Quantitative and Temporal Analysis of Gene Silencing in Tumor Cells Induced by Small Interfering RNA or Short Hairpin RNA Expressed from Plasmid Vectors

YUKI TAKAHASHI, KIYOSHI YAMAOKA, MAKIYA NISHIKAWA, YOSHINOBU TAKAKURA

Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

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ABSTRACT: Vector-based RNA interference (RNAi) has attracted great interest, because of its more prolonged gene silencing effect compared with small interfering RNA (siRNA). However, the intensity and duration of vector-based RNAi effect has received little attention. In this study, the gene silencing kinetics of short hairpin RNA (shRNA)-expressing plasmid DNA (pDNA) driven by U6, H1 or tRNA promoter (pU6-shLuc, pH1-shLuc, and ptRNA-shLuc) was studied in melanoma cells expressing firefly luciferase. A bootstrap method-based moment analysis was performed to statistically and quantitatively evaluate the profile of gene silencing. The analysis showed that pU6-shLuc induced a significantly greater and longer gene silencing than that produced by other promoter-driven shRNA expression vectors. In addition, it was found that pU6–shLuc was at least 100-fold more potent in gene silencing than siRNA targeting the same gene on a numerical basis. These statistical considerations demonstrated that U6 promoter-driven shRNA expressing pDNA is the most effective in inducing gene silencing effect as far as the intensity and duration of RNAi effect is concerned. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 98:74–80, 2009

Keywords: RNA interference; RNA polymerase III promoter; moment analysis; bootstrap method

INTRODUCTION

Small interfering RNA (siRNA) molecules are a powerful inducer of a sequence-specific gene silencing event called RNA interference (RNAi).[1,2] siRNA is expected to be used as a new treatment for various diseases as well as an experimental tool, because its intensity of gene silencing is greater than that of other conventional molecules, such as antisense oligodeoxynucleotides and ribozymes.[3,4] The temporal nature of siRNA-mediated gene silencing has led to the development of methods to extend the duration of its action, such as chemical modification of siRNA and the use of vectors expressing siRNA or short hairpin RNA (shRNA). In the latter case, the promoter that drives shRNA is a major factor determining the intensity and duration of gene silencing.[5,6]

The RNAi effect has often been evaluated using the maximum inhibition of the expression of target genes. Although this approach is effective for selecting the target sequence for effective induction of RNAi, it is not suitable for selecting suitable RNAi effectors. Instead, a kinetic analysis of the time-course of RNAi can be a useful

Correspondence to: Yoshinobu Takakura (Telephone: +81-75-753-4615; Fax: +81-75-753-4614; E-mail: takakura@pharm.kyoto-u.ac.jp)

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method to select a suitable molecule for gene silencing. We and other groups have already reported about quantitative analytical methods for the kinetics of siRNA-mediated RNAi.[7–9] Although shRNA-expressing plasmid DNA (pDNA) shares the mRNA degradation pathway with siRNA, it requires additional steps, such as transcription of shRNA from pDNA in the nucleus, export of shRNA to the cytoplasm and the processing of shRNA into siRNA. More importantly, some of these processes are saturable ones and dependent on the concentration of pDNA, shRNA, or siRNA. Therefore, such model-dependent analyses as those of Bartlett et al.[8] and Raab et al.[9] are difficult to apply to the evaluation of the kinetics of shRNA-expressing vectors.

In our previous study, we solved this problem by applying a model-independent moment analysis, and succeeded in a quantitative evaluation of the kinetics of siRNA-mediated gene silencing.[7] We defined the area under the concentration curve (AUC) and the mean residence time (MRT) of gene silencing after siRNA transfection as the indicators of the intensity and duration of gene silencing, respectively. Statistical comparisons were successfully performed by calculating the mean, standard deviation (SD) and standard error (SE) of the AUC and MRT, using a computer program MOMENT(BS).[10]

In the present study, this model-independent moment analysis was applied to the kinetics of shRNA-expressing pDNAs after transfection to cultured cells. Three types of frequently used RNA polymerase III promoters, human small nuclear RNA U6 (U6), human RNase P RNA H1 (H1), and human $tRNA^{val}$ (tRNA) promoters, were selected and their activity was statistically compared in terms of the intensity and duration of gene silencing. A melanoma B16-BL6 clone that stably expresses firefly and renilla luciferases was used to evaluate the gene silencing effect by simply calculating the ratio of the firefly and renilla luciferase activities.[11]

MATERIALS AND METHODS

Cell Culture

A murine melanoma cell line B16-BL6 and a murine colon carcinoma cell line Colon26 were obtained from the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo, Japan). B16-BL6/dual Luc, a cell line that expresses both firefly and renilla luciferases, was constructed from B16-BL6 cells by genetically labeling with firefly and renilla luciferase genes as described previously.[11] B16-BL6/dual Luc cells were cultured in Dulbecco's modified Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/L-glutamine (PSG) at 37°C and 5% CO_2. The firefly and renilla luciferase activities of B16-BL6/dual Luc cells were about 3 and 10 RLU/s/cell, respectively.

shRNA-Expressing pDNA and siRNA

pDNA expressing shRNA targeting firefly luciferase under the control of human U6 promoter (pU6-shLuc) was constructed from piGENE hU6 vector (iGENE Therapeutics, Tsukuba, Japan) according to the manufacturer's instructions as described previously.[11] pU6-shLuc transcribes a single-stranded RNA 5'-GUG CGU UGU UGG UGU UAA UCC UUC AAG AGA GGG UUG GCA CCA GCA GCG CAC UUU U-3', which forms stem-loop-structured shRNA, targeted to firefly luciferase mRNA (targeted sequence: GTG CGC TGC TGG TGC CAA CCC), with loop sequences of UUCAAGAGA. Plasmids expressing the same shRNA under the control of human H1 promoter (pH1-shLuc) or tRNA promoter (ptRNA-shLuc) were constructed by subcloning the shRNA sequence into the *Bam*H I/*Kpn* I site of pBAsi-hH1 DNA (Takara Bio, Otsu, Japan) or into the *Bam*H I/*Pst* I site of piGENE tRNA Hyg vector (iGENE Therapeutics), respectively. Empty piGENE hU6 vector was used as a control pDNA throughout the present study. Each pDNA was amplified in the DH5α strain of *Escherichia coli* and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Synthetic siRNA targeting the mRNA of firefly luciferase (target sequence: GTG CGC TGC TGG TGC CAA CCC) was purchased from Takara Bio.

Transfection

B16-BL6/dual Luc cells were plated on 24-well culture plates (at a density of 2×10^4 cells/well). After an overnight incubation, transfection of pDNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, 1 μg pDNA

was mixed with 3 μg Lipofectamine 2000 at a final concentration of 2 μg pDNA/mL dissolved in OPTI-MEM I (Invitrogen). Control pDNA was used to adjust the amount of pDNA. The resulting complex was added to the cells and the cells were incubated with the complex for 4 h. Cells were washed with PBS and further incubated with the culture medium as described above for specified time periods.

Luciferase Assay

To determine luciferase activity, B16-BL6/dual Luc cells were lysed using the cell lysis buffer of an assay kit (PiccageneDual, Toyo Ink, Tokyo, Japan) at the indicated time points after transfection. Then, samples were mixed with the luciferase assay buffer of the kit, and the chemiluminescence produced was measured in a luminometer (Lumat LB9507, EG and G Berthold, Bad Wildbad, Germany).

Data Analysis

The firefly luciferase activity of a sample was divided by the renilla luciferase activity of the same sample, which was then normalized using the value of control cells to obtain the parameter R_{GE}, the ratio of gene expression. Differences in each pair of R_{GE} values were statistically evaluated by Student's t-test with a significance level at $p < 0.05$. The time-course of $(1 - R_{GE})$ was used to calculate the area under the curve of the inhibitory effect (AUC_{IE}) and the MRT of the inhibitory effect (MRT_{IE}) as described previously.[7] For the calculation of AUC_{IE} and MRT_{IE}, R_{GE} values over 1 were assumed to be 1. The mean and SE of AUC_{IE} and MRT_{IE} were calculated using the bootstrap method.[10]

To detect any significant differences in AUC_{IE} and MRT_{IE} between different groups, the normal distribution test was performed using the following equation:

$$Z_0 = \frac{|\overline{\Phi}_1 - \overline{\Phi}_2|}{\sqrt{SE_1^2 + SE_2^2}} \tag{1}$$

where $\overline{\Phi}_1$ and $\overline{\Phi}_2$ are the means of a parameter, and SE_1 and SE_2 are the SEs in group 1 and 2, respectively. If $Z_0 > 1.96$ (confidence interval $p < 0.05$), the difference between groups 1 and 2 is assumed to be significant.

Taking shRNA-expressing pDNA as a drug and AUC or MRT as the response to the drug, we assumed that AUC_{IE} and MRT_{IE} versus the initial concentration of shRNA-expressing pDNA (C_0) could be expressed by the following linearized equation, which is known to describe the dose-response curves of drugs.

$$\frac{C_0}{AUC_{IE}} \text{ or } \frac{C_0}{MRT_{IE}} = \frac{C_0}{a} + \frac{b}{a} \tag{2}$$

The parameters a and b were estimated by linearizing the plots. The correlation constant a corresponds to the maximum response, that is, AUC_{max} and MRT_{max}. The correlation constant b corresponds to the required dose of RNAi effector to obtain the response half of AUC_{max} or MRT_{max}; $C_{1/2}$. IC_{50}, a concentration required to obtain R_{GE} value of 0.5, was calculated by assuming that concentration-R_{GE} curves between the experimental values which across R_{GE} value of 0.5were linear.

RESULTS

Time-Courses of Gene Silencing after Transfection of shRNA-Expressing pDNA

Figure 1 shows the time-courses of the ratio of gene expression (R_{GE}) in B16-BL6/dual Luc cells after transfection of shRNA-expressing pDNAs at an indicated initial concentration (C_0). When pH1-shLuc and ptRNA-shLuc were transfected at a C_0 of 0.01 or 0.1 nM, the R_{GE} decreased with time, and reached a minimum at 2 days after transfection. At the highest concentration of 1 nM for pH1-shLuc or 0.7 nM for ptRNA-shLuc, the R_{GE} exhibited an almost flat trough from days 1 to 5 for pH1-shLuc and from days 1 to 6 for ptRNA-shLuc, respectively. Because the plasmid size of ptRNA-shLuc was about 1.5-fold larger than those of pU6-shLuc and pH1-shLuc, the highest concentration of ptRNA-shLuc was set at 0.7 nM. In the case of pU6-shLuc, at a C_0 of 0.1 or 1 nM, the R_{GE} exhibited an almost flat trough from days 1 to 8 for 0.1 nM and from days 1 to 10 for 1 nM, respectively, indicating that almost all target mRNA was degraded under these conditions. In other words, a fraction of siRNA produced would not contribute to the gene silencing, so that the level of gene silencing does not reflect the amount of effective siRNA within cells. Therefore, AUC_{IE} may not be proportional to the initial concentration of shRNA-expressing pDNA. The suppression

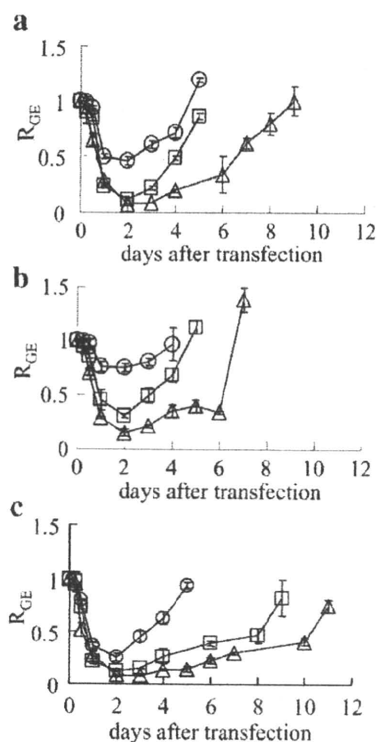

Figure 1. Time-courses of the ratio of gene expression (R_{GE}) following transfection of shRNA-expressing pDNAs. B16-BL6/dual Luc cells were transfected with pDNA expressing shRNA under the control of H1 (a), tRNA (b), or U6 (c) promoter at different initial concentrations: (circle symbols) 0.01 nM, (square symbols) 0.1 nM, and (triangle symbols) 0.7 nM (ptRNA-shLuc) or 1 nM (pH1-shLUC, pU6-shLuc). Luciferase activities were determined at the indicated times after transfection. The results are expressed as mean ± SD ($n = 3$).

was significant ($p < 0.05$) until days 4, 4, and 3 for 0.01 nM, days 8, 4, and 4 for 0.1 nM, and days 10, 7, and 6 for the highest concentration of C_0 of pU6-shLuc, pH1-shLuc, and ptRNA-Luc, respectively.

Moment Parameters for the Kinetics of Gene Silencing by shRNA-Expressing pDNAs

Figure 2 shows the means and SE of AUC_{IE} and MRT_{IE}. All the shRNA-expressing pDNAs showed a C_0-dependent increase in both AUC_{IE} and MRT_{IE}. Table 1 summarizes the significant differences ($p < 0.05$) between two groups. pU6-shLuc showed a greater AUC_{IE} and MRT_{IE} compared with the other shRNA-expressing pDNAs except for the MRT_{IE} at a C_0 of 0.01 nM. For all shRNA expressing pDNA, the AUC_{IE} and

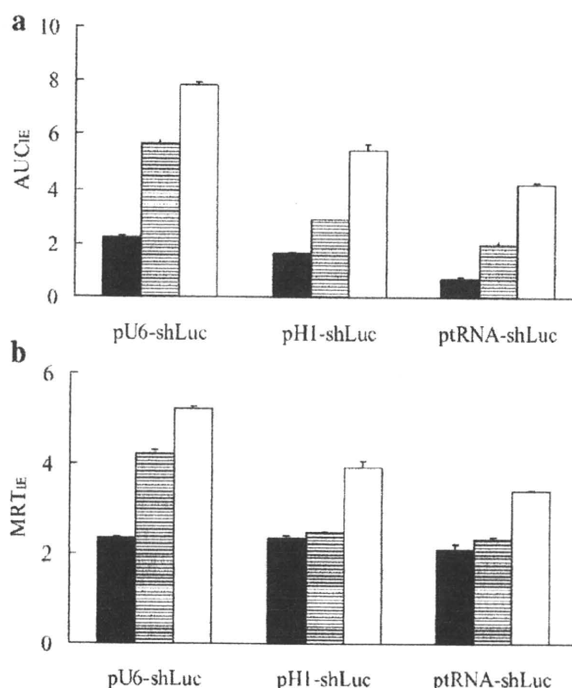

Figure 2. AUC_{IE} and MRT_{IE} of gene silencing by shRNA-expressing pDNAs. (a) AUC_{IE} and (b) MRT_{IE} of the gene silencing by shRNA-expressing pDNAs were calculated using MOMENT(BS), at different initial concentrations: (closed bars) 0.01 nM, (striped bars) 0.1 nM and (open bars) 0.7 nM (ptRNA-shLuc) or 1 nM (pU6-shLUC, pH1-shLuc). Results are expressed as the computer-calculated mean ± SE.

MRT_{IE} increased with an increasing concentration of pDNA. pU6-shLuc showed the greatest values for both parameters, suggesting its superiority over the other vectors.

Linearized Plot of C_0/AUC_{IE} and C_0/MRT_{IE} Versus C_0

Figure 3 shows the plots of C_0/AUC_{IE} and C_0/MRT_{IE} against the C_0 of three different shRNA-expressing pDNAs. The correlation coefficients (r) of both C_0/AUC_{IE} and C_0/MRT_{IE} versus C_0 were more than 0.99 in all cases, indicating successful linearization of C_0/AUC_{IE} and C_0/MRT_{IE} versus C_0 by Eq. (2). The concentration-dependent increase in AUC_{IE} and MRT_{IE} was clearly represented in the inserts of Figure 3. Table 2 summarizes the parameters AUC_{max}, MRT_{max}, and $C_{1/2}$ calculated from the linearized plots. In all cases examined, $C_{1/2}$ values calculated were between 0.01 and 0.1 nM, two of the three initial concentrations used in the present study.

Table 1. Statistical Comparison of Two Groups by the Normal Distribution Test

Pairs for Comparison: Promoter of pDNA	Concentration (nM)	AUC_{IE}	MRT_{IE}
U6 vs. H1	0.01	6.29*	0.0658
	0.1	18.2*	20.3*
	1	10.1*	7.66*
U6 vs. tRNA	0.01	15.9*	1.89
	0.1	20.6*	18.3*
	1 (0.7)	7.34*	4.91*
H1 vs. tRNA	0.01	10.9*	1.81
	0.1	8.81*	2.17*
	1 (0.7)	5.79*	3.32*
U6	0.1 vs. 0.01	20.7*	20.3*
	1 vs. 0.1	11.5*	9.07*
H1	0.1 vs. 0.01	18.7*	1.61
	1 vs. 0.1	12.4*	9.23*
tRNA	0.1 vs. 0.01	11.2*	1.54
	0.7 vs. 0.1	18*	16.0*

*The difference was assumed to be significant ($p < 0.05$) between the groups, based on the normal distribution test described in Eq. (1) ($Z_0 > 1.96$). The highest concentration was set at 0.7 nM for ptRNA-siLuc and 1 nM for the others.

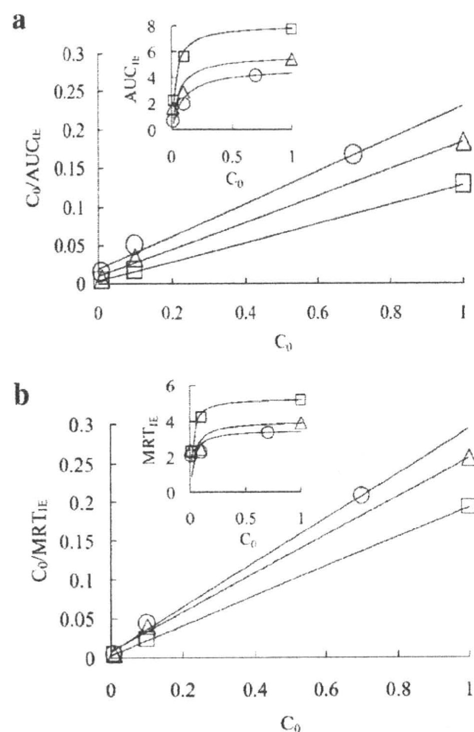

Figure 3. Linear plots of C_0/AUC_{IE} or C_0/MRT_{IE} versus C_0. (a) C_0/AUC_{IE} and (b) C_0/MRT_{IE} were plotted against the initial concentration: (square symbols) pU6-shLuc, (triangle symbols) pH1-shLuc, (circle symbols) ptRNA-shLuc. Coefficients a (AUC_{max}, MRT_{max}) and b ($C_{1/2}$), which were calculated from the straight regression lines, are shown in Table 2. In the inserts, (a) AUC_{IE} and (b) MRT_{IE} were plotted against the initial concentration of shRNA-expressing pDNA.

Therefore, the parameters obtained can be considered reliable to be used for the comparison of the gene silencing by various types of shRNA-expressing pDNA. pU6-shLuc showed a greater AUC_{max} and MRT_{max}, and a smaller $C_{1/2}$ for both AUC_{IE} and MRT_{IE} compared with the other shRNA-expressing pDNAs, suggesting that the U6 promoter generates a greater numbers of shRNA for a longer period of time compared with the other promoters.

These parameters of shRNA-expressing pDNA were compared with those of siRNA targeting firefly luciferase (siLuc). To this end, the parameters AUC_{max}, MRT_{max}, and $C_{1/2}$ of siLuc were calculated using the experimental results reported in our previous paper.[7] The AUC_{max} and MRT_{max} of siLuc were calculated to be 6.73 and 4.21, respectively, both of which lay between those of pU6-shLuc and pH1-shLuc (Table 2).

Table 2. Maximum Response and Concentration Required for Half of the Maximum Response Calculated from the Linearized Plots

	AUC_{IE}			MRT_{IE}		
RNAi Effector	a (AUC_{max})	b ($C_{1/2}$)	r	a (MRT_{max})	b ($C_{1/2}$)	r
pU6-shLuc	8.05	0.0341	0.999	5.3	0.0186	0.999
pH1-shLuc	5.74	0.0595	0.998	4.03	0.0343	0.999
ptRNA-shLuc	4.74	0.0959	0.995	3.51	0.0278	0.998
siLuc	6.73	4.35	0.999	4.21	3.2	0.999

a and b are parameters described in Eq. (2). r is the correlation coefficient of the linear plots.

Gene Silencing after Transfection of siLuc and pU6-shLuc

To compare the gene silencing effects of siLuc and shRNA-expressing pDNA, R_{GE} in B16-BL6/dual Luc cells were determined after the transfection of siLuc or pU6-shLuc at various initial concentrations (Fig. 4). The sampling time was fixed to be 2 days after transfection because R_{GE} reaches minimum values at this time point in the both cases. IC_{50} of siLuc and pU6-shLuc were calculated to be 0.0625 and 0.00417 nM, respectively, indicating that the pU6-shLuc is about 15-fold more potent than siLuc as far as the IC_{50} values estimated using the RGE at 2 days after transfection were used for the calculation.

DISCUSSION

Because siRNA-mediated gene silencing is transient, vector-based RNAi attracts much interest as it may achieve prolonged gene silencing. The promoter that drives shRNA expression is a major factor determining the intensity and duration of gene silencing by shRNA-expressing pDNAs. RNA polymerase III (pol III) promoters are frequently used for plasmid-based RNAi, because these promoters are suitable to transcribe large amounts of short RNAs and their sites of transcription initiation and termination are well understood. There have been several literatures concerning about the effect of pol III promoters on the efficiency of gene silencing by shRNA-expressing pDNAs.[5,6,12] Kawasaki et al.[12] reported

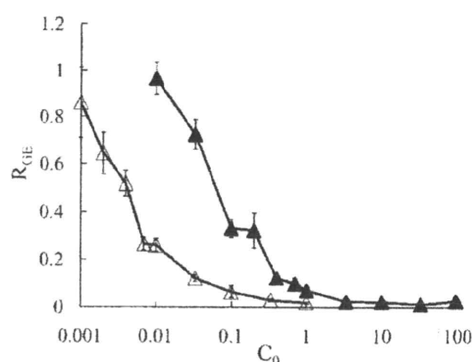

Figure 4. Ratio of gene silencing (R_{GE}) following transfection of siRNA or shRNA-expressing pDNA. B16-BL6/dual Luc cells were transfected with (solid triangles) siLuc or (empty triangles) pU6-shLuc at the indicated initial concentrations (C_0). Luciferase activities were determined at 2 days after transfection. The results are expressed as mean ± SD ($n = 3$).

that tRNA promoter pDNA and U6 promoter pDNA could suppress target gene expression almost equally by using cell lines stably integrated with these pDNAs. Wooddell et al.[5] compared the efficiency of shRNA-expressing pDNA driven by human H1, human U6, or murine U6 promoter, and found that shRNA-expressing pDNA containing the human H1 promoter was significantly less effective in mice than those containing any U6 promoter, although both types of promoter functioned equally in cultured cells. Boden et al.[6] demonstrated that a modified $tRNA^{met}$-derived promoter could induce HIV-1 specific RNAi more efficiently than the other promoters used: U6, H1 and CMV promoters. However, these previous studies compared the promoter-dependent gene silencing effect at a single time point after transfection. Unlike these studies, we have investigated the time-course of gene silencing until the RNAi effect became undetectable with time after transfection. Moreover, we have successfully applied the bootstrap method-based moment analysis to statistically compare the importance and differences of the promoters as far as the intensity and duration of gene silencing is concerned. By calculating the means and SE of parameters for the intensity and duration of gene silencing, AUC_{IE} and MRT_{IE}, we have shown that gene silencing effect of U6 promoter-driven shRNA-expressing pDNA is more potent and durable than those of H1 and tRNA promoter-driven shRNA-expressing pDNA. This result is partly in agreement with previous studies reported by Wooddell et al. and Kawasaki et al., and different from that reported by Bodent et al. The modified $tRNA^{met}$-derived promoter used by Bodent et al. might be more potent than the $tRNA^{val}$-derived promoter used in the present study. In addition, the AUC_{max}, MRT_{max}, and $C_{1/2}$ values estimated also indicated that gene silencing by U6 promoter-driven shRNA-expressing pDNA is more potent than that by H1- or tRNA promoter-driven shRNA-expressing pDNA. Because there parameters are estimated based on the AUC_{IE} and MRT_{IE} values that were obtained after a single transfection of each shRNA-expressing pDNA to cells, AUC_{max} and MRT_{max} values could depend on the stability of pDNA, transcription activity of promoters, intracellular behavior of shRNA and target sequence of shRNA. These pharmacokinetic considerations of this current study suggest that U6 promoter generates more shRNA molecules than the other promoters under the same experimental conditions.

Calculation of the parameters AUC_{max}, MRT_{max}, and $C_{1/2}$ of siLuc revealed that AUC_{max}, MRT_{max} of siLuc was comparable to those of shRNA expressing pDNAs. These results indicate that vector-based RNAi does not always induce a prolonged gene silencing compared with siRNA-mediated RNAi. On the other hand, a large difference was detected in the $C_{1/2}$ of AUC_{IE} and MRT_{IE} between siRNA and shRNA-expressing pDNAs. The largest difference in $C_{1/2}$ was observed between siLuc and pU6-shLuc. The $C_{1/2}$ of AUC_{IE} and MRT_{IE} of siLuc were about 120- and 170-fold greater than those of pU6-shLuc, respectively. However, the IC_{50} of siLuc was calculated to be only about 15-fold greater than that of pU6-shLuc. This discrepancy would be explained by the fact that the $C_{1/2}$ of AUC_{IE} and MRT_{IE} were obtained from the total profile of gene silencing whereas the IC_{50} values were calculated using data at a single time point. Therefore, these results clearly indicated from the kinetic view point that comparison of gene silencing effect by different RNAi-effectors at one time-point is not suitable for the evaluation of the intensity and duration of gene silencing effect.

In conclusion, we have successfully applied the bootstrap method-based moment analysis to quantitatively compare the gene silencing by pDNA expressing shRNA under the control of different promoters. We found that the U6 promoter-driven shRNA-expressing pDNA showed a significantly greater and longer gene silencing effect than the H1- and tRNA promoter-driven ones. Moreover, moment analysis was proven to be useful for the quantitative comparison between siRNA and shRNA-expressing pDNA, and we clearly showed that the U6 promoter-driven shRNA-expressing pDNA possesses at least 100-fold greater gene silencing activity compared with siRNA on a numerical basis. Our statistical evaluations of gene silencing can be a powerful method to optimize RNAi effectors and other conditions that may affect the final output of gene silencing for pursuing effective application of RNAi-based gene silencing.

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Enhancement of antiproliferative activity of interferons by RNA interference-mediated silencing of *SOCS* gene expression in tumor cells

Yuki Takahashi, Haruka Kaneda, Nana Takasuka, Kayoko Hattori, Makiya Nishikawa and Yoshinobu Takakura[1]

Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

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The suppressor of cytokine signaling (SOCS) proteins, negative regulators of interferon (IFN)-induced signaling pathways, is involved in IFN resistance of tumor cells. To improve the growth inhibitory effect of IFN-β and IFN-γ on a murine melanoma cell line, B16-BL6, and a murine colon carcinoma cell line, Colon26 cells, *SOCS-1* and *SOCS-3* gene expression in tumor cells was downregulated by transfection of plasmid DNA expressing short hairpin RNA targeting one of these genes (pshSOCS-1 and pshSOCS-3, respectively). Transfection of pshSOCS-1 significantly increased the antiproliferative effect of IFN-γ on B16-BL6 cells. However, any other combinations of plasmids and IFN had little effect on the growth of B16-BL6 cells. In addition, transfection of pshSOCS-1 and pshSOCS-3 produced little improvement in the effect of IFN on Colon26 cells. To understand the mechanism underlining these findings, the level of *SOCS* gene expression was measured by real time polymerase chain reaction. Addition of IFN-γ greatly increased the *SOCS-1* mRNA expression in B16-BL6 cells. Taking into account the synergistic effect of pshSOCS-1 and IFN-γ on the growth of B16-BL6 cells, these findings suggest that IFN-γ-induced high *SOCS-1* gene expression in B16-BL6 cells significantly interferes with the antiproliferative effect of IFN-γ. These results indicate that silencing *SOCS* gene expression can be an effective strategy to enhance the antitumor effect of IFN under conditions in which the *SOCS* gene expression is upregulated by IFN. (*Cancer Sci* 2008; 99: 1650–1655)

Cytokine-supported tumor immunotherapy is one of the most promising strategies for cancer therapy.[1] Interferons (IFN) and other cytokines are important therapeutic cytokines that exert antitumor activity against a variety of tumor cells,[2–4] and some of them have already been used as anticancer agents in clinical practice. We have shown that IFN-based cancer gene therapy is an effective approach to suppressing tumor cell growth in mice.[4] Furthermore, sustained expression of IFN was shown to be highly effective in inhibiting experimental pulmonary metastasis of colon carcinoma cells.[5]

One of the major problems associated with cytokine-supported tumor immunotherapy is the development of cytokine resistance, which has often been observed in cytokine-based tumor therapy. Resistance of tumor cells to cytokines has been reported for interleukin-6 (IL-6),[6] transforming growth factor-β1,[7] IFN[8] and tumor necrosis factor-α.[9] Because these cytokines induce various changes in tumor cells, cytokine resistance could be the consequence of changes in protein expression.

The suppressor of cytokine signaling (SOCS) proteins compose a family of negative regulators of cytokine signaling that inhibit cytokine action by inhibiting the Janus kinases (JAK)/signal transducer and activators of transcription factor (STAT) pathways.[10] *SOCS* gene expression is also regulated by the cytokine signaling pathway, and the induced SOCS proteins work as a negative feedback mechanism to protect cells from excess activation of cytokine signaling. Up to now, eight structurally-related SOCS family members have been identified. Of those, SOCS-1 and SOCS-3 have been well-characterized, and are the most potent negative regulators of the signals induced by IFN family proteins and IL-6.[11] Recently, a high level of *SOCS-1* and *SOCS-3* gene expression was observed in tumor cells compared with normal cells, and the level of SOCS-1 expression in primary melanomas correlated well with their invasion level. Moreover, it has been found that a forced expression of SOCS-3 in chronic myelogenous leukemia cells and T-cell lymphoma cells endowed them with resistance to IFN-α-mediated growth inhibition.[12,13] These lines of evidence suggest that a high level of *SOCS-1* or *SOCS-3* gene expression in tumor cells may be the key issue in the cytokine resistance.

In the present study, overcoming IFN resistance of tumor cells was examined by downregulating *SOCS-1* or *SOCS-3* gene expression using RNA interference (RNAi), a post-transcriptional gene silencing event in which small interfering RNA (siRNA) degrade target mRNA in a sequence-specific manner.[14–16] Short hairpin RNA (shRNA)-expressing plasmid DNA (pDNA), not siRNA, was selected because shRNA-expressing pDNA produce a more sustained gene silencing effect than siRNA (Yuki Takahashi *et al.*, unpublished data, 2007). The results of IFN treatment following the transfection of pDNA expressing shRNA targeting *SOCS-1* or *SOCS-3* provided experimental evidence that increased SOCS gene expression is associated with IFN resistance in tumor cells, and that silencing *SOCS* gene expression can be a promising strategy to enhance the sensitivity of tumor cells to IFN-mediated growth inhibition.

Materials and Methods

Plasmid DNA and IFN shRNA-expressing pDNA driven by human U6 promoter were constructed from the piGENE hU6 vector (iGENE Therapeutics, Tsukuba, Japan) according to the manufacturer's instructions. Target sites in the murine genes encoding *SOCS-1* and *SOCS-3* were as follows: *SOCS-1* for sites 1–3 were 5′-CTACCTGAGTTCCTTCCCC-3′, 5′-GCCAGGACCTGAATTCCAC-3′ and 5′-GACCTGAATTCCACTCCTA-3′, respectively; *SOCS-3* for sites 1–3 were 5′-GGGGAATCTTCAAACTTTC-3′, 5′-GGCAGGACCTGGAATTCGT-3′ and 5′-GAAGAGAGCTATACTGGTG-3′, respectively. These pDNA transcribe stem loop-type RNA with loop sequences of ACG UGU GCU GUC CGU. pshLuc and pshGFP, shRNA-expressing pDNA targeting *firefly luciferase* mRNA and *GFP* mRNA were constructed as reported previously.[17] The empty piGENE hU6 vector was used as a control pDNA throughout the present study.

[1]To whom correspondence should be addressed.
E-mail: takakura@pharm.kyoto-u.ac.jp

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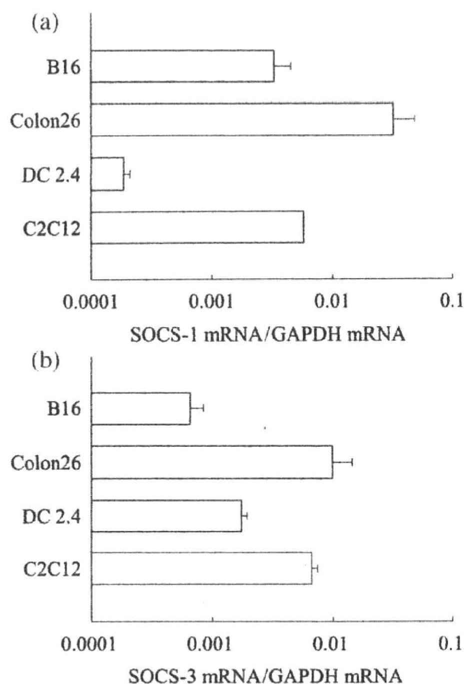

Fig. 1. mRNA expression of *SOCS-1* and *SOCS-3* in murine cell lines. (a) *SOCS-1* and (b) *SOCS-3* mRNA in B16-BL6, Colon26, DC2.4 or C2C12 cells was determined by quantitative reverse transcription polymerase chain reaction. The results are expressed as the mean ± standard deviation of three independent determinations. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

pGL4.74 (hRluc/TK) (phRL-TK), a pDNA that expresses sea pansy luciferase under the control of herpes simplex virus TK promoter, was purchased from Promega (Madison, WI, USA). Three copies of the IFN stimulation response element (ISRE) (5′-CGAAGTACTTTCAGTTTCATATTAGG-3′) were subcloned into the Bgl II/Hind III site of pLuc-MCS (Stratagene, La Jolla, CA, USA) to construct an IFN responsive reporter pDNA, pISRE-Luc.

Each pDNA was amplified in the DH5α strain of *Escherichia coli* and purified using a Qiagen Endofree Plasmid Giga Kit (Qiagen, Hilden, Germany).

Mouse IFN-β and -γ were kind gifts from Dr Yoshihiko Watanabe (Graduate School of Pharmaceutical Sciences, Kyoto University).

Cell culture. A murine melanoma cell line, B16-BL6, a murine lung carcinoma cell line, LLC, and a murine colon carcinoma cell line, Colon26, were obtained from the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo, Japan). A murine myoblast cell line, C2C12, was obtained from the RIKEN Cell Bank (Ibaraki, Japan). A murine dendritic cell line, DC2.4, was a gift from Dr Kenneth Rock (Department of Pathology, University of Massachusetts Medical School, MA, USA).[18] A murine renal cell carcinoma cell line, Renca, a murine bladder tumor cell line, MBT-2, a murine neuroblastoma cell line, and SA1, a murine Fibrosarcoma cell line, were kind gifts from Dr Yoshihiko Watanabe, Kyoto University. Colon26 and DC2.4 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/L-glutamine (PSG) at 37°C and 5% CO_2. The other cell lines were cultured in Dulbecco's modified Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS and PSG at 37°C and 5% CO_2.

***In vitro* transfection.** Cells were plated on culture plates and incubated overnight. Transfection of pDNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, 1 µg pDNA was mixed with 3 µg Lipofectamine 2000 at a final concentration of 2 µg pDNA/mL, and the resulting complex was added to the cells.

mRNA quantification. Total RNA was isolated using MagExtractor MFX-2100 and a MagExtractor RNA kit (Toyobo, Osaka, Japan) following the manufacturer's protocol. To eliminate DNA contamination, the total RNA was treated with DNase I (Takara Bio, Otsu, Japan) prior to reverse transcription (RT). RT was performed using a SuperScript II (Invitrogen) and dT-primer following the manufacturer's protocol. For quantitative mRNA expression analysis, real time polymerase chain reaction (PCR) was carried out with total cDNA using a LightCycler instrument (Roche Diagnostics, Basel, Switzerland). The sequences of the primers used for amplification were as follows: *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* forward, 5′-CTGCCAAGTATGATGACATCAAGAA-3′, reverse, 5′-ACCAGGAAATGAGCTTGACA-3′; *SOCS-1* forward, 5′-GTGGTTGTGGAGGGTGAGAT-3′, reverse, 5′-CCCAGACACAAGCTGCTACA-3′; and *SOCS-3* forward, 5′-AAGGGAGGCAGATCAACAGA-3′, reverse, 5′-TGGGACAGAGGGCATTTAAG-3′. Amplified products were detected online via intercalation of the fluorescent dye SYBR green (LightCycler-FastStart DNA Master SYBR Green I kit, Roche Diagnostics). The cycling conditions were as follows: initial enzyme activation at 95°C for 10 min, followed by 55 cycles at 95°C for 10 s, 60°C for 5 s and 72°C for 15 s. Gene-specific fluorescence was measured at 72°C. The mRNA expression of target genes was normalized by using the mRNA level of *GAPDH*.

IFN-dependent reporter gene expression assay. Tumor cells seeded on culture plates were transfected with pISRE-Luc (0.8 µg/mL), phRL-TK (0.2 µg/mL) and a control pDNA, pshSOCS-1, pshSOCS-3 or pshGFP (1 µg/mL) using Lipofectamine 2000 as described above. Four hours after transfection, cells were washed with PBS and further incubated with the culture medium supplemented with or without the indicated concentration of IFN-β or IFN-γ for an additional 20 h. Then, cells were lysed with Promega Passive Lysis Buffer. Samples were mixed with a Dual-Luciferase Reporter System (Promega) and the chemiluminescence produced was measured in a luminometer (Lumat LB9507, EG and G Berthold, Bad Wildbad, Germany). Firefly luciferase activity was used as an indicator of ISRE-dependent transcription, and sea pansy luciferase activity was used to normalize the transfection efficiency. The ratios of the IFN-added cells were normalized to give x-fold values relative to those of the unstimulated group cultured in the absence of IFN.

Growth inhibition of tumor cells by IFN. B16-BL6 and Colon26 cells were plated on 24-well culture plates (at a density of 1×10^3 cells/well and 2×10^3 cells/well, respectively). The medium was replaced with growth medium containing IFN-β (10, 10^2, 10^3 IU/mL) or IFN-γ (10^0, 10, 10^2, 10^3 IU/mL) and cultured for 5 days. The number of cells was evaluated by MTT assay as described previously.[19]

To evaluate the IFN-mediated growth inhibitory effect on cells subjected to shRNA-expressing pDNA transfection, cells were harvested by trypsinization at 24 h after the transfection and plated again at a density of 1×10^4 cells/well on new 24-well plates, and treated with the indicated concentrations of IFN for 4 days, and cell numbers were determined by MTT assay.

Statistical analysis. Differences were statistically evaluated by Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

mRNA expression of *SOCS-1* and *SOCS-3* in murine cell lines. Quantitative RT-PCR was performed to determine the mRNA expression levels of *SOCS-1* and *SOCS-3* in tumor cell lines, B16-BL6 and Colon26 cells, and in normal cell lines, DC2.4 and C2C12 cells. As shown in Fig. 1(a), the constitutive mRNA

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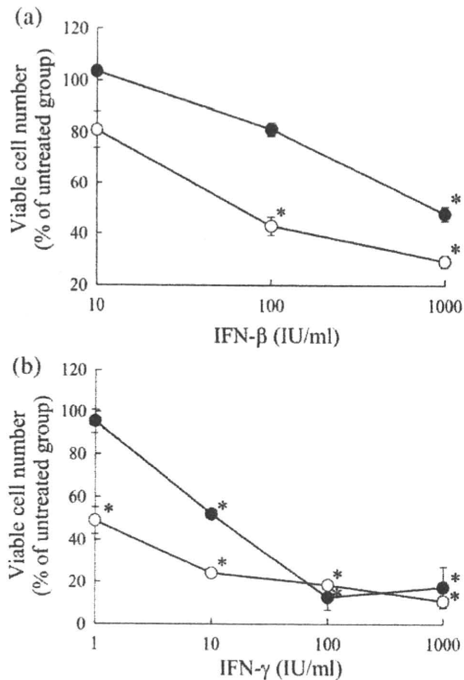

Fig. 2. Anti-proliferative effect of interferons (IFN) against tumor cells. B16-BL6 (●) and Colon26 (○) cells plated on 24-well culture plates (at a density of 1×10^3 cells/well and 2×10^3 cells/well, respectively) were cultured with the indicated concentrations of (a) IFN-β or (b) IFN-γ for 5 days. The number of cells were evaluated by MTT assay. The results are expressed as the mean ± standard deviation of three independent determinations. *$P < 0.05$ for Student's *t*-test compared with the cells cultured with no IFN.

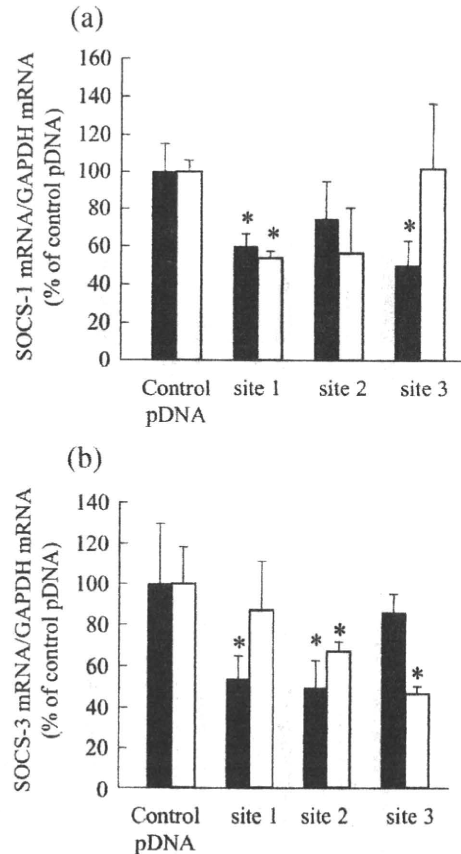

Fig. 3. Effect of transfection of tumor cells with shRNA-expressing plasmid DNA (pDNA) on *SOCS* mRNA expression. Tumor cells seeded on culture plates were transfected with control plasmid DNA (pDNA), pshLuc, pshSOCS-1 (sites 1, 2 and 3) or pshSOCS-3 (sites 1, 2 and 3). Three different sequences in the *SOCS-1* gene (site 1, CTACCTGAGTTCCTTCCCC; site 2, GCCAGGACCTGAATTCCAC; site 3, GACCTGAATTCCACTCCTA) and in the *SOCS-3* gene (site 1, GGGGAATCTTCAAACTTTC; site 2, GGCAGGACCTGGAATTCGT; site 3, GAAGAGAGCTATACTGGTG) were targeted. (a) *SOCS-1* and (b) *SOCS-3* mRNA in B16-BL6 (■) and Colon26 (□) cells was determined 48 h after transfection. The results are expressed as the mean percentage ± standard deviation (percentage of the control group) of three independent determinations. *$P < 0.05$ for Student's *t*-test compared with their corresponding control groups. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

expression level of *SOCS-1* in Colon26 was higher than those in the other cell lines. *SOCS-1* mRNA expression in DC2.4 cells was much lower than that in the other cell lines. The constitutive mRNA expression level of *SOCS-3* in Colon26 and C2C12 cells were higher than those in B16-BL6 and DC2.4 cells (Fig. 1b).

Sensitivity of B16-BL6 and Colon26 cells to IFN-β and IFN-γ. To investigate the growth inhibitory effects of IFN-β and IFN-γ on B16-BL6 and Colon26 cells, cells were added with various concentrations of IFN and cultured for 5 consecutive days. Without addition of IFN, the proliferation rates of B16-BL6 cells and Colon26 cells were not significantly different to each other. Both IFN-β and IFN-γ inhibited the proliferation of B16-BL6 and Colon26 cells in a concentration-dependent manner (Fig. 2). B16-BL6 cells required higher concentrations of IFN for the inhibition compared with Colon26 cells, indicating that B16-BL6 cells are more resistant to IFN-mediated growth inhibition than Colon26 cells.

Silencing of SOCS gene expression for the enhancement of growth inhibitory effect of IFN. Transfection of B16-BL6 and Colon26 cells with shRNA-expressing pDNA reduced the corresponding target gene expression at the mRNA level (Fig. 3). For each target gene, one of the shRNA-expressing pDNA was selected for the following studies based on the inhibitory effect in the real-time PCR analysis (site 1 for SOCS-1 and site 2 for SOCS-3, respectively), and named pshSOCS-1 and pshSOCS-3, respectively.

Because we found that transfection of pshSOCS-1 and pshSOCS-3 reduces the target gene expression, the effects of SOCS gene silencing on the antiproliferative effect of IFN were examined. To this end, IFN-β or IFN-γ were added to the cell medium 2 days after the transfection of shRNA-expressing pDNA. The number of viable cells was determined by MTT assay 4 days after the initiation of IFN treatment (Fig. 4). Transfection of Colon26 cells with pshSOCS-1 or pshSOCS-3 induced no significant changes in the number of cells treated with IFN. On the other hand, transfection of B16-BL6 cells with pshSOCS-1 inhibited the proliferation of IFN-γ-treated cells, whereas transfection with pshSOCS-3 hardly affected the proliferation of IFN-γ-treated cells. The antiproliferative effect of IFN-β on B16-BL6 cells was not improved by transfection of any of the shRNA-expressing pDNA.

To examine whether silencing SOCS gene expression is effective in enhancing the antiproliferative activity of IFN in other cell lines than B16-BL6, similar experiments to those in Fig. 4 were performed using various types of tumor cell lines (Table 1). Silencing of SOCS-1 gene expression was effective in enhancing the antiproliferative activity of IFN-γ on LLC, Renca and MBT-2 cells and that of IFN-β on Renca and MBT-2 cells. In addition, silencing of SOCS-3 gene expression was effective in enhancing antiproliferative activity of IFN-γ on Renca and SA1 cells and that of IFN-β on Renca cells.

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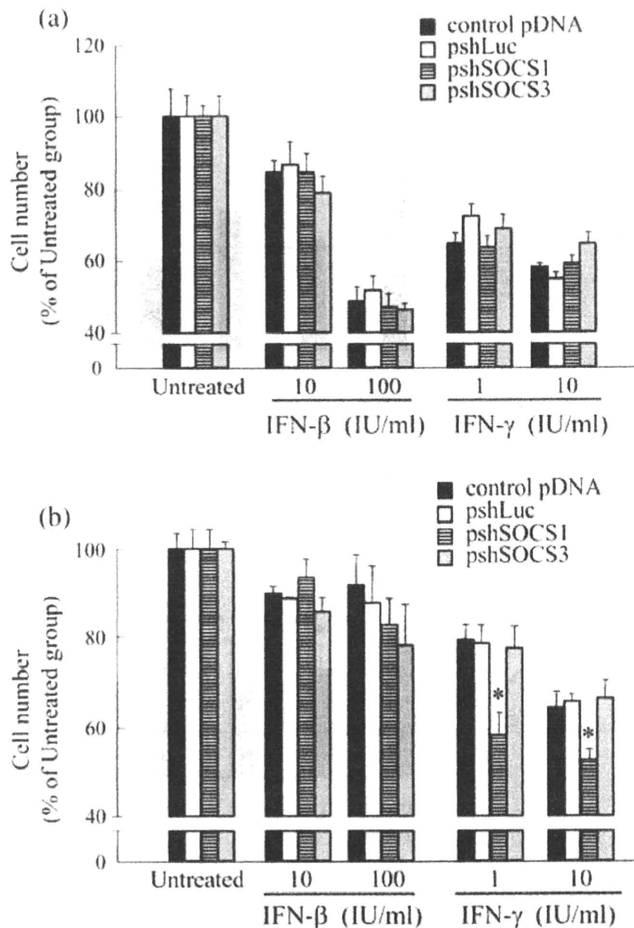

Fig. 4. Anti-proliferative effect of interferons (IFN) against tumor cells transfected with short hairpin (shRNA)-expressing plasmid DNA (pDNA). Tumor cells seeded on culture plates were transfected with control pDNA, pshLuc, pshSOCS-1 or pshSOCS-3. One day after transfection, cells were reseeded on new 24-well culture plates at a density of 1×10^4 cells/well. Twenty-four hours after reseeding, cells were washed with phosphate-buffered saline, followed by addition of the indicated concentrations of IFN and cultured for a further 4 days. The numbers of (a) Colon26 and (b) B16-BL6 cells were evaluated by MTT assay. The results are expressed as the mean ± standard deviation of four independent determinations. *$P < 0.05$ for Student's *t*-test compared with other groups treated with the same concentration of IFN.

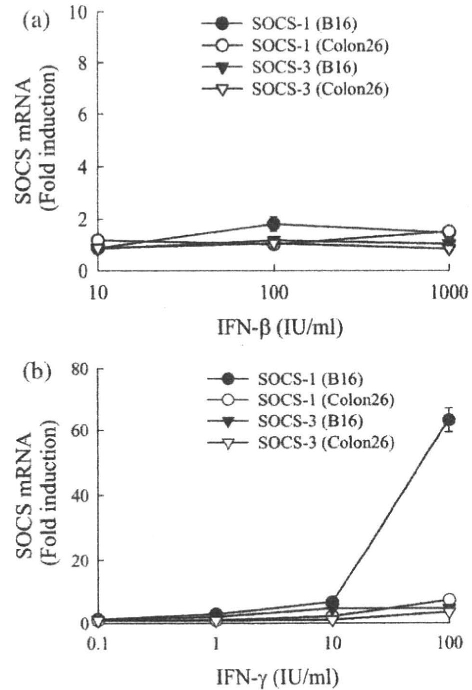

Fig. 5. Effect of interferons (IFN) on *SOCS* mRNA expression in tumor cells. B16-BL6 or Colon26 cells seeded on 12-well culture plates (at a density of 2×10^5 cells/well) were cultured with indicated concentrations of (a) IFN-β or (b) IFN-γ for 24 h. *SOCS-1* and *SOCS-3* mRNA in tumor cells was determined by quantitative reverse transcription polymerase chain reaction and the x-fold induction compared with untreated cells was calculated. The results are expressed as the mean ± standard deviation of three independent determinations.

IFN-induced increase in *SOCS* gene expression in B16-BL6 and Colon26 cells. To examine whether *SOCS* gene expression is upregulated in tumor cells by addition of IFN, we measured the amount of *SOCS* mRNA in B16-BL6 and Colon26 cells incubated with indicated concentrations of IFN for 24 h. A quantitative RT-PCR analysis revealed that addition of IFN-γ greatly increased the *SOCS-1* mRNA expression in B16-BL6 cells (Fig. 5). In contrast, the *SOCS-3* mRNA expression in B16-BL6 cells and *SOCS-1* and *SOCS-3* mRNA expression in Colon26 cells showed only moderate changes, less than fivefold of the untreated values.

Table 1. Effect of interferons (IFN) on the proliferation of tumor cells transfected with short hairpin RNA (shRNA)-expressing plasmid DNA (pDNA)

Cell line	Origin	pDNA	IFN-β (IU/mL)		IFN-γ (IU/mL)	
			10	100	1	10
LLC	Lung carcinoma	Control pDNA	107.8 ± 15.2	81.1 ± 10.7	95.0 ± 3.5	90.2 ± 4.7
		pshSOCS1	98.0 ± 3.0	74.9 ± 3.8	84.0 ± 1.2*	83.6 ± 2.4*
		pshSOCS3	101.6 ± 1.9	78.3 ± 3.3	95.8 ± 0.7	86.8 ± 2.0
Renca	Renal cell carcinoma	Control pDNA	48.8 ± 3.4	44.2 ± 5.4	50.8 ± 4.8	42.1 ± 4.5
		pshSOCS1	30.0 ± 5.3*	29.9 ± 0.9*	31.8 ± 6.9*	21.5 ± 5.2*
		pshSOCS3	29.1 ± 0.6*	25.4 ± 1.8*	27.5 ± 2.6*	13.6 ± 2.4*
MBT-2	Bladder tumor	Control pDNA	73.2 ± 3.2	45.2 ± 0.8	96.8 ± 6.0	88.1 ± 6.3
		pshSOCS1	62.9 ± 0.8*	39.2 ± 0.5*	83.2 ± 2.2*	77.2 ± 4.7*
		pshSOCS3	79.5 ± 1.3*	50.1 ± 0.4*	96.9 ± 0.9	91.9 ± 1.7
SA1	Fibrosarcoma	Control pDNA	41.5 ± 6.0	27.1 ± 8.2	34.7 ± 4.9	31.6 ± 9.3
		pshSOCS1	47.0 ± 4.6	27.2 ± 3.5	30.9 ± 3.7	35.1 ± 4.8
		pshSOCS3	51.9 ± 19.8	24.1 ± 4.7	20.2 ± 3.1*	18.1 ± 2.0*

Tumor cells were treated in a similar manner to that described in the legend of Fig. 4. The results are expressed as the mean percentage of untreated group ± standard deviation of six independent measurements. *$P < 0.05$ for Student's *t*-test compared with the control pDNA and IFN-treated group.

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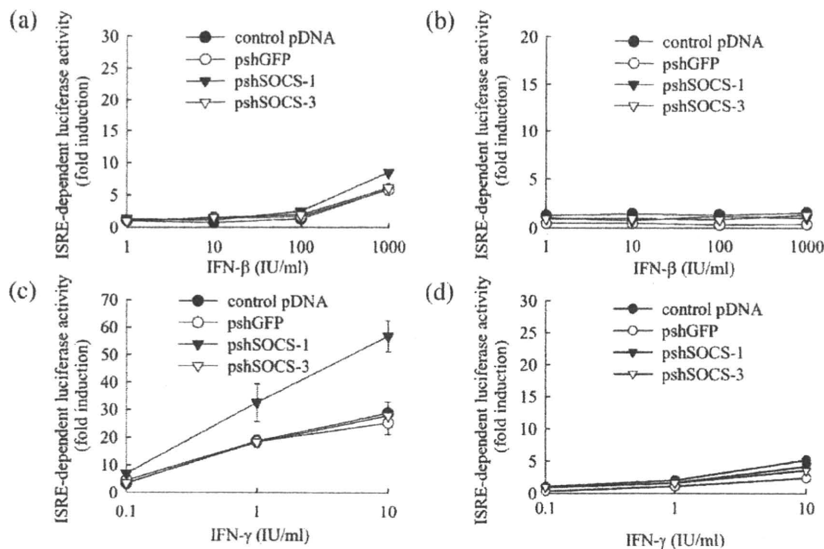

Fig. 6. Effect of transfection of tumor cells with short hairpin RNA (shRNA)-expressing plasmid DNA (pDNA) on interferon (IFN)-induced ISRE-dependent transgene expression. (a,c) B16-BL6 or (b,d) Colon26 cells were transfected with pISRE-Luc, phRL-TK and one of the following: control pDNA, pshGFP, pshSOCS-1 or pshSOCS-3. Four hours after transfection, cells were washed with phosphate-buffered saline, followed by addition of the indicated concentrations of (a,b) IFN-β and (c,d) IFN-γ and cultured for a further 20 h. Then, the luciferase activities of cell lysate were measured and the x-fold induction in luciferase activity compared with untreated cells was calculated. The results are expressed as the mean ± standard deviation of three independent determinations. *$P < 0.05$ for Student's *t*-test compared with other groups treated with the same concentration of IFN.

Increase in ISRE-dependent gene expression by silencing *SOCS-1* gene expression. The IFN signaling pathway recruits several transcription factors that bind to the ISRE, and this binding leads to the activation of the transcription of ISRE-controlled genes. Transfection of pISRE-Luc, pDNA expressing firefly luciferase under the control of ISRE, and the following luciferase assay were used to assess the degree of activation of the IFN signaling pathway in the cells. Thus, B16-BL6 and Colon26 cells were co-transfected with pLuc-ISRE, phRL-TK and one of the shRNA-expressing pDNA: control pDNA, pshSOCS-1, pshSOCS-3 or pshGFP. Then, cells were incubated with the indicated concentration of IFN.

Addition of IFN-β or IFN-γ to Colon26 cells increased the ISRE-dependent firefly luciferase activity to approximately 1.5- and 3-fold of the untreated values, respectively (Fig. 6). Colon26 cells transfected with any of the shRNA-expressing pDNA showed almost an identical ability to increase the ISRE-dependent luciferase expression by IFN.

In B16-BL6 cells transfected with control pDNA, pshGFP or pshSOCS-3, addition of IFN-β and IFN-γ increased the ISRE-dependent luciferase activity to approximately 5- and 20-fold of the untreated value, respectively. On the other hand, in pshSOCS-1-transfected B16 cells, ISRE-dependent luciferase expression was increased to approximately 8- and 50-fold of the unstimulated value by the addition of IFN-β and IFN-γ, respectively, indicating that the IFN-dependent gene expression in B16-BL6 cells was greatly increased by the transfection of pshSOCS-1.

Discussion

An increasing number of studies have demonstrated the roles of SOCS in cytokine signaling, including the immune suppression and cytokine resistance of tumor cells. Sakai *et al.* reported that a forced expression of SOCS-3 in a leukemia cell line renders the cells resistant to the antiproliferative effect of IFN-α.[12] Zitzmann *et al.* recently showed that silencing SOCS-1 expression in neuroendocrine tumor cells enhanced the antitumor activity of IFN-α and IFN-β.[20] The present study investigated whether suppressing *SOCS-1* or *SOCS-3* gene expression in tumor cells can enhance the antiproliferative effect of IFN-β and INF-γ on tumor cells. Although the efficiency of gene silencing was moderate in both B16-BL6 and Colon26 cells (Fig. 3), we obtained significant effects on the antiproliferative effect of IFN (Fig. 4 and Table 1) and activation of ISRE-dependent luciferase expression in B16 cells (Fig. 6) by silencing *SOCS-1* and *SOCS-3* gene expression, suggesting no further reduction was necessary to obtain the silencing effects. In the present study, IFN-resistant cell lines were not used, because a previous report by Fleischmann *et al.* demonstrated that an artificial IFN-resistant B16 cell line showed an increased sensitivity to IFN when inoculated into mice.[21]

Of the murine cell lines used, Colon26 cells showed higher levels of *SOCS* mRNA expression compared with B16-BL6 cells and several types of normal cells (Fig. 1). However, the *SOCS* gene expression was greatly increased by IFN in B16-BL6 cells compared with that in Colon26 cells (Fig. 5). Combining these data with the fact that B16-BL6 cells were more resistant to the growth inhibitory effect of IFN than Colon26 cells, these results strongly support the following two suggestions: (i) IFN-induced *SOCS* gene expression is one of the most important factors for IFN resistance of tumor cells; and (ii) the constitutive *SOCS* gene expression level is not always correlated with the IFN resistance. Recently, Fojtova *et al.* found that melanoma cells which are resistant to the antitumor effect of IFN-γ were different from IFN-sensitive melanoma cells in terms of the constitutive and induced levels of *SOCS* gene expression.[22] IFN-resistant cells had a high constitutive level of *SOCS-3* gene expression and weak SOCS-1 and SOCS-3 induction by IFN-γ. In the present study, we have clearly shown that IFN-mediated induction of *SOCS* gene expression was greater in the IFN-resistant B16-BL6 cells than in the IFN-sensitive Colon26 cells. Although Fojtova *et al.* concluded that a constitutively high level of *SOCS-3* gene expression is a major reason for the resistance of tumor cells to the antitumor effect of IFN,[22] our results suggest that the constitutive level of *SOCS* gene expression is of little importance. In contrast to the study by Fojtova *et al.*, we showed that the IFN-induced SOCS-1 expression, not the constitutive SOCS-1 expression, is a key factor determining the enhancement of the antiproliferative activity of IFN by silencing SOCS-1 expression. Use of IFN-resistant and sensitive cells with the same cell line may underscore the importance of the inductive level of SOCS gene expression.

The IFN-dependent antitumor effect is initiated by the binding of IFN to their cognate receptors followed by phosphorylation of STAT proteins, recruitment of transcription factors and the expression of IFN-dependent gene products. Tumor cells have been reported to become resistant to IFN by reducing the number of IFN receptors on their cell surface, inhibiting STAT phosphorylation or expressing genes that exhibit neutralizing

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effects on IFN-dependent gene products.[23] One of the frequently observed characteristics of IFN-resistant tumor cells is the reduced phosphorylation of STAT proteins after IFN stimulation.[24,25] Although these previous studies made no mention of the relationship between SOCS and IFN resistance, they suggest the involvement of SOCS in IFN resistance because SOCS proteins are inhibitors of STAT phosphorylation. Involvement of an induced *SOCS* gene expression in the inhibition of IFN signaling has recently been reported by Evans *et al.* who showed that IFN-γ-induced *SOCS-1* gene expression terminated the activation of IFN signaling in breast cancer cells through the inhibition of STAT phosphorylation.[26] These results are in good agreement with the findings of our present study, in which *SOCS-1* gene expression in B16 cells was markedly induced by IFN-γ and silencing the *SOCS-1* gene expression enhanced the antiproliferative effect of IFN-γ. In addition, an antiproliferative effect of IFN should be exerted by IFN-dependent gene products, the expression cassette of which usually contains the ISRE sequence.[27] Therefore, we used pISRE-Luc, a plasmid-expressing firefly luciferase under the control of ISRE, to examine whether IFN signaling is upregulated in pshSOCS-1-transfected B16 cells. Luciferase assay of cells clearly demonstrated that only pshSOCS-1 increased ISRE-dependent luciferase expression in the IFN-γ-treated-B16-BL6 cells (Fig. 6). However, we found a discrepancy between the antiproliferative activity and induction in ISRE-dependent luciferase expression by IFN. As the reason for this discrepancy, we speculate less sensitivity of the luciferase-dependent reporter assay in Colon26 cells. In our previous study investigating the relationship between hypoxia and tumor metastasis, we found that hypoxia-responsible luciferase expression in Colon26 cells was induced by hypoxia less than that in B16-BL6 cells despite the fact that vascular endothelial growth factor, a hypoxia-responsible endogenous product, was almost equally induced by hypoxia in both cells.[28] Therefore, the ISRE-dependent luciferase assay may underestimate the endogenous ISRE-dependent gene expression in Colon26 cells. Our result showing that SOCS-1 plays an important role in IFN resistance in B16 melanoma cells is also in agreement with previous results reported by Li *et al.* who showed that melanoma cells express SOCS-1 protein whereas normal melanocytes do not.[29] These authors concluded that SOCS-1 is a progression marker of melanoma and may downregulate cytokine-induced biological responses. However, they did not directly investigate the role of *SOCS-1* gene expression in melanoma cells in cytokine resistance and tumor progression. Contrary to this previous report, we confirmed that SOCS knockdown is an effective approach to improving the therapeutic potency of IFN against a variety type of tumor cells.

In conclusion, we found that RNAi-mediated silencing of *SOCS-1* gene expression in tumor cells enhances the growth inhibitory effect of IFN-γ under conditions where the SOCS-1 expression is upregulated by IFN-γ. Thus, silencing of the SOCS-1 expression offers a promising approach to optimizing IFN-based cancer therapy.

Acknowledgments

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Analysis of the Complete Open Reading Frame of Hepatitis C Virus in Genotype 2a Infection Reveals Critical Sites Influencing the Response to Peginterferon and Ribavirin Therapy.

Makoto Kadokura[1)], Shinya Maekawa[1)], Ryota Sueki[1)], Mika Miura[1)], Kazuki Komase[1)], Hiroko Shindo[1)], Fumitake Amemiya[1)], Tomoyoshi Uetake[1)], Taisuke Inoue[1)], Minoru Sakamoto[1)], Mina Nakagawa[2)], Naoya Sakamoto[2)], Mamoru Watanabe[2)], Nobuyuki Enomoto[1)]

1) First Department of Internal Medicine, Faculty of Medicine, University of Yamanash; 1110, Shimokato, Chuo, Yamanashi 409-3898, Japan.

2) Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University; 1-5-45, Yushima, Bunkyo, Tokyo, 113-8510, Japan

Short title: PEG-IFN/RBV response in HCV-2a

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Correspondence : Shinya Maekawa M.D./Ph.D.

First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi

1110, Shimokato, Chuo, Yamanashi 409-3898, Japan.

Tel: +81-5-5273-9584

Fax: +81-5-5273-6748

E-mail: maekawa@yamanashi.ac.jp

Abbreviations

EVR: Early Virological response

IFN: Interferon

IRF-1: Interferon Regulatory Factor 1

IRRDR: Interferon Ribavirin Resistance Determinant Region

ISDR: Interferon Sensitivity Determinant Region

ORF: Open Reading Frame

PEG-IFN: Pegylated-Interferon

PePHD: PKR -eIF2 Phosphorylation Homology Domain

PKR-BD: Double-Stranded RNA-activated Protein Kinase Binding Domain

RBV: Ribavirin

RVR: Rapid Virological Response

SVR: Sustained Virological Response

ABSTRACT

Purpose: A proportion of patients infected with genotype 2a hepatitis C virus (HCV) cannot achieve a sustained virological response (SVR) to pegylated-interferon plus ribavirin therapy (PEG-IFN/RBV) but the reason remains unclear. The present study aimed to clarify the possible correlation between viral sequence variations and final outcome.

Methods: The pretreatment complete open reading frame (ORF) sequences of genotype 2a HCV were determined by direct sequencing for two independent groups of patients (43 patients as test; group 1 and 35 as validation; group 2), and the correlation with the final outcome was explored.

Results: Patients with SVR (n=58) and with non-SVR (n=20) differed significantly in pretreatment HCV RNA level ($p=0.002$), fibrosis score ($p=0.047$), and cumulative ribavirin dosage ($p=0.003$). By comparison of all amino acid positions in the complete HCV ORFs, threonine at amino acid (aa) 110 in the core region was remarkably frequent in SVR ($p=0.01$ for group 1, $p=0.004$ for group 2, and $p=5E-05$ for combined). A sliding window analysis revealed that the total numbers of amino acid variations within the NS5A aa 2258 to 2306 region were significantly high in SVR compared to non-SVR patients ($p=0.01$ for group 1, $p=0.006$ for group 2, and $p=0.0006$ for combined). Multivariate analyses revealed that core aa 110 ($p=0.02$), NS5A aa 2258-2306 ($p=0.03$), and cumulative ribavirin dosage ($p=0.02$) were identified as independent variables associated with the final outcome.

Conclusions: The outcome of PEG-IFN/RBV therapy is significantly influenced by variation in the core and NS5A regions in genotype 2a HCV infection.

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INTRODUCTION

Worldwide, 180 million of people are estimated to be infected with hepatitis C virus (HCV), and HCV is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1). In HCV-infected patients with chronic hepatitis, treatment with interferon (IFN) can result in viral clearance and biochemical and histological improvements(2). The response to the therapy varies according to HCV genotype and pretreatment HCV RNA level (3-4).

The currently recommended treatment for patients infected genotype 2a HCV with high viral load is pegylated-interferon (PEG-IFN) plus ribavirin (RBV) for 24 weeks (1). Approximately 80% of patients infected with genotype 2a HCV can achieve a sustained virological response (SVR) with this regimen (5-6), although much lower percentages of patients infected with other genotypes can achieve SVR, especially with genotype 1(1). Because of its high response rate, shorter treatment duration was suggested by some studies, although an agreement has not been reached yet (7-8). On the other hand, about 20% of patients infected with this genotype cannot achieve SVR and it remains elusive which patients show poor responses.

Previous studies have reported that amino acid variations in the NS5A-ISDR (9), NS5A-IRRDR(10), NS5B(11), PKR-eIF2 phosphorylation homology domain (PePHD) of E2(12), and core (13-14) correlate with clinical outcome of IFN-based therapy, including PEG-IFN/RBV therapy in patients infected with genotype 1b HCV. Recent full HCV open reading frame analysis for genotype 1 also have reported that core, NS3, and NS5A were associated with early viral response and the outcome in PEG-IFN/RBV therapy (15-16). However, in genotype 2a infection, only a few studies have investigated the association between HCV sequence variation and treatment

response (17-19), and the role of viral factors has not been established yet, especially in the era of PEG-IFN/RBV therapy. Moreover, these previous studies investigated only several isolated HCV genomic regions, and comprehensive analysis of the full HCV open reading frame (ORF) has not been undertaken so far.

In the present study, to assess comprehensively the influence of viral variations on response to the PEG-IFN/RBV therapy in genotype 2a HCV infection, we determined the complete pretreatment HCV ORFs from Japanese patients and investigated viral amino acid variation and their correlation with the response to the combination therapy of PEG-IFN plus RBV.

PATIENTS & METHODS

Study Population

A total of 103 adult Japanese patients infected with genotype 2a HCV, who received the combination therapy with PEG-IFN (PEGINTRON®. Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2005 and 2008 at University of Yamanashi, Tokyo Medical and Dental University, and related institutions were first included in the study. They all fulfilled following criteria: (1) negative for hepatitis B surface antigen, (2) high viral load (≥100 KIU/ml), (3) absence of hepatocellular carcinoma, (4) no other form of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease, (5) free of co-infection with human immunodeficiency virus. Informed consent was obtained from each patient. The study was approved by the ethics committees of all the participating universities and hospitals. The therapy was performed according to the standard treatment protocol of PEG-IFN/RBV therapy for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan (PEG-IFNα-2b 1.5 μg/kg body weight, once weekly subcutaneously, and RBV 600-800 mg daily per os for 24 weeks). To clearly disclose the non-SVR viral characteristics, we have considered those patients who achieved total drug administration of 60% or more for both PEG-IFN and RBV, with the completion of the standard treatment duration. Moreover, although we excluded the patients with extended therapy to make the studied population uniform, we have included non-SVR patients with extended therapy to clarify the specific characteristics of non-SVR patients, a minor population group. As a result, 25 patients were excluded for the following reasons: 4 patients received insufficient dose, 8 patients were discontinued from the therapy within 12 weeks, and 13 SVR patients received