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# Long non-coding RNA AFAP1-AS1 accelerates invasion and predicts poor prognosis of glioma

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**Abstract. – OBJECTIVE: The role of AFAP1-AS1 in glioma is not fully known. This study aims at investigating the expression of AFAP1-AS1 in glioma and its underlying mechanism.**

**PATIENTS AND METHODS: AFAP1-AS1 expressions in brain tissues from 52 cases of glioma and 5 cases of traumatic brain injury were assessed using quantitative fluorescence polymerase chain reaction (PCR). The clinicopathological features of glioma were first recorded. The correlation between AFAP1-AS1 expression and prognosis of glioma was discussed. AFAP1-AS1 expressions in the glioma cell lines were further detected. After knockdown of AFAP1-AS1 in U87MG and U251 glioma cells, cell invasion was assessed by transwell assay. MMP2 and MMP9 expressions in glioma cells, which were important indicators of cell invasive-ness, were determined by Western blot.**

**RESULTS: AFAP1-AS1 was upregulated in the glioma tissues compared with that in the control tissues, and the expression level was correlated with glioma grading and KPS scores. After knockdown of AFAP1-AS1, the invasion capacities of the glioma cells declined significantly, and the expressions of invasion-related proteins MMP2 and MMP9 also decreased significantly.**

**CONCLUSIONS: lncRNA AFAP1-AS1 can considerably facilitate the invasion of glioma cells and acts as an independent predictor of malignancy and prognosis, which may also serve as a potential therapeutic target.**

Key Words
Glioma, LncRNA, AFAP1-AS1, Prognosis.

## Introduction

Glioma is one of the most common and malignant tumors of the brain. Surgery, postoperative chemotherapy and radiotherapy remain the major options for glioma[1]. However, the prognosis of glioma is still poor in spite of the advances in chemotherapy[2]. The five-year survival of glioblastoma patients remains the lowest among all types of glioma. The median survival of glioblastoma is less than 10 months, since it is the most aggressive tumor of all gliomas with WHO grade IV[3]. Current researches mainly focus on exploring molecular markers and the target of glioma in order to improve the treatment efficiency[4].

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts with over 200 nucleotides in length. As byproducts of transcription, lncRNAs cannot code for any proteins in the presence of RNA polymerase II[5]. It is believed that lncRNAs are involved in various biological processes, including epigenetics, transcriptional regulation and post-transcriptional translation[6]. LncRNAs are particularly noted for their roles in the occurrence and development of cancers[7]. AFAP1-AS1, a newly discovered lncRNA, is upregulated in many cancers, including esophageal cancer[8], nasopharyngeal cancer[9], lung cancer[10], pancreatic cancer[11] and gastric cancer[12]. As a prognostic factor, upregulation of AFAP1-AS1 can promote invasion and migration of tumor cells[10,13]. However, the role of AFAP1-AS1 in the biological behaviors and prognosis of glioma is not fully understood. This study aims at clarifying the significance of lncRNA AFAP1-AS1 in the occurrence, development and prognosis of glioma, which provides the foundation for targeted therapy of glioma.

## Patients and Methods

### Tissue Samples

From January 2009 to October 2015, glioma tissues were collected from 52 cases (6 cases of grade I, 10 cases of grade II, 15 cases of grade III and 21 cases of grade IV according to WHO grading system) receiving neurosurgery at the Second Affiliated Hospital of Nantong Univer-

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sity. Besides, 5 non-tumor control cases with traumatic brain injury in the same period were collected as controls. None of the cases received postoperative chemotherapy or radiotherapy, and all the resected tissues were pathologically confirmed as glioma by two experienced pathologists independently. The tissue samples were preserved in liquid nitrogen at -80°C. The clinical data were collected from the recruited cases, including age, gender, tumor size, position and grading. Follow-up was carried out regularly every 3 months in the first 2 years after surgery, and then reduced to once every 6 months from the third year. The last follow-up was performed in October 2015. The investigation was approved by the Ethics Committee of Second Affiliated Hospital of Soochow University. The informed consent was obtained from all cases.

### Cell Culture

Glioma cell lines (U87MG, U251, SHG-44, A172) and normal human astrocyte cell line 1800 were purchased from Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Rockville, MD, USA) or RPMI-1640 medium (Hyclone, South Logan, UT, USA) containing 10% Fetal Bovine Serum (FBS), respectively. The cells were cultured at 37°C with 5% $CO_2$ in a humidified incubator. The medium was replaced every 2 days. Cell passage was performed when the cell confluence was up to 80%-90%.

### Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the tissues or cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary Deoxyribose Nucleic Acid (cDNA) was obtained from total RNA by reverse transcription using the PrimeScript Reverse Transcription Kit (TaKaRa, Dalian, China). The samples were preserved at -20°C. Then, qPCR reactions were carried out using cDNA as the template and qPCR Kit (TaKaRa, Dalian, China). Primers were designed using Primer Premier 5.0 software and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The reaction system consisted of the followings: 2 μl cDNA as the template, 10 μL of 2×SYBR® Premix Ex TaqTM II, 0.6 μL of forward primer of AFAP1-AS1 and 0.6 μL of reverse primer. Sterile distilled water was added to dilute the system with a total volume of 20 μL. The forward primer of AFAP1-AS1 was 5′-ACTGAAGAGGAACCAGGGACAG-3′, and the reverse primer was 5′-GGGAAACTGAAATCAATAAG-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The forward primer of GAPDH was 5'-AACGGATTTGGTCGTATTGG-3', and the reverse primer was 5'-TTGATTTTGGAGGGATCTCG-3'. The reaction conditions were as follows: 95°C for 60 s, 95°C for 15 s, 60°C for 30 s, 72°C for 60 s and 72°C for 5 s, for a total of 40 cycles. $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression. The experiments were totally repeated 3 times.

### siRNA Synthesis and Transfection

The siRNA sequence targeting AFAP1-AS1 was synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China), which was 5'-CCAGACCACUACUGAAUAUTT-3'. The siRNA dry powder was diluted with RNase-free water to a concentration of 20 μmol/L. One day prior to the transfection, log phase cells were harvested, digested with trypsin and counted. Cells were inoculated to 6-well plates, with $1.5\times10^5$ cells in each well and 60% cell density. Transfection was performed using the Lipofectamine TM2000 reagent (Invitrogen, Carlsbad, CA, USA). The cells were divided into two groups: (1) blank control group, treated by Lipofectamine TM2000 and (2) experimental group, transfected with AFAP1-AS1 siRNA. The independent experiments were performed at least 3 times.

### Western Blotting

Cells were collected at 48-72 h after transfection and washed with pre-cooled phosphate-buffered saline (PBS) three times. The cells were added with radioimmunoprecipitation assay (RIPA)/PMSF (phenylmethylsulfonyl fluoride) lysis buffer (Beyotime, Shanghai, China) on ice at 4°C for 30 min. The proteins were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, Shanghai, China) and blotted to the polyvinylidene difluoride (PVDF) membranes (Beyotime, Shanghai, China). The membranes were incubated with 5% bovine serum albumin at room temperature on a shaker for 2 h. Primary antibodies [β-Actin, 1:2000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MMP2 (Affinity, Shanghai, China) and MMP9 (Affinity, Shanghai, China), with 1:1000

dilution] were added to incubate the membranes at 4°C overnight. Then the membranes were washed with Tris-buffered saline-Tween (TBS-T; Beyotime, Shanghai, China) three times, 10 min each time. HRP-labeled secondary antibodies were added (1:5000 dilution, Beyotime, Shanghai, China) to incubate the membranes for 1 h at room temperature. The membranes were washed with Tris-buffered Saline and Tween 20 (TBST) three times,10 min each time. Finally, enhanced chemiluminescence (ECL) Western blotting substrate was added, and the proteins were analyzed by a gel imager. The independent experiments were performed at least 3 times.

### Transwell Invasion Assay

Cells were collected at 48 h after transfection and washed with serum-free DMEM twice. Then the cells were resuspended in serum-free DMEM to adjust the cell concentration to $1\times10^6$/mL. DMEM containing 10% Fetal Bovine Serum (FBS) was added to the lower chamber of the transwell (Corning, Tewksbury, MA, USA) along with the pre-coated Matrigel (BD Bioscience, San Jose, CA, USA). 200 μL of cell suspension was added to the upper chamber. The chambers were taken out after incubation for 24 h. The non-invading cells in the upper chamber were removed with a dry cotton swab and then removed with PBS-wetted cotton swab twice. The chambers were fixed in 4% paraformaldehyde at room temperature for 30-60 min. After staining with 0.1% crystal violet for 30-60 min, the chambers were washed with PBS several times and air-dried at room temperature. Five randomly selected fields were captured under the microscope at the magnification of 200×for cell counting. The experiments were totally repeated 3 times.

### Statistical Analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). Measurements were compared between two groups using *t*-test. Multi-group comparisons were performed using one-way ANOVA, followed by Post-Hoc Test (Least Significant Difference). Correlations between the AFAP1-AS1 expression and clinicopathological features of the patients were analyzed with chi-square test. Kaplan-Meier survival curves were plotted and the difference in survival between two groups was analyzed by the log-rank test. $p<0.05$ was considered significant.

## Results

### LncRNA AFAP1-AS1 Was Upregulated in Glioma Tissues and Cell Lines

Many researchers have found that lncRNA AFAP1-AS1 is upregulated in many cancers, including esophageal cancer, nasopharyngeal cancer, lung cancer, pancreatic cancer and colorectal cancer. According to the detection by qRT-PCR (Figure 1), AFAP1-AS1 was upregulated in all glioma tissues (19.76±1.533) compared with that of the control tissues (3.204±0.754) ($p=0.0016$).

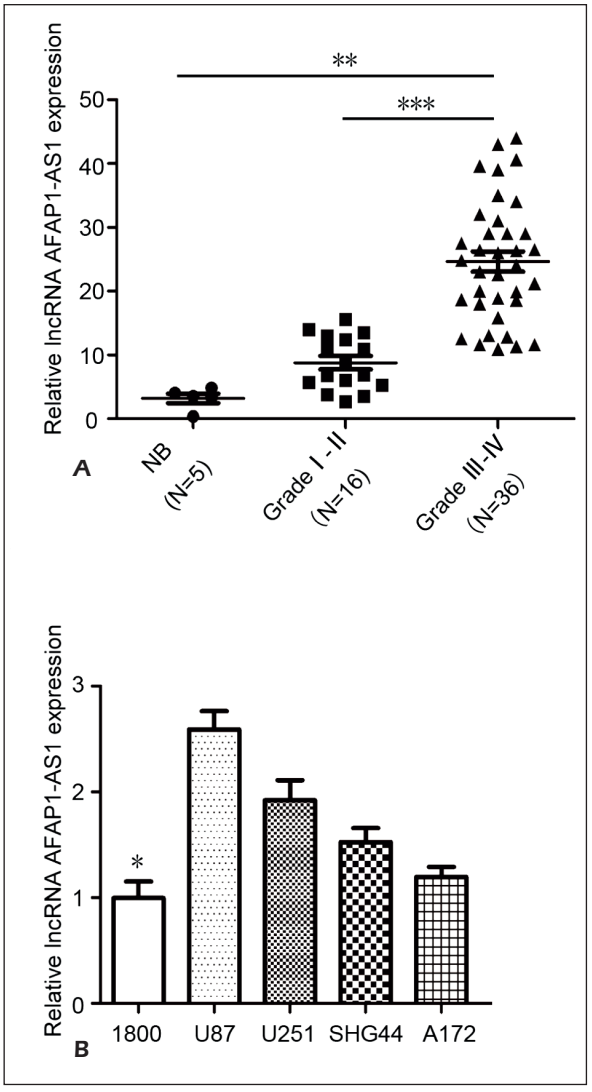


**Figure 1.** Relative expression of lncRNA AFAP1-AS1 in glioma tissues with different grades (n=52), normal brain tissues (n=5) (**A**), glioma cell lines (U87, U251, SHG55, A172) and astrocyte cells (1800) (**B**). The results of three independent experiments were expressed as mean±SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

**Table I.** Correlation between lncRNA AFAP1-AS1 expression and clinicopathological features of glioma patients.

| Clinical Characteristic | No. of patients | No. of Patients | | $\chi^2$ | *p* |
|---|---|---|---|---|---|
| | | Low expression of lncRNA AFAP1-AS1 | High expression of lncRNA AFAP1-AS1 | | |
| ***Gender*** | | 26 | 26 | | |
| Male | 30 | 16 | 14 | 0.315 | 0.575 |
| Female | 22 | 10 | 12 | | |
| ***Age*** | | | | | |
| <40 | 28 | 14 | 14 | 0 | 1 |
| ≥40 | 24 | 12 | 12 | | |
| ***Tumor diameter*** | | | | | |
| <5cm | 19 | 10 | 9 | 0.083 | 0.773 |
| ≥5cm | 33 | 16 | 17 | | |
| ***WHO Grade*** | | | | | |
| I-II | 16 | 12 | 4 | 4.424 | 0.035 |
| III-IV | 36 | 14 | 22 | | |
| ***KPS*** | | | | | |
| >80 | 26 | 19 | 7 | 11.077 | 0.001 |
| ≤80 | 26 | 7 | 19 | | |
| ***Tumor location*** | | | | | |
| Frontal | 21 | 11 | 10 | 0.08 | 0.777 |
| Temporal or others | 31 | 15 | 16 | | |

Moreover, the expression level of AFAP1-AS1 in the high-grade glioma tissues (grade III-IV) (24.64±1.589) was significantly higher than that of the low-grade glioma tissues (grade I-II) (8.788±1.050) ($p$<0.001). However, there is no difference in the transcriptional level of AFAP1-AS1 between WHO I and WHO II or WHO III and WHO IV grades (data were not shown). AFAP1-AS1 expression in the glioma cell lines (U87MG, U251, SHG-44 and A172) and normal human astrocyte cell line 1800 was also detected by qRT-PCR. The expression level in glioma cell lines (U87MG, U251, SHG-44 and A172) (1.809±0.170) was significantly higher than that of normal human astrocyte cell line 1800 (0.997±0.157) ($p$=0.040).

### Correlations Between the AFAP1-AS1 Expression and Clinicopathological Features of the Glioma Patients

We analyzed the correlation between the AFAP1-AS1 expression and clinicopathological features in 52 glioma patients (Table I). It was found that AFAP1-AS1 expression was closely associated with glioma grading and Karnofsky Performance Status (KPS) scores, but not correlated to age, gender, tumor size and tumor position.

### AFAP1-AS1 Expression Predicted Poor Prognosis

A total of 52 patients were divided into the following two groups according to AFAP1-AS1 expression with the median as the cutoff, namely high expression group (26 patients) and low expression group (26 patients). The correlation between the AFAP1-AS1 expression and overall survival (OS) was analyzed in 52 glioma patients using Kaplan-Meier analysis and log-rank test (Figure 2). The result showed that AFAP1-AS1 expression predicted worse prognosis in glioma patients as compared with those with low expression (HR =3.618, 95 % CI=1.534-8.532, $p$<0.001).

### Silencing of AFAP1-AS1 Inhibited the Invasion Abilities of U87MG and U251 Cell Lines

To identify the role of AFAP1-AS1 in glioma, AFAP1-AS1-specific siRNAs were constructed to knock down AFAP1-AS1 expression in U87MG and U251 cells. After transfection, the mRNA expression of AFAP1-AS1 was determined using qRT-PCR. The result (Figure 3) showed that compared with the control group, AFAP1-AS1 expression was inhibited after transfection of AFAP1-AS1-specific siRNA.

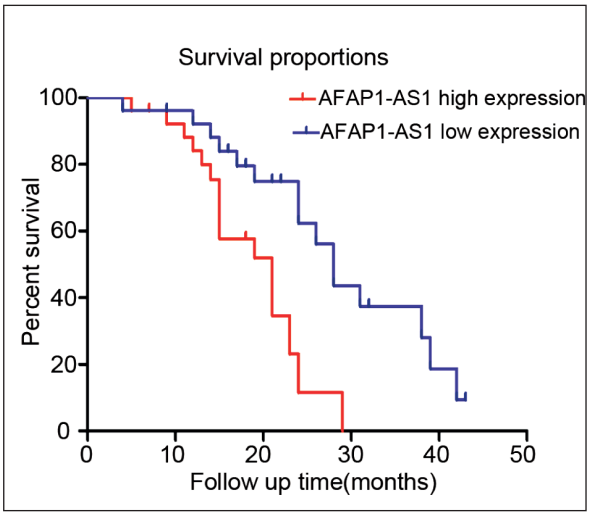


**Figure 2.** Kaplan-Meier method showed that AFAP1-AS1 expression was negatively correlated with the overall survival of glioma patients ($p<0.001$).

Transwell assay was used to analyze the effect of AFAP1-AS1 on the glioma cells. The result (Figure 4) showed that siRNA AFAP1-AS1 downregulated AFAP1-AS1 in U87MG and U251 glioma cells, leading to a reduction in their migration abilities. Western blot (Figure 5) suggested that knockdown of AFAP1-AS1 decreased the expressions of the invasion-related proteins MMP2 and MMP9. It is suggested that lncRNA AFAP1-AS1 promoted the invasion abilities of glioma cell lines.

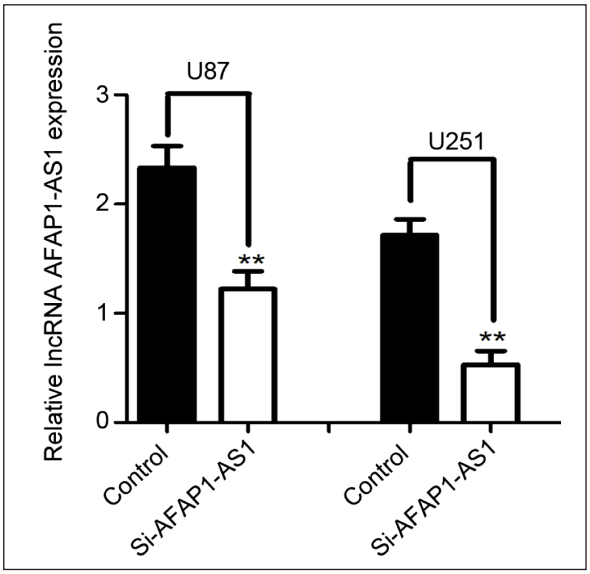


**Figure 3.** si-AFAP1-AS1 transfection downregulated AFAP1-AS1 compared to the control (**$p<0.01$).

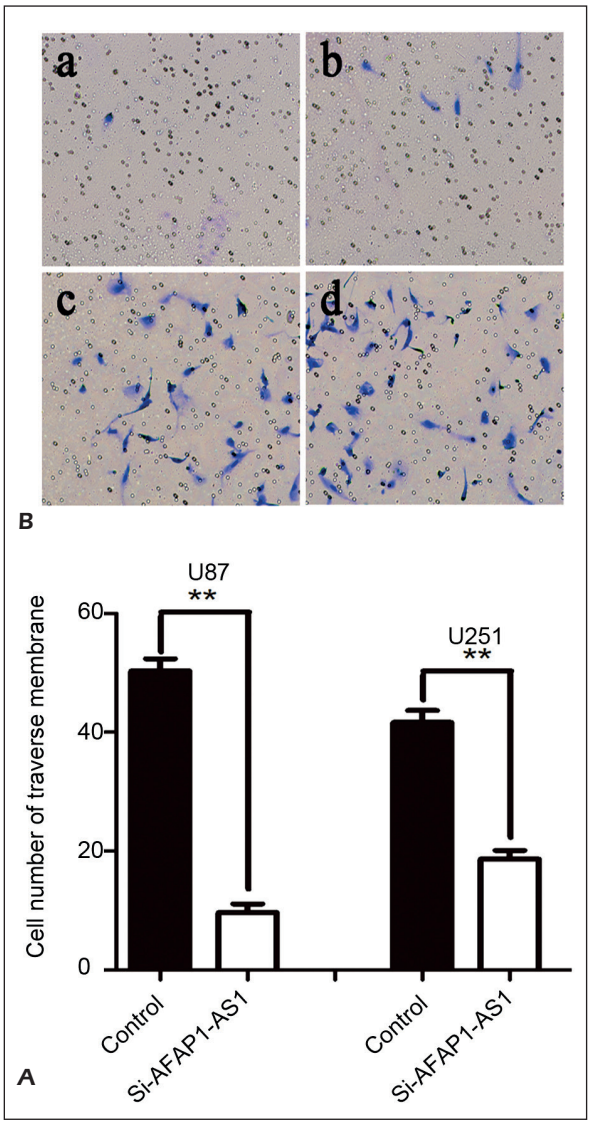


**Figure 4.** Effects of AFAP1-AS1 knockdown on invasion of glioma cells. **A**, The numbers of U87MG and U251 cells that traversed the Matrigel basement membrane af-ter transfection of si-AFAP1-AS1. **B**, A graphical representation of the numbers of U87MG and U251 cells that traversed the Matrigel basement membrane (**$p<0.01$).

## Discussion

LncRNA was considered as a non-functional byproduct initially[5]. Recent studies have indicated the significant roles of lncRNAs in many diseases, including cancers[9]. Differentially expressed lncRNAs in some cancers can provide the reference for diagnosis, treatment and prognosis of cancer[2]. Therefore, identifying the abnormally expressed lncRNAs in tumor and analyzing the consequences can benefit the treatment and prognostic prediction.

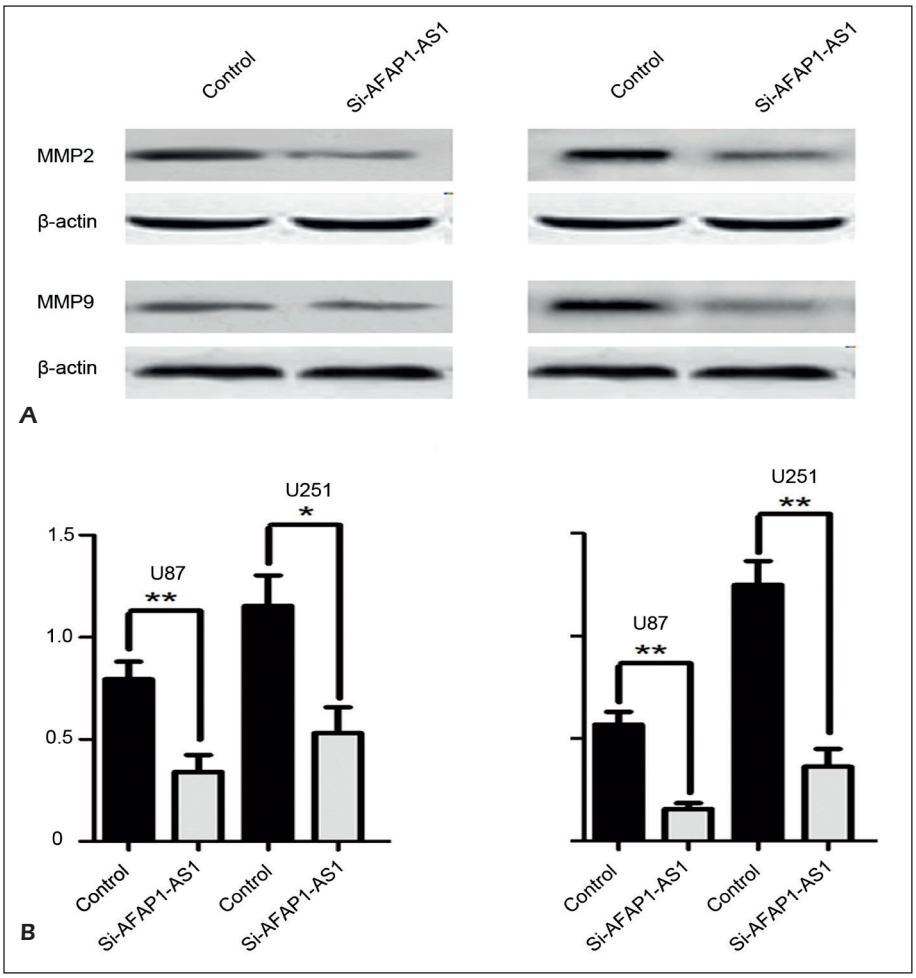


**Figure 5.** Expressions of the invasion-related proteins MMP2 and MMP9 in glioma cell lines (U87 and U251) after transfecting si-AFAP1-AS1. **A**, The expressions of MMP2 and MMP9 in U87 cells and U251 cells transfected with si-AFAP1-AS1 or siRNA controls. β-actin was used as a loading control. **B**, A graphical representation of the protein expressions of MMP2 and MMP9 (**$p$<0.01, ***$p$<0.001).

AFAP1-AS1 was the first lncRNA studied in Barrett's esophagus and esophageal cancer[10]. As an antisense lncRNA coding for the AFAP1 gene, AFAP1-AS1 is lowly methylated and highly expressed in Barrett's esophagus and esophageal cancer. AFAP1-AS1 is highly expressed in many cancers, including esophageal cancer[10], lung cancer[13], pancreatic cancer[10], nasopharyngeal cancer[1], liver cancer[14] and colorectal cancer[7], which predicts poor prognosis as an oncogene. However, the role of AFAP1-AS1 in glioma is barely known.

This study aims at analyzing the expression of AFAP1-AS1 in glioma with different tumor grades and its potential mechanism. It was found that AFAP1-AS1 was upregulated in the glioma tissues and its expression level was correlated to the grading and KPS scores of glioma patients, but not correlated to age, gender, tumor size and tumor position. AFAP1-AS1 upregulation predicted a poor prognosis in glioma. Meanwhile, we found that the expression level in glioma cell lines was higher than that of normal human astrocyte cell line. AFAP1-AS1 knockdown inhibited the migration abilities of the U87MG and U251 glioma cells, and the expression of MMP2 and MMP9 protein were also downregulated in the two cell lines. These results indicated the role of AFAP1-AS1 as a prognostic predictor for glioma. The existing studies have also reported that AFAP1-AS1 is overexpressed in esophageal cancer compared with that of the normal esophageal tissues. AFAP1-AS1 expression predicted a poor prognosis of glioma. The Knockdown of AFAP1-AS1 can inhibit the proliferation and migration abilities of esophageal adenocarcinoma cells[8,15]. According to Bo et al[9], AFAP1-AS1 was upregulated in the nasopharyngeal cancer tissues, and its expression was closely related to the clinical staging, lymph node metastasis, distant metastasis and poor prognosis of patients. Overall survival and disease-free survival of glioma pa-

tients with higher expression of AFAP1-AS1 and AFAP1-AS1 knockdown can inhibit the invasion and migration abilities of nasopharyngeal cancer cells. Later Ye et al[11] applied lncRNA microarray technique in the screening of differentially expressed lncRNAs in pancreatic ductal adenocarcinoma. They found that AFAP1-AS1 expression was closely associated with migration, invasion and poor prognosis of pancreatic ductal adenocarcinoma[11]. Other researches have also indicated AFAP1-AS1 is highly expressed in colorectal cancer[16], lung cancer[10], and liver cancer[14], which acts as an oncogene. Therefore, AFAP1-AS1 may serve as a predictor for malignancy and prognosis of glioma patients.

## Conclusions

Our investigation showed that AFAP1-AS1 was upregulated in the glioma tissues, which was associated with higher malignancy and worse prognosis. AFAP1-AS1 knockdown inhibited the migration and invasion abilities of glioma cells as represented by the downregulation of MMP2 and MMP9. Hence, AFAP1-AS1 is a likely predictor of malignancy and prognosis of glioma *via* regulating invasion-related proteins MMP2 and MMP9. However, the specific mechanism remains unknown and requires further investigations.

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**Conflict of Interests:**

The authors declare they have no conflict of interest.

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