Downregulation of microRNA-431 by human interferon-β inhibits viability of medulloblastoma and glioblastoma cells via upregulation of SOCS6

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Abstract. miRNAs are small non-coding RNAs that inhibit gene expression by cleaving or hindering the translation of target mRNAs. In this study, we focused on miR-431, which mediated inhibition of cell viability by human interferon-β (HuIFN-β). We aimed to demonstrate an antineoplastic effect of HuIFN-β via miR-431 expression against medulloblastoma and glioblastoma, because HuIFN-β is frequently used in adjuvant therapy of these tumors. Addition of HuIFN-β to medulloblastoma and glioblastoma cells reduced viability, significantly decreased miR-431 expression, upregulated expression of SOCS6 (putative miR-431 target genes) and inhibited Janus kinase (JAK) 1 and signal transducer and activator of transcription (STAT) 2. The mitogen-activated protein kinase (MAPK) pathway, but not the phosphoinositide 3-kinase (PI3K)-Akt pathway, was downregulated in medulloblastoma cells, whereas the PI3K-Akt pathway, but not the MAPK pathway, was downregulated in glioblastoma cells. Addition of HuIFN-β and transient transfection with miR-431 to medulloblastoma and glioblastoma cells did not reduce viability, downregulated expression of SOCS6, and concomitantly activated the JAK1 and STAT2. We propose that, in medulloblastoma and glioblastoma cells, HuIFN-β decreases miR-431 expression and upregulates SOCS6 expression, and consequently inhibit cell proliferation by suppressing the JAK-STAT signaling pathway.

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Introduction

miRNAs are small non-coding RNAs that regulate gene expression by associating with the nucleotides in the 3' untranslated regions (UTRs) of target mRNAs and inhibiting protein translation (1) or by directing mRNA degradation (2). miRNAs play important roles in cell proliferation, differentiation, cell cycle and apoptosis (3,4). Each miRNA can potentially regulate hundreds of mRNAs. In particular, miRNAs that regulate cell proliferation are associated with various disorders including cancer (5). Numerous studies have revealed differences in the expression of various miRNAs between tumors and normal tissues (6), suggesting that miRNAs can function as tumor suppressors or oncogenes in human cancers.

Human interferons (HuIFNs) are a family of cytokines that have pleiotropic biological functions, including antiviral and antitumor effects (7-9). They also regulate basic cellular functions including growth, differentiation, and immunoreactivity (10-12). HuIFNs induce a cascade of events leading to an increase in the expression of various genes, including those responsible for the biological effects of IFNs (10-14). Based in part on the differential use of unique receptors through which they mediate their biological effects, three classes of HuIFNs have been distinguished: HuIFN-α and -β are grouped together as type I IFN; HuIFN-γ is a type II IFN; and HuIFN-λ is a type III IFN. HuIFN-β is useful as an antineoplastic drug sensitizer when administered in combination with nitrosoureas (15), although the role of miRNAs in this function is not clear.

In a previous study, we reported that inhibition of cell viability by HuIFN-β was mediated by miR-431 (16). Medulloblastoma is the most common malignant tumor of the central nervous system in children (17). Conventional treatment of medulloblastoma consists of surgery, radiation and chemotherapy. Despite successful treatments, aggressive adverse effects result in neurological and endocrine complications in many survivors (18). Glioblastoma is highly invasive, proliferative and vascularized (19). Despite aggressive treatment, such as surgery, radiotherapy and chemotherapy, the

median survival is <16 months (20). Therefore, it is important to understand the mechanisms associated with the development and progression of glioblastoma.

In this study, we focused renewed attention on the miR-431. We investigated the relationship between miR-431 expression and the antineoplastic effects of HuIFN-β using medulloblastoma and glioblastoma cell lines, because HuIFN-β is used clinically as an antineoplastic drug. Our goal was to identify antineoplastic mechanism of miR-431 associated with antineoplastic effects of HuIFN-β in medulloblastoma and glioblastoma.

Materials and methods

Cell lines and culture conditions. Human medulloblastoma cell line TE671 was provided by the Department of Molecular Genetics, Kitasato University Graduate School of Medical Sciences. Another medulloblastoma cell line, ONS-76, and glioblastoma cell lines A172 and U251-MG were purchased from the Japanese Health Science Research Resources Bank. ONS-76 cell line was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Sanko Junyaku Co., Ltd., Tokyo, Japan). TE671 and A172 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. U251-MG cell line was maintained in Eagle's minimal essential medium supplemented with 10% FBS. Cell lines were treated with recombinant HuIFN-β (PeproTech., Rocky Hill, NJ, USA) dissolved in medium just before use. Cells were grown in medium containing $0.01x10^5$, $0.5x10^5$ or $1.0x10^5$ IU/ml recombinant HuIFN-β for 24 h or 48 h, with replacement of fresh medium and recombinant HuIFN-β every 24 h. All cell lines were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

Measurement of cell viability. Cell viability was measured from the average of 6 MTS assays per sample, quantified by absorbance at 490 nm in a microplate colorimeter using the CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions, for each time point.

RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

Evaluation of HuIFN-β-induced changes in miRNA expression levels using quantitative RT-PCR. Quantitative RT-PCR for miR-431 was carried out using the Taqman MicroRNA Assay (Applied Biosystems, Foster City, CA, USA). Thirty nanograms of total RNA from medulloblastoma and glioblastoma cells were reverse transcribed using the TaqMan Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. For normalization, each miRNA was amplified on the same plate with the reference miRNA, RNU6B, and we calculated changes in expression levels relative to this standard.

Transfection of miRNA molecules. miRNA precursors that mimic miR-431 and a control nonspecific miRNA (Pre-miR Negative Control) were obtained from Ambion (Austin, TX, USA). Using siPORT NeoFX (Ambion), miRNA precursors were transfected into ONS-76, TE671, A172 and U251-MG cells, according to the manufacturer's protocol.

Estimation of miR-431 target gene expression. Medulloblastoma (ONS-76 and TE671) and glioblastoma (A172 and U251-MG) cell lines were grown in medium containing $1.0x10^5$ IU/ml recombinant HuIFN-β for 48 h. RNA was extracted at 48 h of treatment, cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen), and predicted targets gene for miR-431 were identified using TargetScan (http://www.targetscan.org). Steady-state expression levels of SOCS6 mRNA was evaluated by quantitative RT-PCR using Fast SYBER Green Master Mix and the following oligonucleotide primers and annealing temperatures: *SOCS6*, 5'-AAGAATTCATCCCTTGGATTAGGT AAC-3' (forward) and 5'-CAGACTGGAGGTCGTGGAA-3' (reverse) at 60˚C. Expression of each gene was normalized to a GAPDH control: 5'-ACCCACTCCTCCACCTTTG-3' (forward) and 5'-CTCTTGTGCTCTTGCTGGG-3' (reverse).

Western blotting. Cells grown to 80% confluence were treated with HuIFN-β or pre-miR-431. After treatment, cells were washed three times with ice-cold PBS. Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (ATTO, Tokyo, Japan). Membranes were blocked in 5% non-fat dried milk in PBS-Tween-20 and incubated with primary antibody. The following antibodies were used: SOCS6, JAK1 and total Akt1/2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and STAT1 p-STAT1, STAT2, p-STAT2, p-Akt (phospho-Akt, Ser^{473}), p44/42 MAPK (Erk1/2), and phosphor-p44/42 MAPK (Erk1/2) (Cell Signaling Technology, Danvers, MA, USA). GAPDH (Sigma, St. Louis, MO, USA) was used as an internal control. Secondary antibodies were conjugated to horseradish peroxidase and immunoreactive proteins were detected using the ECL-plus system (Amersham, Piscataway, NJ, USA).

Results

Effect of HuIFN-β on viability of medulloblastoma and glioblastoma cells. HuIFN-β was added to culture medium at a concentration $0.01x10^5$, $0.5x10^5$ or $1.0x10^5$ IU/ml for 24 h or 48 h. HuIFN-β suppressed viability of medulloblastoma and glioblastoma cell lines in a dose- and time-dependent manner (Fig. 1).

Effect of HuIFN-β on miR-431 expression. Based on the results shown in Fig. 1, we analyzed the expression levels of miR-431 in medulloblastoma and glioblastoma cell lines after 48 h treating with HuIFN-β ($1.0x10^5$ IU/ml). HuIFN-β significantly decreased miR-431 expression in all cell lines compared with untreated control cell lines (Fig. 2).

Examination of the role of miR-431 in cell viability. In order to examine whether decreased expression of miR-431 results in reduced cell viability, medulloblastoma and glioblastoma cells were treated with HuIFN-β ($1.0x10^5$ IU/ml) and transiently transfected with miR-431. Cell proliferation was not suppressed at 48 h (Fig. 3).

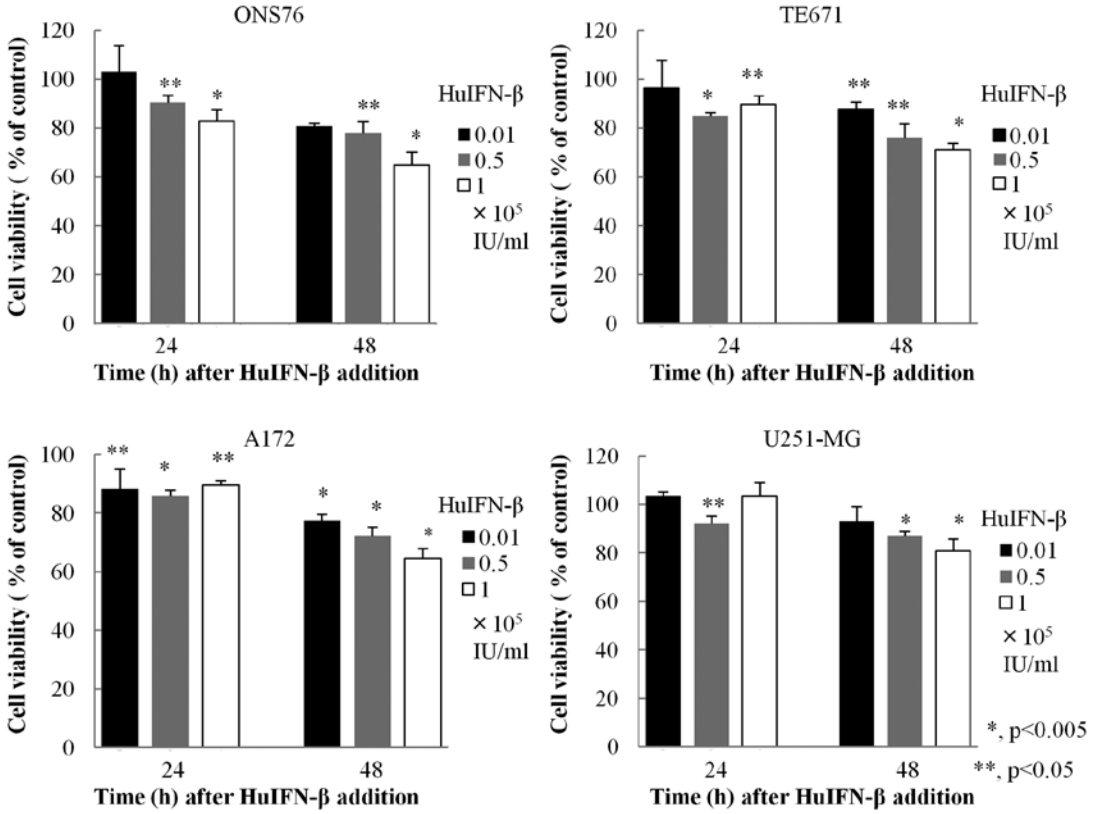

Figure 1. Effect of HuIFN-β treatment on cell viability. HuIFN-β was added to the culture medium of medulloblastoma and glioblastoma cells, at 0.01x10^5 IU/ml (black square), 0.5x10^5 IU/ml (grey square) or 1x10^5 IU/ml (white square). Results are presented as means ± SD. *p<0.005, **p<0.05.

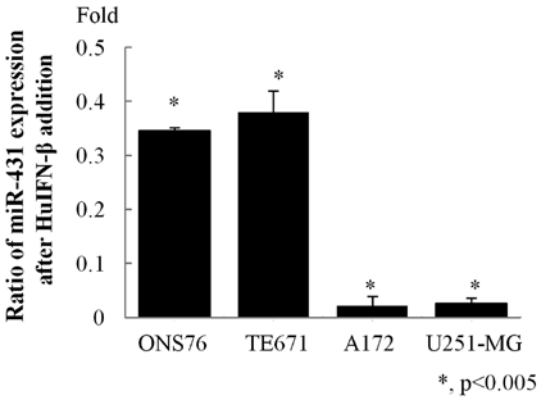

Figure 2. Effect of HuIFN-β treatment on expression levels of miR-431. Quantitative RT-PCR was performed to examine the effects of 48 h treatment with HuIFN-β (1x10^5 IU/ml) on miR-431 expression in medulloblastoma and glioblastoma cells. miR-431 expression is presented as a fold difference with respect to control cells containing no HuIFN-β, with the value for control cells set at a fold change of 1. Results are presented as means ± SD. *p<0.005.

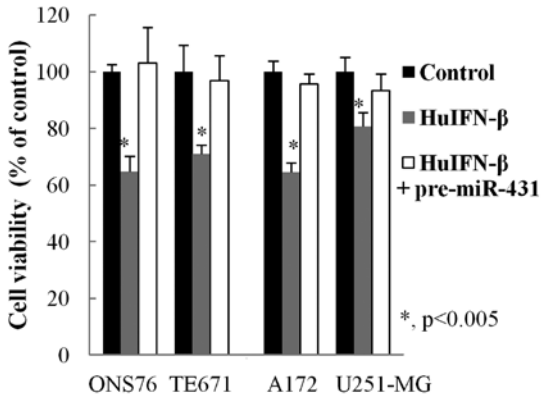

Figure 3. Effects of HuIFN-β treatment with or without pre-miR-431 addition on cell viability. Changes in cell viability in medulloblastoma and glioblastoma cells treated with HuIFN-β with or without pre-miR-431. Results are presented as means ± SD. *p<0.005.

Search for miR-431 target genes. Predicted target genes for miR-431 were identified with TargetScan. There were 166 conserved targets for miR-431. Among these genes, we focused on SOCS6, which is related to cell viability. A schematic representation of *SOCS6* mRNAs, showing the predicted miR-431 binding sites located in their 3' UTR, is shown in Fig. 4A.

Upregulation of miR-431 target genes. Treating medulloblastoma and glioblastoma cells with HuIFN-β (1.0x10^5 IU/ml) also significantly increased *SOCS6* expression after 48 h relative to control cells (Fig. 4B). However, when these cells were treated with both HuIFN-β (1.0x10^5 IU/ml) and transiently transfected with miR-431, *SOCS6* expression was significantly suppressed (Fig. 4B).

Examination of JAK-STAT signaling pathways. When medulloblastoma and glioblastoma cells were treated with HuIFN-β (1.0x10^5 IU/ml), an increase in protein levels of SOCS6 was consistently observed after 48 h (Figs. 5 and 6). The addition of HuIFN-β (1.0x10^5 IU/ml) and transiently transfected

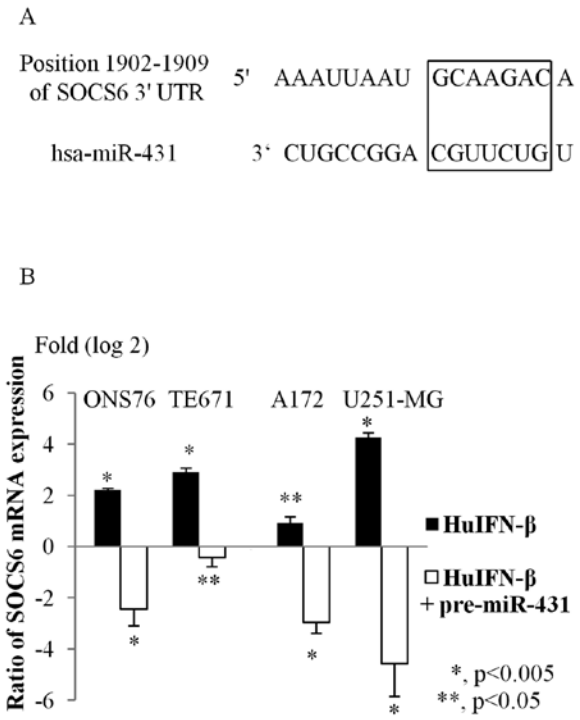

Figure 4. SOCS6 mRNA expression. (A) Sites of miR-431 seed matching the SOCS6 3' UTRs. (B) SOCS6 mRNA level was analyzed by quantitative RT-PCR at 48 h after treatment with HuIFN-β ($1x10^5$ IU/ml) (■) in combination with pre-miR-431 (□). mRNA expression levels are presented as a fold difference with respect to control cells containing no HuIFN-β and pre-miR-431 which was set at a fold change of 1. Results are presented as means ± SD. $^*p<0.005$, $^{**}p<0.05$.

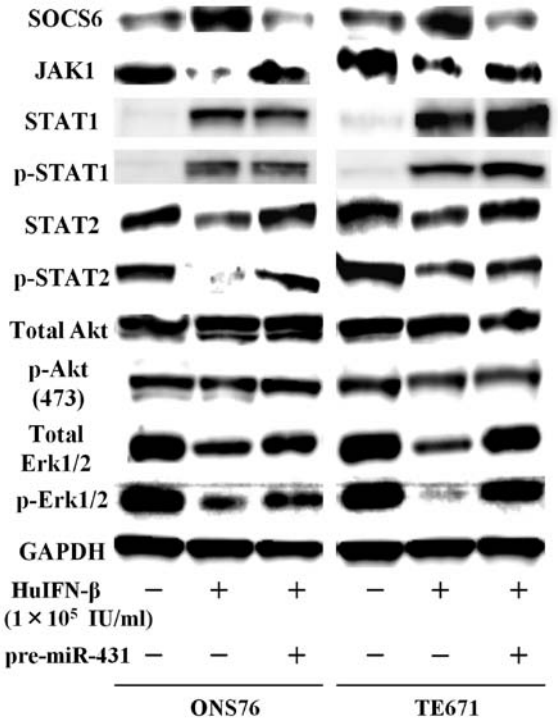

Figure 5. Effects of HuIFN-β and miR-431 on SOCS6 expression and JAK-STAT signaling pathway in medulloblastoma cells. Immunoblot of SOCS6, JAK1, STAT1, p-STAT1, STAT2, p-STAT2, total Akt, p-Akt (Ser^{473}), total Erk1/2, and p-Erk1/2 protein in medulloblastoma cells treated with or without HuIFN-β ($1x10^5$ IU/ml) and pre-miR-431 treatment for 48 h. GAPDH was used as a loading control.

with miR-431 significantly reduced expression of SOCS6 in medulloblastoma and glioblastoma cells (Figs. 5 and 6). We investigated the effects of HuIFN-β-mediated *SOCS6* upregulation via suppression of miR-431 on JAK-STAT, phosphoinositide 3-kinase (PI3K)-Akt and mitogen-activated protein kinase (MAPK) phosphorylation. In medulloblastoma and glioblastoma cells, protein levels of JAK1, STAT2 and p-STAT2 were reduced after 48 h treatment with HuIFN-β ($1.0x10^5$ IU/ml), while protein levels of STAT1 and p-STAT1 were not reduced (Figs. 5 and 6). As for PI3K-Akt and MAPK phosphorylation, when ONS76 and TE671 cells were treated with HuIFN-β ($1.0x10^5$ IU/ml), there was a reduction in total extracellular signal-regulated kinase (Erk)1/2 and p-Erk1/2, while the levels of total Akt and p-Akt Ser^{473} were not significantly reduced (Fig. 5). When A172 and U251-MG cells were treated with HuIFN-β ($1.0x10^5$ IU/ml), there was a reduction in the levels of total Akt and p-Akt Ser^{473}, while the levels of total Erk1/2 and p-Erk1/2 were not significantly reduced (Fig. 6). Upregulation of miR-431 by transient transfection with miR-431 and simultaneous treatment with HuIFN-β ($1.0x10^5$ IU/ml) reversed the suppression of expression of these genes (Figs. 5 and 6).

Discussion

miRNAs are known as playing an important role in antitumor activity. Differential expression of miRNAs is observed in a variety of cancers. Changes in miRNA expression in cancer lead to dysregulation of cell proliferation, which results in tumorigenesis (21,22). Several studies have reported dysregulation of cell proliferation in medulloblastoma and glioblastoma via downregulation of target genes by changes in miRNA expression. For example, in medulloblastoma, miR-124, miR-129 and miR-383 inhibit cell proliferation through downregulation of their respective target genes, which are SLC16A1 for miR-124 (23), CDK6 for miR-124 and miR-129 (24,25), and PRDX3 for miR-383 (26). In glioblastoma, miR-124, miR-134 and miR-137 inhibit cell proliferation through downregulation of their respective target genes, which are PPP1R13L or SOS1 for miR-124 (27,28), Nanog for miR-134 (29), and RTVP-1 for miR-137 (30).

IFN-β is used as a therapeutic agent for a variety of neoplasms. For example, it is frequently used to treat melanoma, medulloblastoma and glioblastoma in adjuvant therapy. Although its molecular mechanisms remain unclear, Chawla-Sarkar *et al* suggested that one of the antitumor effects of IFN-β in melanoma cells involves apoptosis (31). Yoshino *et al* also suggested that IFN-β mediated cytotoxicity, including apoptosis, in glioblastoma cells might involve upregulation of IFN regulatory factor (IRF)-1 and IRF-2 (9).

Based on these conclusions, we investigated the interaction between expression of miRNAs and treatment with IFN-β, using medulloblastoma and glioblastoma cell lines, because these factors both have antitumor effects. We previously reported that miR-431 expression was upregulated by addition of HuIFN-β, using a non-cancer HuIFN-β sensitive cell line (RSa) and its variant HuIFN-β resistant cell line (F-IFr) (16). The change in miR-431 expression induced by addition of HuIFN-β was

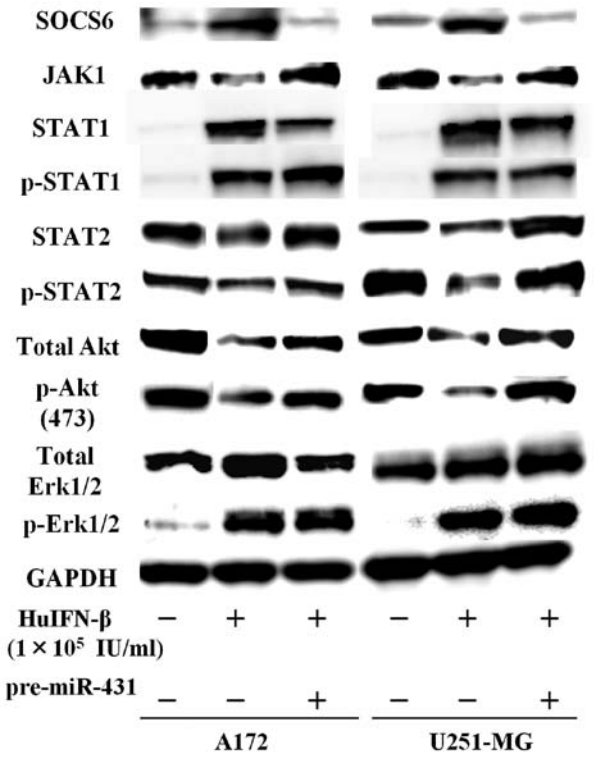

Figure 6. Effects of HuIFN-β and miR-431 on SOCS6 expression and JAK-STAT signaling pathway in glioblastoma cells. Immunoblot of SOCS6, JAK1, STAT1, p-STAT1, STAT2, p-STAT2, total Akt, p-Akt (Ser^{473}), total Erk1/2, and p-Erk1/2 protein in glioblastoma cells treated with or without HuIFN-β ($1x10^5$ IU/ml) and pre-miR-431 treatment for 48 h. GAPDH was used as a loading control.

involved in inhibition of cell viability (16). Therefore, we focused renewed attention on the function of miR-431 in medulloblastoma and glioblastoma cells.

First, we confirmed the effect of HuIFN-β on inhibition of cell proliferation in medulloblastoma and glioblastoma cells. Then, we showed using quantitative RT-PCR that miR-431 expression in both cell lines treated with HuIFN-β was significantly decreased.

In addition, we confirmed that cell proliferation was not suppressed after 48 h treatment with HuIFN-β and transient transfection with miR-431. These results suggest that miR-431 plays an important role in regulating proliferation by HuIFN-β in medulloblastoma and glioblastoma cells. We then sought to determine whether miRNA-regulated signaling pathways modulated cell viability. Previous studies revealed that some signaling pathways that affect cell viability are regulated by miRNA expression (32-35). We decided to focus on one of these, the JAK-STAT signaling pathway. The factors that regulate JAK-STAT signaling pathways and are involved in cell proliferation in cancer have been investigated by others (36,37). For numerous cancers, activation of JAK-STAT signaling pathway contributes to cell proliferation. Therefore, suppression of this pathway should inhibit cancer cell proliferation. In gastric cancer, OPB-31121, a novel small molecular inhibitor, inhibits JAK-STAT signaling pathway and has antitumor effects (37). Thus, JAK-STAT signaling pathway can affect cell proliferation.

In the present study, we examined the miR-431 target genes that are suspected of suppressing cell viability, and focused on SOCS6, which is a functional target of miR-431. SOCS6 is a suppressor of cytokine signaling that belongs to the cytokine-induced STAT inhibitors, and it mediates cytokine-induced signaling. According to quantitative RT-PCR analysis and western blot analysis, although SOCS6 is upregulated in medulloblastoma and glioblastoma cells treated with HuIFN-β, it is downregulated in cells treated with HuIFN-β and transiently transfected with miR-431. These observations suggest that decreased miR-431 expression increases SOCS6 expression in medulloblastoma and glioblastoma cells treated with HuIFN-β. Therefore, we hypothesized that miR-431-mediated SOCS6 upregulation would be accompanied by the inhibition of JAK-STAT signaling. HuIFN-β suppressed this signaling pathway in medulloblastoma and glioblastoma cells via suppression of JAK1 and STAT2. In addition, we also considered the involvement of the PI3K-Akt and MAPK pathways, as part of the JAK-STAT signaling pathway, in cell proliferation. The MAPK pathway was only inhibited in medulloblastoma cells. In contrast, the PI3K-Akt pathway was only inhibited in glioblastoma cells.

There are several clinical studies on the therapeutic use of IFN-β for various malignant tumors. For example, NC65 tumors (a human renal cell carcinoma) treated with recombinant HuIFN-β did not shrink and failed to undergo apoptosis (38). This study indicates that there are many hurdles facing the use of HuIFN-β as an anticancer agent. In contrast, combination therapy with IFN-β and ranimustine has been particularly useful for the treatment of malignant gliomas in Japan (39). A recent study investigated IFN-β monotherapy and combination therapy with IFN-β and temozolomide, a relatively new alkylating agent. This combination therapy was significantly associated with a favorable outcome (40). Our results suggest that decreased miR-431 expression in medulloblastoma and glioblastoma cells, in combination with a PI3K-Akt or MAPK inhibitor, may be able to suppress tumor cell proliferation more effectively.

In conclusion, our results demonstrate that miR-431, which is downregulated by HuIFN-β in medulloblastoma and glioblastoma cells, contributes to suppression of the JAK1 and STAT2 via upregulation of SOCS6. Combination therapy with downregulation of miR-431 and a PI3K-Akt or MAPK inhibitor may represent a more effective strategy for the treatment of IFN-β resistant medulloblastoma and glioblastoma.

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