박사 학위 논문

Snail and ZEB2 play an oncogenic role in pediatric and adult glioblastoma cells through the induction of epithelial mesenchymal transition like process

교모세포종에서 상피중간엽 이행을 통한 Snail 및 ZEB2의 종양형성역할에 관한 연구

2015 년 2 월

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의학과 병리학 전공

명 재 경

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Snail and ZEB2 play an oncogenic role in pediatric and adult glioblastoma cells through the induction of epithelial mesenchymal transition like process

By Jae Kyung Myung

A thesis submitted to the Department of Pathology in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science(Pathology) at Seoul National University College of Medicine

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Approved by Thesis Committee:

Professor _____**Chairman**

Professor _____**Vice chairman**

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Abstract

Snail and ZEB2 play an oncogenic role in pediatric and adult glioblastoma cells through the induction of epithelial mesenchymal transition like process

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Background: The factors affecting adult and pediatric glioblastoma progression are of great clinical importance since dismal outcomes have been observed for glioblastoma patients. Here, we focused on Epithelial mesenchymal transition (EMT) related factors as a key molecular factor in determining clinical outcome. The Snail and ZEB2 genes are known to coordinate the regulation of tumor progression in diverse tumors through induction of EMT; however, its role in pediatric and adult 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 and ZEB2 expression in pediatric glioblastoma (KNS42) and adult glioblastoma cell lines (U87, and U373). Specific inhibition of both Snail and ZEB2 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 adult and pediatric glioblastoma cells as well as increased the number of cells in the G1 phase (G1 phase arrest). Also, inhibition of ZEB2 significantly reduced invasion and migration of both pediatric and adult glioblastoma cells. Interestingly, in pediatric glioblastoma, but not in adult glioblastoma cells, silencing of ZEB2 reduced cell proliferation, cell viability, and changed cell cycle progression of tumor cells, but these findings were not evoked in adult glioblastoma cells. Immunehistochemical staining for Snail and ZEB2 showed no difference between pediatric glioblastoma and adult glioblastoma. However, comparing to the immunoreactions of normal astrocytes and low grade glial tumors, glioblastoma cells showed relatively strong Snail and ZEB2 expression pattern. Also, mRNA for ZEB2 was higher in adult and pediatric glioblastoma cells than normal tissues.

Conclusions: Knockdown of Snail suppressed the proliferation, viability, migration, and invasion of adult and pediatric glioblastoma cells as well as inhibited cell cycle progression by promoting EMT like process. Also, inhibition of ZEB2 significantly reduced invasion and migration of both pediatric and adult glioblastoma cells. However, in pediatric glioblastoma cells, but not in adult glioblastoma cells, silencing of ZEB2

reduced cell proliferation, cell viability, and cell cycle progression of tumor cells. Our study demonstrated that Snail and ZEB2 play an oncogenic role in pediatric and adult glioblastoma by promoting EMT like process. However, unlike Snail, ZEB2 was shown to act differently on the cell proliferation, cell viability and cell cycle progression of pediatric and adult glioblastoma cells, which suggests that pediatric and adult glioblastoma may be different in certain biology and oncogenic process.

Keywords

Epithelial mesenchymal transition (EMT), Glioblastoma, small interfering RNA (siRNA), Snail, ZEB2

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Introduction

Glioblastomas are poorly differentiated astrocytic tumors, which are relatively common in adults, but rare in children [1]. Pediatric glioblastoma and adult glioblastoma share morphological characteristics, however, the molecular pathologies of these two entities are known to be different [2]. Unlike in adult glioblastoma, EGFR gene amplification and EGFRvIII mutations and deletions were rare in pediatric glioblastoma [3]. In addition, PDGFR-α gene amplification was low in pediatric glioblastoma compared to adult glioblastoma [4]. Although it is clear that different molecular pathologies may exist in these two types of glioblastomas, their clinical outcome is similar. They are clinically aggressive tumors and usually show resistance to chemotherapy, radiation therapy or both. Despite conventional advancements in modern therapeutic methodologies such as surgery, chemotherapy, and radiation therapy, dramatic improvement in the survival of pediatric and adult glioblastoma has not yet been achieved [5]. These limitations of current therapeutic modalities in pediatric and adult 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 pediatric and adult glioblastoma progression in order to overcome the major therapeutic obstacles during

treatment [6-10]. Tumor progression is a complicated process occurring via a coordinated series of cellular and molecular processes [11]. The invasiveness, high cell proliferation rate 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 in epithelial tumor invasion since invasion and metastatic cascade of tumor cells are achieved by EMT induction [11, 12]. Cancer cells acquire diverse abilities such as migration ability, treatment resistance, and stem cell properties through EMT induction. Tumor cells that acquire mesenchymal phenotypes through EMT induction readily invade surrounding tissues and result in aggressive behavior and poor clinical outcome [13, 14]. Recently, cancer stem cell theories have been identified as a major cause for recurrence and resistance to therapy [15]. In other words, cancer cells harboring stem cell properties can remain alive despite intensive treatment, giving rise to tumor recurrence and therapy failure. Interestingly, the fact that EMT induction enhanced cancer stem cell property was well known. Therefore, greater understanding of the role of EMT induction in pediatric and adult glioblastoma would be beneficial to develop optimal treatment strategies. However, only a few studies have been performed on the role of the EMT-activating transcription factor in adult glioblastoma. This is because

EMT process begins with the functional loss of E-cadherin, but the expression of E-cadherin was absent or low in brain tissue. Therefore, the use of the term “EMT” in central nervous system (CNS) tumors is controversial, because it is impossible to define the epithelial nature of the CNS. So, we are referring to “EMT-like processes” instead of “EMT”. Regardless of the controversy surrounding EMT mechanisms and E-cadherin expression in the CNS, a few studies until date define the significant role of EMT like process and EMT-activating transcription factors such as Snail, Slug, ZEB2 in the oncogenesis of adult glioblastoma cells. In other words, it has been reported that EMT-activating transcription factors such as SNAI1 (Snail), SNAI2 (Slug) and ZEB2 induce aggressive behavior in adult glioblastoma cells [8, 16-19], suggesting that EMT like process is one of the key mechanisms in regulating aggressive behavior and invasive properties of adult glioblastoma cells. However, regardless of outstanding achievement of a few studies about the important role of EMT like process in glial tumors, only a few studies have revealed the association between expression of the EMT-activating transcription factor and glioblastoma progression [7, 13, 14]. Consequently, the EMT-activating transcription factor should be studied to ascertain pediatric and adult glioblastoma progression.

Among several EMT-activating transcription factors, we focused on Snail and ZEB2. The Snail (also known as SNAI1 *or* Snail1) gene was firstly characterized in *Drosophila melanogaster* [8, 20], and currently, more than 50 family members of the SNAI1 gene have been isolated from metazoans [8, 21]. The expression of Snail is known to be associated with various physiological functions such as gastrulation, neural crest formation, and various developmental processes [8, 11, 21]. 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 [8, 22-40]. 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 adult glioblastoma cells [8]. They demonstrated that Snail induced the proliferation and migration of adult glioblastoma cells.

ZEB2 (also known as SIP1: Smad-interacting protein 1) belongs to the zinc finger E-box binding protein (ZEB) family and was characterized for the first time from a *Drosophila* complementary DNA (cDNA) expression library [10, 41]. Previous studies revealed that ZEB2 expression is closely related to aggressive clinicopathological features, including histological grade and overall survival in various tumors. These results were mainly

observed in epithelial tumors such as ovarian carcinoma, gastric cancer, pancreatic cancer, and squamous cell carcinoma [10, 17, 22, 23, 39, 42-46]. According to a recent study by Xia *et al,* ZEB2 was expressed in adult glioblastoma cells, and it regulated the migration and invasive ability of glioblastoma cells [10]. These results taken together to verify the role of Snail and ZEB2 in pediatric and adult glioblastoma will provide new insights into the molecular mechanisms of glioblastoma progression. Our studies intended to evaluate the effect of the knockdown of Snail and ZEB2 on the invasion, migration, viability and proliferation of cells as well as on the cell cycle of pediatric and adult glioblastoma cells in vitro. On the basis of these results, our study has generated new insights into the molecular mechanisms of glioblastoma progression.

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℃ in a humidified atmosphere of 5% CO_2 and the medium was replaced every 2–3 days.

Patients

104 patients with glial tumors (60 adult glioblastoma, 40 pediatric glioblastoma, 2 adult diffuse astrocytoma, 2 pediatric pilocytic astrocytoma) were included in the present study. Immunohistochemistry for Snail and ZEB2 protein were performed with these patient samples after production of tissue microarray. Fresh frozen tissues of adult glioblastomas (n=6), pediatric glioblastomas (n=8) and non-neoplastic brain tissue (control brain tissue, n=2) were used for real–time polymerase chain reaction (PCR)

analysis to detect ZEB2 mRNA.

Immunohistochemistry

Tissue microarray was generated by obtaining a 2-millimeter diameter core from each donor block and transferring them to a recipient block using a trephine apparatus (Superbiochips laboratories, Seoul, Republic of Korea). Four micrometer thick sections were obtained from the TMA blocks and they were stained with Snail polyclonal antibody (Abcam, Cambridge, UK) using a 1:400 dilution and ZEB2 polyclonal antibody (Abcam, Cambridge, UK) using a 1:200 dilution. Immunohistochemical staining was carried out using the standard avidin-biotin-peroxidase complex (ABC) method. After deparaffinization, endogenous peroxidase activity was quenched by immersing the sections in absolute alcohol (100%) containing 0.3% H2O2 for 15 minutes. The sections were immersed in 10nM citric acid buffer (pH 6.0) and heated in a conventional microwave to unmask the antigens. Subsequent detergent treatment was done by immersing the slides in phosphate-buffered saline for 15minutes. The sections were then treated with normal serum for 30 minutes to reduce background staining. After this, treatment of primary antibodies was done at room temperature for 1 hour followed by

treatment with biotinylated secondary antibodies (biotinylated anti-mouse immunoglobulin antibody, DAKO Co., Ltd., Kyoto, Japan) for 25 minutes. Subsequently, detergent treatment was done using phosphate-buffered saline for 15 min. The reaction products were allowed to develop by immersing the sections in 3.3`-diaminobenzidine tetrahydrochloride solution containing 0.03% H2O2. Nuclei were counterstained using Meyer's hematoxylin for 3 minutes.

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Knockdown of Snail and ZEB2 expression

To inhibit endogenous Snail and ZEB2 gene in pediatric and adult glioblastoma cell lines, small interfering RNAs (siRNA) were used. Snail-siRNA, ZEB2 siRNA and "scrambled siRNA (negative control)" were purchased (Thermo Fisher Scientific, Waltham, USA or Bioneer, Daejeon, Korea). Two kinds of siRNAs to target one gene were described in Table 1. Routinely, cells were transfected with 25–50 nM siRNAs for Snail, ZEB2 and scrambled siRNA by using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer`s instructions. Knockdown efficiency was measured at the mRNA level by quantitative RT-qPCR. The transfected cells were used for various assays such as western blot and CCK-8 and BrdU assays, 48–72 h after

transfection

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’
ZEB2 (1)	5-GAACAGACAGGCUUACUUA(dTdT)-3’	5’-GAAGCUACGUACUUUAAUA(dTdT)-3’
ZEB2 (2)	5-CACUAGACUUCAAUGACUA(dTdT)-3’	5’-UAGUCAUUGAAGUCUAGUG(dTdT)-3’

RNA extraction and RT-qPCR

Total RNA was extracted from transfected cell lysates and fresh frozen tissues using TRIzol reagent (Invitrogen) and PureLink RNA mini kit (Invitrogen) according to the manufacturer`s protocols. cDNA was subsequently synthesized using EcoDry Premix-Oligo (dT) (Clontech, Mountain View, CA) from 1 μg 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, by using gene-specific primers (Table 2). Tumor samples were analyzed in triplicates and gene expression levels were normalized against that of GAPDH.

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

Gene	Sense	Antisense
Snail	TTTCTGGTTCTGTGTCCTCTGC	CCTGTAGCTCAAAGCAGCTGTA
ZEB2	GCCGTTAGCTCCCAACAGTA	AGCCTGAGAGGAGGATCACA
E-Cadherin	TGCCCAGAAAATGAAAAAGG	GTGTATGTGGCAATGCGTTC
Vimentin	GAGAACTTTGCCGTTGAAGC	GCTTCCTGTAGGTGGCAATC
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG

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× LDS sample buffer (Invitrogen) and heating them at 70℃ for 10 min. Equal amounts of the proteins (depending on experiment) were loaded on NuPage 4-12% gradient polyacrylamide gels (Invitrogen). The proteins were transfered i-Blot system (Invitrogen) using polyvinylidine 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) ZEB2 (1:1,000; ProSci, CA), E-Cadherin (1:200; Abcam, Cambridge, UK) and Vimentin (1:1,000; Cell Signaling) either rocking for overnight at 4℃. 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 visualized by exposing the autoradiographic film (Kodak, Rochester, NY).

CCK and BrdU assay

Cell viability was determined using CCK-8 (Dojindo Laboratories, Japan). About 4 × 10^3 cells were seeded in 96-well culture plates and transfected with scrambled siRNA and specific siRNA. Following 24–72 h of incubation, CCK-8 reagent was added and incubated for 2 h; then, the absorbance of each well was measured at 540 nm with a micro-ELISA reader (Molecular Devices, Sunnyvale, CA).

Proliferation of transfected cells was determined using a BrdU proliferation ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany). BrdU was added to the cells, the mixture was incubated for 2 h, and the cells were treated according to the manufacturer's instructions before measuring the optical density at 370 nm by using an ELISA plate reader. Percentage of cell survival was determined on the basis of the relative absorbance of transfected cells with specific siRNA versus cells with scrambled siRNA. All assays were performed in triplicates.

FACS analysis

To assess cell cycle distribution, flow cytometry was performed. The cells were transfected with scrambled siRNA, Snail and *ZEB2*-specific siRNA. Three days after transfection, the cells were harvested, washed in phosphate-buffered saline (PBS), and

fixed in 70% ethanol for 1 h at 4℃. Then, the cells were washed with PBS, rehydrated, and resuspended with 0.5 mg/ml RNase A (Sigma-Aldrich) in PBS buffer for 30 min at 37℃. The cells were stained with 10 μg/ml propidium iodide solution (Sigma-Aldrich) in the dark. The stained cells were then analyzed for DNA content with a flow cytometer (FACS Caliber, BD, Heidelberg, Germany).

Invasion assay and wound healing assay

Invasion of tumor cells was analyzed using Cell Invasion Assay Kit (8 μm 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 (10μg/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 noninvading 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 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^{TM} 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 ± 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 comparison, was used. A result with a p value of < 0.01 and <0.05 was considered statistically significant.

Results

Downregulation of Snail and ZEB2 expression reduced mesenchymal features in adult and pediatric glioblastoma cells

Molecular features of EMT are the down regulation of E-cadherin expression and up regulation of mesenchymal markers, such as vimentin. We compared the mRNA and protein expression of E-cadherin, and vimentin after Snail and ZEB2 inhibition with specific siRNAs in adult and pediatric glioblastoma cell lines. Inhibition of Snail and ZEB2 significantly increased the expression of E-cadherin at both mRNA and protein levels in adult glioblastoma and pediatric glioblastoma cells. In contrast, vimentin mRNA and protein levels were reduced in both adult and pediatric glioblastoma cells after inhibition of Snail and ZEB2 expression (Fig. 1 and Fig. 2). Snail and ZEB2 are well-known EMT-activating transcription factors through the regulation of E-cadherin expression. Based on these changes in the expression of EMT markers after down-regulation of Snail and ZEB2 expressions, we confirmed that mesenchymal features were reduced in both adult and pediatric glioblastoma cells.

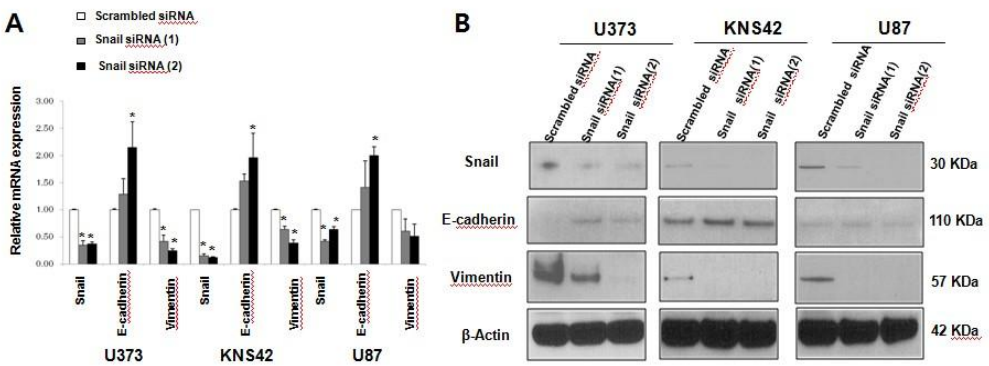


Figure 1. Relative mRNA and protein expression of Snail, E-cadherin, and vimentin after Snail inhibition with specific siRNA in adult and pediatric glioblastoma cell lines. U373, KNS-42, and U87 cells were transfected with scrambled siRNA and two Snail-specific siRNA. The total mRNA and protein were extracted from each transfected cell. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) analysis of the mRNA expression of Snail, E-cadherin, and vimentin in each cell line (A). Western blotting of the protein of Snail, E-cadherin, and vimentin expression in each cell line (B). 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

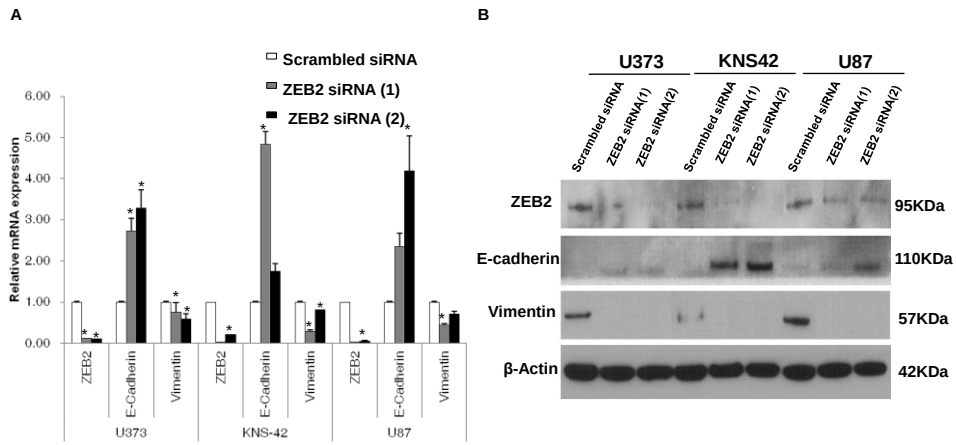
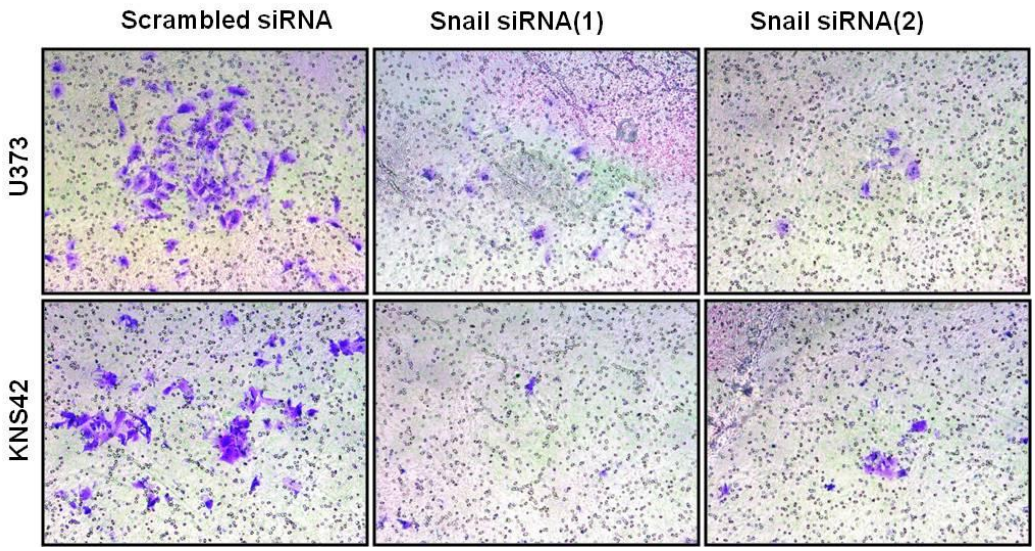


Figure 2. . Comparative analysis of mRNA and protein expression for EMT markers after *ZEB2* siRNA and scrambled siRNA transfection in adult and pediatric glioblastoma cell lines. Relative mRNA expression of ZEB2, E-cadherin, and vimentin after transfection of scrambled siRNA and two *ZEB2*-specific siRNAs (A). Protein expression levels of ZEB2, E-cadherin, and vimentin after transfection of scrambled siRNA and two *ZEB2*-specific siRNAs (B). β-actin loading control is shown in the bottom panel. *$p < 0.05$ *vs.* scrambled siRNA control

Inhibition of Snail and ZEB2 reduced the invasive ability and migration of glioblastoma cells

In addition to increased expression of the mesenchymal marker after EMT like process induction, cells acquired more motile and invasive phenotype. To evaluate these characteristics, we evaluated the invasion and migration ability based on Snail and ZEB2 expression. Inhibition of Snail expression reduced the invasive ability and migration ability of U373 and KNS42 glioblastoma cells (Fig. 3 and Fig. 4). Also, the inhibition of ZEB2 expression reduced the invasive ability of all of these cells (U373, U87 and KNS42 cells) (Fig. 5 and Fig. 6).


Scrambled siRNA
Snail siRNA(1)
Snail siRNA(2)
U373
KNS42

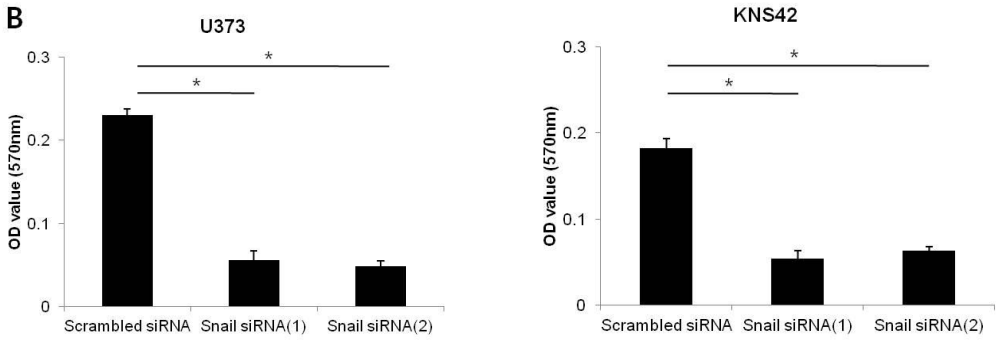

B
U373
KNS42
OD value (570nm)
0.3
0.2
0.1
0
*
*
Scrambled siRNA
Snail siRNA(1)
Snail siRNA(2)

Figure 3, Cell invasion assay after Snail inhibition with specific siRNA in the U373 and KNS42 cells. Snail siRNA gene knockdown significantly decreased cell invasion. Images displaying the bottom side of the filter inserts with cells that invaded through the filter pores (A). Columns graph represent cell count analysis (B). Data shown correspond to one representative experiment out of the three performed. *$p < 0.01$ *vs.* scrambled siRNA control

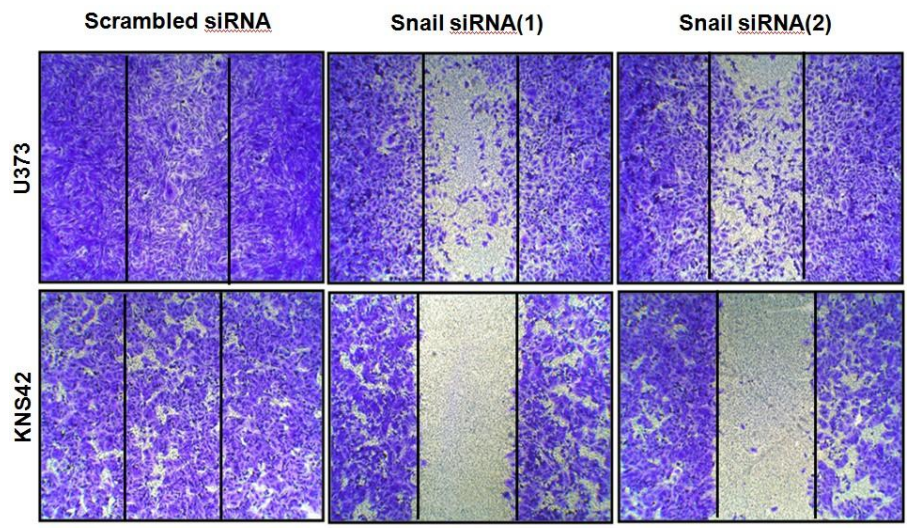
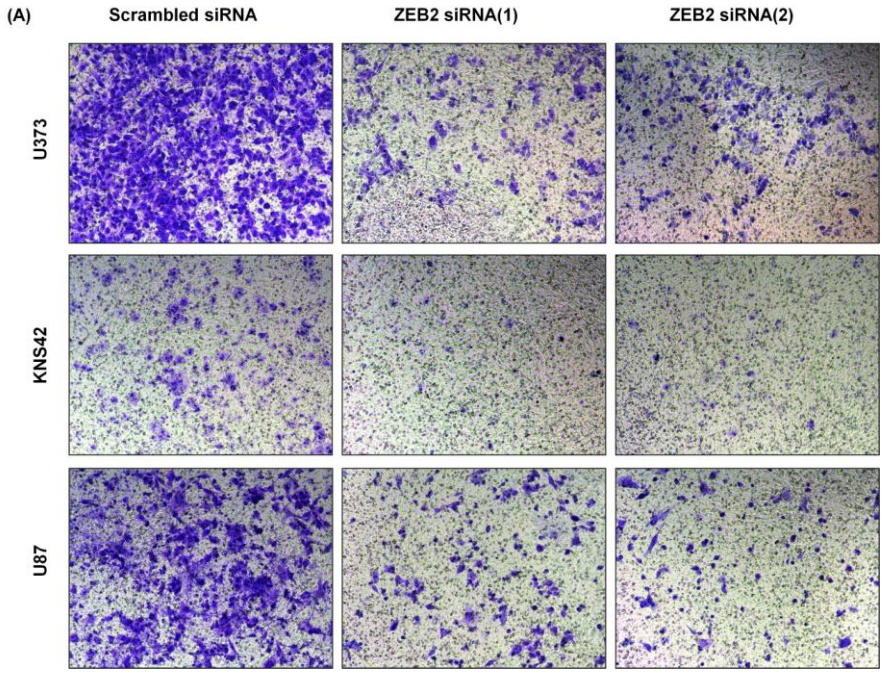


Figure 4. Cell migration assay after inhibition with specific siRNA in adult and pediatric glioblastoma cells. Snail siRNA gene knockdown significantly decreased cell migration of adult and pediatric glioblastoma cells as compared with universal scrambled siRNA. Representative images of decreased migration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


(A)
Scrambled siRNA
ZEB2 siRNA(1)
ZEB2 siRNA(2)
U373
KNS42
U87

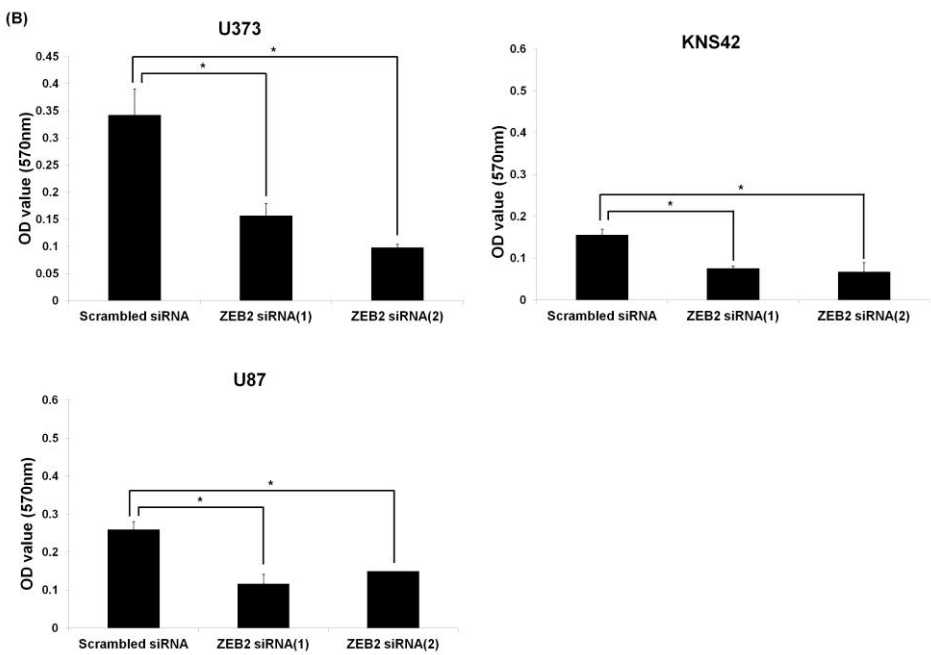

(B)
U373
KNS42
U87
OD value (570nm)
Scrambled siRNA
ZEB2 siRNA(1)
ZEB2 siRNA(2)
*

Figure 5. Invasion assay after ZEB2 siRNA and scrambled siRNA transfection in adult glioblastoma and pediatric glioblastoma cell lines. Images displaying the cells that invaded through the filter pores after transfection of scrambled siRNA and two ZEB2-specific siRNAs (A). Column graph summarizing the average total number of invaded cells quantified using imaging software (B). *$P < 0.05$, vs. scrambled siRNA (control).

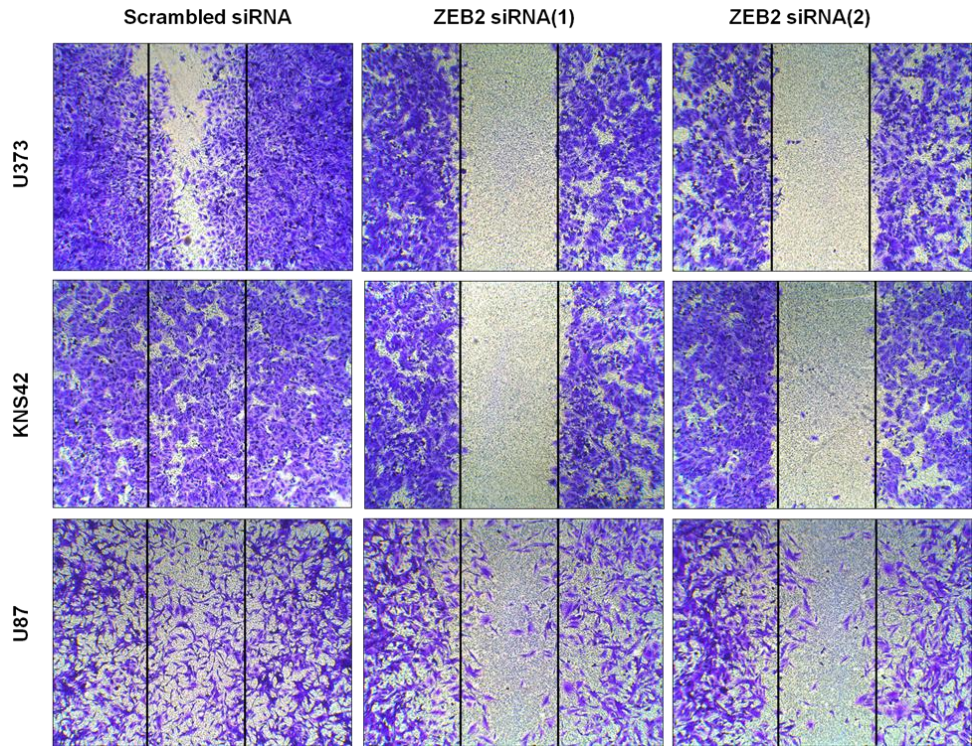


Figure 6. Cell migration assay of adult glioblastoma and pediatric glioblastoma cell lines in response to ZEB2 siRNA and scrambled siRNA transfection. Cells were cultured and wound lines were introduced using a microtip. After incubation, gap closure was increased in cells transfected with scrambled siRNA compared to those transfected with ZEB2-specific siRNA. These results were common in pediatric glioblastoma and adult glioblastoma cells.

Inhibition of Snail expression reduced the proliferation and viability of adult and pediatric glioblastoma cell lines

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 on cell survivals, 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 adult and pediatric glioblastoma cell lines. Also, the CCK assay demonstrated the effect of Snail expression on cell viability reduction in Snail-specific siRNA transfected cells as compared to that for scrambled siRNA transfected cells. The results are shown in Figure 7.

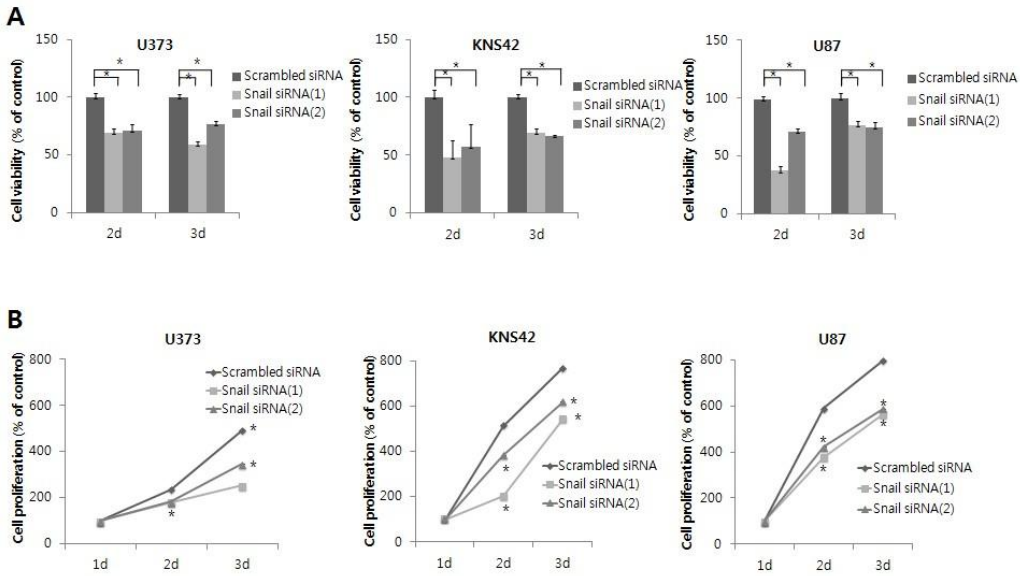


Figure 7. Cell proliferation and viability assay after Snail inhibition with specific siRNA for Snail. Column graphs showed the effects of Snail inhibition on the viability of U373, KNS 42, and U87 cells as compared with scrambled siRNA, determined using a CCK assay at days 1, 2, and 3 post siRNA transfection (A). Line graph showed the effects of Snail inhibition on the proliferation 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). Data shown correspond to one representative experiment out of the three performed. *$p < 0.01$ *vs* scrambled siRNA control.

Inhibition of ZEB2 expression reduced the proliferation and viability of pediatric glioblastoma cells

To determine the effect of ZEB2 on the proliferation and viability of glioblastoma cells, we performed proliferation and viability assays. Inhibition of ZEB2 expression significantly reduced the proliferation and viability of KNS 42 cell, while the proliferation and viability of U87 and U373 cells were not affected. Therefore, the effect of regulation of ZEB2 expression on cell proliferation and viability was different in pediatric glioblastoma and adult glioblastoma cells, and only pediatric glioblastoma cells were influenced by ZEB2 regulation. The results are shown in Figure 8.

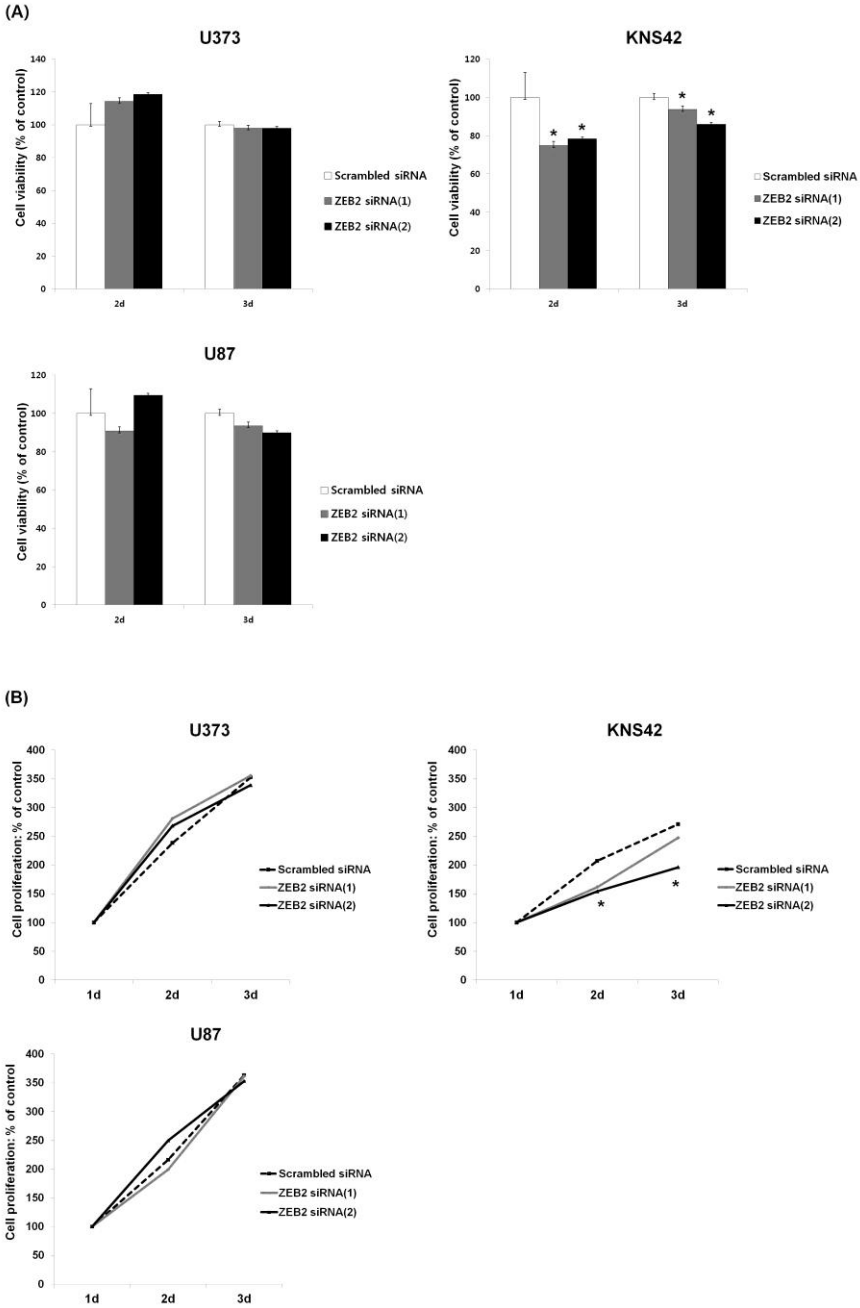

(A)
U373
KNS42
U87
Cell viability (% of control)
Scrambled siRNA
ZEB2 siRNA(1)
ZEB2 siRNA(2)
2d
3d
(B)
Cell proliferation: % of control
1d
2d
3d

Figure 8. Cell proliferation and viability assays after ZEB2 siRNA and scrambled siRNA transfection in adult and pediatric glioblastoma cell lines. Column graphs showed the effects of ZEB2 inhibition on the viability of adult and pediatric glioblastoma cell lines (A). Interestingly, decreased cell viability was only observed in pediatric glioblastoma cell after ZEB2 inhibition. *P < 0.05, vs. scrambled siRNA (control). Line graphs showed the effects of ZEB2 inhibition on the proliferation of adult and pediatric glioblastoma cell lines (B). Considerably decreased proliferation of cells after ZEB2 inhibition was only observed in pediatric glioblastoma cells. *P < 0.05, vs. scrambled siRNA (control).

Inhibition of Snail gene expression induced G1-phase cell cycle arrest in adult and pediatric glioblastoma cells

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 9, the cells exhibited a significant increase in the fraction of cells in the G1 phase (G1 phase arrest) and a corresponding reduction in the fraction of cells in the S and G2/M phase. This result is commonly observed in pediatric and adult glioblastoma cells

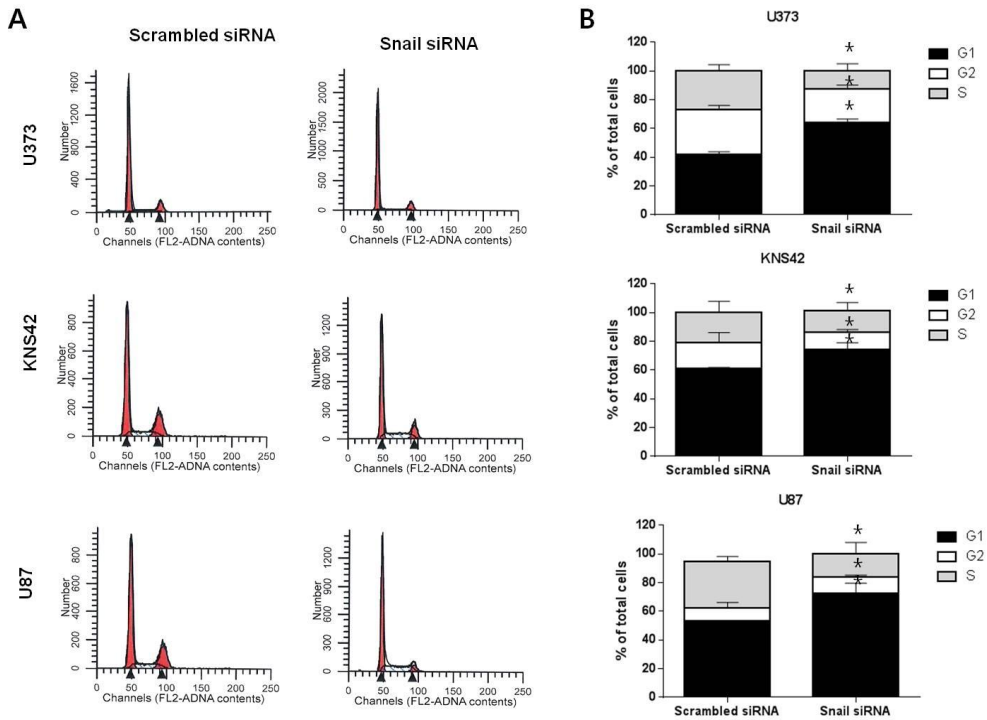


Figure 9. FACS analysis of propidium iodide-stained U373, KNS42, and U87 cells after Snail siRNA tranfection. U373, KNS42, and U87 cells were grown for 3 days on either Snail siRNA transfection or scrambled siRNA (A). Fluorescence analysis of the DNA content (propidium iodide staining; FL2-A) was performed. Different cell distribution in the G1, S, and G2/M phases of the cell cycle was observed in pediatric and adult glioblastoma cells, respectively. Quantitative assessment of the percentage of cellular population was associated with each phase of the cell cycle (B). A significantly large number of G1 phase cells (G1 phase arrest) 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

Inhibition of ZEB2 gene expression induced G1-phase cell cycle arrest in pediatric glioblastoma cells

To determine the effect of ZEB2 on cell cycle distribution, we performed cell cycle analyses. Different distributions in the G1, S, and G2/M phases of the cell cycle were observed in adult and pediatric glioblastoma cells. Cell cycle analysis demonstrated that the inhibition of ZEB2 expression significantly increased the proportion of cells in G1 (G1 phase arrest), while the proportion of cells in G2/S was significantly reduced. This result was observed only for KNS42 cells, while U87 and U373 cells did not reveal significant changes in the proportion of cells in G1 and G2/S phases (Fig. 10). Similar to the results obtained in the proliferation and viability assays, the effect of inhibition of ZEB2 expression had different effects on pediatric and adult glioblastoma cells. These results indicate that ZEB2 downregulation induced mesenchymal changes in both pediatric and adult glioblastoma cells, while cell proliferation, viability, and cell cycle progression were differently affected in adult and pediatric glioblastoma cells

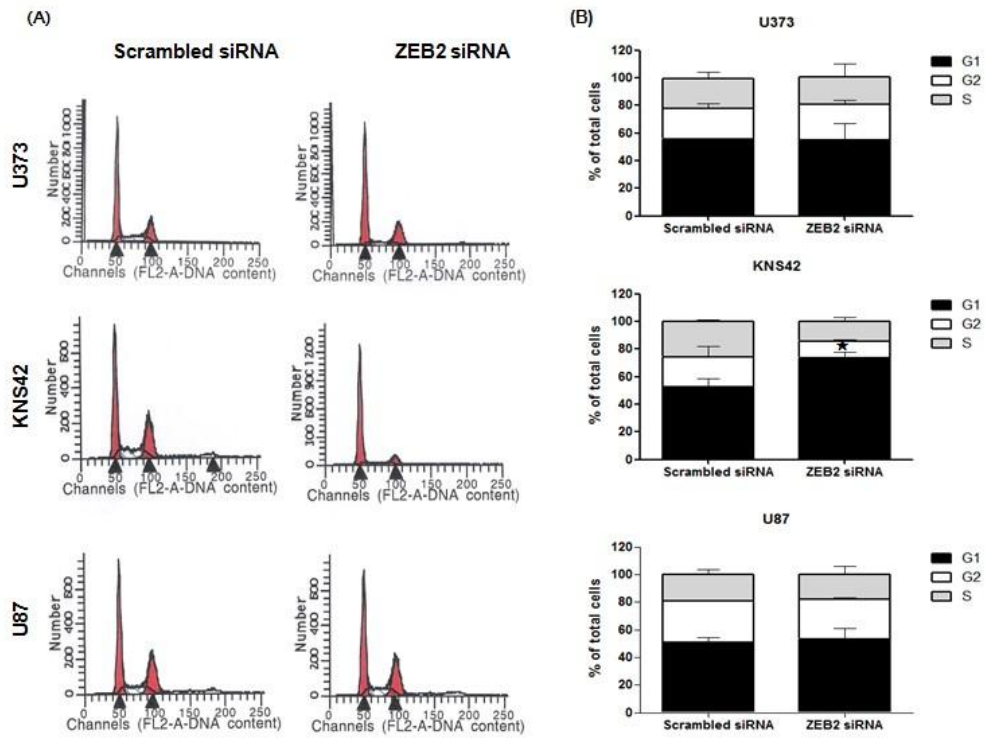


Figure 10. FACS analysis after ZEB2 siRNA and scrambled siRNA transfection in adult and pediatric glioblastoma cell lines. DNA content analysis (propidium iodide staining; FL2-A) by flow cytometry revealed different cell cycle distributions in G1, S, and G2/M phases for adult and pediatric glioblastoma cell lines (A). Considerably increased proportions of G1 phase cells (G1 phase arrest) were only observed in pediatric glioblastoma cell. Quantification of cell cycle population (in percentage) (B). *P < 0.05 vs. scrambled siRNA (control).

Immunohistochemical analysis of Snail and ZEB2 protein expression in glioma and normal brain tissue samples

The expression level of the Snail protein in normal brain tissue and different WHO-grade formalin fixed paraffin-embedded glioma samples was determined using immunohistochemical staining (Fig. 11). The expression of Snail was higher in glioma samples than in normal brain tissue. Furthermore, adult and pediatric glioblastomas showed higher expression of the Snail protein than low-grade gliomas (2 pilocytic astrocytoma, 2 diffuse astrocytoma). There was no apparent difference in Snail expression in pediatric glioblastoma and adult glioblastoma.

In normal brain parenchyma, astrocytes and neurons were weakly immunoreactive for ZEB2. Comparing to this, pediatric glioblastoma and adult glioblastoma tissue sections showed strong staining patterns for ZEB2. However, there was no apparent difference in ZEB2 expression in pediatric glioblastoma and adult glioblastoma like Snail staining. In addition, we evaluated the expression levels of ZEB2 in low-grade glioma and highly malignant gliomas (WHO grade IV). Both adult (diffuse astrocytoma) and pediatric (pilocytic astrocytoma) low-grade glioma tested positive for ZEB2 along the nuclear

membrane. Although the intensity was faint, the expression levels were greater than normal astrocyte. In comparison, glioblastoma sections exhibited more pronounced staining patterns for ZEB2 than low-grade pediatric and adult glioma sections (Fig. 12).

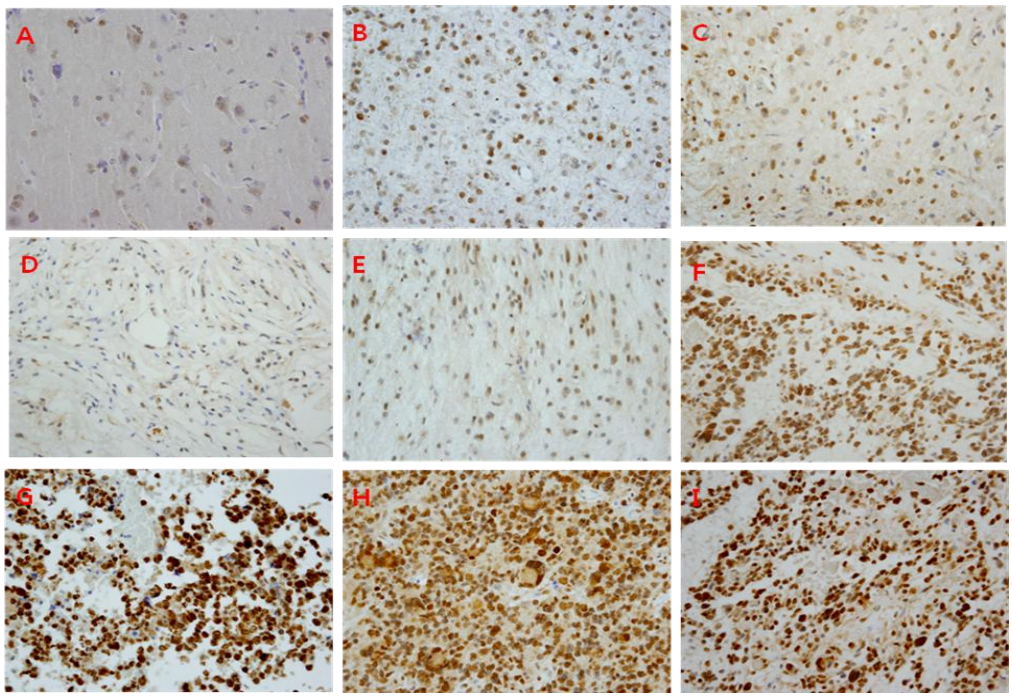


Figure 11. Immunohistochemical study of normal brain tissue, adult low-grade glioma, pediatric low-grade glioma, adult glioblastoma, and pediatric glioblastoma. Weak immunoreaction for Snail was localized on astrocytes and neurons in normal brain tissue (A). Adult low-grade glioma cases showed very weak expression of Snail (B, C). Pediatric low-grade glioma cases also showed weak immunoreactions for Snail (D, E). High levels of ZEB2 expression were observed for adult glioblastoma (F, G) and pediatric glioblastoma cases (H, I). (ZEB2 immunostaining; original magnification, 400×).

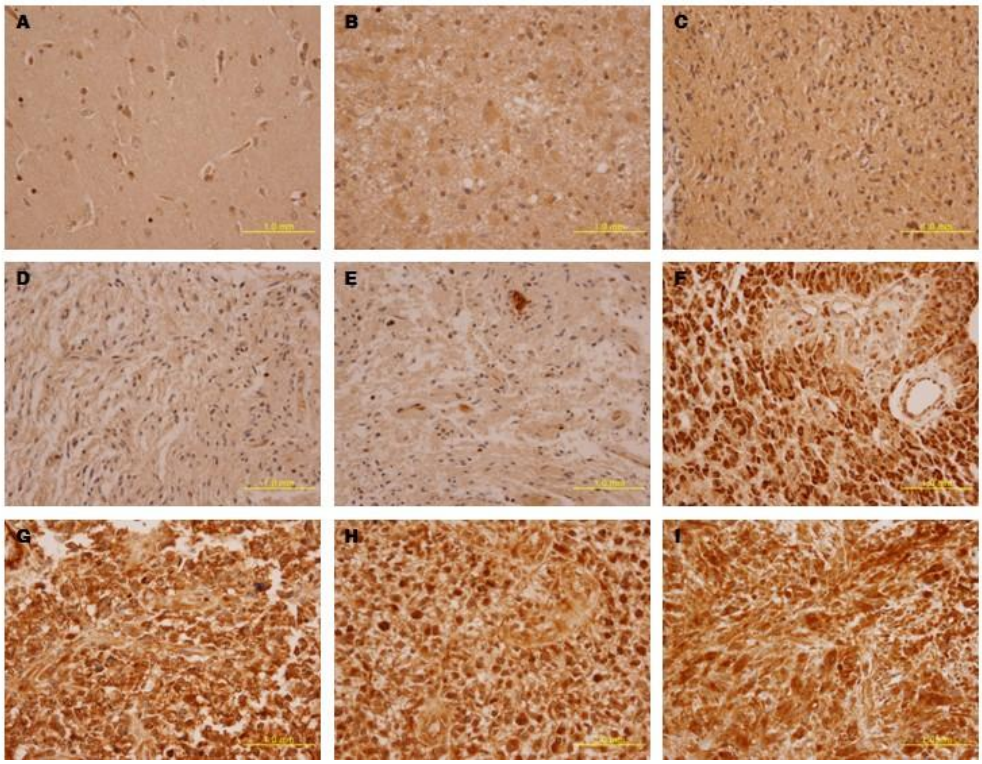


Figure 12. Immunohistochemical study of normal brain tissue, adult low-grade glioma, pediatric low-grade glioma, adult glioblastoma, and pediatric glioblastoma. Weak immunoreaction for ZEB2 was localized on astrocytes and neurons in normal brain tissue (A). Adult low-grade glioma cases showed very weak expression of ZEB2 (B, C). Pediatric low-grade glioma cases also showed weak immunoreactions for ZEB2 (D, E). High levels of ZEB2 expression were observed for adult glioblastoma (F, G) and pediatric glioblastoma cases (H, I). (ZEB2 immunostaining; original magnification, 400×).

mRNA expression of ZEB2 in normal brain, pediatric glioblastoma and adult glioblastoma tissues of the human patients

Adult glioblastoma patients showed a higher level of ZEB2 mRNA (ZEB2 mRNA/GAPDH mRNA) (mean±s.d.:2.689±1.351) than non-neoplastic brain tissues (0.961±0.105), and the difference reached statistical significance (p=0.011). Pediatric glioblastoma patients also showed a slightly high level of ZEB2 mRNA (1.030±0.3260) than non-neoplastic brain tissues, but the difference did not show statistical significance (p=0.515). Adult glioblastoma patients expressed a higher level of ZEB2 mRNA than pediatric glioblastoma patients, and the difference was statistically significant (p=0.008) (Fig. 13).

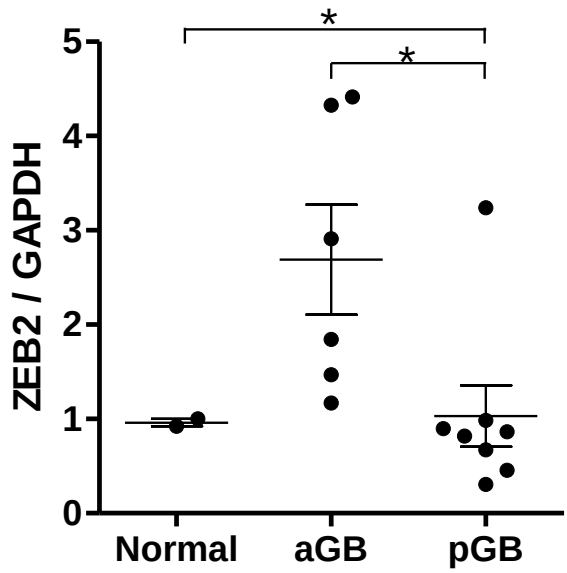


Fig. 13 Semiquantitative real time PCR analysis for ZEB2 mRNA expression in fresh-frozen normal brain tissues, adult glioblastoma tissues, and pediatric glioblastoma tissues. ZEB2 mRNA expression was significantly higher in adult glioblastoma tissues than pediatric glioblastoma tissues or normal brain tissues.

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. 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 adult glioblastoma cells as well as epithelial tumors[8-10, 16]. 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 glioblastoma 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 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 EMT related factors`s expression on glioblastoma progression, and the present study will serve to expand current knowledge on the new oncogenic role of EMT activating factors, such as Snail and ZEB2 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 [23, 24, 34, 40], ovarian [23, 38, 39], hepatocellular [25, 27, 36, 42], and colorectal carcinomas [33, 45]. 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 [19]. 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 expression for Snail protein 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 adult and pediatric glioblastoma cells. Including our previous study, most study which revealed the important role of EMT related factors in glioblastoma only used the adult glioblastoma cells or patients samples. Although different pathogenesis between adult and pediatric glioblastoma was suggested by many studies, but the clinical outcome was similar. Therefore we included pediatric glioblastoma cell line to compare the different role of Snail in pediatric and adult glioblastoma. Knockdown of Snail gene expression showed

increased E-cadherin and decreased vimentin expression at the transcriptional and protein level in adult and pediatric glioblastoma cells. 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 adult and pediatric glioblastoma cells. Inhibition of Snail gene expression was found to induce G1 phase 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 adult and pediatric glioblastoma cells by promoting EMT like process. 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 by promoting EMT like process. Also, we showed that Snail gene expression was significantly related to the highly invasive ability in adult and pediatric glioblastoma cells. Based on these results, Snail expression commonly effected on

invasive and proliferation ability of pediatric and adult glioblastoma cell, and these features also commonly exerted on the poor prognosis of adult and pediatric glioblastoma. Our results about role of Snail in adult glioblastoma were similar with Han *et al.*`s [8] results. And our results also additionally suggested the important oncogenic role of Snail in pediatric glioblastoma as well as adult glioblastoma.

Adult glioblastoma and pediatric glioblastoma are aggressive brain tumors; they display histological similarities, but their molecular pathologies are yet unclear. Diverse molecular factors or mechanisms have been introduced to explain the cause of poor prognosis. In the present study, we studied the role of Snail and ZEB2 expression in glioblastoma cells, with particular emphasis on the differences between pediatric and adult glioblastoma cells. In other words, the effects on invasiveness, migration ability, cell proliferation, viability and cell cycle distribution of Snail expression were not different in pediatric and adult glioblastoma cells. However, ZEB2 expression showed some discrepancies in viability, proliferation and cell cycle between pediatric and adult glioblastoma cells.

The ZEB2, which was originally identified as a Smad-interacting protein, is known to control the migration and invasion of cancer cells. The molecular hallmark of EMT is the

loss of or decrease in E-cadherin expression and upregulation of mesenchymal markers. ZEB2 is well known for its role as an EMT-activating transcription factor through the regulation of E-cadherin expression. Furthermore, ZEB2 was reported as an E-cadherin repressor and major regulator of tumor cell invasion [23, 39, 43, 44]. Therefore, we aimed to define EMT-like processes and association between ZEB2 gene expression and tumor progression in pediatric and adult glioblastoma cells. Previously, mesenchymal change induced by ZEB2 expression was studied only for adult glioblastoma cells [17]. According to Qi *et al*, ZEB2 downregulation resulted in the restoration of E-cadherin expression and suppression of vimentin expression in U87 and U251 glioma cell lines. Consequently, we hypothesized that inhibition of ZEB2 expression would result in increased E-cadherin and decreased vimentin expression. This was indeed confirmed by our studies at both mRNA and protein levels. Although we detected the molecular hallmarks of EMT at the mRNA and protein levels in pediatric and adult glioblastoma cells, it was necessary to evaluate whether these encompassed phenotypic changes of mesenchymal type as well. To evaluate this, we compared the migration ability of cells after siRNA-mediated ZEB2 knockdown. Inhibition of ZEB2 gene expression significantly reduced the migration abilities in pediatric and adult glioblastoma cells.

Based on these results, we conclude that pediatric and adult glioblastoma cells undergo mesenchymal change at both molecular and phenotypic levels. Since there are no recent findings about mesenchymal-like changes in pediatric glioblastoma cells, our results are the first to demonstrate EMT like process in pediatric glioblastoma cells.

In terms of cell proliferation, viability, and growth cycle distribution, our study revealed different results for pediatric and adult glioblastoma cells after ZEB2 gene inhibition. ZEB2 knockdown by siRNA resulted in significantly reduced cell proliferation and viability of pediatric glioblastoma cells and an increased cell population during G1 phase arrest. These results, however, were not observed in adult glioblastoma cells. In contrast, ZEB2 gene inhibition in adult glioblastoma cells caused no changes in cell proliferation and viability. This difference was also observed in cell cycle analysis. In adult glioblastoma cells, the proportion of cells in G1 phase arrest did not increase after ZEB2-specific inhibition. These results differed from those obtained by Qi *et al.* They provided experimental evidence for ZEB2-regulated cell cycle progression, migration, invasion, and apoptosis in U251 and U87 glioma cells. Although the effects on invasion and migration are comparable with our results, the influence of ZEB2 inhibition on cell cycle progression is different. Our analyses revealed significant effects on cell cycle

progression in only pediatric glioblastoma cells, not in adult glioblastoma cells. Similarly, in contrast to Qi *et al*, we found that ZEB2 inhibition did not affect cell proliferation or viability in the adult glioblastoma cell lines U87 and U373. Thus, we concluded that although ZEB2 downregulation induced significant reduction of EMT-like properties in both adult glioblastoma and pediatric glioblastoma cells, ZEB2-dependent effects on cell proliferation, viability, and growth cycle distribution were apparent only in pediatric glioblastoma, but not in adult glioblastoma, cells. One of the reasons for these differences may be that different downstream molecules and/or genetic factors underlie ZEB2 gene regulation in adult and pediatric glioblastoma cells, explaining the different pathologies in these two types of tumors.

. In addition, we performed immunohistochemical staining of ZEB2 on various formalin fixed paraffin embedded tissues, including glioblastoma, low-grade gliomas, and normal brain specimens from both pediatric and adult cohorts. As a result, normal astrocytes and low-grade glioma cells showed very weak ZEB2 expression; however, strong staining patterns were revealed for adult glioblastoma and pediatric glioblastoma. Although there were no visible differences in ZEB2 protein expression between adult and pediatric glioblastoma and among the same WHO grade using immunohistochemistry, but there

was significant different mRNA expression of ZEB2 between pediatric and adult glioblastoma. Also distinct different protein expression of ZEB2 in immunohistochemistry were observed between low-grade and high-grade gliomas. In corroboration with our results, Qi *et al* correlated ZEB2 protein expression with the status of WHO grade. We propose that these results reflect the role of ZEB2 in glial tumors. High-grade gliomas typically demonstrate more invasive properties and greater resistance to treatment than low-grade gliomas, which is similar to the signature pattern observed in many epithelial cancers following EMT induction. It seems likely that high-grade glioma cells acquired these properties by EMT like process induction through the upregulation of ZEB2 expression, and that these features had individual effects on pediatric glioblastoma and adult glioblastoma cells.

In conclusion, we found that Snail and ZEB2 expression fulfills an oncogenic function in both pediatric glioblastoma and adult glioblastoma cells. Snail gene expression enhanced the proliferation, viability, and invasion ability of glioblastoma cells by promoting EMT like process and there was no different role between adult glioblastoma and pediatric glioblastoma. However, the expression of ZEB2 exerts different effects on cell proliferation, viability, and cell cycle progression in pediatric glioblastoma and adult

glioblastoma cells.

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국문초록

교모세포종에서 상피중간엽 이행을 통한 Snail 및 ZEB2의 종양성 역할에 관한 연구

명 재 경

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배경

종양의 진행과정에 대한 이해는 치료 측면에 있어서 매우 중요하며, 특히 임상적으로 나쁜 예후를 보여주는 교모세포종의 경우 그 중요성이 더욱 크다. 상피중간엽 이행과 관련된 인자 중 Snail 및 ZEB2 유전자는 상피중간엽 이행과정의 조절을 통해서 현재 다양한 종류의 종양의 진행에 영향을 미치는 것으로 알려졌다. 하지만 교모세포종에서는 이러한 역할에 대해 알려진 바 없어 이를 밝히고, 소아 및 성인의 교모세포종 세포주에서 다르게 작용하는지, 어떻게 다르게 작용하는지를 밝히고자 연구를 계획하였다.

방법 및 결과

RNAi 기술을 이용하여 소아 교모세포종 세포주 (KNS42) 및 성인 교모세포종 세포주(U87, U373)에서 Snail 및 ZEB2 유전자 발현을 억제 한 후 세포주의 증식, 생존, 침습 및 이동, 세포주기에 미치는 영향을 관찰하였다. Snail 유전자 발현 억제는 E-cadherin 의 발현을 증가시켰으며 vimentin 발현을 감소시켰다. 또한 Snail 유전자 발현 억제는 성인 및 소아 교모세포종 세포주의 증식, 생존, 침습 및 이동을 유의하게 감소시켰으며 또한 세포주기상 G1 시기에 있는 세포의 분포를 증가시켜 G1기 정체를 일으켰다. ZEB2 유전자 발현 억제는 Snail과 마찬가지로 성인 및 소아의 교모세포종 세포주에서 E-cadherin의 발현을 증가시켰으며 vimentin 발현을 감소시켰고, 성인 및 소아의 모든 세포주에서 증식능력, 침습능력 및 이동능력의 감소를 일으켰다. 그러나 ZEB2 유전자 발현 억제가 세포의 생존능력 및 세포주기에 미치는 영향을 보았을 때 소아 교모세포종에서는 생존 능력의 감소와 G1기 세포수의 증가가 뚜렷하게 관찰 되어 G1기 정체를 보였으나 성인 교모세포종 세포주에서 이러한 변화가 없었다. 이러한 결과는 Snail 발현 억제의 결과와 차이를 보이는 것이다. 면역 조직화학 염색상 Snail 및 ZEB2의 발현은 성인 및 소아의 교모세포종에서 과발현되었으나 두 그룹간에 차이를 보이지는 않았고 저등급의 뇌교종과 고등급의 뇌교종간에는 확실한 발현 차이를 보였다. 또한 조직에서의

ZEB2 mRNA 발현은 성인 및 소아 교모세포종에서 정상조직보다 높았다.

결론

Snail 및 ZEB2 발현은 교모세포종 세포주에서 상피중간엽이행과 같은 과정을 유도해 공히 성인 및 소아의 교모세포종세포주의 침습 및 이동에 영향을 미쳐 종양진행을 촉진하는 역할을 한다는 사실을 확인하였다. 그러나 교모세포종 세포주의 증식능력, 생존능력 및 세포 주기의 변화를 보았을 때, Snail 발현 억제는 소아와 성인의 교모세포종에서 공히 증식능력 및 생존능력 억제 및 G1기 세포 수의 증가를 보였으나 ZEB2의 발현억제는 소아의 교모세포종 세포주에서만 이러한 영향이 나타났고 성인의 세포주에서는 별 변화가 없었다. 이러한 결과의 원인은 성인과 소아의 교모세포종이 서로 다른 병리 생태를 가지는 것을 의미하는 것으로 그간 임상경험에서 주장되었던 소아의 교모세포종과 성인의 교모세포종과 생물학적 특성 및 예후가 다르다는 것을 뒷받침한다. 따라서 소아의 교모세포종의 예후 예측 및 치료는 성인의 교모세포종과 다르게 이루어져야 함을 시사하고 있다.

Keywords

Snail, ZEB2, small interfering RNA (siRNA), epithelial mesenchymal transition (EMT), Glioblastoma