

Original Article

Snail plays an oncogenic role in glioblastoma by promoting epithelial mesenchymal transition

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Abstract: Background: The factors affecting glioblastoma progression are of great clinical importance since dismal outcomes have been observed for glioblastoma patients. The Snail gene is known to coordinate the regulation of tumor progression in diverse tumors through induction of epithelial mesenchymal transition (EMT); however, its role in glioblastoma is still uncertain. Therefore, we aimed to further define its role in vitro. Methods and results: The small interfering RNA (siRNA) technique was employed to knock down Snail expression in three glioblastoma cell lines (KNS42, U87, and U373). Specific inhibition of Snail expression increased E-cadherin expression but decreased vimentin expression in all cell lines. In addition, inhibition of the expression of Snail significantly reduced the proliferation, viability, invasion, and migration of glioblastoma cells as well as increased the number of cells in the G1 phase. Conclusions: Knockdown of Snail suppresses the proliferation, viability, migration, and invasion of cells as well as inhibits cell cycle progression by promoting EMT induction. The findings suggest that expression of this gene facilitates glioblastoma progression. Therefore, these results indicate the clinical significance of Snail for use as a potential therapeutic target for glioblastoma.

Keywords: Epithelial mesenchymal transition (EMT), glioblastoma, small interfering RNA (siRNA), Snail

Introduction

Glioblastomas are commonly occurring brain tumors and are difficult to treat. Despite conventional advancements in modern therapeutic methodologies such as surgery, chemotherapy, and radiation therapy, dramatic improvement in the survival of patients with glioblastomas has not yet been achieved [1]. These limitations of current therapeutic modalities in patients with glioblastoma are germane to remarkable resistance to cell death and a distinct invasive nature. Many researchers focused on various molecular and genetic mechanisms implicated in glioblastoma progression in order to overcome the major therapeutic obstacles during treatment [2-6].

Tumor progression is a complicated process occurring via a coordinated series of cellular and molecular processes [7]. The invasiveness and higher cell proliferation and viability are

important factors in tumor progression. One of the mechanisms that can explain tumor progression is the epithelial mesenchymal transition (EMT), which is the first step [8, 9] in epithelial tumor invasion since invasion and metastatic cascade of tumor cells are achieved by EMT induction [7, 10]. However, only a few studies have been performed on the role of the EMT-activating transcription factor in glioblastoma. Limited studies have been conducted due to the absence or low expression of E-cadherin. Therefore, it is yet to be ascertained whether EMT induction is directly responsible for the invasive mechanism of brain tumors as in epithelial tumors [11]. This is because EMT begins with the functional loss of E-cadherin. However, only a few studies have revealed the association between expression of the EMT-activating transcription factor and glioblastoma progression [3, 8, 9]. Consequently, the EMT-activating transcription factor should be studied to ascertain glioblastoma progression [3].

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Table 1. Sequences for two kinds of siRNAs

Gene	Sense	Antisense
Snail (1)	5'-GCGUGGGUUUUUGUAUCCA(dTdT)-3'	5'-GACUGUGAGUAAUGGCUGU(dTdT)-3'
Snail (2)	5'-GCGUGGGUUUUUGUAUCCA(dTdT)-3'	5'-GACUGUGAGUAAUGGCUGU(dTdT)-3'

Table 2. Primer sequences for reverse transcription and quantitative real-time polymerase chain reaction

Gene	Sense	Antisense
Snail	TTTCTGGTTCTGTGCCTCTGC	CCTGTAGCTCAAAGCAGCTGTA
E-Cadherin	TGCCAGAAAATGAAAAGG	GTGTATGTGGCAATGCGTTC
Vimentin	GAGAACTTTGCCGTTGAAGC	GCTTCCTGTAGTGGCAATC
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG

The Snail (also known as *SNAI1* or Snail1) gene was firstly characterized in *Drosophila melanogaster* [4, 12], and currently, more than 50 family members of the *SNAI1* gene have been isolated from metazoans [4, 13]. The expression of Snail is known to be associated with various physiological functions such as gastrulation, neural crest formation, and various developmental processes [4, 7, 13]. In addition, Snail is a well-known factor related to the regulation of the invasion in various carcinomas, such as breast tumors, gastric cancers, hepatocellular carcinomas, and colon cancers [4, 14-32]. Various studies that revealed an association between Snail expression and regulation of tumor progression were documented with carcinomas. Recently, Han *et al.* demonstrated the role of Snail in the progression of glioblastoma cells [4]. They demonstrated that Snail induced the proliferation and migration of glioblastoma cells. These results taken together to verify the role of Snail in EMT process regulation will provide new insights into the molecular mechanisms of glioblastoma progression. Our studies intended to evaluate the effect of the knockdown of Snail on the invasion, migration, viability and proliferation of cells as well as on the cell cycle of glioblastoma cells *in vitro*.

Materials and methods

Cell culture

Human pediatric glioblastoma cell line KNS42 (cordially provided by Japan Health Science Research Resources Bank) and adult glioblastoma cell lines U87 and U373 were obtained from the American Type Culture Collection

(Manassas, VA). Cells were maintained Dulbecco's modified Eagle's medium (DMEM) (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS, Invitrogen, NY), penicillin (50 U/ml), and streptomycin (50 U/ml). The cells were kept at 37°C in a humidified atmosphere of 5% CO₂ and the medium was replaced every 2-3 days.

Immunohistochemistry

The primary antibodies used in the immunohistochemical study of formalin-fixed, paraffin-embedded sections is Snail (polyclonal, Abcam, Cambridge, UK) using a 1:400 dilution. Immunohistochemistry was performed by the avidin-biotin peroxidase (ABC) method, based on our previous report [33].

Knockdown of Snail

To inhibit endogenous Snail gene in glioblastoma cell lines, small interfering RNAs (siRNA) were used. Scrambled siRNA (universal negative control siRNA), Snail-siRNA was purchased (Thermo Fisher Scientific, Waltham, USA or Bioneer, Daejeon, Korea). Two kinds of siRNAs to target one gene were described in **Table 1**. Cells were plated 40-60% confluency and transfected 25-50 nM scrambled siRNA, Snail-siRNA using Lipofectamin RNAiMAX reagent (Invitrogen) according to the manufacturer's instruction. Knockdown efficiency was confirmed by RT-qPCR. The transfected cells were used for various assays 48-72 h after transfection to allow effective knockdown of Snail was specified in each experiment.

Reverse transcription- polymerase chain reaction (PCR) and real-time reverse transcription-PCR

Total RNA was extracted from transfected cell lysates using PureLink RNA mini kit (Invitrogen). cDNA synthesis was performed using EcoDry

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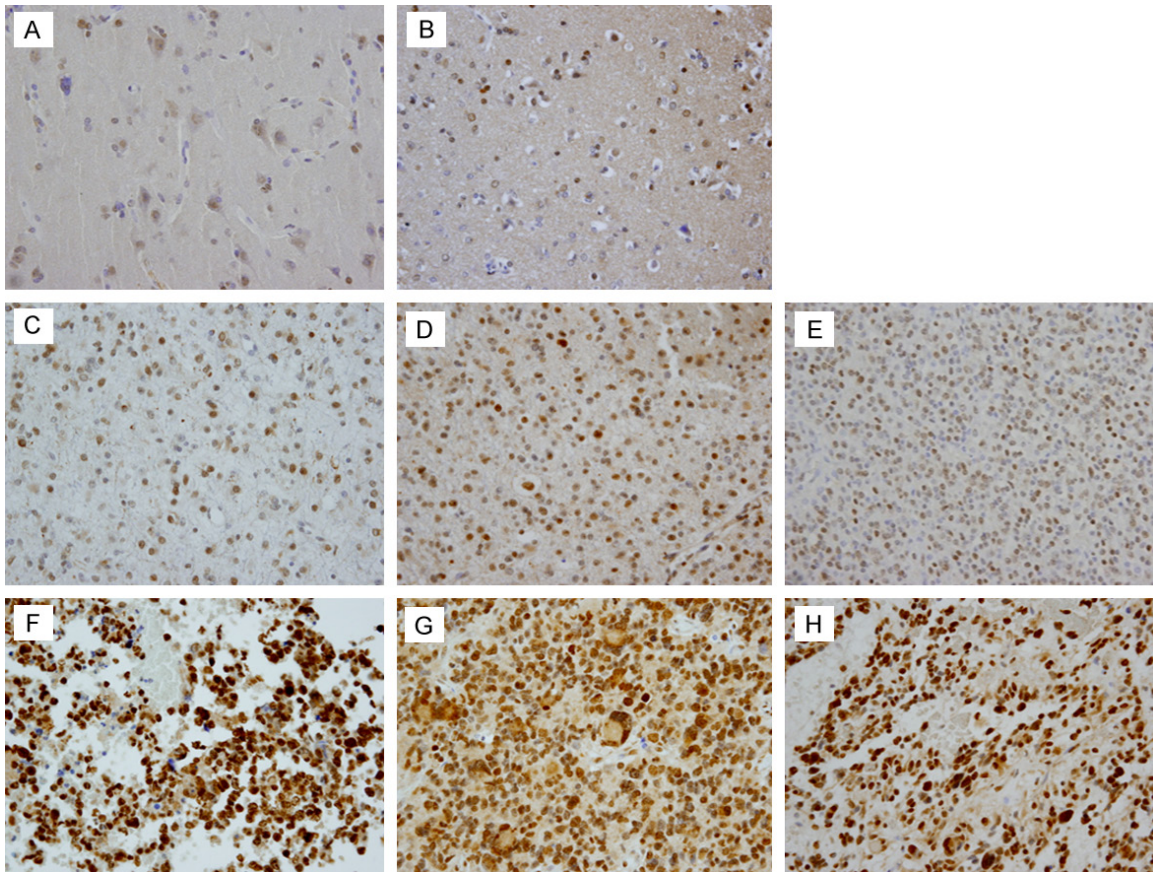


Figure 1. Expression of Snail in normal brain tissue and glioma. A, B. Weak expression of Snail presents in normal brain tissue. Neurons and a few glial cells mainly show very weak immunoreactivity to the Snail protein. C-E. Weak-to-moderate immunoreactivity to the Snail protein along the nuclear membrane was observed in low-grade glial tumors (WHO grade II). F-H. Glioblastomas exhibiting strong staining intensity for Snail protein (Snail immunostaining; original magnification, 400 \times).

Premix-Oligo (dT) (Clontech, Mountain View, CA, USA) from 1 μ g of total RNA. Real-time PCR (power SYBR Green, ABI, UK) analysis was performed using an ABI (Applied Biosystems, Foster City, CA) Prism 7000 Sequence Detector, according to the manufacturer's protocol with the appropriate primers (Table 2). The relative expression of each gene was normalized against GAPDH as described by the manual of ABI prism 7000 Sequence Detection System. These experiments were performed in triplicate and repeated in three independent experiments.

Western blot

Total protein extracts were isolated RIPA buffer (Thermo Scientific, Pierce Biotechnology, Pittsburgh, PA) and quantification was performed using the BCA Assay (Thermo Scientific).

The samples were prepared by adding the NuPage 4 \times LDS sample buffer (Invitrogen) and heating them at 70°C for 10 min. Equal amounts of the proteins (depending on experiment) were loaded on NuPage 4-12% gradient polyacrylamide gels (Invitrogen). The proteins were transferred i-Blot system (Invitrogen) using polyvinylidene fluoride (PVDF) membranes and then, the membrane was blocked for 1 hour at room temperature. The blots were then incubated in the primary antibodies; Snail (1:200; Cell Signaling, USA), E-cadherin (1:200; Abcam, Cambridge, UK) and Vimentin (1:1,000; Cell Signaling) either rocking for overnight at 4°C. HRP-conjugated secondary antibody was then incubated for 1 hour at room temperature. The membranes were developed using the enhanced chemiluminescence detection system (Invitrogen) and visual-

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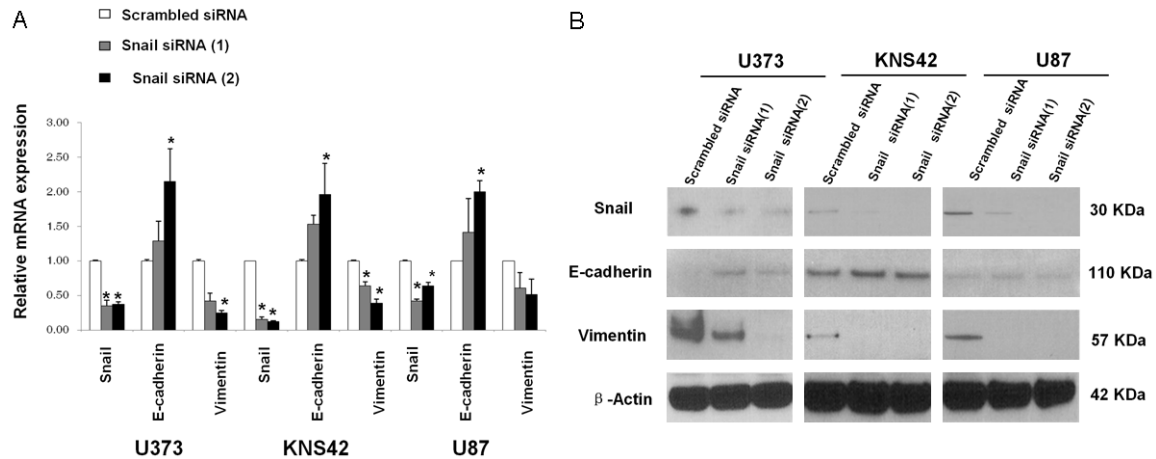


Figure 2. Relative mRNA and protein expression of Snail, E-cadherin, and vimentin after Snail inhibition with specific siRNA in adult and pediatric glioblastoma cell lines. U373, KNS-42, and U87 cells were transfected with scrambled siRNA and two Snail-specific siRNA. The total mRNA and protein were extracted from each transfected cell. A. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) analysis of the mRNA expression of Snail, E-cadherin, and vimentin in each cell line. B. Western blotting of the protein of Snail, E-cadherin, and vimentin expression in each cell line. The β -actin loading control is shown in the lower panel. Data shown correspond to one representative experiment out of the three performed. * $P < 0.05$ vs. scrambled siRNA control.

ized by exposing the autoradiographic film (Kodak, Rochester, NY).

CCK and BrdU assay

An equal density of cells (4×10^3) from transfection with scrambled siRNA and Snail-siRNA were seeded in 96-well plates and incubated for 24-72 hours. The viable cell number was evaluated using a CCK (cell counting kit; Dojindo, Kumamoto, Japan) assay. CCK were added and incubated for 2 hour and absorbance of each well was measured at 540 nm with a micro-ELISA reader (Molecular Devices; Sunnyvale, CA, USA).

Cell proliferation was determined using the Cell Proliferation ELISA BrdU kit (Roche Diagnostics GmbH; Mannheim, Germany). BrdU was added to the cells for 2 hours and the cells were treated according to the manufacture's protocol. The optical density at 370 nm was measured on an ELISA plate reader. Percentage of cell survival was determined by relative absorbance of cells transfected with Snail-siRNA versus cells scrambled-siRNA. All assays were performed in triplicates.

Cell cycle analysis

To assess the cell cycle phase distributions, a flow cytometry analysis of DNA contents of cells

were performed as described. The cells were transfected with scrambled siRNA and Snail-siRNA. Three days after transfection, the cells were harvested, washed in PBS, and fixed in 70% ethanol for 1 h at 4°C. Then, cells were washed in PBS and incubated with 0.5 mg/ml Rnase A (Sigma Aldrich) for 30 min at 37°C and DNA-binding dye 10 μ g/ml propidium iodine solution (Sigma Aldrich) in the dark. DNA contents of the samples were analyzed on a FACS Caliber flow cytometer (BD, Heidelberg, Germany) using a peak fluorescence gate to discriminate aggregates.

Invasion assay and wound healing assay

Invasion of tumor cells was analyzed using Cell Invasion Assay Kit (8 μ m pore size, Chemicon, MA, USA) according to the manufacture's protocol. After transfection, the cells (1×10^5 /well) were suspended in serum-free medium and plated on the upper chamber including mitomycin-C (10 μ g/ml, Sigma Aldrich). The lower chamber was filled with culture medium supplemented with 10% fetal bovine serum as the chemoattractant. After 24, and 72 h, the non-invading cells were removed gently by cotton swab. The cells that are invaded that are present on the lower side of the upper chamber were stained and air dried. The invaded cells were photographed and counted under the light microscope. For quantification, the stained

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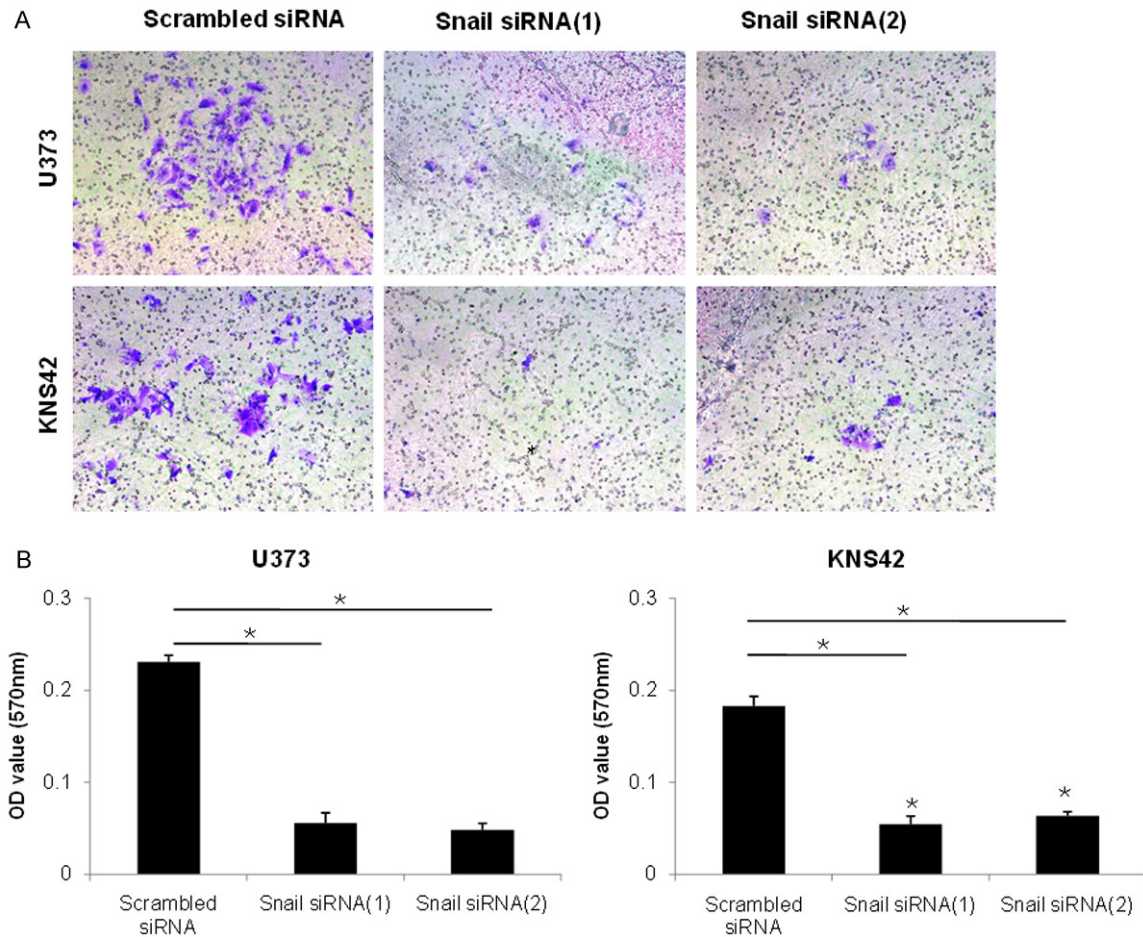


Figure 3. Cell invasion assay after Snail inhibition with specific siRNA in the U373 and KNS 42 cells. Snail siRNA gene knockdown significantly decreased cell invasion. A. Images displaying the bottom side of the filter inserts with cells that invaded through the filter pores. B. Columns graphs represent cell count analysis. Data shown correspond to one representative experiment out of the three performed. * $P < 0.01$ vs. scrambled siRNA control.

cells were dissolved with 10% acetic acid, and absorbance was measured at 560 nm. The assay was performed in triplicates.

The wound healing assay was performed with a CytoSelect™ 24-well cell invasion assay kit (Cell Biolabs, San Diego, CA) according to the manufacturer's protocol. Transfected cells were added to either side of the open ends at the top of the insert. When cells formed a monolayer, the insert were removed to generate a consistent 0.9 mm wound gap in the middle. To analyze of migration distance, the wound gap were observed for each time point. At different time period, cells were fixed and stained with methylene blue and photographed.

Statistical analysis

All statistical data were presented as mean \pm SD and GraphPad Prism software (GraphPad

Software, San Diego, CA) was used for statistical analyses. Statistical significance was determined by Student's *t* test. For comparison of more than three groups, one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons, was used. A result with a *P* value of < 0.01 and < 0.05 was considered statistically significant.

Results

Immunohistochemical analysis of Snail protein expression in glioma and normal brain tissue samples

The expression level of the Snail protein in normal brain tissue and in different WHO-grade paraffin-embedded glioma samples was determined using immunohistochemical staining (**Figure 1**). The expression of Snail was higher in

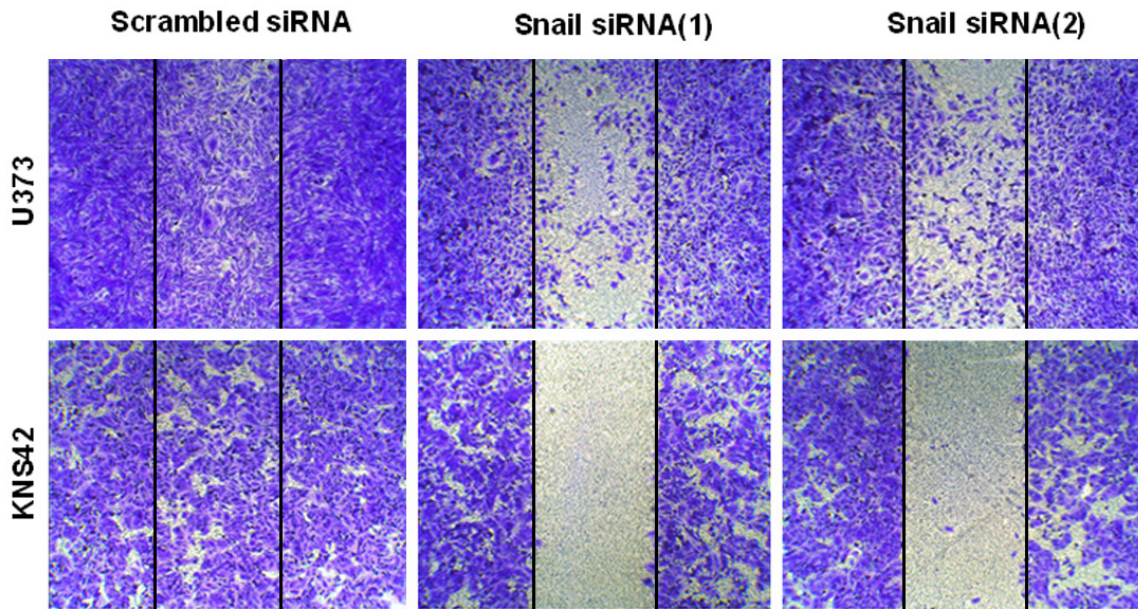


Figure 4. Cell migration assay after inhibition with specific siRNA in U373 and KNS42 cells. Snail siRNA gene knockdown significantly decreased cell migration as compared with universal scrambled siRNA. Representative images of decreased migration in U373 and KNS 42 cells by Snail siRNA gene knockdown in a wound-healing assay.

glioma samples than in normal brain tissue. Furthermore, highly malignant gliomas (WHO grade IV) showed higher expression of the Snail protein than low-grade gliomas (WHO grade II).

Effect of Snail knockdown on E-cadherin and vimentin expression

A molecular feature of EMT is the down regulation of E-cadherin expression and up regulation of a mesenchymal marker, such as vimentin. We compared the mRNA and protein expression of Snail, E-cadherin, and vimentin after Snail inhibition with specific siRNAs in adult and pediatric glioblastoma cell lines. The results are shown in **Figure 2**. Overall, the expression profiling change of E-cadherin and vimentin mRNA and protein after Snail inhibition with specific siRNA was consistent in three cell lines. Real-time quantitative polymerase chain reaction (PCR) demonstrated that knock-down of Snail expression in these three cell lines significantly increased E-cadherin mRNA expression as compared with that in the control group. These results were further simultaneously validated in protein expression. In contrast, the mRNA and protein expression of vimentin significantly decreased as compared to that in the control group after Snail specific siRNA treatment.

Effect of Snail knockdown on invasion and migration ability

In addition to increased expression of the mesenchymal marker after EMT induction, cells acquired a more motile and invasive phenotype. To evaluate these characteristics, we evaluated the invasion and migration ability based on Snail expression. Inhibition of Snail expression reduced the invasive ability of U373 and KNS42 glioblastoma cells (**Figure 3**). Also, Snail specific siRNA treatment significantly reduced the migration ability of U373 and KNS42 glioblastoma cells through EMT induction (**Figure 4**).

Snail knockdown on proliferation and viability

In addition to the well-characterized role of EMT in increased invasion and migration of tumor cells, induction of EMT is also found to be associated with diverse effects such as cell proliferation and viability. Many studies have shown that increased proliferation and viability of cells in EMT-induced cells, results of which are consistent those of the present study. The growth curves determined using the BrdU assay revealed that inhibition of Snail expression significantly reduced glioblastoma cell proliferation in all cell lines. Also, the CCK assay demonstrated the effect of Snail expression cell

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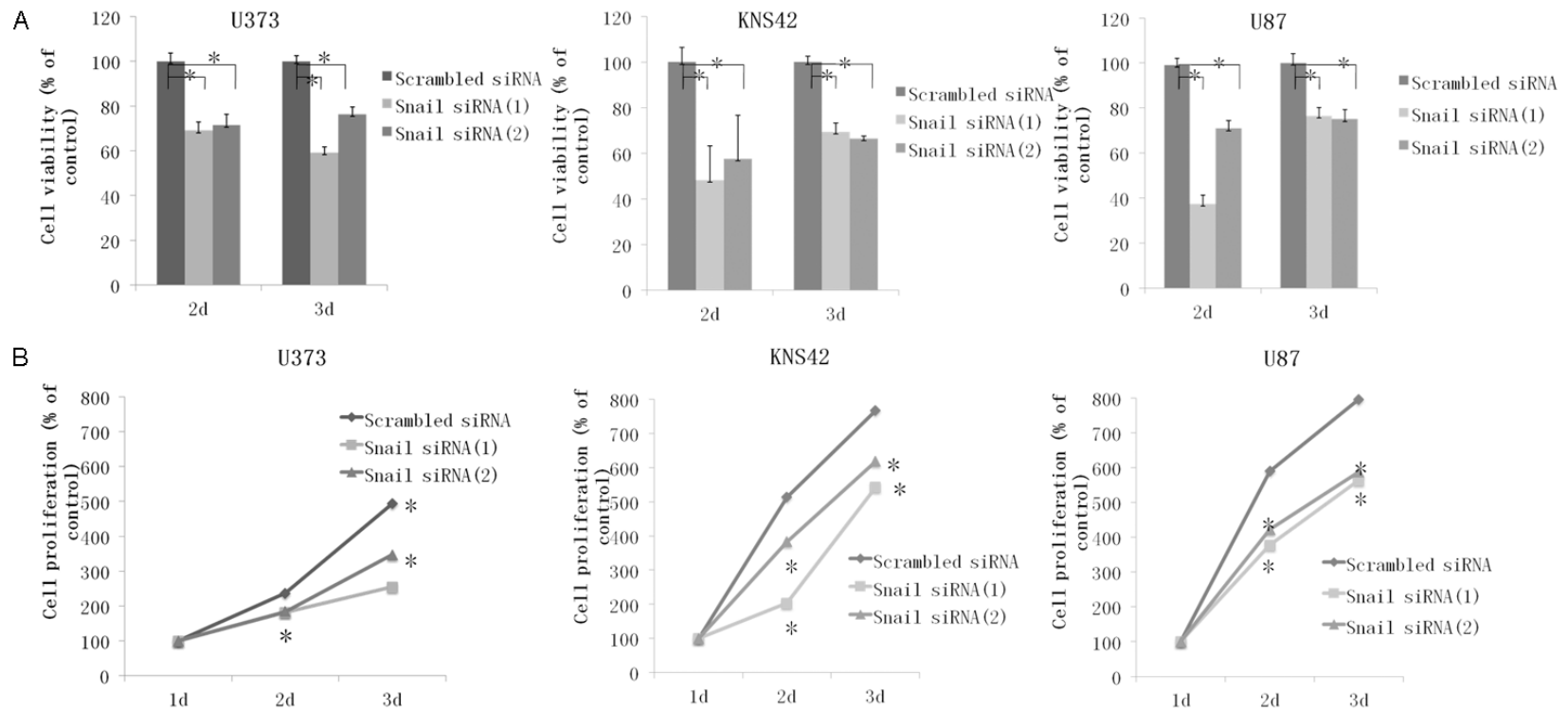


Figure 5. Cell proliferation and viability assay after Snail inhibition with specific siRNA for Snail. A. Column graphs showing the effects of Snail inhibition on the viability of U373, KNS 42, and U87 cells as compared with scrambled siRNA determined by a BrdU proliferation assay at days 1, 2, and 3 post siRNA transfection. B. Line graphs showing the effects of Snail inhibition on the proliferation of U373, KNS 42, and U87 cells as compared with scrambled siRNA, determined using a CCK assay at days 1, 2, and 3 post siRNA transfection. Data shown correspond to one representative experiment out of the three performed. * $P < 0.01$ vs scrambled siRNA control.

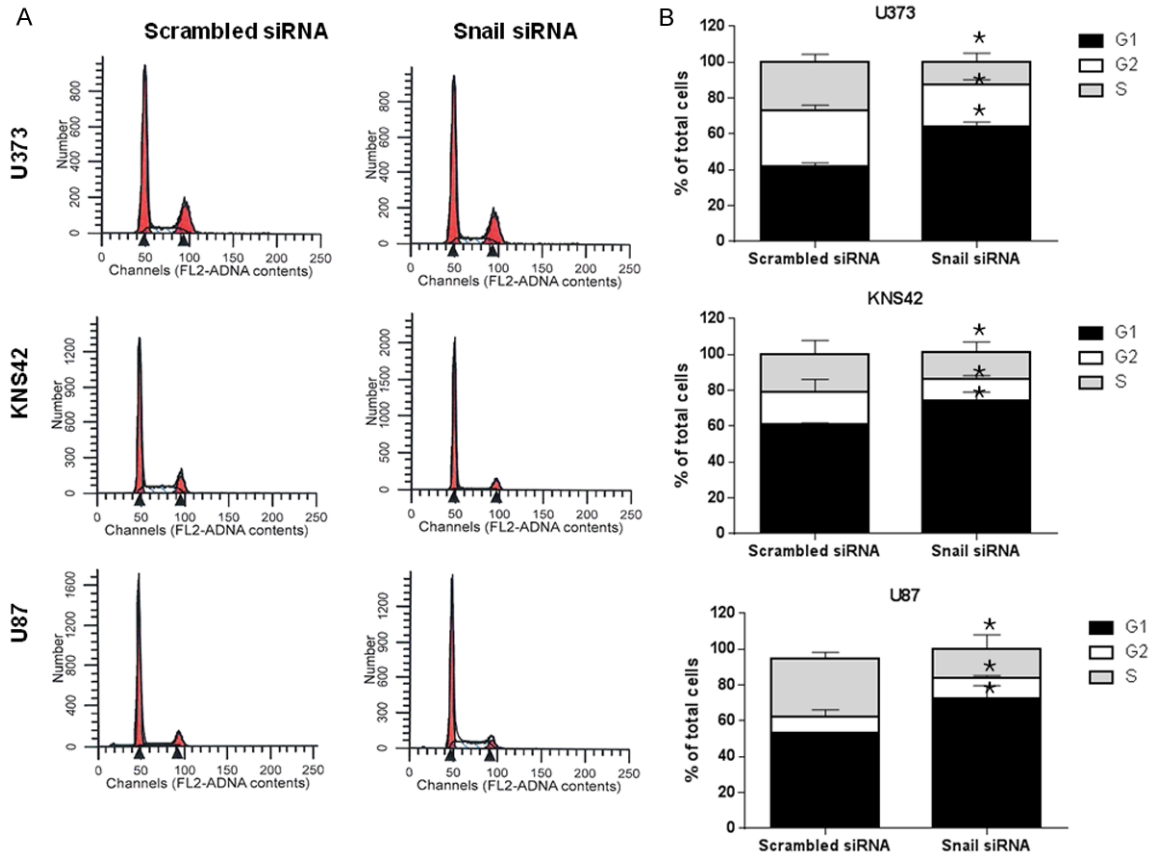


Figure 6. FACS analysis of propidium iodide-stained U373, KNS42, and U87 cells after Snail siRNA transfection. A. U373, KNS42, and U87 cells were grown for 3 days on either Snail siRNA transfection or scrambled siRNA. Fluorescence analysis of the DNA content (propidium iodide staining; FL2-A) was performed. Different cell distribution in the G1, S, and G2/M phases of the cell cycle was observed in U373, KNS42, and U87 cells, respectively. B. Quantitative assessment of the percentage of cellular population associated with each phase of the cell cycle. A significantly large number of G1 phase cells were observed in all cell lines after treatment of Snail siRNA. Data shown correspond to one representative experiment out of the three performed. * $P < 0.01$ vs. scrambled siRNA control.

viability reduction in Snail-specific siRNA transfected cells as compared to that for scrambled siRNA transfected cells. The results are shown in **Figure 5**.

Effect of Snail knockdown on cell cycle

To examine the effects of Snail expression on the cell cycle, we evaluated the cell cycle distribution using flow cytometry at 72 h post transfection in scrambled siRNA and Snail-specific siRNA-treated cells. As shown in **Figure 6**, the cells exhibited a significant increase in the fraction of cells in the G1 phase and a corresponding reduction in the fraction of cells in the S phase. This result is observed in all cell lines.

Discussion

The EMT process included acquisition of mesenchymal and migration properties through the

loss of cell-cell interaction and apico-basal polarity. These processes played a major role in the various developmental processes, tumor cell invasion, and metastasis [7, 10]. With regard to tumor cell invasion, these programs were used to explain the mechanism of invasive processes only in epithelial cancers. However, recent new results demonstrated that EMT-activating transcription factors, such as Snail, Slug, Twist, and ZEB2, enhanced the proliferation, invasion, and migration of glioblastoma cells as well as epithelial tumors [4-6, 34]. These studies proposed a correlation between glioblastoma progression and expression of EMT-activating transcription factors in brain tumors similar to that in epithelial tumors. Based on these findings, further studies are warranted for the role of EMT-activating transcription factors, especially those in glioblasto-

ma progression, in order to enhance the survival rate of patients with glioblastoma and broaden our understanding of glioblastoma pathogenesis. Many researchers may claim that the effect of EMT-activating transcription factors on the progression of glioblastoma was difficult to determine due to the lack of E-cadherin expression in normal brain tissue and in brain tumors. However, in addition to E-cadherin, various diverse genes and proteins exist that regulate EMT processes. Also, some experimental results demonstrated a definite effect of EMT-activating transcription factors on the progression of glioblastomas. Recently, a new experiment was conducted on the molecular classification of glioblastomas, and the results suggested that glioblastomas could be subclassified into four groups based on gene expression. The mesenchymal subtype showed high expression of EMT-related genes as compared to the other subtypes. Therefore, it is important to evaluate the effect of the Snail expression on glioblastoma progression, and the present study will serve to expand current knowledge on the new oncogenic role of Snail in glioblastoma pathogenesis.

Recently, various studies have described the role of Snail in cell proliferation, cell survival, and tumor cell invasion as well as on EMT induction. These recent studies suggested that aberrant expression of Snail is related to other clinicopathological findings, including patient survival in breast [15, 16, 26, 32], ovarian [15, 30, 31], hepatocellular [17, 19, 28, 35], and colorectal carcinomas [25, 36]. Analysis of these results clarified the influence of Snail on patient survival; however, no study has evaluated the association between patient survival and the aberrant expression of Snail in glioblastoma patients. Interestingly, in our previous study, we first confirmed that the level of Snail protein was higher in glioma samples than in normal brain tissue using immunohistochemistry. Further, we determined a statistically significant positive correlation between Snail protein expression and the WHO grade of glioma [33]. In that study, we tried to ascertain a significant correlation between Snail expression and various clinicopathological factors, and a positive correlation was determined between Snail expression and the WHO grade of glioma. Glioblastoma, WHO grade IV, showed the highest protein expression as compared to other

low-grade glial tumors. Based on these results, we hypothesize that Snail expression in glioblastoma is clinically significant. Therefore, to verify this assumption, we evaluated the effect of Snail gene expression on the proliferation, viability, cell cycle, and invasion and migration ability of glioblastoma cells. Knockdown of Snail gene expression showed increased E-cadherin and decreased vimentin expression at the transcriptional and protein level. Also, various functional studies demonstrated that inhibition of Snail expression reduced the proliferation and viability of glioblastoma cells and decreased the invasion and migration ability of glioblastoma cells. Inhibition of Snail gene expression was found to induce G1 arrest in cell cycle analysis. On comparison with our previous result, we ascertained that the association between Snail protein expression and the WHO grade of glial tumors may be due to the fact that increased Snail gene expression enhanced the proliferation, viability, and invasion ability of glioblastoma cells by promoting EMT induction. The glioblastoma was highly invasive and destructive, had higher proliferation activity, and was resistant to chemotherapy and radiotherapy. Although there were many factors associated with the proliferation, viability, and invasiveness of glioblastoma cells, we suggested that the expression of the Snail gene could be one of the regulation factors for determination of high proliferation activity and viability of glioblastoma cells. Also, we showed that Snail gene expression was significantly related to the highly invasive or destructive nature of glioblastomas. Therefore, our study revealed that Snail target therapy can be used for glioblastoma treatment and also suggested other possible target molecules for glioblastoma treatment.

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Disclosure of conflict of interest

None.

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