factors. In our previous studies, we reported the construction of a plasmid by reducing the number of CpG motifs and optimizing promoter and enhancer regions in the vector, such that it allows sustained transgene expression [1]. We also demonstrated the potential use of this novel vector for increasing the therapeutic effects of interferon gene transfer against tumor metastasis, atopic dermatitis or hepatitis. The drawback of sustained transgene expression, however, is that it might increase the risk of inducing an immune response to the transgene product.

Induction of an immune response to the transgene product or, in other words, the encoded therapeutic protein, is a major concern in gene therapy because it can cause serious adverse effects and affect the transgene expression profile [2]. Induction of the immune response depends on several factors, including the antigenicity of the protein, types of transgene-expressing cells, transgene expression profile, and subcellular localization or secretion of the product. A previous study suggested that transgene expression in antigen-presenting cells (APCs) is a risk factor for eliciting an immune response [3]. On the other hand, studies investigating the association between the transgene expression profile and the immune response to the product are limited. Bates *et al.* [4] reported that hydrodynamic injection of plasmid DNA expressing firefly luciferase (fLuc) driven by a cytomegalovirus (CMV) promoter, which resulted in transient luciferase expression, scarcely induced anti-fLuc antibody production, whereas administration of an fLuc expression vector driven by a ubiquitin promoter, which generated sustained gene expression, induced antibody production in the serum. The same group also investigated whether incorporation of target sites for APC-specific microRNA (miRNA) in the plasmid and the use of cell type-specific promoters would be effective approaches for reducing the risk of immune responses to the transgene product [5]. In general, the reduction in transgene expression could be accounted for by two different phenomena: the removal of transgene-expressing cells as a consequence of the immune response or a reduced efficiency of transgene expression by processes such as promoter inactivation. It is necessary to distinguish between these two phenomena to clearly understand the association between the transgene expression profile and the immune response. This issue, however, has not been fully addressed in previous studies.

In the present study, we investigated the effect of the transgene expression profile on the induction of an immune response to the transgene product. Accordingly, different types of plasmid vectors were administered by hydrodynamic injections to obtain transient and sustained transgene expression profiles. fLuc was selected as a model antigen because (i) the amount of fLuc can be quantitatively determined using the luciferase assay and (ii) fLuc is immunogenic and elicits an immune response [6,7]. The immune response to fLuc-expressing cells was evaluated using *Gaussia* luciferase (gLuc) as a reporter, which was accomplished by co-administering a gLuc-expressing plasmid along with the fLuc-expressing vectors. This approach was employed for several reasons: (i) our preliminary study indicated that hydrodynamic administration of the gLuc-expressing vector, pROSA-gLuc, resulted in sustained, high gLuc expression for more than 1 year (Takahashi Y and Matsui Y, unpublished data); (ii) the distribution of two vectors co-administered by hydrodynamic injection almost completely overlapped [8]; (iii) the expression levels of gLuc can be quantitatively and reproducibly determined by measuring serum gLuc activity without sacrificing the mice; and (iv) the use of gLuc protein as a marker for transgene-expressing cells enables us to monitor the removal of these cells as a consequence of the immune response to fLuc because the reduction in serum gLuc activity is ascribable to the removal of the transgene-expressing cells themselves and not to any phenomenon reducing the transgene expression levels in the cells. Plasmids that express fLuc stably (pCpG-fLuc) or transiently (pCMV-fLuc) were co-administered with the pROSA-gLuc vector to mice by hydrodynamic injections. The cytotoxic immune response to fLuc-expressing cells was continuously evaluated by measuring serum gLuc activity without sacrificing the mice.

Materials and methods

Plasmid DNA

pCpG-mcs was purchased from Invivogene (San Diego, DA, USA). The fLuc-expressing plasmids, pCMV-fLuc and pCpG-fLuc, were constructed as described previously [1]. pROSA-gLuc and pROSA-fLuc, plasmid expressing gLuc and fLuc, respectively, were constructed using In-fusion Advantage polymerase chain reaction. pCMV-fLuc was amplified in the *Escherichia coli* strain DH5α, whereas pCpG-mcs, pCpG-fLuc and pROSA-gLuc were amplified in the *E. coli* strain GT115 Plasmid DNA was purified using JETSTAR 2.0 Plasmid MAXI Plasmid Purification Kits (GENOMED GmbH, Löhne, Germany). Characteristics of the plasmid DNAs used are summarized in Table 1.

Mice and plasmid DNA administration

Four-week-old female ICR mice and 6-week-old female C57/BL6 mice, weighing approximately 20 g each, were purchased from Japan SLC (Shizuoka, Japan). C57/BL6 mice were used only in the experiment in which interferon (IFN)-γ secretion from splenocytes was evaluated. All animal experiments were subject to deliberation and approval by the Ethics Committee for Animal Experiments

Table 1. Properties of plasmid DNA.

Plasmid	Size (kbp)	Number of CpG*	Enhancer	Promoter	cDNA
pCMV-fLuc	7.1	846	hCMV	hCMV	Firefly luciferase
pCpG-mcs	3.0	0	hCMV	hEF1	None
pCpG-fLuc	4.7	194	hCMV	hEF1	Firefly luciferase
pROSA-gLuc	5.8	598	None	hROSA26	*Gaussia* luciferase
pROSA-fLuc	6.7	622	None	hROSA26	Firefly luciferase

*The number of CpG dinucleotides in plasmid DNA is indicated. hCMV, human cytomegalovirus; hEF1, human elongation factor 1.

at the Graduate School of Pharmaceutical Sciences, Kyoto University. Plasmid DNA was administered to the mice using a hydrodynamics-based procedure, in which plasmid DNA dissolved in saline solution (whose volume equaled 8% of the total body weight) was injected into the tail vein of the mice within 5 s using a 26-gauge needle.

Luciferase assay

Blood was collected from the tail vein of the mice at the specified time points and incubated at 4°C for 2 h to allow clotting. Centrifugation was performed at 8000 *g* for 20 min to obtain the serum samples. Luciferase activity in the liver was then measured. The liver was harvested and homogenized in lysis buffer (0.1 M Tris, 0.05% Triton X-100, 2 mM ethylenediaminetetraacetic acid, pH 7.8), using a volume equaling 10 ml buffer/gram weight of the liver. The homogenates were centrifuged at 12 000 *g* for 10 min at 4°C. The supernatant was mixed with luciferase assay buffer (PicageneDual, Toyo Ink, Tokyo, Japan) and chemiluminescence was measured using a luminometer with integration periods of 10 s (Lumat LB 9507; EG and G Berthold, Bad Wildbad, Germany). Luciferase activities of samples collected from mock-transfected mice were very low and less than 500 RLU.

Quantitation of antibody titers

Serum samples were obtained as described above. fLuc-specific antibodies were measured using an enzyme-linked immunosorbent assay (ELISA), as described previously [9]. In brief, 96-well flat-bottom polystyrene plates were coated with 0.2 mg/ml of fLuc (Promega, San Luis Obispo, CA, USA) followed by overnight incubation at 4°C. The wells were blocked with phosphate-buffered saline (PBS) containing 0.5% Tween 20 (PBS-T) and 5% bovine serum albumin for 1 h at 37°C and then washed with PBS-T. Serial dilutions of the serum samples were added to the wells, and the plate was incubated for 2 h at 37°C, followed by washing. Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (dilution 1:2000; Zymed Lab, San Francisco, CA, USA) was added to each well. After 1 h of incubation at 37°C and subsequent washing, freshly prepared o-phenylenediamine dihydrochloloride (Wako, Tokyo, Japan) solution containing H_2O_2 was added to each well, and the plates were incubated for 10 min at room temperature. The reaction was stopped by the addition of 10% H_2SO_4 to each well, and absorbance was measured at 490 nm.

IFN-γ secretion from splenocytes

C57BL6 mice received two injections of pROSA-gLuc with pCpG-mcs or pCpG-fLuc with a 3-week interval. One week after the second injection, splenocytes were isolated, purified and cultured in the presence of mitomycin C-treated murine melanoma B16BL6 cells or mitomycin C-treated B16BL6 cells stably expressing fLuc (B16BL6/fLuc) in 96-well culture plates for 2 days. The concentration of IFN-γ in supernatant was determined by an ELISA (Ready-SET-Go! Mouse IFN-γ ELISA; eBioscience, San Diego, CA, USA).

Measurement of serum alanine aminotransferase (ALT) activity

At the indicated time points after plasmid DNA administration, serum was collected as described above. Serum ALT level was measured using a quantification kit (Transaminase CII test Wako; Wako Pure Chemical, Osaka, Japan).

Hematoxylin and eosin staining

Mice were euthanized by cutting the vena cava and the liver was gently infused with 2 ml of saline through the portal vein to remove the remaining blood. The liver was then fixed in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). The stained sections were examined using a microscope (Biozero BZ-8000; Keyence, Osaka, Japan).

Histochemical analysis of the liver

The liver, obtained from the euthanized mice as described above, was embedded in Tissue-Tek OCT embedding

compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan), frozen in liquid nitrogen, and stored in 2-methyl butanol at −80°C. Frozen liver sections (thickness 10 μm) were cut using a microtome cryostat (Jung Frigocut 2800E; Leica Microsystems AG, Wetzlar, Germany), in accordance with a standard procedure, and fixed with 4% paraformaldehyde in PBS.

For detection of $CD8^+$ and $CD4^+$ cells in the liver, the fixed sections were first blocked with 20% fetal bovine serum in PBS for 1 h at 37°C, followed by incubation with biotinylated antibodies specific for mouse CD8 (Acris Antibody, Herford, Germany) or CD4 (eBioscience, San Diego, CA, USA) for 1 h at 37°C. The samples were examined under a fluorescence microscope (Biozero BZ-8000).

Spleen removal and macrophage depletion

The mice were anaesthetized and shaved, and a 2-cm incision was made in the skin at the left flank. The peritoneal membrane was cut open, and the entire spleen was removed intact. The peritoneal membrane and the skin were then closed separately using surgical silk thread. This procedure ensures that the spleen is completely removed and that no splenic fragments are left behind [10].

Clodronate-encapsulated liposomes (clodronate liposomes), prepared as described previously [11], were used for macrophage depletion. The mice were administered clodronate liposomes 1 and 3 days before plasmid DNA administration, and the clodronate liposome administration was repeated every 2 days.

Statistical analysis

The results were evaluated using Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Hydrodynamic injection of pROSA-gLuc resulted in stable gLuc activity in the serum

To evaluate the stability of gLuc expression after hydrodynamic injection of pROSA-gLuc, serum gLuc activity was measured over time after hydrodynamic administration of 0.1, 1 or 10 μg of pROSA-gLuc, without sacrificing the mice. As shown in Figure 1, stable gLuc activity was observed for more than 1 year, irrespective of the pROSA-gLuc dose administered. On the other hand, hydrodynamic injection of pCMV-gLuc (pCMV-based gLuc expressing plasmid) or pCpG-gLuc (pCpG-based gLuc expressing plasmid) also resulted in a long-term gLuc expression, although the time-dependent decline in gLuc activity was greater than that after pROSA-gLuc administration (data not shown).

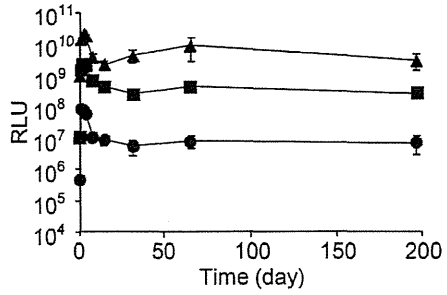

Figure 1. Serum gLuc activity. Time course of gLuc activity in serum after hydrodynamic injections of 0.1 (circle), 1 (square) and 10 μg (triangle) of pROSA-gLuc. The results are expressed as the mean ± SD of five mice.

Co-administration of fLuc-expressing plasmid affected gLuc activity from pROSA-gLuc

pROSA-gLuc was co-administered with 10 μg of pCMV-fLuc or 0.1, 0.3, 1, 3 or 10 μg of pCpG-fLuc, and serum gLuc activity was measured as an indicator of the number of cells expressing both gLuc and fLuc (Figure 2A). The time course of serum gLuc activity after co-administration of pROSA-gLuc with 10 μg of pCMV-fLuc or 0.1 μg of pCpG-fLuc was similar to that obtained after administration of pROSA-gLuc alone. On the other hand, serum gLuc activity showed a sharp decline approximately 1 week after co-administration of pROSA-gLuc with 1, 3 or 10 μg of pCpG-fLuc. This reduction in serum gLuc activity was initiated earlier as the dose of co-administered pCpG-fLuc was increased. A slight reduction in serum gLuc activity was also observed in mice that received pROSA-gLuc with 0.3 μg of pCpG-fLuc. These results suggest that sustained fLuc expression from the co-administered pCpG-fLuc induces the reduction in serum gLuc activity. Sustained expression of fLuc, an antigenic protein, is capable of inducing a fLuc-specific immune response; we therefore hypothesized that the number of fLuc-expressing cells (which also simultaneously express gLuc) is reduced because of the removal of the transgene-expressing cells by the fLuc-specific immune response, which in turn is induced by sustained fLuc expression.

The activities of both the luciferases (gLuc and fLuc) in the liver were measured 17 days after co-administration (Figure 2B). fLuc activity in the liver was higher in mice that received a low dose (0.1 or 0.3 μg) of pCpG-fLuc or 10 μg of pCMV-fLuc than in mice that received a higher

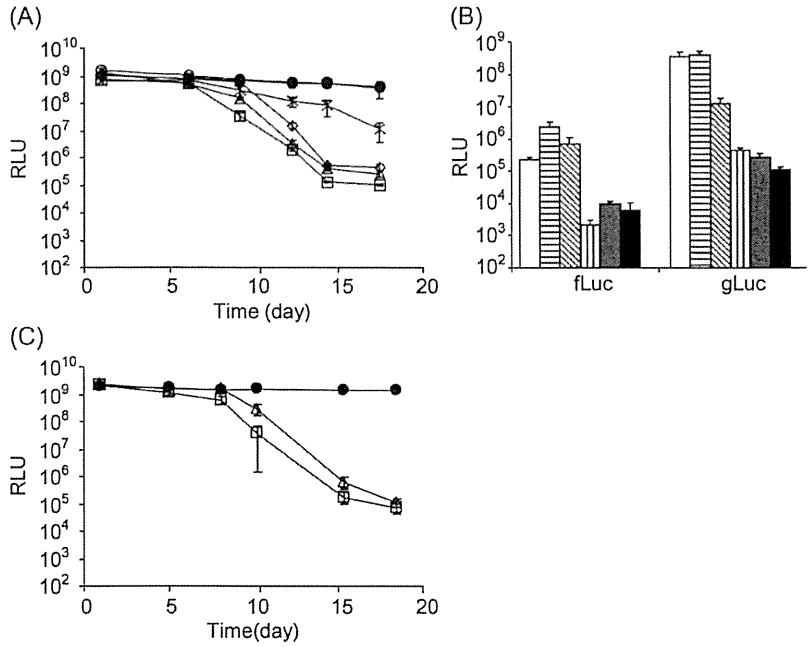

Figure 2. gLuc and fLuc activities in the serum and liver after co-administration of the respective plasmids by hydrodynamic injection. (A) Time course of serum gLuc activity after co-administration of 10 μg of pROSA-gLuc with 10 μg of pCMV-fLuc (closed circle) or 0.1 (open circle), 0.3 (asterisk), 1 (open diamond), 3 (open triangle) or 10 μg (open square) of pCpG-fLuc. (B) fLuc and gLuc activities in the liver 17 days after co-administration of 10 μg of pROSA-gLuc with 10 μg of pCMV-fLuc (open column) or 0.1 (horizontal lines), 0.3 (skewed lines), 1 (vertical lines), 3 (grey) or 10 μg (closed column) of pCpG-fLuc. (C) Time-course of gluc activity in the serum after co-administration of 10 μg of pROSA-gLuc with 10 μg of pCpG-mcs (closed circle), pROSA-fLuc (open triangle) or pCpG-fLuc. The results are expressed as the mean ± SD of five mice.

dose (1, 3 or 10 μg) pCpG-fLuc, which suggests that the initial high and sustained expression obtained by a high dose pCpG-fLuc might induce a fLuc-specific immune response that removed the fLuc expressing cells. Similarly, gLuc activity in the liver of the mice that received pROSA-gLuc with 10 μg of pCMV-fLuc or 0.1 μg of pCpG-fLuc was much higher than the corresponding activity in mice that received higher doses of co-administered pCpG-fLuc, which strongly suggests the removal of transgene-expressing cells by fLuc-specific immune response.

Next, pROSA-gLuc was coadministered with pROSA-fLuc. Serum gLuc activity showed a sharp decline approximately 10 days after the co-administration of pROSA-gLuc with pROSA-fLuc (Figure 2C).

fLuc-specific humoral and cellular immune response were induced in mice that received hydrodynamic injections of pCpG-fLuc

As a next step, we evaluated the fLuc-specific immune response in mice that received hydrodynamic injections of the fLuc-expressing plasmid DNA by measuring fLuc-specific antibodies in the serum 14 days after pCpG-fLuc or pCMV-fLuc administration. As shown in Figure 3A, fLuc-specific antibodies were detected in the serum of mice that had been administered with 1, 3 or 10 μg of pCpG-fLuc. On the other hand, fLuc-specific antibodies were scarcely detected in the serum of mice receiving 10 μg of pCMV-fLuc.

To evaluate the cellular immune response specific for fLuc, splenocytes were collected. Splenocytes of mice receiving pCpG-fLuc produced significantly higher amounts of IFN-γ in response to fLuc than the splenocytes of mice receiving pCpG-mcs or pCMV-fLuc (Figure 3B).

Reduction in serum gLuc activity immediately after co-administration of pROSA-gLuc with pCpG-fLuc in mice pre-administered with pCpG-fLuc

We hypothesized that the fLuc-specific immune response induced by sustained fLuc expression eliminated fLuc-expressing cells, thereby also resulting in decreased gLuc activity. We tested this hypothesis by pre-administering fLuc-expressing plasmid DNA, and evaluated whether the fLuc-specific immune response thereby induced affects the profile of gLuc expression from pROSA-gLuc

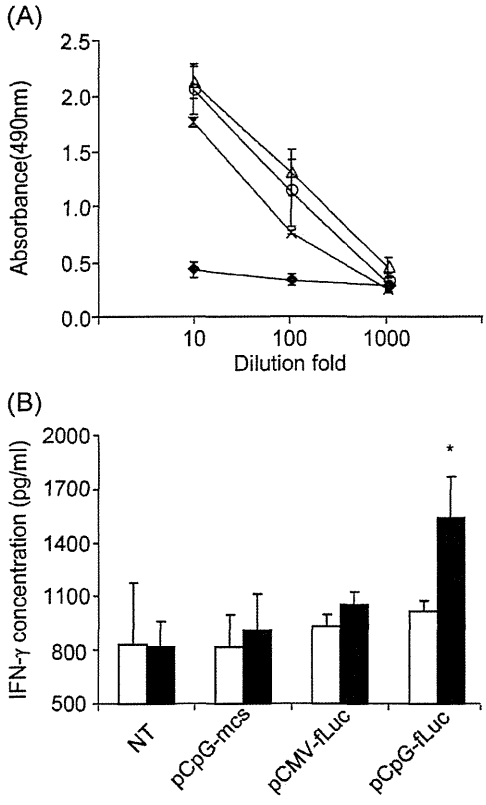

Figure 3. Induction of humral and cellular immune response specific for fLuc after hydrodynamic injection of fLuc-expression vector. (A) Mice received hydrodynamic injections of 10 μg of pCMV-fLuc (closed diamond) or 1 (asterisk), 3 (open triangle) or 10 μg (open circle) of pCpG-fLuc. Fourteen days after plasmid DNA administration, serum samples from the mice were collected, and antibody titers were measured by ELISA. The results are expressed as the mean ± SD of five mice. (B) Splenocytes collected from untreated mice or mice receiving pDNA administration were cocultured with B16BL6 (open columns) or B16BL6/fLuc cells (closed columns) for 2 days. IFN-γ concentration in the culture medium was measured by ELISA. The results are expressed as the mean ± SD of five mice. *$p < 0.05$ compared to the B16BL6-stimulated group.

that was subsequently co-administered with fLuc-expressing plasmid DNA. Specifically, saline, pCpG-fLuc or pCMV-fLuc were pre-administered through hydrodynamic injections, followed by co-administration of pROSA-gLuc with pCpG-fLuc or pCMV-fLuc 14 days later. As shown in Figure 4, a reduction in the gLuc activity was observed as early as 2 days after pCpG-fLuc co-administration in mice pre-administered with pCpG-fLuc. In addition, serum gLuc activity began to decrease 7 and 9 days after co-administration of pROSA-gLuc with pCpG-fLuc in mice pre-administered with pCMV-fLuc or saline, respectively. On the other hand, co-administration of pROSA-gLuc with pCMV-fLuc in mice pre-administered with pCpG-fLuc scarcely altered serum gLuc activity.

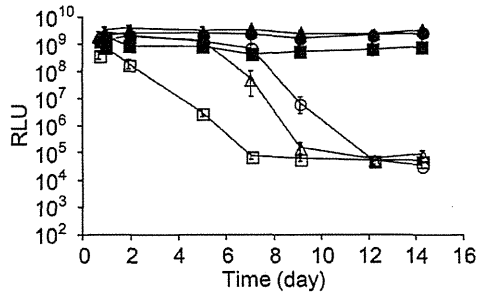

Figure 4. Time course of serum gLuc activity after repeated hydrodynamic injections. The mice first received hydrodynamic injections of saline (circle), 10 μg of pCMV-fLuc (triangle) or 10 μg of pCpG-fLuc (square). Fourteen days after the first administration, the mice were co-administered 10 μg of pROSA-gLuc with 10 μg of pCMV-fLuc (closed symbols) or pCpG-fLuc (open symbols). The results are expressed as the mean ± SD of five mice.

Serum gLuc activity decreased only upon simultaneous co-administration of pROSA-gLuc and pCpG-fLuc

We investigated whether co-expression of fLuc and gLuc in the same cell was required for the reduction in gLuc expression observed after co-administration of pROSA-gLuc with pCpG-fLuc. To avoid co-expression of fLuc and gLuc in the same cells, we sequentially injected the two plasmids instead of a simultaneous injection because it was shown that the former protocol resulted in the expression of the transgenes in different cells with little overlap [8]. Mice were first co-administered pROSA-gLuc with pCpG-fLuc. Fourteen days after the first injection, pROSA-gLuc and pCpG-fLuc were co-administered or sequentially administered; the latter was administered with a 12-h interval (Figure 5A). Serum gLuc activity decreased in mice that received co-administration and not sequential administration. On the other hand, fLuc activity in the liver 14 days after the second injection was comparable between these two groups (Figure 5B), which suggests that the fLuc-specific immune response was induced in both groups.

We further investigated whether reduction in gLuc activity after co-administration of pROSA-gLuc with pCpG-fLuc accompanies the reduction in gLuc expression from separately administered pROSA-gLuc. The mice first received hydrodynamic administration of 1 μg of pROSA-gLuc. One week after the first injection, the mice were administered saline (without any plasmid DNA) or co-administered 10 μg of pROSA-gLuc with pCpG-mcs or pCpG-fLuc (Figure 5C). Co-administration of pCpG-mcs hardly affected the gLuc expression from pROSA-gLuc. Serum gLuc activity was enhanced by the second administration of pROSA-gLuc, which reflects the fact that the dose of pROSA-gLuc was 10-fold higher in the second administration than in the first administration. In the mice

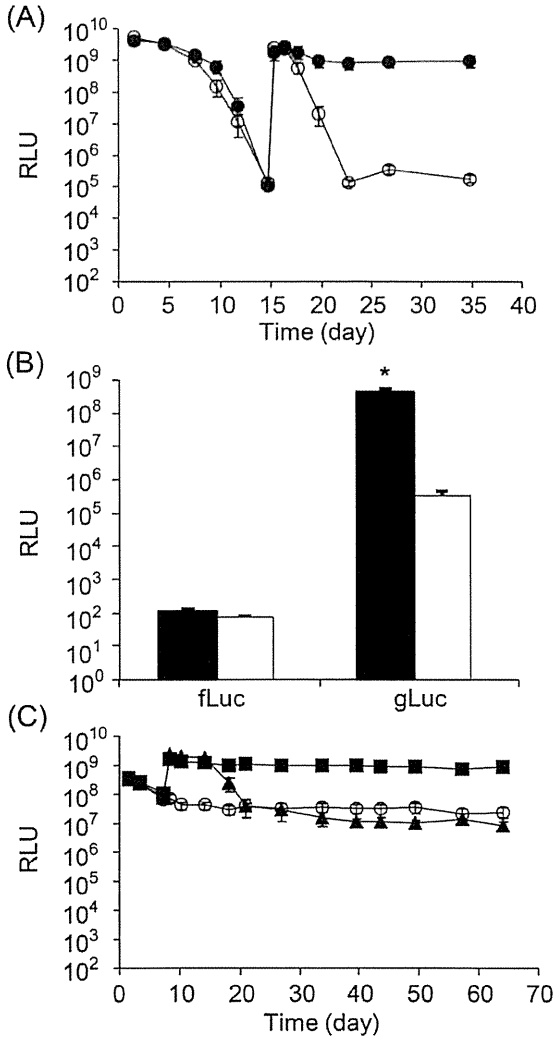

Figure 5. Time course of serum gLuc activity and activities of both gLuc and fLuc in the liver after simultaneous or sequential hydrodynamic delivery. (A) The mice initially received hydrodynamic injection of 10 μg of pROSA-gLuc with 10 μg of pCpG-fLuc. Fourteen days after the first injection, the mice received co-injection of 10 μg of pROSA-gLuc with 10 μg of pCpG-fLuc (open circles) or a sequential injection of 10 μg of pROSA-gLuc followed by 10 μg of pCpG-fLuc after a 12-h interval (closed circles). The results are expressed as the mean ± SD of five mice. *$p < 0.05$ compared to the simultaneous injection group. (B) fLuc and gLuc activities in the liver 21 days after simultaneous (open column) or sequential (closed column) injections of pROSA-gLuc with pCpG-fLuc. The results are expressed as the mean ± SD of five mice. •$p < 0.05$ compared to the simultaneous injection group. (C) Mice initially received hydrodynamic injection of 1 μg of pROSA-gLuc. Seven days after the first injection, the mice received saline injection (open circle) or co-administration of 10 μg of pROSA-gLuc with 10 μg of pCpG-mcs (closed square) or pCpG-fLuc (closed triangle). The results are expressed as the mean ± SD of five mice.

that received a second injection of pROSA-gLuc with pCpG-fLuc, serum gLuc activity began to decrease 7 days after the second injection and reached the levels found in the control mice that received saline during the second administration. This result implies that gLuc-expressing cells generated by the first pROSA-gLuc administration were not affected by the immune response induced by the second injection of pROSA-gLuc with pCpG-fLuc.

Inflammatory cells, including CD8$^+$ cells, were detected in the liver after pCpG-fLuc administration

Mice were co-administered pROSA-gLuc with pCpG-fLuc or pCpG-mcs twice with an interval of 2 weeks. The livers were collected from the mice 2, 4 or 6 days after the second injection, and liver sections were prepared to evaluate the effect of the fLuc-specific immune response. HE staining of the liver sections (Figure 6A) revealed a large number of infiltrating cells in the liver 2 days after the second administration of pCpG-fLuc. The number of infiltrating cells declined on day 4 and returned to a level comparable to that found in the pCpG-mcs group by day 6. The liver sections were stained with CD4- or CD8-specific antibodies to characterize the type of infiltrating cells (Figures 6B and 6C). A slight difference was observed in the number of CD4$^+$ cells between the pCpG-mcs and pCpG-fLuc groups. By contrast, the number of CD8$^+$ cells was higher in the pCpG-fLuc group than in the pCpG-mcs group 2 days after the second administration; the number decreased on day 4 and returned to a level comparable with that found in the pCpG-mcs group by day 6.

Because it was hypothesized that the removal of transgene-expressing hepatocytes by the infiltrating cells was related to hepatic injury, the time-course of serum ALT level was measured. As previously reported, an increase in serum ALT level was detected immediately after hydrodynamic injection, irrespective of the types of plasmid DNAs, indicating a transient increase in the permeability of cellular membrane by the injection. At 7 and 9 days after injection, the serum ALT level of mice receiving pCpG-fLuc was significantly higher than that of mice receiving pCpG-mcs. This result also implies that hepatocytes expressing fLuc are damaged by the fLuc-specific immune response.

Spleen removal and macrophage depletion did not change the profile of serum gLuc activity after co-administration of pROSA-gLuc with pCpG-fLuc

Although hepatocytes are the chief cell type expressing transgenes after hydrodynamic injections of plasmid DNA, transgene expression also occurs in nonparenchymal cells in the liver including Kupffer cells and in other organs such as the spleen [12]. Kupffer cells and splenic

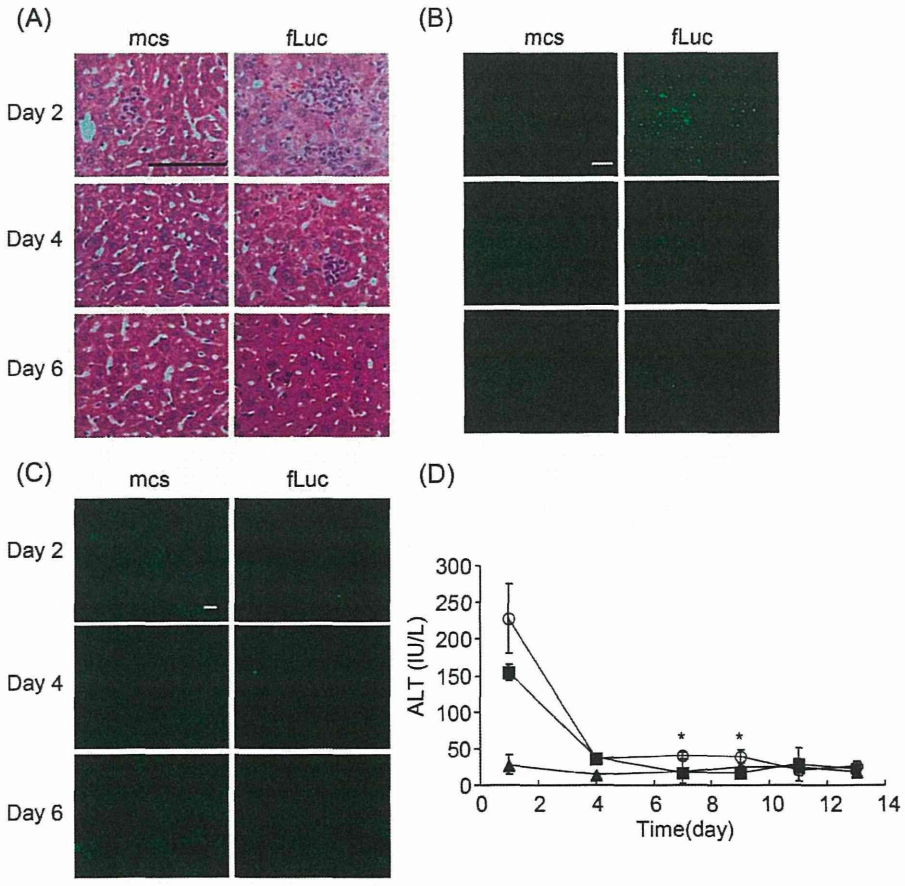

Figure 6. Histological analysis of the liver after repeated hydrodynamic delivery of fLuc-expressing plasmids. (A–C) Mice received hydrodynamic co-injections of 10 μg of pROSA-gLuc with 10 μg of pCpG-mcs or pCpG-fLuc twice with an interval of 2 weeks. Livers were collected from the mice 2, 4 or 6 days after the second injection. The liver sections were subjected to HE staining (A) or immunofluorescenc staining using CD8- (B) or CD4-specific antibodies (C). Scale bar = 100 μm. (D) Time-course of ALT levels in the serum in untreated mice (closed triangle) or mice receiving hydrodynamic injection of 10 μg of pCpG-fLuc (open circle) or pCpG-mcs (closed square). The results are expressed as the mean ± SD of five mice. *$p < 0.05$ compared to the pCpG-mcs injected group.

macrophages are known to function as APCs; therefore, the fLuc-specific immune response induced after hydrodynamic injection of pCpG-fLuc might be a result of fLuc expression in these cells. To test this hypothesis, the mice were given hydrodynamic injections of pROSA-gLuc with pCpG-fLuc or pCpG-mcs after spleen removal, macrophage depletion, or a combination of spleen removal and macrophage depletion (Figure 7). Neither treatment was found to affect the time course of serum gLuc activity after administration of pROSA-gLuc with pCpG-mcs (data not shown). However, the serum gLuc activity after co-administration of pROSA-gLuc with pCpG-fLuc decreased in splenectomized or macrophage-depleted mice. Moreover, even in mice subjected to both spleen removal and macrophage depletion, serum gLuc activity was observed to decrease 1 week after administration of pROSA-gLuc with pCpG-fLuc. On the other hand, there was a tendency that the degree of reduction in gLuc activity was slightly smaller in mice receiving splenectomy or macrophage depletion than untreated mice, and the treatments of splenectomy and macrophage depletion appeared to have an additive effect.

Discussion

In the present study, single pROSA-gLuc administration was found to result in serum gLuc activity that was stable for more than 1 year, implying that sustained gLuc expression did not induce a gLuc-specific immune response such as anti-gLuc antibody production. On the other hand, sustained fLuc expression induced a fLuc-specific immune response, suggesting that the antigenicity of fLuc protein is higher than that of the gLuc protein.

Co-administration of high doses of pCpG-fLuc with pROSA-gLuc resulted in a reduction in serum gLuc activity,

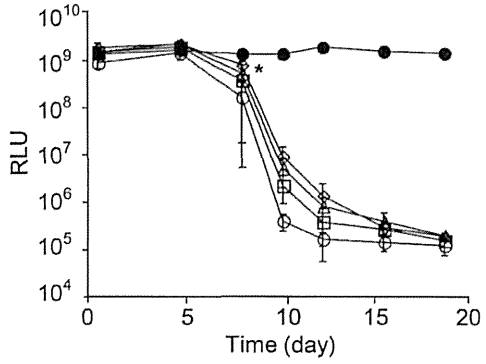

Figure 7. Time course of serum gLuc activity with hydrodynamic injection after spleen removal and macrophage depletion. Mice were left untreated (circle) or subjected to macrophage depletion (triangle), spleen removal (square) or both treatments (diamond). The mice were then co-administered 10 μg of pROSA-gLuc with 10 μg of pCpG-mcs (closed symbols) or pCpG-fLuc (open symbols). The results are expressed as the mean ± SD of five mice. *$p < 0.05$ compared to untreated mice.

which is likely caused by the removal of transgene-expressing cells as a consequence of the fLuc-specific immune response induced by sustained fLuc expression. On the other hand, co-administration of high doses of pCMV-fLuc, which results in a high but transient transgene expression, induced neither a detectable fLuc-specific immune response, nor a reduction in the serum gLuc activity. This suggests that the duration of fLuc expression would be an important factor for eliciting the fLuc-specific immune response, which in turn eliminates the fLuc-expressing cells. The results obtained in the present study indicate that long-term expression of a transgene has a higher risk of eliciting an immune response than short-term expression.

Aubert *et al.* [13] demonstrated that a cytotoxic immune response was the chief mechanism responsible for the removal of transgene-expressing cells after retroviral-mediated β-galactosidase gene transfer in the liver. In addition, an *ex vivo* study suggested that a cytotoxic immune response resulted in the removal of transgene (green fluorescent protein)-expressing cells in concert with the helper T cells after an epidermal gene transfer [14]. In the present study, hydrodynamic injection of pCpG-fLuc induced a cellular immune response specific to fLuc. In addition, $CD8^+$ cell infiltration was observed in the liver of the mice that received pCpG-fLuc administration. These results suggest that fLuc-specific cytotoxic T lymphocytes play an important role in eliminating the fLuc-expressing cells, which is in agreement with previous studies [13]. In addition, hydrodynamic injection of pCpG-fLuc and pROSA-gLuc resulted in the increase in serum ALT levels at approximately 1 week after the administration, when the decline in gLuc activity occurred, also suggesting that hepatocytes expressing both fLuc and gLuc were damaged. Furthermore, fLuc-specific antibodies may be involved in the removal of the fLuc-expressing cells.

A reduction in serum gLuc activity was observed only when pROSA-gLuc was co-administered with pCpG-fLuc. On the other hand, gLuc expression from pre-administered pROSA-gLuc was scarcely affected by the second injection of pROSA-gLuc with pCpG-fLuc. From these results, it is likely that the decrease in serum gLuc activity after the simultaneous injection of pROSA-gLuc and pCpG-fLuc is caused by the elimination of cells expressing both gLuc and fLuc, as a consequence of the fLuc-specific immune response. This fact suggests that co-administration of pROSA-gLuc with a vector expressing a gene of interest could be a useful method for monitoring the effect of an immune response to the product of interest in transgene-expressing cells. However, there is a possibility that transgene expression level from a plasmid vector may be affected by the simultaneously delivered plasmid vector via the interference in transgene expression process [15]. Detailed molecular studies would be required to exclude this possibility.

When the transgene product is a secretory protein, the transgene-expressing cells secrete the products so that they may be taken up by APCs to elicit an immune response [16–19]. fLuc is a nonsecretory protein and is scarcely released from the fLuc-expressing cells. Therefore, a fLuc-specific immune response induced after pCpG-fLuc administration is likely a result of the direct gene delivery of plasmid DNA into APCs. By using a target sequence for miRNA-142-3p, a miRNA highly expressed in APCs, Brown *et al.* [20] demonstrated that transgene expression in APCs, resulting in sustained transgene expression, is the chief reason for the induction of an immune response to the transgene products. Hydrodynamic injection also delivers plasmid DNA into Kupffer cells in the liver and spleen cells, both of which function as APCs; we therefore evaluated the role of Kupffer and splenic cells in the induction of the immune response. We found that, even in mice that had been subjected to both spleen removal and macrophage depletion, serum gLuc activity declined after hydrodynamic administration of pROSA-gLuc with pCpG-fLuc, which implied that the fLuc-specific immune response could eliminate fLuc-expressing cells in the absence of macrophages in the liver (Kupffer cells) or spleen (splenic macrophages), or in fact any splenic cells. It is known that the liver has some dendritic cells; gene transfer to the dendritic cells might occur after hydrodynamic injection of plasmid DNA [21] and these dendritic cells might function as APCs. In addition, hepatocytes might also function as APCs after hydrodynamic injection because hepatocytes have been previously reported to exhibit antigen presenting ability [22–24]. Further studies are required for determining which types of cells functioned as APCs in the induction of the fLuc-specific immune response after hydrodynamic injection of pCpG-fLuc.

Although induction of an immune response to a transgene product is a serious problem in gene therapy, eliciting the immune response is, in turn, desirable for DNA vaccination. Our results demonstrate that single pCpG-fLuc administration could induce a strong cytotoxic immune response against fLuc; therefore, hydrodynamic administration of the recombinant pCpG vector encoding an antigenic protein might prove to be potent as a DNA vaccine because it also shows sustained transgene expression. On the other hand, in the development of DNA vaccine by hydrodynamic gene delivery, the administration of adjuvant is desirable to induce stronger immune response because the degree of immune activation is limited even after hydrodynamic delivery of CpG-rich plasmid DNA [25].

In conclusion, the present study demonstrates that high levels of sustained expression of a transgenic antigen induce an immune response and that the cells expressing the transgene product are eliminated, probably as a result of the transgene product-specific immune response.

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ORIGINAL INVESTIGATION

Genome-wide association study identifies a *PSMD3* variant associated with neutropenia in interferon-based therapy for chronic hepatitis C

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Abstract Cytopenia during interferon-based (IFN-based) therapy for chronic hepatitis C (CHC) often necessitates reduction of doses of drugs and premature withdrawal from therapy resulting in poor response to treatment. To identify genetic variants associated with IFN-induced neutropenia, we conducted a genome-wide association study (GWAS) in 416 Japanese CHC patients receiving IFN-based therapy. Based on the results, we selected 192 candidate single nucleotide polymorphisms (SNPs) to carry out a replication analysis in an independent set of 404 subjects. The SNP rs2305482, located in the intron region of the *PSMD3* gene on chromosome 17, showed a strong association when the results of GWAS and the replication stage were combined (OR = 2.18, $P = 3.05 \times 10^{-7}$ in the allele frequency model). Logistic regression analysis showed that rs2305482 CC and neutrophil count at baseline were independent predictive factors for IFN-induced neutropenia (OR = 2.497, $P = 0.0072$ and OR = 0.998, $P < 0.0001$, respectively). Furthermore, rs2305482 genotype was associated with the doses of pegylated interferon (PEG-IFN) that could be tolerated in hepatitis C virus genotype 1-infected patients treated with PEG-IFN plus ribavirin, but not with treatment efficacy. Our results suggest that genetic

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testing for this variant might be useful for establishing personalized drug dosing in order to minimize drug-induced adverse events.

Introduction

Chronic hepatitis C virus (HCV) infection is a significant risk factor for progressive liver fibrosis and hepatocellular carcinoma. Antiviral treatment improves the natural course in chronic hepatitis C (CHC) (George et al. 2009; Yoshida et al. 2004). Newly-developed treatments involving direct-acting antivirals (DAAs), including nonstructural (NS) 3/4A protease inhibitors have shown promising outcomes in combination with pegylated interferon (PEG-IFN) plus ribavirin (RBV) in several clinical trials. Thus, >70 % of patients infected with HCV genotype 1 are reported to achieve sustained virological responses (SVR) (Jacobson et al. 2011; Poordad et al. 2012; Zeuzem et al. 2011). Furthermore, interferon-free (IFN-free) therapies are expected to be useful especially in IFN-resistant patients and may become the standard of care in the near future. However, IFN-based regimens have been standard-of-care therapies over the last couple of decades.

IFN-based therapies are associated with various adverse effects. Cytopenia is common due to bone marrow suppression cased by IFN or DAA and hemolysis by RBV. This is particularly the case in patients with advanced hepatic fibrosis, but can sometimes also occur in those with mild fibrosis. This then often necessitates dose reduction or premature withdrawal from therapy, resulting in poor response to treatment. For instance, it was reported that rates of viral clearance were significantly reduced in patients who could not be maintained on at least 80 % of their drug doses for the duration of PEG-IFN/RBV therapy (McHutchison et al. 2002). Therefore, pre-treatment prediction of possible adverse effects in order to avoid them and undergo therapy safely is desirable.

Recent genome-wide association studies (GWASs) have identified two important host genetic variants influencing CHC treatment: (1) single nucleotide polymorphisms (SNPs) near the interleukin-28B (*IL28B*) gene, which are strongly associated with response to therapy for chronic HCV genotype 1 infection (Ge et al. 2009; Suppiah et al. 2009; Tanaka et al. 2009), and (2) SNPs in the inosine triphosphatase (*ITPA*) gene, which accurately predict RBV-induced anemia in European–American (Fellay et al. 2010) and Japanese population (Ochi et al. 2010). We validated the association between this *ITPA* genetic variant and RBV-induced anemia (Sakamoto et al. 2010), and reported that the *ITPA* genotype affects the tolerated doses of RBV and treatment response in a stratified group (Kurosaki et al. 2011; Matsuura et al. 2014). Additionally, our GWAS showed that *DDRGK1/ITPA* variants are strongly associated with IFN-induced thrombocytopenia as well as anemia during PEG-IFN/RBV therapy (Tanaka et al. 2011). Thompson et al. (2012) also reported that the *ITPA* genetic variant was associated with anemia and thrombocytopenia during PEG-IFN/RBV therapy. However they identified no genetic determinants of IFN-induced neutropenia at the level of genome-wide significance by their GWAS in populations of European Americans, African Americans and Hispanics.

Hence, to identify genetic variants associated with IFN-induced neutropenia, we conducted a GWAS in Japanese CHC patients.

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Materials and methods

Patients

From 2007 to 2012, samples for the GWAS were obtained from 416 CHC patients who were treated at 22 hospitals (liver units with hepatologists) throughout Japan. In the following stage of replication analysis, samples were collected in an independent set of 404 Japanese CHC patients. Most patients were treated with PEG-IFN-α2b (1.5 μg/kg body weight subcutaneously once a week) or PEG-IFN-α2a (180 μg once a week) plus RBV (600–1,000 mg daily according to body weight) for 48 weeks for HCV genotype 1 and 24 weeks for genotype 2. Treatment duration was extended in some patients up to 72 weeks for genotype 1 and 48 weeks for genotype 2 according to physicians' preferences. Other patients were treated with PEG-IFN-α2a or IFN monotherapy, or IFN-α2b plus RBV in standard doses of the regimens. The doses of drugs were reduced according to the recommendations on the package inserts or the clinical conditions of the individual patients. Erythropoietin or other growth factors were not given. Patients chronically infected with hepatitis B virus or human immunodeficiency virus, or with other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis, were excluded from this study. Written informed consent was obtained from all individual participants in this study and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Inclusion criteria of neutropenia

In the initial stage of GWAS, we defined the inclusion criteria of the case group as minimum neutrophil counts of $<750/mm^3$ at week 2 or 4 during IFN-based therapy, since the dose reduction of IFN is recommended at those levels on the package inserts. Thereafter we did it as minimum neutrophil counts of $<600/mm^3$ at week 2 or 4 in the following GWAS and the replication stages.

SNP genotyping and data cleaning

We conducted two stages of GWAS using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc. Santa Clara, CA) according to the manufacturer's instructions. The cut-off value was calculated to maximize the difference, which was also close to median change. At GWAS, the average overall call rate of patients in the case and the control group reached 98.66 and 98.79 %, respectively. We then applied the following thresholds for SNP quality control (QC) in data cleaning: SNP call rate ≥95 % for all samples, minor allele frequency (MAF) ≥1 % for all samples. A total of 601,578 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots of SNPs showing $P < 0.0001$ in association analyses by comparing allele frequencies in both groups were checked by visual inspection and SNPs with ambiguous genotype calls were excluded. In the replication study, the genotyping of 192 candidate SNPs in an independent set of 404 Japanese HCV-infected patients was carried out using the DigiTag2 assay (Nishida et al. 2007). Successfully genotyped SNPs in the replication analysis had a >95 % call rate, and cleared Hardy–Weinberg equilibrium (HWE) $P \geq 0.001$. One SNP could not be genotyped, and hence we obtained data on 191 SNPs including rs9915252. Three SNPs, rs4794822, rs3907022, and rs3859192 located around the proteasome 26S subunits non-ATPase 3 (*PSMD3*) gene and rs8099917 near the *IL28B* gene were genotyped by TaqMan SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol.

Laboratory and histological tests

Blood samples were obtained at baseline and at appropriate periods after the start of therapy and for hematologic tests, blood chemistry, and HCV RNA. Fibrosis was evaluated on a scale of 0–4 according to the METAVIR scoring system. The SVR was defined as an undetectable HCV RNA level by Roche COBAS Amplicor HCV Monitor test, v.2.0 (Roche Molecular Diagnostics, Pleasanton, CA) with a lower detection limit of 50 IU/ml or Roche COBAS AmpliPrep/COBAS TaqMan HCV assay (Roche Molecular Diagnostics, Pleasanton, CA) with a lower detection limit of 15 IU/ml 24 weeks after the completion of therapy. Serum granulocyte colony-stimulating factor (G-CSF) levels were analyzed using Human G-CSF Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN).

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Expression quantitative trait locus analysis

Expression quantitative trait locus analysis (eQTL) was conducted using the web-based tool, Genevar (http://www.sanger.ac.uk/resources/software/genevar) (Yang et al. 2010). We evaluated the correlations between rs2305482 genotypes and the expression of transcripts of *PSMD3* or colony-stimulating factor 3 (*CSF3*) by the Spearman's rank correlation coefficient.

Statistical analysis

In the GWAS and the replication stages, the observed association between a SNP and neutropenia induced by IFN-based therapy was assessed by the Chi square test with a two-by-two contingency table in three genetic models: the allele frequency model, the dominant-effect model and the recessive-effect model. Significance levels after Bonferroni correction for multiple testing were $P = 8.31 \times 10^{-8}$ (0.05/601,578) in the GWAS stage and $P = 2.62 \times 10^{-4}$ (0.05/191) in the replication stage. Categorical variables were compared between groups by the Chi square test, and non-categorical variables by the Student's t test or the Mann–Whitney U test. Multivariate logistic regression analysis with stepwise forward selection was performed with $P < 0.05$ in univariate analysis as the criteria for model inclusion. To evaluate the discriminatory ability of neutrophil counts at baseline to predict neutropenia during IFN-based therapy, receiver operating characteristic curve (ROC) curve analysis was conducted. Changes of serum G-CSF levels from baseline to the period with neutropenia during IFN-based therapy were compared by the repeated measure analysis of variance (ANOVA). Correlations between neutrophil counts and serum G-CSF levels were analyzed using Pearson's correlation coefficient test. $P < 0.05$ was considered significant in all tests.

Results

Genetic variants associated with IFN-induced neutropenia

We conducted two stages of GWAS by changing the terms of neutrophil counts, followed by the replication analysis (Fig. 1). The characteristics of the patients in each group for the GWAS and the replication stage are summarized in Table 1. At the first stage of GWAS (GWAS-1st), we genotyped 416 Japanese CHC patients with minimum neutrophil counts of <750/mm^3 (Case-G1, $n = 114$) and ≥1,000/mm^3 (Control-G, $n = 302$) at week 2 or 4 during IFN-based therapy. Here there may still be mixed with undesirable samples that should be removed from the case group. Therefore, we designed and carried out the second stage of GWAS (GWAS-2nd) comparing the patients with more severe neutropenia to the control group: in patients with minimum neutrophil counts of <600/mm^3 (Case-G2, $n = 50$) and ≥1,000/mm^3 (Control-G, $n = 302$) at week 2 or 4 using the same samples as used in GWAS-1st. Supplementary Fig. 1 shows a genome-wide view of the single-point association data based on allele frequencies in GWAS-1st and GWAS-2nd. No association between SNPs and IFN-induced neutropenia reached a genome-wide level of significance [Bonferroni criterion $P < 8.31 \times 10^{-8}$ (0.05/601,578)]. Therefore, we selected the candidate SNPs principally

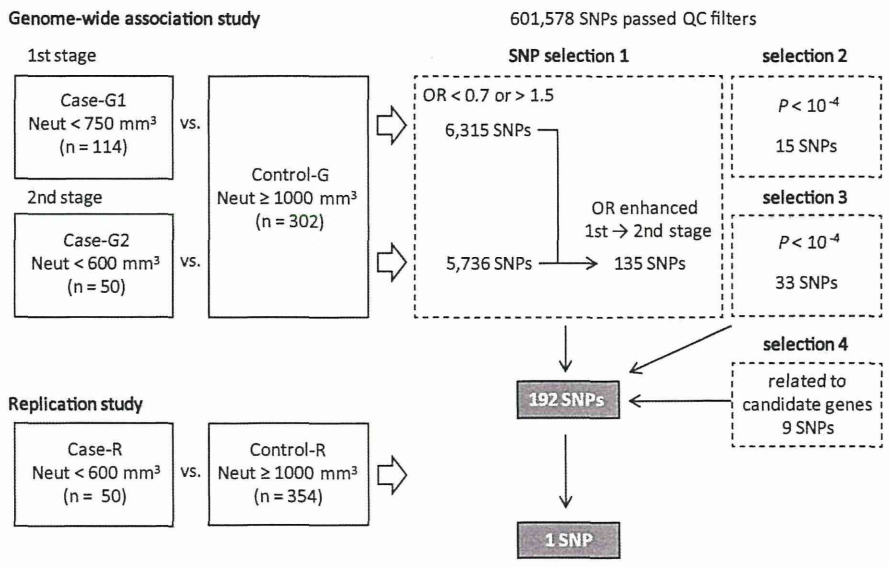

Fig. 1 Outline of the study design. *Neut* neutrophil counts, *SNP* single nucleotide polymorphism, *QC* quality control, *OR* odds ratio

Table 1 Clinical characteristics of patients in GWAS and the replication study

	GWAS			Replication study	
	Case-G1 ($n = 114$)	Case-G2 ($n = 50$)	Control-G ($n = 302$)	Case-R ($n = 50$)	Control-R ($n = 354$)
At baseline					
Gender, male/female	48/66	21/29	170/132	24/26	208/146
Age, years	57.9 (8.7)	57.1 (8.3)	57.2 (11.2)	59.1 (10.2)	56.7 (9.6)
Neutrophil count, /mm^3	1,800 (777)	1,662 (897)	2,750 (984)	1,570 (552)	2,724 (985)
Hemoglobin, g/dL	13.6 (1.3)	13.5 (1.3)	14.2 (1.5)	13.6 (1.6)	14.3 (1.5)
Platelet count, $\times 10^9$/L	141 (42)	132 (46)	164 (54)	140 (47)	162 (60)
ALT, IU/L	82.9 (88.6)	70.4 (53.1)	81.5 (77.9)	87.8 (82.7)	85.2 (71.1)
HCV genotype, 1/2/ND	95/18/1	40/10/0	250/51/1	45/5/0	277/77/0
HCV RNA, log IU/mL	5.9 (0.8)	5.9 (1.0)	6.1 (0.8)	6.1 (0.9)	6.1 (0.8)
Liver fibrosis, F0-2/F3-4/ND	62/22/30	25/10/15	168/70/64	21/6/23	229/87/38
rs8099917, TT/TG + GG/ND	74/39/1	35/15/0	189/109/4	31/17/2	278/70/6
Regimen					
PEG-IFN + RBV/IFN + RBV/PEG-IFN/IFN mono	112/0/0/2	48/0/0/2	277/9/9/7	44/4/2/0	351/0/3/0
At week 4					
Neutrophil count, /mm^3	606 (126)	496 (104)	1,551 (501)	501 (89)	1,533 (484)

Data are expressed as number for categorical data or the mean (standard deviation) for non-categorical data

GWAS genome-wide association study, *ALT* alanine transaminase, *ND* not determined, *PEG-IFN* pegylated interferon, *IFN* mono, interferon monotherapy, *RBV* ribavirin

by comparing between GWAS-1st and GWAS-2nd as follows. There were 6,315 and 5,736 SNPs with odds ratios (ORs) <0.7 or >1.5 at GWAS-1st and GWAS-2nd, respectively. Of these, the ORs of 135 SNPs were more notable at GWAS-2nd than at GWAS-1st. In addition to the 135 SNPs, we selected 15 and 33 SNPs with $P < 10^{-4}$ at GWAS-1st and GWAS-2nd, and added 9 SNPs which are located around the candidate genetic regions identified by the GWAS stage and are non-synonymous or related to diseases in previous reports. Consequently, we carried out the replication analysis focusing on this total of 192 SNPs.

In the subsequent replication analysis, we carried out genotyping of the 192 candidate SNPs in an independent set of 404 Japanese HCV-infected patients with minimum neutrophil counts of <600/mm^3 (Case-R, $n = 50$) and ≥1,000/mm^3 (Control-R, $n = 354$) at week 2 or 4 during IFN-based therapy (Table 1; Fig. 1). The results in the replication stage combined with GWAS-2nd are shown in Supplementary Table 1. Several SNPs such as rs11743919 and rs2457840 showed strong associations with low P value, however, the MAF of them were <5 %. In general, low frequent SNPs tend to show unsettled associations, especially in statistical analysis with small number of samples. Therefore, we excluded these SNPs from the final candidates. Consequently, we determined the SNP rs2305482, located in the intron of *PSMD3* gene on chromosome 17, as the most promising candidate, which showed a strong association with IFN-induced neutropenia in the combined results of GWAS-2nd and the replication stage (OR = 2.18; 95 % CI = 1.61–2.96, $P = 3.05 \times 10^{-7}$ in the allele frequency model) (Table 2).

Association of SNPs located in *PSMD3-CSF3* with neutropenia

A previous GWAS showed that rs4794822 located between the *PSMD3* and *CSF3* genes was associated with neutrophil counts in Japanese patients including 14 different disease groups (Okada et al. 2010). As shown in Fig. 2, rs4794822 is in strong linkage disequilibrium (LD) with rs2305482 which we identified in the present study. Thus, the pairwise LD (r^2) in the HapMap JPT: Japanese in Tokyo, Japan, is 0.66. Because the SNP rs4794822 is not included in the Affymetrix Genome-Wide Human SNP Array 6.0, we additionally genotyped it together with three other SNPs (rs9915252, rs3859192 and rs3907022) located in the same LD block around the *PSMD3* gene (Fig. 2). The allele frequency of each SNP was compared between patients with minimum neutrophil counts of <600/mm^3 (Case-G2 + R: Case-G2 plus Case-R, $n = 100$) and ≥1,000/mm^3 (Control-G + R: Control-G plus Control-R, $n = 656$) at week 2 or 4 during IFN-based therapy. This showed that, rs4794822 was also strongly associated with neutropenia during IFN-based therapy (OR = 2.24; 95 % CI = 1.63–3.07, $P = 3.63 \times 10^{-7}$ in the allele frequency model) (Table 3).

Table 2 SNP associated with interferon-induced neutropenia

dbSNP rsID	Nearest gene	Risk allele	Allele (1/2)	Stage	Case			Control			OR[a] (95 % CI)	*P* value[b]
					11	12	22	11	12	22		
rs2305482	*PSMD3*	C	C/A	GWAS-1st	23 (20.4)	52 (46.0)	38 (33.6)	26 (8.6)	143 (47.4)	133 (44.0)	1.61 (1.17–2.20)	2.95×10^{-3}
				GWAS-2nd	12 (24.5)	28 (57.1)	9 (18.4)	26 (8.6)	143 (47.4)	133 (44.0)	2.37 (1.54–3.65)	6.47×10^{-5}
				Replication	12 (24.4)	20 (40.8)	17 (34.7)	33 (9.5)	136 (39.1)	179 (51.4)	1.99 (1.30–3.06)	1.46×10^{-3}
				Combined[c]	24 (24.5)	48 (49.0)	26 (26.5)	59 (9.1)	279 (42.9)	312 (48.0)	2.18 (1.61–2.96)	3.05×10^{-7}

Data of allele distribution represent number (%). Data of subjects whose genotypes were not determined were excluded

SNP single nucleotide polymorphism

[a] Odds ratio for the allele frequency model

[b] *P* value by the Chi square test for the allele frequency model

[c] Allele distributions in GWAS-2nd and replication were combined

Predictive factors for IFN-induced neutropenia

The following analyses were carried out for rs2305482 and rs4794822 using the subjects in Case-G2 + R and Control-G + R. Neutrophil counts at baseline correlated with rs2305482 and rs4794822 genotypes (Supplementary Fig. 2), and strongly affected IFN-induced neutropenia as shown by ROC analysis (area under the curve = 0.860) (Supplementary Fig. 3). Furthermore, gender, hemoglobin level, and platelet count at baseline were also significantly associated with IFN-induced neutropenia by univariate analysis (Table 4). Therefore, we analyzed pretreatment predictive factors for IFN-induced neutropenia in logistic regression models that included the following variables: gender, neutrophil count, platelet count, and rs2305482 or rs4794822 genotypes. In addition to neutrophil count, rs2305482 CC was an independent predictive factor for IFN-induced neutropenia (OR = 2.497; 95 % CI = 1.281–4.864, $P = 0.0072$) (Table 5) as was rs4794822 CC (OR = 2.272; 95 % CI = 1.337–3.861, $P = 0.0024$) (Supplementary Table 2).

Impact of *PSMD3-CSF3* SNPs on tolerated drug doses and treatment efficacy

To evaluate the impact of *PSMD3-CSF3* SNPs on doses of drugs given, and on treatment efficacy, we selected 380 HCV genotype 1-infected patients treated with PEG-IFN/RBV for 48 weeks. They were selected as having information available on the doses of PEG-IFN/RBV that they had received (Supplementary Table 3). It was reported that rates of viral clearance were significantly reduced in patients who could not be maintained on at least 80 % of their drug doses for the duration of PEG-IFN/RBV therapy (McHutchison et al. 2002). In reference to this result, we stratified the patients into three groups according to the doses of PEG-IFN or RBV administered, as follows: <60 %, ≥60 to <80 %, ≥80 % of the planned doses for 48 weeks. The proportion of patients in the <60 % group for PEG-IFN was significantly higher in patients possessing rs2305482 CC than in those with AA/AC ($P = 0.005$), whereas there was no association for RBV (Fig. 3). The same results were found in the analysis of rs4794822 (Supplementary Fig. 4). However, the univariate analysis of pretreatment factors associated with SVR showed that there was no association between SVR and rs2305482 or rs4794822 genotypes (Supplementary Table 3).

Candidate SNP-gene association analysis in IFN-induced neutropenia

To investigate whether the SNPs associated with neutropenia affect the expression of nearby genes, we conducted

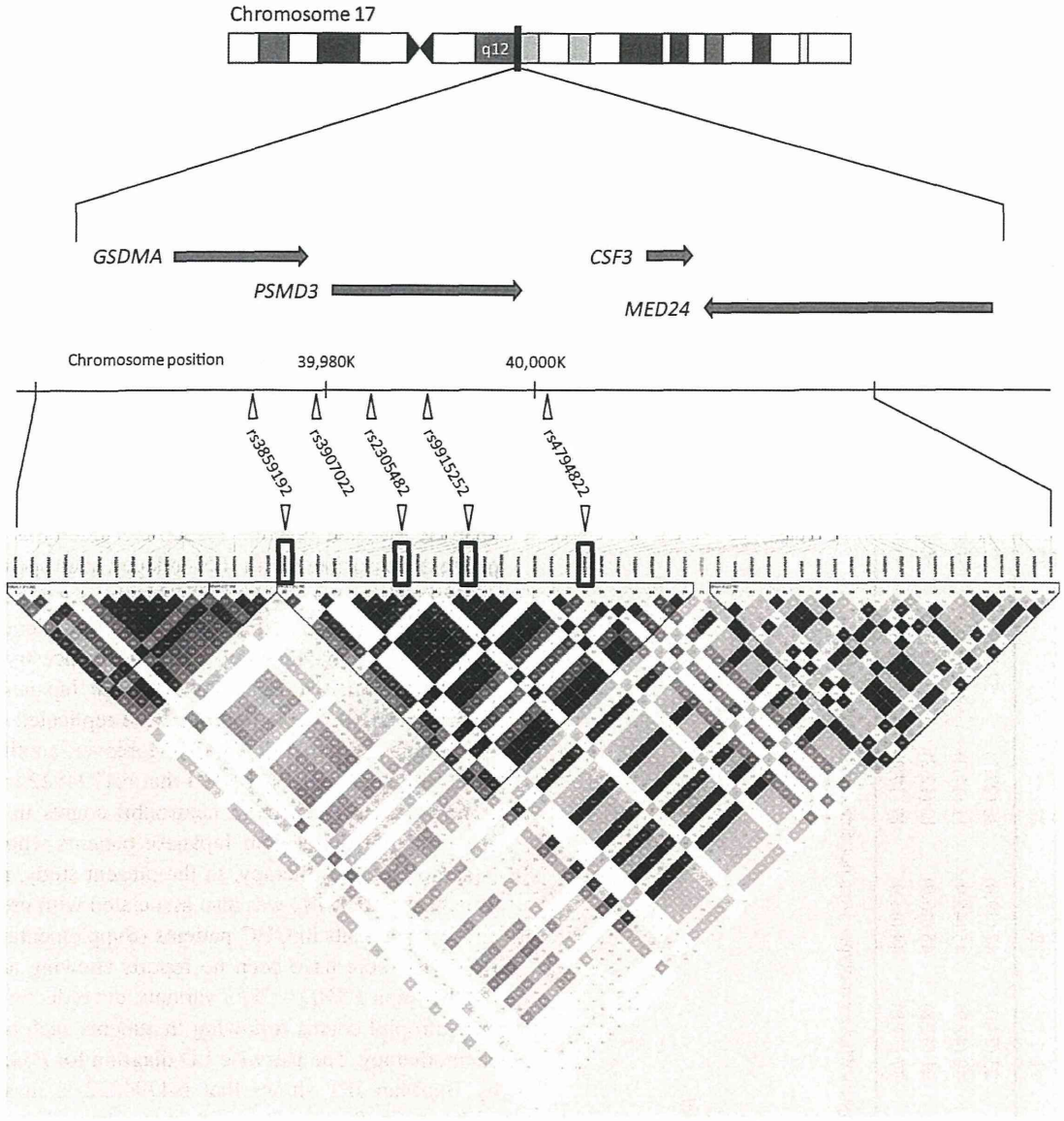

Fig. 2 Position on chromosome and pairwise linkage disequilibrium (r^2) diagrams in the HapMap JPT around the *PSMD3-CSF3* locus

an eQTL analysis. The C allele of rs2305482, a risk for neutropenia, was associated with higher expression levels of *PSMD3* in the populations of LWK: Luhya in Webuye, Kenya (rho = 0.30, $P = 0.006$), and MEX: Mexican ancestry in Los Angeles, California (rho = 0.36, $P = 0.015$) (Supplementary Fig. 5a), whereas it was associated with lower expression levels of *CSF3* in CHB: Han Chinese in Beijing, China, in the probe of ILMN_1655639 (rho = −0.48, $P = 5.5 \times 10^{-6}$) (Supplementary Fig. 5b), and in MEX in that of ILMN_1706852 (rho = −0.33, $P = 0.028$) (Supplementary Fig. 5c).

CSF3 encodes a cytokine, known as G-CSF which is produced by different type of cells such as macrophages, monocytes, stromal cells in the bone marrow, fibroblast, and endothelial cells. The eQTL analysis is based on the whole-genome gene expression variations in lymphoblastoid cell lines derived from HapMap individuals. Therefore, it was still necessary to analyze gene expression in G-CSF producing cells, as well as expression at the protein level. Hence, we measured serum G-CSF levels at baseline and week 2 or 4 (at the time of minimum neutrophil counts) in 127 CHC patients receiving IFN-based therapy. There were no differences in serum G-CSF levels at baseline and the time of minimum neutrophil counts as well as in their changes according to rs2305482 or rs4794822 genotypes (Supplementary Fig. 6a, b). In addition, neutrophil counts

Table 3 Association of SNPs located in *PSMD3-CSF3* with interferon-induced neutropenia

dbSNP rsID	Nearest gene	Risk allele	Allele (1/2)	Case-G2 + R[a] ($n = 100$)			Control-G + R[b] ($n = 656$)			OR[c] (95 % CI)	*P* value[d]
				11	12	22	11	12	22		
rs9915252	*PSMD3*	G	G/C	23 (24.0)	47 (49.0)	26 (27.1)	57 (8.9)	276 (43.3)	304 (47.7)	2.13 (1.57–2.89)	9.64×10^{-7}
rs4794822	*PSMD-CSF3*	C	C/T	42 (42.9)	45 (45.9)	11 (11.2)	130 (21.2)	308 (50.2)	176 (28.7)	2.24 (1.63–3.07)	3.63×10^{-7}
rs3907022	*GSDMA-PSMD*	A	A/G	41 (41.8)	45 (45.9)	12 (12.2)	129 (21.3)	306 (50.6)	170 (28.1)	2.11 (1.54–2.89)	2.31×10^{-6}
rs3859192	*GSDMA*	C	C/T	37 (37.8)	44 (44.9)	17 (17.3)	123 (19.9)	313 (50.7)	181 (29.3)	1.82 (1.34–2.48)	1.04×10^{-4}

Data of allele distribution represent number (%). Data of subjects whose genotypes were not determined were excluded

SNP single nucleotide polymorphism

[a] Case-G2 + R: Case-G2 plus Case-R

[b] Control-G + R: Control-G plus Control-R

[c] Odds ratio for the allele frequency model

[d] *P* value by the Chi square test for the allele frequency model

did not correlate with serum G-CSF levels at baseline and the time of minimum neutrophil counts (Supplementary Fig. 7a), and there was no difference in the changes of serum G-CSF levels from baseline to the time of minimum neutrophil counts between patients with minimum neutrophil counts of $\geq 1{,}000/mm^3$ and $<600/mm^3$ (Supplementary Fig. 7b).

Discussion

The present GWAS first showed a strong association between genetic variant and IFN-induced neutropenia, namely, with rs2305482 in *PSMD3* on chromosome 17. Although neutrophil counts at baseline were associated with the rs2305482 genotype and the incidence of neutropenia during IFN-based therapy, the logistic regression analysis revealed that the rs2305482 genotype was independently associated with IFN-induced neutropenia.

Intriguingly, the *PSMD3-CSF3* locus was reported to be associated with total white blood cell (WBC) counts based on GWAS of populations with European ancestry (Crosslin et al. 2012; Soranzo et al. 2009) and in Japanese (Kamatani et al. 2010). These findings were replicated in African Americans (Reiner et al. 2011). Moreover, another GWAS by Okada et al. (2010) showed that rs4794822 in *PSMD3-CSF3* was associated with neutrophil counts in 14 different groups of diseases in Japanese patients who were not undergoing chemotherapy. In the present study, rs4794822 as well as rs2305482 was also associated with pretreatment neutrophil counts in CHC patients (Supplementary Fig. 2). However, there have been no reports showing an association between *PSMD3-CSF3* variants and reduction of WBC or neutrophil counts following treatments such as IFN and chemotherapy. The pairwise LD diagram for *PSMD3-CSF3* by HapMap JPT shows that rs4794822 is in strong LD with rs2305482, which we identified here (Fig. 2). In the present study, both rs2305482 and rs4794822 were associated with IFN-induced neutropenia. Collectively, previous reports together with our results imply that the *PSMD3-CSF3* locus is associated with neutropenia in CHC patients under IFN-based therapy as well as with neutrophil counts in healthy individuals and patients without bone marrow suppressive therapy.

In further clinical investigation, the rs2305482 and rs4794822 genotypes were associated with the doses of PEG-IFN that could be given to HCV genotype 1-infected patients treated with PEG-IFN/RBV (Fig. 3; Supplementary Fig. 4). Unfortunately, we could not collect the detailed information about the reason for the reduction of PEG-IFN in this group. However, we highly suppose that these SNPs affected the doses of PEG-IFN through neutropenia in some cases, since neutropenia is one of the major

Table 4 Univariate analysis of pretreatment factors associated with interferon-induced neutropenia

	Case-G2 + R[a] ($n = 100$)	Control-G + R[b] ($n = 656$)	P value[c]
Gender, male/female	45/55	378/278	0.018
Age, years	58.1 (9.3)	56.9 (10.4)	0.262
Neutrophil count, /mm^3	1,614 (735)	2,742 (979)	<0.001
Hemoglobin, g/dL	13.5 (1.5)	14.2 (1.5)	<0.001
Platelet count, $\times 10^9$/L	136 (46)	163 (57)	<0.001
ALT, IU/L	79.1 (69.7)	83.5 (74.3)	0.574
HCV RNA, log IU/ml	6.0 (0.9)	6.1 (0.8)	0.164
Liver fibrosis, F0-2/F3-4/ND	46/16/38	397/157/102	0.674
rs2305482, AA + AC/CC/ND	74/24/2	591/59/6	<0.001
rs4794822, TT + TC/CC/ND	56/42/2	484/130/42	<0.001

Data are expressed as number for categorical data or the mean (standard deviation) for non-categorical data

ALT alanine transaminase, *ND* not determined

[a] Case-G2 + R: Case-G2 plus Case-R

[b] Control-G + R: Control-G plus Control-R

[c] Categorical variables were compared between groups by the Chi square test and non-categorical variables by the Student's t test

Table 5 Logistic regression analysis of pretreatment factors associated with interferon-induced neutropenia

	OR (95 % CI)	P value
Gender, female	1.229 (0.734–2.059)	0.4331
Neutrophil count, /mm^3	0.998 (0.997–0.998)	<0.0001
Platelet count, $\times 10^9$/L	1.005 (0.953–1.059)	0.8604
rs2305482, CC	2.497 (1.281–4.864)	0.0072

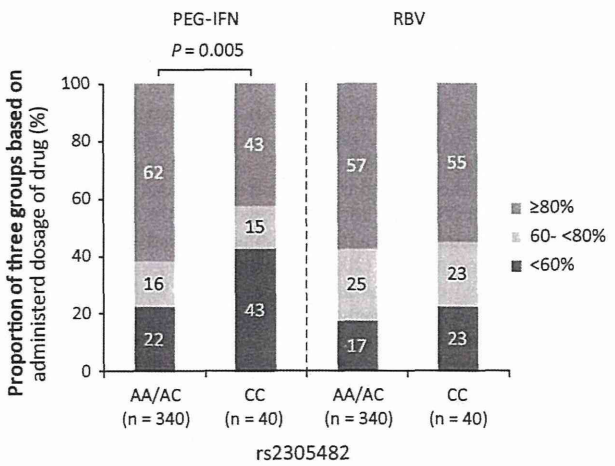

Fig. 3 Administered doses of PEG-IFN and RBV according to rs2305482 genotypes. The patients were stratified into three groups according to the doses of PEG-IFN or RBV administered, as follows: <60 %, ≥60 to <80 %, ≥80 % of the planned doses for 48 weeks. The proportion of patients receiving <60 % of the PEG-IFN doses was significantly higher in patients with rs2305482 CC than in those with AA/AC ($P = 0.005$, by the Chi square test). *PEG-IFN* pegylated interferon, *RBV* ribavirin

reasons for the dose reduction of PEG-IFN in PEG-IFN/RBV therapy. While, there were no associations between SVR and rs2305482 or rs4794822 genotypes (Supplementary Table 3).

PSMD3 encodes the proteasome 26S subunit, non-ATPase 3, a member of the 26S proteasome family, and is involved in the control of cell cycle transition via the ubiquitin–proteasome pathway (Bailly and Reed 1999). *CSF3* encodes G-CSF, which controls the production, differentiation, and function of granulocytes (Nagata et al. 1986). Recombinant G-CSF is widely used to treat patients with severe neutropenia during chemotherapy. Therefore, we hypothesize that *PSMD3-CSF3* variants may influence neutrophil counts through affecting the process of endogenous G-CSF synthesis during IFN-based therapy or other bone marrow suppressive therapies. However, eQTL analysis by Okada et al. (2010) showed that rs4794822 was significantly associated with the expression level of *PSMD3*, rather than that of *CSF3* in the JPT and CHB populations. Our eQTL analysis showed that the risk allele for neutropenia at rs2305482 correlated with higher expression levels of *PSMD3* in LWK and MEX populations (Supplementary Fig. 5a), whereas with lower expression levels of *CSF3* in MEX and especially in CHB populations (Supplementary Fig. 5b, c). However, these results were not replicated in the other probe of *CSF3*. Additionally, we analyzed serum G-CSF levels in CHC patients receiving IFN-based therapy. Although serum G-CSF levels were thought to be increased in response to neutropenia regardless of rs2305482 and rs4794822 genotypes, there was no evidence that they were lower in patients with a risk allele of these SNPs at baseline and during the neutropenic period (Supplementary Fig. 6). Moreover, neutrophil counts did not correlate with serum

G-CSF levels at baseline and the time of minimum neutrophil counts (Supplementary Fig. 7a). Further functional analyses of these genes and polymorphisms are required to elucidate the reason for the association between *PSMD3-CSF3* and IFN-induced neutropenia as well as neutrophil counts in healthy individuals.

In previous reports, *PLBC4*, *DARC*, *CXCL2*, and *CDK5* loci have also been associated with neutrophil or WBC counts in healthy individuals or patients who were not under chemotherapy (Crosslin et al. 2012; Kamatani et al. 2010; Okada et al. 2010; Reiner et al. 2011). However, there were no associations with these loci discernible in our GWAS.

The important limitation of this study is that the association between rs2305482 and IFN-induced neutropenia was not statistically significant in a genome-wide level. Thompson et al. (2012) also identified no genetic determinants of IFN-induced neutropenia during PEG-IFN/RBV therapy at the level of genome-wide significance by their GWAS. Unlike our study design, they analyzed the association between the reduction of neutrophil counts at week 4 and any SNPs. Indeed, we analyzed the association between the reduction of neutrophil counts at week 2 or 4 and rs2305482 or rs4794822, but there was no significant association. Therefore, further independent replication analyses which are designed in the similar way as our study are desirable.

IFN-free therapies are expected to be useful especially in IFN-resistant patients and may become the standard of care in the near future. However, combination therapies of DAA and IFN will continue to be used for some time. Our findings contribute to our understanding of the genetic factors influencing IFN-induced neutropenia. Furthermore, these genetic variants may be associated with neutropenia during chemotherapies for various malignant diseases as well as IFN-based therapy for CHC. Therefore, genetic testing of these variants might be useful for establishing personalized doses of such therapies to minimize drug-induced adverse events. Additionally, our results might contribute to the elucidation of the mechanism of drug-induced neutropenia.

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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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