were administered 600 mg of ribavirin per day, those weighing $>60$ and $\leq 80$ kg were given 800 mg per day, and those weighing $>80$ kg were administered 1000 mg per day. The PEG-IFN and ribavirin doses were modified based on the manufacturer's recommendations. All patients were scheduled to undergo 48 weeks of treatment. The treatment duration was extended up to 72 weeks in some patients. In addition, treatment was discontinued before 48 weeks in some patients who had a low likelihood of achieving an eradication of HCV due to the presence of serum HCV RNA at 24 weeks after starting therapy.

A sustained virologic response was defined as undetectable serum HCV RNA at 24 weeks after ending the therapy. A patient was considered to have relapsed when serum HCV RNA was detectable between the end of treatment and 24 weeks after completing treatment, although serum HCV RNA was undetectable during and at the end of therapy. Patients were considered to have non-response if serum HCV RNA was detectable at 24 weeks after initiating therapy (i.e., null response or partial response according to the American guidelines [Ghany et al., 2009]). Patients were considered to have a rapid virologic response if they had undetectable serum HCV RNA at 4 weeks after starting therapy. An early virologic response was defined as the disappearance or decrease in serum HCV RNA levels by at least 2 $\log_{10}$ at 12 weeks after starting therapy. Patients were considered to have a complete early virologic response if serum HCV RNA was undetectable at 12 weeks after starting therapy and a partial early virologic response if the serum

HCV RNA levels had decreased by at least 2 $\log_{10}$ at 12 weeks after initiating therapy. Patients were considered not to have an early virologic response if their HCV RNA levels did not decrease by more than 2 $\log_{10}$ at 12 weeks compared to the pretreatment levels. Patients were considered to have a slow virologic response if the serum HCV RNA became undetectable between 12 and 24 weeks.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of the Ogaki Municipal Hospital and the Nagoya University School of Medicine. Prior to initiating the study, each patient provided written informed consent to use the laboratory data, analyze genetic polymorphisms near the *IL28B* gene, and test stored serum samples.

### Assessments of Serum HCV RNA Levels and Genetic Polymorphisms Near the *IL28B* Gene

After a patient provided informed consent, serum samples were obtained at the patient's regular hospital visits, just prior to initiating treatment, every 4 weeks during the treatment period, and during the 24-week follow-up period after treatment. Serum samples were stored at −80°C until further use. The HCV RNA levels were measured using a quantitative real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System).

Genotyping of rs 8099917 polymorphisms near the *IL28B* gene was performed using the TaqMan SNP assay (Applied Biosystems, Foster City, California) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C__11710096_10, Applied Biosystems).

***Statistical analyses.*** Quantitative values are reported as the mean ± SD. In between-group differences were analyzed by the chi-square test. Univariate and multivariate analyses using a logistic regression model were performed to identify factors that predict a sustained virologic response, including age, sex, body weight, serum alanine aminotransferase activity, serum aspartate aminotransferase activity, serum gamma-glutamyl transpeptidase levels, serum alkaline phosphatase values, serum albumin levels, total serum bilirubin values, white blood cell counts, hemoglobin, platelet counts, hepatitis activity grade (A0 and A1 vs. A2 and A3), liver fibrosis grade (F0 and F1 vs. F2 and F3), pretreatment HCV RNA levels ($\geq 6.5 \log_{10}$ vs. $< 6.5 \log_{10}$), reduction in peginterferon dose and ribavirin dose, reduction in HCV RNA levels at 4 weeks after starting therapy ($\geq 3 \log_{10}$ vs. $< 3 \log_{10}$), and the type of an early virologic response. All *P*-values are two-tailed, and $P < 0.05$ was considered significant statistically.

## RESULTS

The characteristics of the patients examined in this study are shown in Table I. Liver histology was evaluated according to the METAVIR score [The French METAVIR Cooperative Study Group, 1994]. Although some patients had a reduction in their PEG-IFN and ribavirin doses during therapy, respectively, all patients except for those who discontinued the therapy had more than 80% adhesion to both the PEG-IFN and ribavirin regimens. No patients discontinued the therapy because of adverse effects. The treatment duration was extended up to 72 weeks in 51 of 71 patients (71.8%) who exhibited a slow virologic response. As a final outcome, 118 patients (43.4%) achieved a sustained virologic response, 84 patients (30.9%) relapsed, and the remaining 70 patients (25.7%) had no response.

TABLE I. Characteristics of all Study Patients (n = 272)

| | |
|---|---|
| Age (years) | 56.0 ± 10.9 |
| Sex (female/male) | 139 (51.1)/133 (48.9) |
| Body weight (kg) | 57.8 ± 10.5 |
| Alanine aminotransferase (IU/L) | 64.6 ± 56.4 |
| Aspartate aminotransferase (IU/L) | 53.9 ± 42.7 |
| Gamma-glutamyl transpeptidase (IU) | 48.5 ± 43.9 |
| Alkaline phosphatase (IU/L) | 267.9 ± 101.3 |
| Albumin (g/dl) | 4.04 ± 0.37 |
| Total bilirubin (mg/dl) | 0.79 ± 0.30 |
| White blood cell count (/μl) | 4892 ± 1333 |
| Hemoglobin (g/dl) | 14.0 ± 1.3 |
| Platelet count ($\times 10^3$/μl) | 163 ± 51 |
| Liver histology-activity (A0/A1/A2/A3)* | 3 (1.2)/136 (55.3)/ 92 (37.4)/15 (6.1) |
| Liver histology-fibrosis (F0/F1/F2/F3)* | 27 (11.0)/114 (46.3)/ 70 (28.5)/35 (14.2) |
| Pretreatment HCV RNA concentration ($\log_{10}$ IU/ml) | 6.35 ± 0.79 |
| Reduction in the peginterferon dose | 81 (29.8) |
| Reduction in the ribavirin dose | 130 (47.8) |
| Final outcomes (sustained virologic response /relapse/ no response) | 118 (43.4)/ 84 (30.9)/70 (25.7) |

HCV, hepatitis C virus.
Percentages are shown in parentheses.
*Liver biopsy was not performed in 26 patients.

### Reduction in Serum HCV RNA Levels at 4 Weeks after Starting Therapy and Treatment Outcome According to Genetic Polymorphisms Near the *IL28B* Gene

An analysis of genetic polymorphisms at rs8099917 near the *IL28B* gene indicated that 207 patients (76.1%) had a TT genotype, 3 patients had a GG genotype (1.1%), and the remaining 62 patients were TG heterozygote (22.8%). Table II shows the comparison of the background characteristics between patients with the favorable TT genotype and those with the unfavorable TG/GG genotype. As reported previously [Abe et al., 2010], gamma-glutamyl transpeptidase level was higher significantly in patients with the TG/GG genotype. As a final outcome, the rate of a sustained virologic response was higher significantly in patients with the TT genotype. Among 207 patients with the TT genotype, serum HCV RNA became undetectable in 19 patients (9.2%) at 4 weeks after starting therapy (a rapid virologic response). In the remaining 188 patients, the decrease in serum HCV RNA levels at 4 weeks after starting therapy ranged from 0.12

TABLE II. Characteristics of Study Patients According to the Genetic Polymorphisms Near the *IL28B* Gene

| | Patients with TT genotype of rs8099917 (n = 207) | Patients with TG/GG genotype of rs8099917 (n = 65) | *P*-value |
|---|---|---|---|
| Age (years) | 56.5 ± 10.4 | 54.4 ± 12.4 | 0.4112 |
| Sex (female/male) | 107 (51.7)/100 (48.3) | 32 (49.2)/33 (50.8) | 0.8384 |
| Body weight (kg) | 57.8 ± 10.9 | 57.8 ± 9.4 | 0.8361 |
| Alanine aminotransferase (IU/L) | 65.1 ± 53.3 | 62.8 ± 65.6 | 0.2548 |
| Aspartate aminotransferase (IU/L) | 53.6 ± 34.8 | 54.7 ± 62.0 | 0.3339 |
| Gamma-glutamyl transpeptidase (IU) | 44.2 ± 37.1 | 62.3 ± 59.0 | 0.0003 |
| Alkaline phosphatase (IU/L) | 263.1 ± 90.3 | 282.8 ± 129.9 | 0.3875 |
| Albumin (g/dl) | 4.04 ± 0.36 | 4.05 ± 0.43 | 0.8020 |
| Total bilirubin (mg/dl) | 0.79 ± 0.30 | 0.76 ± 0.32 | 0.3010 |
| White blood cell count (/μl) | 4826 ± 1333 | 5100 ± 1320 | 0.1608 |
| Hemoglobin (g/dl) | 13.9 ± 1.3 | 14.1 ± 1.4 | 0.3339 |
| Platelet count ($\times 10^3$/μl) | 161 ± 49 | 169 ± 57 | 0.3871 |
| Liver histology-activity (A0/A1/A2/A3)* | 2 (1.1)/98 (52.4)/ 74 (39.6)/13 (6.9) | 1 (1.7)/38 (64.4)/ 18 (30.5)/2 (3.4) | 0.3241 |
| Liver histology-fibrosis (F0/F1/F2/F3)* | 21 (11.2)/83 (44.4)/ 57 (30.5)/26 (13.9) | 6 (10.2)/31 (52.5)/ 13 (22.0)/9 (15.3) | 0.6401 |
| Pretreatment HCV RNA concentration ($\log_{10}$ IU/ml) | 6.37 ± 0.85 | 6.29 ± 0.55 | 0.0582 |
| Reduction in the peginterferon dose | 61 (29.5) | 20 (30.8) | 0.9644 |
| Reduction in the ribavirin dose | 101 (48.8) | 29 (44.6) | 0.5565 |
| Final outcomes (sustained virologic response /relapse/ no response) | 106 (51.2)/ 69 (33.3)/32 (15.5) | 12 (18.4)/15 (23.1)/ 38 (58.5) | <0.0001 |

HCV, hepatitis C virus.
Percentages are shown in parentheses.
*Liver biopsy was not performed in 26 patients.

$\log_{10}$ to 5.71 $\log_{10}$ (mean, 3.12 $\log_{10}$). The reduction in serum HCV RNA levels was $\geq 3 \log_{10}$ in 98 patients (47.3%), $< 3 \log_{10}$ and $\geq 2 \log_{10}$ in 52 patients (25.1%), $< 2 \log_{10}$ and $\geq 1 \log_{10}$ in 23 patients (11.1%), and $< 1 \log_{10}$ in 15 patients (7.3%). Figure 1A shows the rate of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy in patients with the TT genotype. The rates were higher significantly in patients who achieved a rapid virologic response or had a $\geq 3 \log_{10}$ decrease in

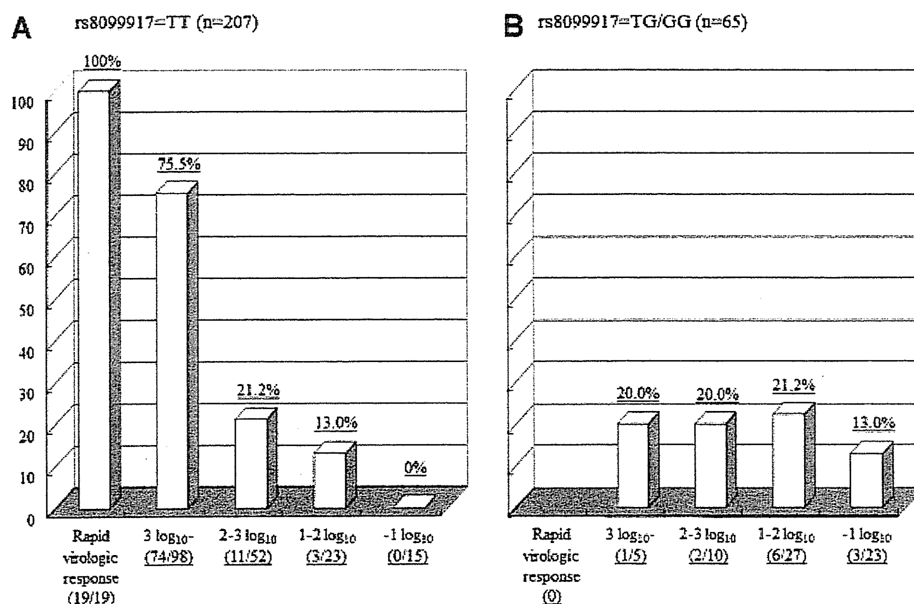


Fig. 1. The rate of sustained virologic responses (%) based on the reduction in serum HCV RNA levels at 4 weeks after starting therapy. A: Patients with the TT genotype for rs8099917, (B) patients with the TG/GG genotype for rs8099917.

serum HCV RNA levels at 4 weeks compared to those with a $<3$ $\log_{10}$ decrease in serum HCV RNA levels ($P < 0.0001$). When a 3 $\log_{10}$ decrease in serum HCV RNA levels was defined as the cut-off point, 56.5% of patients were considered to have a $\geq 3$ $\log_{10}$ decrease in serum HCV RNA levels. The sensitivity, specificity, positive predictive value, and negative predictive value for a sustained virologic response were 86.8, 75.2, 78.6, and 84.4%, respectively.

Among the 65 patients who had the TG/GG genotype, no patient achieved a rapid virologic response at 4 weeks after initiating therapy. The decrease in serum HCV RNA levels at 4 weeks after starting therapy ranged from 0.11 $\log_{10}$ to 4.75 $\log_{10}$ (mean, 1.66 $\log_{10}$). The reduction in serum HCV RNA levels at 4 weeks after starting the therapy were smaller in patients with the TG/GG genotype than those with the TT genotype ($1.66 \pm 1.02$ $\log_{10}$ in patients with the TG/GG genotype vs. $3.12 \pm 1.37$ $\log_{10}$ in patients with TT genotype excluding RVR, $P < 0.0001$). The reduction in serum HCV RNA levels was $\geq 3$ $\log_{10}$ in five patients (7.7%), $<3$ $\log_{10}$ and $\geq 2$ $\log_{10}$ in 10 patients (15.4%), $<2$ $\log_{10}$ and $\geq 1$ $\log_{10}$ in 27 patients (41.5%), and $<1$ $\log_{10}$ in 23 patients (35.4%). Figure 1B shows the rates of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy in patients with the TG/GG genotype. There were no differences in the rate of a sustained virologic response based on the reduction in HCV RNA levels at 4 weeks after starting therapy; the rate of a sustained virologic response remained at 20% approximately regardless of the reduction in HCV RNA levels in 42 patients with a $\geq 1$ $\log_{10}$ reduction in serum HCV RNA levels.

### Association Between an Early Virologic Response at 12 Weeks and Treatment Outcome Based on Genetic Polymorphisms Near the *IL28B* Gene

Figure 2 shows the rate of patients with the TT genotype or TG/GG genotype for rs8099917 who achieved a complete early virologic response, a partial early virologic response, and those who did not achieve early virologic response at 12 weeks after starting therapy based on the reduction in serum HCV RNA level at 4 weeks after initiating therapy. Nearly 75% of patients with the TT genotype whose HCV RNA levels were reduced by $\geq 3$ $\log_{10}$ at 4 weeks after starting the therapy achieved a complete early virologic response. In contrast, 80% of patients with the TG/GG genotype whose HCV RNA levels were reduced by $\geq 3$ $\log_{10}$ at 4 weeks after starting the therapy showed a partial early virologic response. The majority of patients with the TT or TG/GG genotypes achieved a partial early virologic response when their reduction in HCV RNA levels was $<3$ $\log_{10}$ and $\geq 2$ $\log_{10}$ or $<2$ $\log_{10}$ and $\geq 1$ $\log_{10}$.

Figure 3 shows the rates of a sustained virologic response according to the type of early virologic response in patients with the TT genotype (Fig. 3A) and TG/GG genotype (Fig. 3B). Among patients with the TT genotype, the rate of sustained virologic response was significantly higher in patients with a complete early virologic response than in those with a partial early virologic response ($P < 0.0001$). In contrast, there was no difference in the rate of a sustained virologic response between patients with a complete early virologic response and those with a partial early virologic response ($P = 0.8917$) among patients with

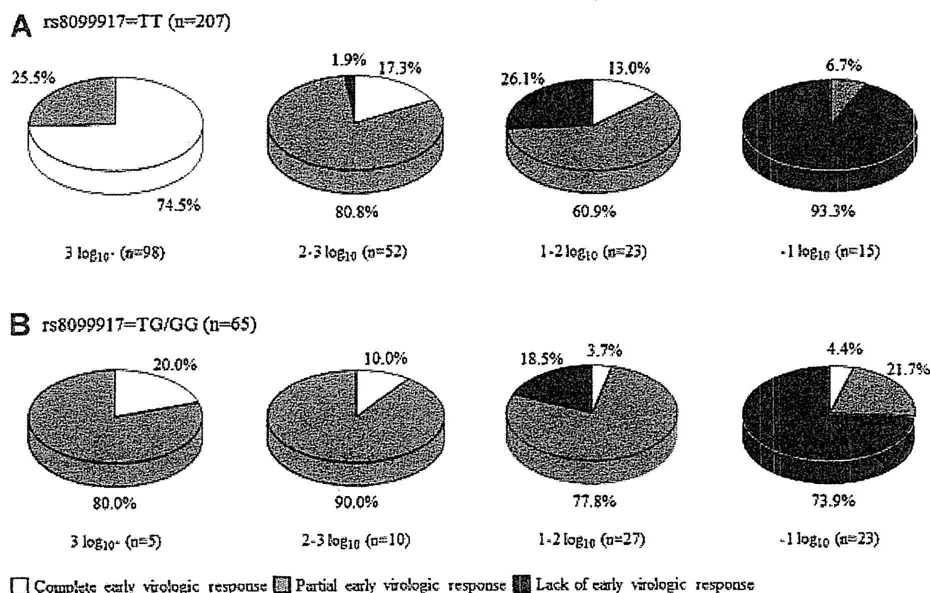


Fig. 2. The association between the virologic responses at 12 weeks after starting therapy and the reduction in serum HCV RNA levels at 4 weeks after starting therapy. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

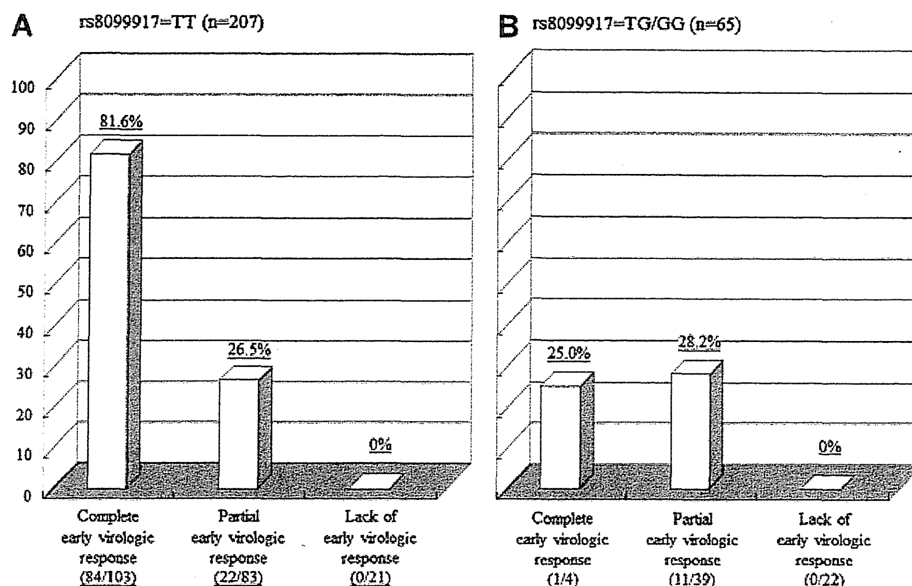


Fig. 3. The rate of sustained virologic responses based on the type of early virologic response. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

the TG/GG genotype. None of the patients with the TT genotype or TG/GG genotype who yielded a lack of an early virologic response reached a sustained virologic response.

### Univariate and Multivariate Analyses for Factors Associated With a Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients With the TT and the TG/GG Genotype for the rs8099917

Univariate and multivariate analyses were conducted for factors associated with a sustained virologic response based on different genetic polymorphisms near the *IL28B* gene. In patients with the TT genotype, the factors that were associated with a sustained virologic response included serum alkaline phosphatase levels, serum albumin, platelet counts, hepatitis activity grade, liver fibrosis grade, reduction in HCV RNA levels at 4 weeks after starting therapy, and a complete early virologic response based on a univariate analysis (Table IIIA). In a multivariate analysis, the serum albumin levels, reduction in HCV RNA levels 4 weeks after starting therapy, and a complete early virologic response were independent factors that were significantly associated with a sustained virologic response (Table IIIB). A reduction in HCV RNA levels 4 weeks after starting therapy was the strongest factor that affected a sustained virologic response. In patients with the TG/GG genotype, the factors that were associated with a sustained virologic response included patient age, platelet counts, and pretreatment HCV RNA levels based on a univariate analysis (Table IIIA). A reduction in the HCV RNA levels at 4 weeks after starting therapy was not associated with a sustained virologic response. In a multivariate analysis, patient age and pretreatment HCV RNA levels were independent factors that were significantly associated with a sustained virologic response (Table IIIC).

### Characteristics of Patients who Achieved a Sustained Virologic Response to the Combination Therapy Despite the Unfavorable TG/GG Genotype Near the *IL28B* Gene

Table IV shows the characteristics of 12 patients who achieved a sustained virologic response despite having the unfavorable TG/GG genotype for rs8099917 near the *IL28B* gene. All but one patient was under 60 years old and had liver fibrosis not more than grade 2 (one patient did not undergo a liver biopsy). Except for one patient, the reduction in the serum HCV RNA levels at 4 weeks after starting therapy was less than 3 $\log_{10}$ and all but one patient showed a partial early virologic response at 12 weeks after starting the therapy. In all 11 patients with a partial early virologic response, the serum HCV RNA was undetectable up to 24 weeks after starting the therapy. All but one patient extended the treatment duration from 48 to 72 weeks (two patients discontinued therapy at 60 weeks during the extended treatment period). When the characteristics of patients who achieved a sustained virologic response were compared between those with the unfavorable TG/GG genotype and those with the favorable TT genotype, patients with the TG/GG genotype were younger ($41.8 \pm 14.4$ years vs. $55.1 \pm 10.4$ years, $P = 0.0023$) and had lower pretreatment HCV RNA levels ($5.91 \pm 0.44$ $\log_{10}$ IU/ml vs. $6.21 \pm 1.05$ $\log_{10}$ IU/ml, $P = 0.0199$).

TABLE III. Univariate and Multivariate Analyses for Factors Associated With a Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients With the TT and the TG/GG Genotype for the rs8099917

| (A) Univariate analyses | *P*-value | |
|---|---|---|
| | Patients with TT genotype of rs8099917 (n = 207) | Patients with TG/GG genotype of rs8099917 (n = 65) |
| Age (years) | 0.0505 | 0.0007 |
| Sex (female/male) | 0.1830 | 0.2296 |
| Body weight (kg) | 0.6891 | 0.2456 |
| Alanine aminotransferase (IU/L) | 0.7988 | 0.4032 |
| Aspartate aminotransferase (IU/L) | 0.5021 | 0.1705 |
| Gamma-glutamyl transpeptidase (IU) | 0.6340 | 0.6648 |
| Alkaline phosphatase (IU/L) | 0.0315 | 0.0599 |
| Albumin (g/dl) | 0.0002 | 0.6594 |
| Total bilirubin (mg/dl) | 0.2929 | 0.7130 |
| White blood cell count (/μl) | 0.2508 | 0.5549 |
| Hemoglobin (g/dl) | 0.0847 | 0.2289 |
| Platelet count ($\times 10^3/\mu l$) | 0.0454 | 0.0411 |
| Liver histology-activity (A0–1/A2–3) | 0.0445 | 0.1117 |
| Liver histology-fibrosis (F0–1/F2–3) | 0.0002 | 0.2283 |
| Pretreatment HCV RNA concentration ($\geq$6.5 $\log_{10}$ vs. <6.5 $\log_{10}$) | 0.5279 | 0.0379 |
| Reduction in the peginterferon dose | 0.4316 | 0.5563 |
| Reduction in the ribavirin dose | 0.1823 | 0.4272 |
| Reduction in HCV RNA levels at 4 weeks after starting the therapy ($\geq$3 $\log_{10}$ vs. < 3 $\log_{10}$) | <0.0001 | 0.9265 |
| Early virologic response (complete vs. partial) | <0.0001 | 0.9777 |
| Early virologic response (partial vs. non) | 0.8632 | 0.0686 |

| (B) Multivariate analyses: Patients with TT genotype of rs8099917 | *P*-value | Odds ratio (95% confidence interval) |
|---|---|---|
| Alkaline phosphatase (IU/L) | 0.2617 | |
| Albumin (g/dl) | 0.0365 | 28.287 (1.4107–755.41) |
| Platelet count ($\times 10^3/\mu l$) | 0.2599 | |
| Liver histology-activity (A0–1/A2–3) | 0.6678 | |
| Liver histology-fibrosis (F0–1/F2–3) | 0.2307 | |
| Reduction in HCV RNA levels at 4 weeks after starting the therapy ($\geq$3 $\log_{10}$ vs. <3 $\log_{10}$) | <0.0001 | 16.029 (6.8593–40.406) |
| Early virologic response (complete vs. partial) | 0.0224 | 0.3685 (0.1557–0.8749) |

| (C) Multivariate analyses: Patients with TG/GG genotype of rs8099917 | *P*-value | Odds ratio (95% confidence interval) |
|---|---|---|
| Age (years) | 0.0022 | 0.0034 (0.0000–0.0840) |
| Platelet count ($\times 10^3/\mu l$) | 0.3344 | |
| Pretreatment HCV RNA concentration ($\geq$6.5 $\log_{10}$ vs. <6.5 $\log_{10}$) | 0.0304 | 0.0548 (0.0020–0.4950) |

HCV, hepatitis C virus.

## DISCUSSION

Several previous studies reported that patients who achieved a rapid virologic response, in which serum HCV RNA become undetectable at 4 weeks after starting therapy, had a high likelihood of achieving a sustained virologic response [Martinez-Bauer et al., 2006; Poordad et al., 2008; de Segadas-Soares et al., 2009; Martinot-Peignoux et al., 2009]. In addition, several recent studies reported the predictive value of the degree of reduction in serum HCV RNA levels at 4 weeks after starting therapy [Yu et al., 2007; Huang et al., 2010; Toyoda et al., 2011]. Therefore, the viral dynamics of HCV at 4 as well as 12 weeks after starting therapy is important for response-guided therapy.

Genetic polymorphisms near the *IL28B* gene have emerged as the strongest predictive factor of a sustained virologic response in patients infected with HCV genotype 1 [Hayes et al., 2011; Kurosaki et al., 2011]. In addition, Thompson et al. [2010 reported that genetic polymorphisms near the *IL28B* gene were associated strongly with early viral dynamics during PEG-IFN and ribavirin combination therapy. These findings raised an important issue of whether response-guided therapy, based on the reduction in serum HCV RNA levels at 4 or 12 weeks after starting

TABLE IV. Patients who Achieved a Sustained Virologic Response Despite the TG/GG Genotype for the rs8099917

| | Age (years) | Sex | Liver histology | Pretreatment HCV RNA level ($\log_{10}$ IU/ml) | HCV RNA reduction at 4 weeks | Response at 12 weeks | HCV RNA became undetectable (weeks) | Treatment duration (weeks) |
|---|---|---|---|---|---|---|---|---|
| 1. | 31 | Female | A1/F1 | 6.13 | 2.19 | partial EVR | 20 | 48 |
| 2. | 55 | Male | A1/F1 | 5.80 | 1.77 | partial EVR | 16 | 72 |
| 3. | 57 | Female | A1/F1 | 5.58 | 3.01 | partial EVR | 16 | 72 |
| 4. | 57 | Female | A1/F1 | 6.21 | 1.81 | partial EVR | 20 | 72 |
| 5. | 62 | Male | N.D. | 6.23 | 1.13 | partial EVR | 24 | 72 |
| 6. | 21 | Male | A1/F2 | 6.04 | 1.83 | partial EVR | 24 | 72 |
| 7. | 42 | Male | A1/F1 | 6.27 | 0.57 | partial EVR | 24 | 72 |
| 8. | 29 | Female | A1/F2 | 5.83 | 1.83 | partial EVR | 20 | 60 |
| 9. | 52 | Male | A1/F0 | 5.91 | 2.12 | complete EVR | 12 | 48 |
| 10. | 40 | Male | A2/F1 | 5.84 | 1.34 | partial EVR | 20 | 72 |
| 11. | 27 | Male | N.D. | 5.63 | 0.42 | partial EVR | 24 | 72 |
| 12. | 28 | Male | A1/F0 | 6.59 | 0.76 | partial EVR | 20 | 60 |

N.D., not done; HCV, hepatitis C virus; EVR, early virologic response.

therapy, retains a predictive value when considering genetic polymorphisms near the *IL28B* gene.

In the present study, the predictive value of the decrease in serum HCV RNA levels was evaluated at 4 and 12 weeks after starting therapy in Japanese patients infected with HCV genotype 1b based on genetic polymorphisms near the *IL28B* gene. Consistent with previous reports, patients with the TG/GG genotype for rs8099917 had a smaller reduction in serum HCV RNA levels at 4 weeks after starting treatment ($P < 0.0001$), which indicates an unfavorable response to the combination therapy. Patients with the TT genotype for rs8099917, which is associated with a favorable response to the combination therapy, exhibited a significant difference in the rate of a sustained virologic response based on the reduction in serum HCV RNA levels at 4 weeks after initiating the therapy. Patients with a rapid virologic response or with a $\geq 3$ $\log_{10}$ reduction in HCV RNA levels had a higher likelihood of achieving a sustained virologic response.

In contrast, these factors did not have any predictive value in patients with the TG/GG genotype. Only 18.5% of patients achieved a sustained virologic response (12 of 65 patients), and it was difficult to identify these patients based on the reduction in HCV RNA levels at 4 weeks or the type of an early virologic response at 12 weeks after starting therapy. Patients who achieved a sustained virologic response, despite the TG/GG genotype for rs8099917, were identified among those with a $<2$ $\log_{10}$ and $\geq 1$ $\log_{10}$ or even $<1$ $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy. Interestingly and paradoxically, the possibility of a sustained virologic response can be expected in patients with a $<1$ $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy only when they have the unfavorable TG/GG genotype.

In the evaluation at 12 weeks after starting therapy, patients with the TT genotype who achieved a complete early virologic response had a higher rate of a sustained virologic response significantly than patients who achieved a partial early virologic response, whereas this difference was not found in patients with the TG/GG genotype. No patients who failed to achieve an early virologic response achieved a sustained virologic response regardless of the genetic polymorphisms near the *IL28B* gene. Thus, the lack of an early virologic response retained a strong predictive value for the failure of achieving a sustained virologic response. This result supports the recommendation in the AASLD guidelines, in which treatment may be discontinued in patients without an early virologic response at 12 weeks of treatment.

The characteristics of patients who achieved a sustained virologic response despite the unfavorable TG/GG genotype were younger in age and lower pretreatment HCV RNA levels. Most patients with the TG/GG genotype who achieved a sustained virologic response showed a partial early virologic response and extended the treatment duration. It was difficult to identify these patients according to viral dynamics at 4 or 12 weeks after starting therapy.

There are several limitations in this study. Some patients with a slow virologic response did not have their treatment period extended from 48 to 72 weeks. This is because the effectiveness of a 72-week combination therapy regimen in patients with HCV genotype 1 with a slow virologic response [Berg et al., 2006; Pearlman et al., 2007] had not been established in Japan in the earlier part of this study. This fact might have influenced the treatment outcome especially in patients with the unfavorable TG/GG genotype. Another limitation is a smaller sample size of patients with the TG/GG genotype in comparison to that of patients with the TT genotype. This sample size could have caused the lack of statistical significance in the rate of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy or according to the type of an early virologic response in patients with the TG/GG genotype. In addition, the data were based on Japanese patients infected with HCV genotype 1b. Therefore, these results should be confirmed in other ethnicities and patients infected with HCV genotype 1a.

In conclusion, among patients infected with HCV genotype 1b with the TT genotype for rs8099917, a rapid virologic response or a $\geq 3$ $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy, or a complete early virologic response indicate strongly that these patients will achieve a sustained virologic response as a final outcome for PEG-IFN and ribavirin combination therapy. Early viral dynamics retain the predictive value in this patient subpopulation. A reduction in HCV RNA levels at 4 weeks after starting therapy or the type of an early virologic response does not predict the likelihood that patients with the TG/GG genotype will achieve a sustained virologic response. In contrast, the lack of an early virologic response retains a strong predictive value for the failure to achieve a sustained virologic response regardless of *IL28B* polymorphisms, which remains useful as a factor to stop therapy.

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HEPATOLOGY

# Clinical impact of HFE mutations in Japanese patients with chronic hepatitis C

Yoji Ishizu, Yoshiaki Katano, Takashi Honda, Kazuhiko Hayashi, Masatoshi Ishigami, Akihiro Itoh, Yoshiki Hirooka, Isao Nakano and Hidemi Goto

Department of Gastroenterology, Nagoya University Graduate School of Medicine, Nagoya, Japan

**Key words**
antiviral therapy, H63D mutation, iron overload.

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**Correspondence**
Yoshiaki Katano, Department of Gastroenterology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Email: ykatano@med.nagoya-u.ac.jp

**Abbreviations**
CHC, chronic hepatitis C; HCV, hepatitis C virus; HH, hereditary hemochromatosis; IFN, interferon; IL28B, interleukin 28B; PCR, polymerase chain reaction; PEG-IFN, pegylated-interferon-alpha 2b; RBV, ribavirin; SNP, single-nucleotide polymorphism; SVR, sustained virological response.

## Abstract

**Background and Aim:** HFE mutations, a common cause of hereditary hemochromatosis (HH), are reportedly associated with hepatic iron overload, severe liver fibrosis, and good response to interferon treatment in European patients with chronic hepatitis C (CHC). HH shows ethnicity-based differences and little is known about the effects of HH mutations on CHC in the Japanese. Thus, the aim of this study was to clarify the clinical influence of HFE mutations in Japanese CHC patients.
**Methods:** In a total of 251 patients with CHC, we analyzed the frequencies of H63D and S65C mutations in the HFE gene, and the influence of these mutations on clinical parameters and response to pegylated-interferon-alpha 2b (PEG-IFN) plus ribavirin therapy.
**Results:** Fourteen patients (5.6%) carried the H63D mutation; all were heterozygotes. No S65C mutations were found. Only hemoglobin levels in the H63D heterozygotes were higher than in wild-type patients. Eleven of 14 H63D heterozygotes achieved sustained virological response (SVR). On univariate analysis, factors associated with SVR were interleukin 28B (IL28B) polymorphism, age, hepatitis C virus (HCV) genotype, HCV viral load, white blood cell count, stage of fibrosis and H63D mutation. All patients with both TT genotype in IL28B (rs8099917) and H63D mutation in HFE ($n = 10$) achieved SVR.
**Conclusions:** The H63D mutation has little impact on the clinical characteristics of CHC, but is related to favorable response to PEG-IFN plus ribavirin therapy, particularly in patients with the TT allele in IL28B.

## Introduction

Hepatitis C virus (HCV) infection is a significant global health problem, affecting 170 million individuals worldwide. HCV infection causes chronic hepatitis that can develop into cirrhosis and hepatocellular carcinoma. Elevated hepatic iron concentration has often been found in patients with chronic hepatitis C (CHC),[1] and this excess iron increases oxidative stress, which can accelerate the progression of fibrosis[2] and may promote hepatic carcinogenesis.[3] Moreover, hepatic iron accumulation is thought to lower the response rate to interferon (IFN)-based therapy in patients with CHC.[4–8]

HFE mutations are the major gene variations in hereditary hemochromatosis (HH),[9] which is a common autosomal recessive disorder associated with iron overload in Caucasians.[10] Therefore, there has been much interest in the roles of HFE mutations in patients with HCV infection. Several studies have been performed in order to assess the correlations among HFE mutations, hepatic iron overload and disease progression in CHC. However, the effects of HFE mutations on hepatic iron concentration and disease severity remain controversial.[11–18] On the other hand, the presence of HFE mutations was reported to be associated with good response to IFN therapy.[11,19] As the prevalence of HFE mutations is lower in Asian populations than in Caucasian populations,[20] most studies on HFE mutations have been conducted in Western countries, with only one small study being conducted in an Asian country.[21] Clarifying the effects of these mutations on iron loading and clinical features in Asian patients may help to further understand the role of HFE mutations in HCV-infected patients.

The aim of this study was to examine the influence of HFE gene variants on iron overload and clinical characteristics, and to investigate whether HFE mutations affect response to pegylated-interferon-alpha 2b (PEG-IFN) plus ribavirin (RBV) therapy in Japanese CHC patients.

## Methods

***Patients.*** A total of 251 Japanese patients infected with HCV and being treated at Nagoya University Hospital were enrolled in this retrospective study. Patients included 143 men and 108 women with a mean age of 53.8 ± 12.3 years. Exclusion criteria


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were as follows: hepatitis B surface antigenemia; human immunodeficiency virus infection; chronic alcohol abuse; autoimmune liver disease; and history of phlebotomy. Degree of inflammatory activity and stage of fibrosis were assessed according to the histological scoring system of METAVIR[22] by pathologists who were blinded to clinical data. Hepatic iron storage was graded with Perls' Prussian blue stain on a scale of 0–4 as follows: grade 0, iron granules absent or barely discernible × 400; grade 1, barely discernible × 250 or easily discernible × 400; grade 2, discrete granules resolved × 100; grade 3, discrete granules resolved × 25; grade 4, massive visible × 10, or naked eye.[23] Patients received subcutaneous injections of PEG-IFN (1.5 μg/kg) once per week plus oral RBV (600 mg for < 60 kg, 800 mg for 60–80 kg, 1000 mg for > 80 kg) daily, in accordance with Japanese guidelines.[24] Sustained virological response (SVR) was defined as undetectable HCV RNA at 24 weeks after withdrawal of treatment. The other patients were considered to have non-SVR. This study was approved by the Nagoya University Hospital ethics committee, and was conducted in accordance with the principles of the 1975 Declaration of Helsinki. Written informed consent was obtained from all patients.

***Genomic analysis.*** Genomic DNA was isolated from peripheral blood leukocytes by standard procedures. HFE mutations at position 63 (histidine to aspartic acid, H63D) and at position 65 (serine to cysteine, S65C) were determined by the polymerase chain reaction (PCR)-restriction fragment length polymorphism method with *Bcl*-I (for H63D) and *Hinf*-I (for S65C), as described previously.[11] Detection of the rs8099917 single-nucleotide polymorphism (SNP) in interleukin 28B (IL28B) was also performed by real-time PCR using custom-designed primers and probes (Taqman SNP Genotyping Assays; Applied Biosystems, Foster, CA, USA). IL28B SNP rs8099917 was amplified and the results were analyzed by real-time PCR in a thermal cycler (7300 Real-time PCR System; Applied Biosystems).

***Statistical analysis.*** Quantitative variables were compared by the Mann–Whitney *U*-test, and are expressed as median values with interquartile range. The distribution of qualitative variables was compared by the $\chi^2$-test or Fisher's exact test, as appropriate. Multiple logistic regression analysis was performed in order to determine the factors contributing to SVR. *P*-values less than 0.05 were considered to be statistically significant and $0.1 > P \geqq 0.05$ were referred to as marginally significant. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

## Results

***Influence of H63D mutation on clinical characteristics.*** Of the 251 patients with CHC, 14 carried the H63D mutation (5.6%); all were heterozygous. Patient characteristics according to the presence of H63D mutation are shown in Table 1. No significant differences in clinical, laboratory, or histological data were observed between H63D heterozygotes and wild-type patients, except that hemoglobin levels were higher in H63D heterozygous patients. There was no correlation between the presence of H63D mutation and genotype of IL28B. Histological evaluation of hepatic iron deposition using Perls' Prussian blue method in nine H63D heterozygous patients demonstrated low levels of iron staining in the liver: six had grade 0, two had grade 1, and one had grade 2 positive staining.

***Influence of H63D mutation on response to PEG-IFN plus RBV therapy in patients with CHC.*** Of the 251 patients who received PEG-IFN plus RBV therapy, 116 (46.2%) achieved SVR. Univariate analysis identified seven factors that influenced SVR: younger age ($P = 0.003$); genotype 2 ($P < 0.001$); lower viral load (< 1000 KIU/mL) ($P < 0.001$); higher white blood cell count ($P = 0.039$); lower stage of fibrosis ($P = 0.002$); genotype TT of IL28B rs8099917 ($P < 0.001$); and presence of H63D heterozygosity ($P = 0.012$)

**Table 1** Comparison of characteristics according to the presence of the H63D mutation

| | H63D heterozygotes ($n$ = 14) | Wild type ($n$ = 237) | *P*-value |
|---|---|---|---|
| Sex (male/female) | 11/3 | 132/105 | 0.093 |
| Age (years) | 53 (44–56.8) | 56 (47–63) | 0.148 |
| Body mass index (kg/m²) | 21.6 (19.6–24.4) | 22.1 (20.3–24.4) | 0.282 |
| Alanine aminotransferase (IU/L) | 35 (28.2–50.1) | 38 (28–68) | 0.520 |
| Aspartate aminotransferase (IU/L) | 53.5 (26.5–103.3) | 46 (29–80) | 0.748 |
| Gamma-glutamyl transpeptidase (IU/L) | 49 (25–62) | 32.0 (21.0–62.3) | 0.327 |
| Total bilirubin (mg/dL) | 1.00 (0.78–1.03) | 0.8 (0.6–1.0) | 0.247 |
| Albumin (g/dL) | 4.1 (4.0–4.3) | 4.1 (3.8–4.3) | 0.292 |
| Serum ferritin (ng/mL) | 110 (82–143) | 89 (40–180) | 0.580 |
| White blood cell counts (/mm³) | 4550 (3950–4950) | 4600 (3900–5400) | 0.652 |
| Hemoglobin (g/dL) | 14.7 (13.5–15.8) | 13.7 (13.0–14.8) | 0.040 |
| Platelet counts (/mm³) | 177 000 (135 000–158 000) | 164 000 (132 000–203 000) | 0.468 |
| HCV genotype (1/2) | 12/2 | 181/56 | 0.326 |
| Viral load (KIU/mL) (< 1000/ 1000) | 5/9 | 56/181 | 0.334 |
| Stage of inflammatory activity (0/1/2/3) | 0/6/5/0 | 4/96/82/4 | 0.592 |
| Stage of fibrosis (0/1/2/3/4) | 0/7/4/0/0 | 28/74/50/26/7 | 0.381 |
| IL28B rs8099917 genotype (TT/TG or GG) | 10/4 | 175/62 | 0.538 |

Data is expressed as median values and (interquartile range).
HCV, hepatitis C virus; IL28B, interleukin 28B.


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**Table 2** Characteristics of SVR and non-SVR in patients with chronic hepatitis C

| | SVR ($n = 116$) | non-SVR ($n = 135$) | *P*-value |
|---|---|---|---|
| Sex (male/female) | 69/47 | 74/61 | 0.456 |
| Age (years) | 54 (43.3–60) | 58 (49–64.5) | 0.003 |
| Body mass index (kg/m$^2$) | 22.0 (20.4–23.8) | 22.3 (20.5–24.9) | 0.140 |
| Alanine aminotransferase (IU/L) | 34 (26–63) | 39.5 (29.3–69.8) | 0.094 |
| Aspartate aminotransferase (IU/L) | 46 (26–94) | 46 (29.3–77) | 0.896 |
| Gamma-glutamyl transpeptidase (IU/L) | 31 (21–53) | 34 (21.8–64.5) | 0.248 |
| Total bilirubin (mg/dL) | 0.80 (0.60–1.00) | 0.8 (0.6–1.0) | 0.736 |
| Albumin (g/dL) | 4.1 (3.8–4.3) | 4.0 (3.8–4.2) | 0.109 |
| Serum ferritin (ng/mL) | 95.5 (40–178) | 86.4 (44.3–161.5) | 0.661 |
| White blood cell counts (/mm$^3$) | 4800 (4000–5800) | 4550 (3900–5200) | 0.039 |
| Hemoglobin (g/dL) | 14.0 (13.0–15.0) | 13.7 (13.0–14.6) | 0.136 |
| Platelet counts (/mm$^3$) | 167 000 (137 000–206 000) | 160 000 (131 000–199 000) | 0.499 |
| HCV genotype (1/2) | 76/40 | 117/18 | < 0.001 |
| Viral load (KIU/mL) (< 1000/≥ 1000) | 42/74 | 18/117 | < 0.001 |
| Stage of inflammatory activity (0/1/2/3) | 1/49/38/1 | 3/53/49/3 | 0.344 |
| Stage of fibrosis (0/1/2/3/4) | 13/45/21/6/1 | 15/36/33/20/6 | 0.002 |
| H63D mutation (present/absent) | 11/105 | 3/132 | 0.012 |
| IL28B rs8099917 genotype (TT/TG or GG) | 101/15 | 82/53 | < 0.001 |

Data are presented as median values and (interquartile range).
HCV, hepatitis C virus; IL28B, interleukin 28B; SVR, sustained virological response.

**Table 3** Factors associated with SVR in patients with chronic hepatitis C by multivariate analysis

| Factor | Category | OR (95%CI) | *P*-value |
|---|---|---|---|
| IL28B rs8099917 | Genotype TT | 7.089 (2.961–16.976) | < 0.001 |
| Age (years) | Younger age (each 1 year decrease) | 1.062 (1.030–1.094) | < 0.001 |
| HCV genotype | Genotype 2 | 2.978 (1.357–6.535) | 0.001 |
| Viral load (KIU/mL) | < 1000 | 4.631 (1.937–11.073) | 0.007 |
| H63D mutation | Present | 5.281 (0.994–28.072) | 0.051 |

Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on multivariate logistic regression analysis are shown.
CI, confidence interval; HCV, hepatitis C virus; IL28B, interleukin 28B; OR, odds ratio; SVR, sustained virological response.

(Table 2). On multivariate analysis, IL28B genotype (TT; $P < 0.001$), age (each 1-year decrease; $P < 0.001$), HCV genotype (2; $P = 0.001$), viral load (< 1000 KIU/mL; $P = 0.007$) and H63D mutation (heterozygosity; $P = 0.051$) were significant or marginal independent predictors of SVR (Table 3). In our study population, the SVR rate among patients with genotype TT in IL28B was 55.8% and that for genotypes TG and GG was 21.7%. In the analysis of IL28B SNP, H63D mutation was considered to improve prediction of SVR, as among 14 patients carrying the H63D mutation, the 10 patients with genotype TT in IL28B all achieved SVR. The characteristics of the patients are shown in Table 4.

**Table 4** Characteristics of patients with both H63D mutation in HFE and genotype TT in IL28B (rs8099917)

| Patient | Sex | Age (years) | HCV genotype | Viral load (KIU/mL) |
|---|---|---|---|---|
| 1 | Male | 52 | 1 | 106 |
| 2 | Male | 57 | 2 | 342 |
| 3 | Male | 44 | 1 | 2080 |
| 4 | Male | 50 | 1 | 4950 |
| 5 | Female | 55 | 1 | 3220 |
| 6 | Male | 25 | 1 | 5100 |
| 7 | Male | 70 | 1 | 100 |
| 8 | Female | 28 | 2 | 2650 |
| 9 | Male | 66 | 1 | 2900 |
| 10 | Female | 57 | 1 | 250 |

HCV, hepatitis C virus; IL28B, interleukin 28B.

## Discussion

The clinical penetrance of the HFE gene mutation is low. A large cohort study reported that, even in male carriers of the homozygous C282Y mutation, which is the most common genotype resulting in HH, only 28% of the subjects showed the clinical expression of iron overload, and in female patients, only 1% developed iron overload-related symptoms, possibly due to iron loss caused by menstruation.[25] With regard to the H63D mutation, its effect on body iron stores was much milder than the C282Y mutation and, particularly in heterozygotes, iron overload was scarcely observed.[20,26,27] However, in patients with chronic HCV infection, which is often associated with hepatic iron deposition, it was unclear whether HFE mutations were associated with iron


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overload.[11–18] These conflicting results may have been due to genetic and environmental factors that affect iron accumulation. The Hemochromatosis and Iron Overload Screening (HEIRS) Study group indicated the importance of considering the effects of ethnic differences on serum iron markers.[28] In this study, H63D heterozygotes with CHC did not show any differences in laboratory data, except for hemoglobin levels, as compared with wild-type patients (Table 1). In Korea, Won *et al.* showed no correlation between serum ferritin levels and the H63D heterozygous state; however, the number of subjects was small.[21] This study also confirmed the lack of correlation between the presence of H63D heterozygosity and serum ferritin levels in Asia. Histological examination of liver iron stores substantiated the notion that H63D heterozygosity had no influence on hepatic iron deposition. We did not analyze the C282Y mutation because of its very low prevalence in Asian countries, including Japan, as reported previously.[10,20,29–33] On the other hand, we assessed the frequency of the S65C mutation, as no reports on this mutation have been published in Japan. However, in line with other studies from Asia,[31–33] none of the present subjects carried the S65C mutation.

The effects of H63D heterozygosity on fibrosis progression in patients with chronic HCV infection also remain unclear.[11,12,14–18] Although we did not find a correlation between the H63D mutation and fibrosis stage, some reports have suggested that the H63D heterozygote state would accelerate progression of fibrosis.[16–18] In these studies, the H63D heterozygotes showed increased hepatic iron deposition. Iron-related oxidative stress can increase hepatic inflammation and promote fibrosis,[2] and the authors of these studies suggested that progression of hepatic fibrosis was due to hepatic iron overload. Our study showed no evidence of excess iron deposition, which explains why H63D heterozygotes showed no correlation with fibrosis stage.

This study demonstrated that H63D heterozygotes with CHC show a good response to PEG-IFN plus RBV therapy. The positive effect of H63D mutation on IFN responsiveness in patients with CHC was reported in 2004.[19] The Hepatitis C Anti-Viral Long-Term Treatment to Prevent Cirrhosis (HALT-C) trial, a large and well-designed study, showed the same results in advanced CHC patients who received PEG-IFN plus RBV therapy.[11] Hepatic iron overload was shown to be associated with lower response rates to interferon therapy[4–8] and recent meta-analysis confirmed the beneficial effects of phlebotomy on response to interferon therapy.[34] Whereas H63D mutation is a potential cause of hepatic iron overload, a better treatment response was observed in H63D mutation carriers. The present study does not confirm the effects of H63D mutation on iron overload, but supports the positive effect of this mutation on IFN responsiveness. The precise mechanism of this effect remains unclear. The immunologic functions of HFE mutations were thought to play an important role.[11,19] The HFE gene is closely linked to the human leukocyte antigen-A3 locus, and some immunological differences, such as decreased cell-surface expression of major histocompatibility complex class I[35] and elevated monocyte chemoattractant protein-1 levels,[36] have been reported in HFE mutation carriers, as compared with wild-type patients. Further studies on the association between HFE mutations and immunologic functions in CHC patients are necessary in order to clarify these issues.

Factors associated with SVR have been widely studied, and several factors, including HCV genotype, viral load, mutations in HCV core and NS5A region, sex, liver fibrosis, ethnicity and age, have been suggested to play important roles in IFN responsiveness. Recently, three genome-wide association studies validated the correlation between SVR and SNP near the IL28B gene.[37–39] A minor allele in this genetic variation strongly predicts the failure of PEG-IFN plus RBV therapy; the SVR rate for our patients with this minor IL28B allele was 21.7%, and the positive predictive value of the major IL28B allele for SVR was 55.8%. Thus, another factor was considered to be necessary for improving SVR prediction.

Our study suggests that the H63D mutation is correlated with the outcome of IFN treatment, and combining IL28B SNP and H63D mutation may improve the predictive value for SVR. All patients with both H63D mutation and the major allele (genotype TT) of IL28B (rs8099917) achieved SVR. Although the frequency of H63D mutations is low in the Japanese population, it is much higher in North American and European populations; this correlation may therefore be more useful as a predictive factor for SVR in Western regions.

In conclusion, 5.6% of patients with CHC in Japan carry the H63D mutation in the HFE gene, and the S65C mutation was not detected. The H63D mutation had no influence on hepatic iron overload, but the presence of this mutation was associated with a good response to PEG-IFN plus RBV therapy.

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**ORIGINAL ARTICLE**

# Effects of interferon-α-transduced tumor cell vaccines and blockade of programmed cell death-1 on the growth of established tumors

R Omori[1], J Eguchi[1], K Hiroishi[1], S Ishii[1], A Hiraide[1], M Sakaki[1], H Doi[1], A Kajiwara[1], T Ito[1], M Kogo[2] and M Imawari[1]

Interferon-alpha (IFN-α) has strong antitumor effects, and IFN-α gene therapy has been used clinically against some cancers. In this study, we evaluated the efficacy of the combination of IFN-α-transduced tumor cell vaccines and programmed cell death 1 (PD-1) blockade, and investigated the mechanisms of the antitumor effects of the combined therapy. A poorly immunogenic murine colorectal cancer cell line, MC38, was transduced to overexpress IFN-α. In a therapeutic model, parental tumor-bearing mice were inoculated with MC38-IFNα cells and an anti-PD-1 antagonistic antibody. Analyses of immunohistochemistry and tumor-specific lysis were performed. The outgrowth of the established tumors was significantly reduced in mice treated with the combination of IFN-α and anti-PD-1. Immunohistochemical analyses of the therapeutic model showed marked infiltration of $CD4^+$ cells and $CD8^+$ cells in the established MC38 tumors of mice treated with both IFN-α and anti-PD-1. Significant tumor-specific cytolysis was detected when splenocytes of mice that were treated with both IFN-α and anti-PD-1 were used as effector cells. These results suggest that blockade of the PD-1 PD-ligand enhanced the Th1-type antitumor immune responses induced by IFN-α. The combination of IFN-α gene-transduced tumor cell vaccines and PD-1 blockade may be a possible candidate for a cancer vaccine for clinical trials.

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**Keywords:** colorectal cancer; cytotoxic T lymphocyte; immunotherapy; interferon-alpha; programmed cell death-1; tumor-based vaccine

## INTRODUCTION

Cellular immune responses are thought to be impaired in patients with advanced malignant tumors, and tumors are thought to escape immune surveillance of patients by several mechanisms.[1,2] To overcome the immune suppression or immune escape of patients with malignant tumors, novel approaches for inducing a strong cellular immune response are needed in such patients. Several studies have revealed that the cytokine gene transduction of tumor cells induces potent antitumor immune responses without systemic adverse effects in murine models. The subcutaneous injection of the transduced cells can induce a local inflammation at the site of tumor by the accumulation of inflammatory cells, such as activated natural killer (NK) cells, macrophages, dendritic cells and T lymphocytes.[3–6] As a result of these phenomena, the clinical application of a vaccination using gene-transduced tumor cells as a vehicle to deliver cytokines is attractive.

Interferon-alpha (IFN-α) has many biologic effects, including the enhancement of IFN-α/β production,[7,8] an antiviral function, the inhibition of cell growth and antiangiogenesis.[9] IFN-α upregulates the expression of major histocompatibility complex class I molecules on the cell surface, enhances the proliferation of type-I helper T cells (Th1),[10] and has an important role in the generation of cytotoxic T lymphocytes (CTLs) in specific antitumor immune responses.[11] We previously reported that IFN-α-expressing tumor cells promote the survival of tumor-specific CTLs by preventing apoptosis,[12] and in addition, the combination of IFN-α gene therapy and either interleukin (IL)-4 or IL-12 gene therapy was found to suppress the outgrowth of established tumors.[13,14] Based on these immunomodulating effects of IFN-α, it has been used to treat patients with tumors, such as melanoma, renal cell carcinoma and leukemia.

The programmed cell death-1 (PD-1) protein was first described as a member of the B7 family of costimulatory molecules that modulate T-cell antigen-specific receptor signaling and control T-cell activation, inactivation and survival.[15] Recently, PD-1 has been identified as a marker of exhausted T cells in chronic infectious disease.[16–22] Among the numerous mechanisms of tumor-induced immunosuppression by which tumors can escape from immune surveillance, a number of studies have suggested a role of the interaction between PD-1 and the programmed death-ligand 1 (PD-L1) in inhibiting the effector functions of antigen-specific $CD8^+$ T cells.[23–26] PD-1 is expressed on tumor-infiltrating $CD8^+$ T cells in tumors or on antigen-specific CD8 T cells in hosts with tumors, and the function of these PD-$1^+$ T cells is impaired.[27,28] PD-L1 is expressed at high levels in several different cancers,[29] and the high levels of expression of PD-L1 on tumors is strongly associated with poor prognosis.[30,31] A recent study showed that the blockade of PD-1–PD-L signaling restored functional T-cell responses in several cancers and, subsequently, improved clinical outcomes.[32–34]

In this study, the antitumor effects of the combination of IFN-α-transfected tumor cell vaccine therapy and PD-1 blockade were evaluated in a poorly immunogenic murine colorectal cancer

[1]Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, Shinagawa-ku, Tokyo, Japan and [2]Promotion Center of Pharmaceutical Education, Showa University School of Pharmacy, Shinagawa-ku, Tokyo, Japan. Correspondence: Dr J Eguchi, Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan.
E-mail: j.eguchi@med.showa-u.ac.jp
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system as a preliminary investigation of the combined therapy before clinical studies. We show that, when parental tumor-bearing mice were injected with IFN-α-overexpressing tumor cells and anti-PD-1 blocking antibody, the outgrowth of the established parental tumors was significantly suppressed. Furthermore, in order to explore the mechanisms of the antitumor effects induced by IFN-α and anti-PD-1 combination therapy, we performed immunohistologic staining of the tumors and tried to induce tumor-specific T lymphocytes.

## MATERIALS AND METHODS

### Mice

Female 6-week-old C57BL/6 (B6) mice were purchased from Sankyo Lab Service (Tokyo, Japan) for use in experiments from 8 to 12 weeks of age. Mice were maintained in an animal care facility at Showa University. This study was approved by the ethical committee for Animal Experiments of Showa University (permission #2011-1111).

### Cell lines, culture medium and reagents

The MC38 murine colorectal adenocarcinoma cell line, the MCA205 fibrosarcoma cell line (both B6 mouse origin) and yeast artificial chromosome-1 (YAC-1) lymphoma cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU ml$^{-1}$ penicillin, 100 μg ml$^{-1}$ streptomycin, 10 mM HEPES buffer, 1 mM minimum essential medium sodium pyruvate and 0.1 mM minimum essential medium nonessential amino acids (complete medium) in a humidified incubator with 5% $CO_2$ in air at 37 °C. All cell culture reagents were purchased from Life Technologies (Gaithersburg, MD). YAC-1 cells were used as target cells in order to assess the nonspecific killing in cytolytic assays.

The MC38 cell line was genetically modified in order to produce murine IFN-α (MC38-IFNα), as described previously.[35] The expression of IFN-α was confirmed by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit according to the manufacturer's instructions (mouse IFN-α ELISA, PBL InterferonSource, New Brunswick, NJ). MC38 cells expressing the neomycin-resistance gene following retroviral transduction with MFG-Neo (MC38-Neo) were used as control cells.[35] Gamma-irradiation (100 Gy for tumor cells) was performed with Gammacell 3000 Elan (Nordion, Kanata, ON, Canada). As reported previously, $1 \times 10^5$ cells of MC38-IFNα produce IFN-α 20.8 ± 0.5 ng/48 h, and MC38-wild type (WT) cells do not produce any IFN-α. IFN-α gene transduction does not affect the growth of tumor cells *in vitro* or the survival of γ-irradiated tumor cells.[36]

### *In vitro* culture of splenocytes from immunized mice stimulated with a genetically modified MC38 or anti-PD-1 antibody

Mice were initially inoculated with $1 \times 10^5$ MC38-IFNα tumor cells on days 0, 7 and 14. Subsequently, MC38-immune mice received challenges of $3 \times 10^5$ MC38-WT cells on day 28. Splenocytes ($3 \times 10^6$ cells ml$^{-1}$) were harvested from these mice on day 35 and then incubated in the presence of irradiated (100 Gy) and genetically modified MC38 ($2 \times 10^5$ cells ml$^{-1}$) or 10 μg ml$^{-1}$ of anti-PD-1 antagonistic antibody (BioLegend, San Diego, CA) either alone or in combination in complete medium. Cells were harvested every 3 days, and cell numbers were determined microscopically. IFN-γ and IL-10 production of splenocytes from immunized mice were observed. After 3 days of incubation, IFN-γ and IL-10 concentrations in the culture supernatant were measured by ELISA using a commercially available kit according to the manufacturer's instructions (mouse IFN-γ ELISA and mouse IL-10 ELISA, R&D Systems, Minneapolis, MN).

### Flow cytometric analyses of lymphocytes from immunized mice stimulated with a genetically modified MC38 or anti-PD-1 antibody

In order to observe the immunogenic effects of IFN-α and anti-PD-1 antibody, flow cytometric analyses was performed using FACSCalibur (Nippon Becton Dickinson, Tokyo, Japan). After 7 days of *in vitro* incubation, splenocytes from MC38-IFNα immunized mice were harvested. CD4$^+$ and CD8$^+$ T cells were separated from splenocytes using CD4 and CD8 microbeads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany), and isolated using an autoMACS Pro Separator (Miltenyi Biotec). These cells were stained with fluorescein isothiocyanate-conjugated and phycoerythrin-conjugated monoclonal antibodies. The monoclonal antibodies used in this assay were anti-H-2K$^b$, CD4, CD8, CD25, 7-AAD (obtained from Nippon Becton Dickinson) and caspase-8 (Medical and Biological Laboratories, Nagoya, Japan) antibodies.

### Therapeutic models

In order to evaluate the potential to treat established tumors, we measured the size of established WT tumors of mice treated with genetically modified MC38 cells and antibody, as previously described.[35] In brief, B6 mice were injected subcutaneously with $1 \times 10^5$ MC38-WT cells in the right flank. In all, 7, 10 and 14 days after the WT inoculation, treatment was performed following the injection of $1 \times 10^5$ genetically modified MC38 or 200 μg of anti-PD-1 antibody either alone or in combination into the contralateral (left) flank. Each experiment involved six mice per group. Tumor size was measured twice a week using vernier calipers. Experiments with the therapeutic model were performed three times.

### *In vivo* antibody-mediated depletion of leukocytes

To determine the role of the immune system in reduction of tumor growth *in vivo*, CD4$^+$ T cells, CD8$^+$ T cells or NK cells were depleted as reported previously.[13] Culture medium of hybridomas producing the following antibodies was used at appropriate dilutions/concentrations: anti-CD4 (GK1.5, TIB207; American Type Culture Collection (ATCC), Manassas, VA), anti-CD8 (2.43, TIB210; ATCC) and anti-asialo-GM1 (anti-NK cells; WAKO, Osaka, Japan). All antibody doses and treatment regimens have been identified for the same batch of antibodies used in preliminary studies. Mice were inoculated with $1 \times 10^5$ cells of MC38-WT 3 days after depletion of leukocytes, and MC38-IFNα and anti-PD-1 antibody combination treatment were performed 7, 10 and 14 days after the WT inoculation. This treatment was confirmed to completely delete the desired cell population for the entire duration of the study, as determined by flow cytometric analysis (data not shown).

### Immunohistologic analysis

B6 mice were injected subcutaneously in the right flank with $1 \times 10^5$ MC38-WT cells. On days 7, 10 and 14, $1 \times 10^5$ MC38-IFNα and/or 200 μg of anti-PD-1 antagonistic antibody were inoculated into the contralateral (left) flank. Tumor tissues were harvested 4 days after the last inoculation (18 days after WT inoculation) and were immediately embedded in optimal cutting temperature compound (Tissue-Tek, Elkhart, IN) and frozen. Serial 5-μm sections were exposed to anti-CD4, anti-CD8a, anti-CD11c and anti-Gr-1 antibodies (Nippon Becton Dickinson) and anti-PDL1-antibody (BioLegend). Rat IgG2a (Nippon Becton Dickinson) was used as a control antibody. Immunostaining was completed with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Immunoreactive cells were visualized using light microscopy (400 ×) and counted in 10 fields in a blinded fashion. Each experiment involved two mice per group.

### Induction of tumor-specific CTL

Mice were initially inoculated with $1 \times 10^5$ MC38-IFNα tumor cells and/or 200 μg of anti-PD-1 antibody on days 0, 7 and 14. Subsequently, MC38-immune mice received challenges of $3 \times 10^5$ MC38-WT cells on day 28. Splenocytes ($3 \times 10^6$ cells ml$^{-1}$) were harvested from these mice on day 42 and then stimulated *in vitro* with irradiated (100 Gy) MC38-IFNα tumor cells ($3 \times 10^5$ cells ml$^{-1}$). Seven days later, responder cells ($1 \times 10^6$ cells ml$^{-1}$) were restimulated with irradiated MC38-IFNα tumor cells (100 Gy, $1 \times 10^5$ cells ml$^{-1}$) and syngeneic dendritic cells ($2 \times 10^5$ cells ml$^{-1}$) in the presence of 50 IU ml$^{-1}$ of recombinant mouse IL-2 (R&D Systems). Dendritic cells were generated from bone marrow cells of B6 mice using murine granulocyte-macrophage colony-stimulating factor (10 ng ml$^{-1}$) and IL-4 (10 ng ml$^{-1}$) that was obtained from PeproTech EC (London, England), as


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reported previously.[36] Cytolytic assays were performed 7 days after the last stimulation using the responder cells as effector cells.

### Cytolytic assays

Tumor-stimulated effector cells were assessed for cytolytic activity against MC38-WT, MCA205 and YAC-1 cells in triplicate in a 4-h $^{51}$Cr-release assay. Target cells ($1 \times 10^6$ cells ml$^{-1}$) were labeled with 3.7 MBq of $Na_2{}^{51}CrO_4$ (GE Healthcare, Tokyo, Japan) for 1 h at 37 °C. Labeled cells were washed and resuspended. Target cells ($5 \times 10^3$) and various numbers of effector cells at the indicated effector to target ratios (E:T) were plated in 200 μl of complete medium in each well of the 96-well round-bottom plates. $^{51}$Cr-release was measured after a 4-h incubation at 37 °C. The percent lysis was determined using the formula (release in assay−spontaneous release) × 100/(maximum release−spontaneous release). Maximum release was determined by the lysis of labeled target cells with 1% Triton X-100. Spontaneous release was measured by incubating target cells in the absence of effector cells and was <15% of maximum release.

### Statistical analyses

Statistical analyses were carried out using means s.d., one-way analysis of variance and Turkey's honestly significant difference *post-hoc* test. Differences between groups were considered significant when the $P$ values were $<0.05$.

## RESULTS

### Anti-PD-1 antibody and IFN-α-overexpressing cells enhanced IFN-γ production and reduced IL-10 production by splenocytes from mice immunized with MC38-IFNα

First, we observed the *in vitro* effects of MC38-IFNα and the anti-PD-1 antibody. Mice were initially inoculated with MC38-IFNα three times. Subsequently, these mice received challenges of MC38-WT cells 14 days after the last immunization. One week later, splenocytes of the treated mice were harvested and stimulated with the MC38-IFNα or anti-PD-1 antibody *in vitro*. After 3 days of incubation, the IFN-γ and IL-10 concentrations of

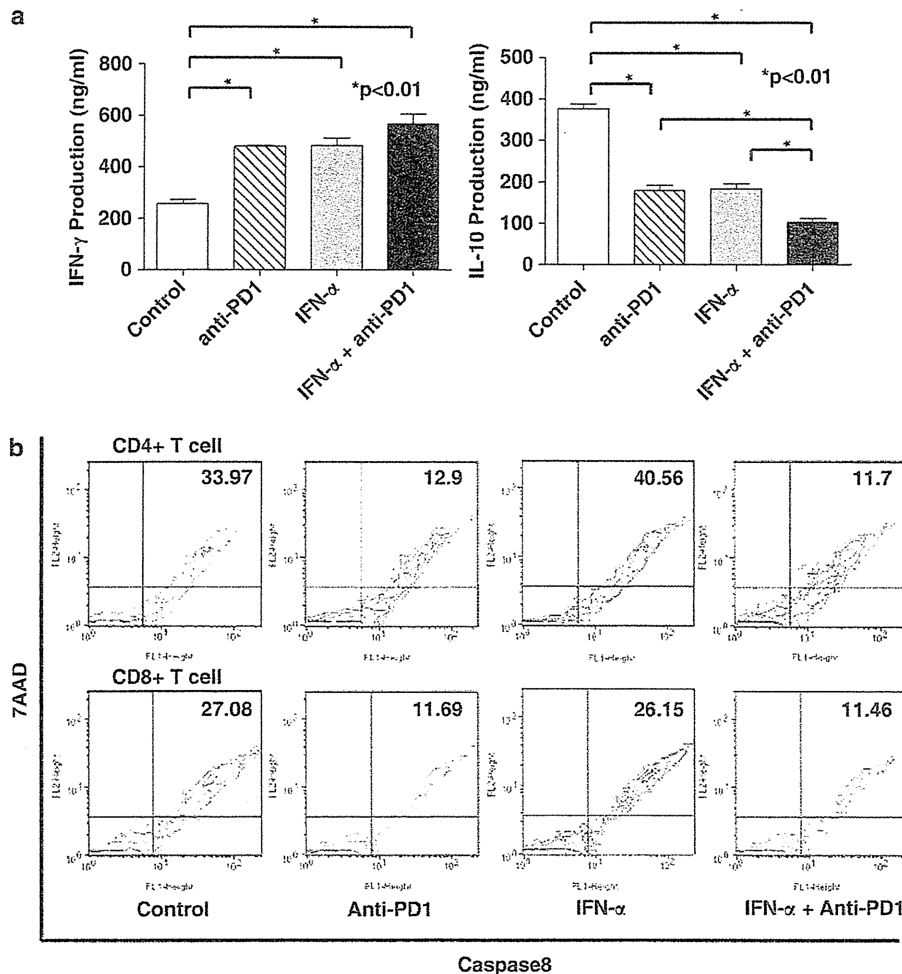


**Figure 1.** (**a**) The combination of interferon (IFN)-α and anti-programmed cell death-1 (PD-1) enhances IFN-γ production but reduces the interleukin (IL)-10 production of splenocytes obtained from mice immunized with MC38-IFNα after *in vitro* stimulation. Mice were inoculated with MC38-IFNα cells three times and then injected with MC38-wild-type (WT) cells 2 weeks after the last MC38-IFNα inoculation. Seven days later, mice were killed, and splenocytes from these mice were stimulated *in vitro* with MC38-IFNα cells alone or in combination with an anti-PD-1 antibody. After 3 days of incubation, IFN-γ and IL-10 concentrations in the culture supernatant were measured by an enzyme-linked immunosorbent assay (ELISA). (**b**) Blockade of PD-1 reduced the level of apoptosis in lymphocytes from mice immunized with MC38-IFNα. Splenocytes from mice immunized with MC38-IFNα were stimulated with MC38-IFNα or anti-PD-1 antibody. After 7 days of incubation, splenocytes were isolated to CD4$^+$ and CD8$^+$ T cells and stained with the phycoerythrin (PE)-conjugated anti-7AAD and fluorescein isothiocyanate (FITC)-conjugated anti-caspase-8. Numbers in each histogram indicate the percentage of 7AAD$^+$/caspase-8$^+$ cells in the total CD4$^+$ or CD8$^+$ population. These experiments were repeated three times, and a representative result is shown.

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**Table 1.** Flow cytometric analyses of lymphocytes from immunized mice stimulated with a genetically modified MC38 or anti-PD-1 antibody

| *Treatment* | *Caspase-8(−)/ 7AAD(−)* | *Caspase-8(+)/ 7AAD(−)* | *Caspase-8(+)/ 7AAD(+)* |
|---|---|---|---|
| *CD4* | | | |
| Control | 55.29 | 10.74 | 33.97 |
| Anti-PD-1 | 74.25 | 12.85 | 12.9 |
| IFN-α | 47.72 | 11.72 | 40.56 |
| IFNα+anti-PD-1 | 80.12 | 8.13 | 11.7 |
| *CD8* | | | |
| Control | 68.32 | 4.14 | 27.08 |
| Anti-PD-1 | 85.66 | 2.61 | 11.69 |
| IFN-α | 71.32 | 2.37 | 26.15 |
| IFNα+anti-PD-1 | 86.35 | 2.16 | 11.46 |

Abbreviations: IFN, interferon; PD-1, programmed cell death-1.
Numbers indicate the percentage of positive cells in the total $CD4^+$ or $CD8^+$ population.

the supernatant of each culture were measured by ELISA. IFN-γ production was clearly increased in the IFN-α and anti-PD-1 antibody-stimulated group compared with controls (IFN + anti-PD-1 vs control, $P = 0.009$; Figure 1a), whereas IL-10 production was suppressed significantly ($P < 0.001$, Figure 1a). In addition, the proliferation rate of the splenocytes did not differ significantly between the control group and the treatment groups (data not shown). These results suggest that IFN-α and anti-PD-1 elicited a potent Th1-type response.

PD-1 blockade prevented the apoptosis of lymphocytes from mice immunized with MC38-IFNα

To determine the immunogenic effects of IFN-α and PD-1 blockade, flow cytometric analyses was performed. Splenocytes from mice immunized with MC38-IFNα were stimulated with MC38-IFNα or anti-PD-1 antibody. After 7 days of incubation, splenocytes were isolated to $CD4^+$ and $CD8^+$ T cells and analyzed by flow cytometry. Treatment with anti-PD-1 antibody decreased the population of caspase-$8^+$ $7AAD^+$ T-cell subsets (Table 1 and Figure 1b). This result indicate that blockade of PD-1 reduced the level of apoptosis in both $CD4^+$ and $CD8^+$ T cell. Furthermore, there was no significant difference on percentage of $CD4^+$ regulatory T cells in every group (data not shown).

Therapeutic inoculations with the combination of IFN-α-transduced MC38 tumor cells and the anti-PD-1 antibody suppressed the *in vivo* growth of established MC38 tumors

We examined the therapeutic effects of MC38-IFNα and the anti-PD-1 antibody. Mice bearing established WT MC38 tumors were treated with an injection of MC38-IFNα alone, anti-PD-1 antibody alone or the combination. Starting 7 days after the inoculation of MC38-WT, mice were injected every 3 days with the MC38-IFNα cells and/or the anti-PD-1 antibody in the opposite flank. A significant suppression of outgrowth of the established tumors was observed in the IFN-α and anti-PD-1 combination treatment group (IFN + anti-PD-1, $174.17 \pm 35.54\,mm^2$ vs control, $328.67 \pm 26.36\,mm^2$ on day 28, $P = 0.020$ vs controls; Figure 2a). Although the mean tumor size of the mice was relatively suppressed compared with the control group, the suppressive effects on the established tumors were not significant when the immunizations were performed with MC38-IFNα alone (IFN, $220.17 \pm 36.70\,mm^2$ vs control, $328.67 \pm 26.36\,mm^2$ on day 28, $P = 0.121$ vs controls) or with the anti-PD-1-antibody alone (anti-PD-1, $277.00 \pm 31.53\,mm^2$ vs control, $328.67 \pm 26.36\,mm^2$ on day 28, $P = 0.636$ vs controls).

$CD4^+$ and $CD8^+$ cells are responsible for antitumor effects induced by combination of IFN-α-transduced MC38 tumor cells and the anti-PD-1 antibody

To characterize immune mechanisms suppressing *in vivo* tumor growth in the therapeutic model, immunocompetent B6 mice were depleted of $CD4^+$ T cells, $CD8^+$ T cells or asialo-$GM1^+$ cells using specific antibodies. Depletion of $CD4^+$ T cells and $CD8^+$ T cells abrogated therapeutic effect induced by MC38-IFNα and anti-PD-1 antibody combination treatment (Figure 2b). On the other hand, depletion of NK cells did not affect to the tumor suppression. These results suggest that both $CD4^+$ and $CD8^+$ T cells are responsible for antitumor response in mice treated with IFN-α and anti-PD-1.

Combined MC38-IFNα and anti-PD-1 antibody treatment induced the infiltration of CD4- and CD8-positive cells in established WT tumors

In order to analyze the antitumor mechanisms that were induced by the combined IFN-α and anti-PD-1 therapy, we performed immunohistochemical staining using the WT tumor tissues of the treated mice. The results showed that marked infiltration of both $CD4^+$ cells and $CD8^+$ cells had occurred by the addition of the anti-PD-1 antibody compared with controls and the group treated with MC38-IFNα alone (Table 2 and Figure 3). In addition, only a few $CD11c^+$ and $Gr\text{-}1^+$ cells could be detected in the WT tumors of every group. These results suggest that the antitumor effects of the IFN-α and anti-PD-1 therapy were dependent on both $CD4^+$ cells and $CD8^+$ cells.

Marked tumor-specific cytolysis was detected when splenocytes from mice treated with both MC38-IFNα and the anti-PD-1 antibody were used as effector cells

Mice were initially inoculated with MC38-IFNα and/or the anti-PD-1 antibody on days 0, 7 and 14. Subsequently, these mice received challenges of MC38-WT cells on day 28. Splenocytes of the treated mice were harvested on day 35 and stimulated weekly *in vitro* with the MC38-IFNα cells twice. Cytolytic assays against the MC38 or the YAC-1 cells, which are sensitive to NK cells, were performed 7 days after the second stimulation. A high specificity for MC38 was observed when treatment was performed with MC38-IFNα alone ($32.5 \pm 7.2\%$ for MC38 and $10.3 \pm 2.6\%$ for YAC-1, E:T = 20, $P = 0.012$; Figure 4a) or the combination of both MC38-IFNα and the anti-PD-1-antibody ($58.1 \pm 6.7\%$ for MC38 and $14.1 \pm 1.7\%$ for YAC-1, E:T = 20, $P < 0.001$). When splenocytes from mice that were treated with only the anti-PD-1 antibody were used as effector cells, comparable cytolysis was detected for both targets of MC38 and YAC-1 cells ($18.6 \pm 5.9\%$ for MC38 and $18.2 \pm 2.7\%$ for YAC-1, E:T = 20, $P = 0.231$). Compared with the IFN-α or anti-PD-1 single treatment groups, significant cytotoxicity against MC38 was observed in the IFN-α and anti-PD-1 combination treatment group ($P < 0.001$ vs anti-PD-1, $P = 0.002$ vs MC38-IFNα; Figure 4b). These findings suggest that anti-PD-1 has a minimal ability to induce tumor-specific cytotoxicity, but it can enhance the strong specific response that is induced by IFN-α.

## DISCUSSION

Gene therapy using tumor cells that are genetically modified to produce cytokines has been studied in several therapeutic models. We have previously reported that the combination of IFN-α gene-transduced tumor-based vaccination therapy and IL-4 or IL-12 gene therapy suppresses the outgrowth of established tumors.[13,14] Although the suppressive effects of established tumors were observed in these cytokine combination therapy models, we


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