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Long non-coding RNA ZEB1-AS1 promotes glioma cell proliferation, migration and invasion through regulating miR-577

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Abstract. – OBJECTIVE: Long non-coding RNA ZEB1-AS1 (ZEB1-AS1) was reported to be implicated and aberrantly expressed in multiple cancers. However, the potential mechanism and clinical significance of ZEB1-AS1 in the carcinogenesis of glioma remain unclear.

PATIENTS AND METHODS: RT-PCR was performed to determine the expression of ZEB1-AS1 in glioma tissues and cell lines. The association between ZEB1-AS1 expression and clinical features and prognosis were statistically analyzed. MTT and transwell assays were used to test the proliferation, invasion, and migration of glioma cells. Luciferase report assay was used to detect the correlation between ZEB1-AS1 and miR-577 in glioma.

RESULTS: Compared with normal brain tissues and cells, ZEB1-AS1 in glioma tissues and cell lines was shown to be expressed at high levels. Clinical association analysis indicated that ZEB1-AS1 expression was closely associated with tumor size (p = 0.014), KPS (p = 0.004) and WHO grade (p = 0.001). In addition, it was observed that high expression level of ZEB1-AS1 was remarkably associated with overall survival and could be an independent prognostic indicator of glioma using univariate and multivariate analysis. Functional experiments demonstrated that down-regulation of ZEB1-AS1 suppressed the proliferation, invasion, and migration of glioma cell *in vitro*. In the mechanism, we found that ZEB1-AS1 acted as a competing endogenous RNA to sponge miR-577. Moreover, miR-577 could reverse the tumor-promotive role of ZEB1-AS1 on glioma cells.

CONCLUSIONS: Our findings demonstrated that ZEB1-AS1 might play an oncogenic role in glioma and was a poor prognostic factor. The ZEB1-AS1/miR-577 axis might be a potential target for the development of effective glioma therapy.

Key Words:
Long noncoding RNA, ZEB1-AS1, miR-577, Proliferation, Migration, Invasion, Prognosis.

Introduction

Gliomas are defined as tumors arising from glial cells and the remarkable character are vascular proliferation and aggressive invasion[1,2]. Gliomas are subdivided into four stages according to the 2016 World Health Organization (WHO) classification system[3]. The most common and aggressive subtype is glioblastoma, which is classified grade IV astrocytic tumor. Even after surgery and postoperative radiotherapy plus chemotherapy, the prognosis of glioblastoma is still relatively poor with a survival rate for 5 years of only 9.8%[4, 5]. The reasons for poor prognosis of glioblastoma are rapid cell growth, distant metastasis, and limited knowledge of its potential mechanisms[6]. Therefore, there exists a crucial need to understand molecular mechanisms underlying glioma progression.

Long noncoding RNAs (lncRNAs), which are longer than 200 nucleotides with no protein-coding potential, have been shown to be involved in the regulation of the proliferation, differentiation and apoptosis[7,8]. A growing number of researches[9,10] have reported that the dysregulation of lncRNAs expression plays critical roles in development and progression of various tumors, including glioma. However, the role and potential molecular of lncRNAs in tumors is still unclear and needs to be further elucidated. Growing evidence[11] has showed that lncRNAs act as 'sponges' to bind specific miRNAs which have been well studied in biological progression. For instance, Xu et al[12] reported that lncRNA TUSC7 suppressed colorectal cancer cells proliferation by sponging miR-211. Xu et al[13] showed that lncRNA HOTAIR promoted esophageal cancer cell pro-

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liferation, metastasis and EMT by acting as a miR-148a sponge. Yao et al[14] revealed that knockdown of lncRNA XIST suppressed glioma cell proliferation, migration and invasion by up-regulating miR-152. Those studies confirmed that lncRNAs have the ceRNA activity by acting as miRNA sponges to be involved in the regulation of tumors behavior.

LncRNA ZEB1 Antisense 1 (ZEB1-AS1) has been reported to be highly expressed and may serve as a tumor promoter in various tumors, including osteosarcoma[15] hepatocellular carcinoma[16] and bladder cancer[17]. Lv et al[18] reported that ZEB1-AS1 expression was markedly increased in glioma tissues and down-regulation of ZEB1-AS1 inhibited glioma cell proliferation and migration, as well as the epithelial-mesenchymal transition. In addition, ZEB1-AS1 expression was significantly associated with poor prognosis of glioma patients. However, the potential molecular mechanism of ZEB1-AS1 underlying metastasis remains largely unclear. In the present work, our attention focused on whether ZEB1-AS1 could act as a sponge for miRNAs to affect the biological processes of glioma.

Patients and Methods

Patients and Tissue Samples

Patients with glioma (n=65) who underwent initial surgery in Pingyi People's Hospital from 2009 to 2011 were retrospectively selected for this study. None of the patients had received chemotherapy or radiotherapy prior to surgery. Tumor tissues were confirmed according to the criteria of World Health Organization (WHO) Classification by two pathologists. The clinical features of these cases have been summarized in Table I. For qRT-PCR and Western blot analysis, tissues were immediately frozen in liquid nitrogen and kept at -80°C until analysis. Written informed consent was obtained from each patient before surgery. This study was approved by the Ethics Committee of the Pingyi People's Hospital.

Cell Lines and Culture

The control human astrocyte cell line (HEB), and human glioma cell lines (A172, U87, T98G and SHG44) were purchased from Shanghai Institute of Cell Biology (Xuhui, Shanghai, China). All cell lines were cultured in Dulbecco's

Table I. Clinical association between ZEB1-AS1 expression and clinicopathological variables in glioma patients.

Variable	Number	ZEB1-AS1 expression		*p*-value
		High	Low	
Age (years)				0.238
< 45	37	17	20	
≥ 45	28	17	11	
Gender				0.839
Male	39	20	19	
Female	26	14	12	
Family history of cancer				0.292
Yes	23	10	13	
No	42	24	18	
Tumor location				0.714
Supratentorial	33	18	15	
Infratentorial	32	16	16	
Tumor size (cm)				0.014
< 3	38	15	23	
≥ 3	27	19	8	
KPS				0.004
< 90	31	22	9	
≥ 90	34	12	22	
WHO grade				0.001
I–II	31	23	8	
III-IV	34	11	23	

Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco®, Grand Island, NJ, USA), 1% streptomycin and penicillin (Beyotime, Haidian, Beijing, China) in a humidified 37°C, 5% CO_2 incubator.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from tumor tissues and cell lines by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Extracted RNA (100 ng) was reverse-transcribed to first-strand cDNA using the High Capacity cDNA Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan). qRT-PCR analysis for ZEB1-AS1 expression was conducted using a standard protocol from Power SYBR Green (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. ZEB1-AS1 expression data were normalized to GAPDH from the same sample. The comparative Ct (ΔΔCt) method was used to determine the expression fold change. The primers for ZEB1-AS1 were 5'-AACCTTGTTGCTAGGGACCG-3'(forward) and 5'-AGTCACTTCCCATCCCGGTT-3'(reverse); the primers for miR-577 were 5'-TGCGGTAGATAAAATATTGG-3' (forward) and 5'-CCAGTGCAGGGTCCGAGGT-3' (reverse); the primers for GAPDH were 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward) and 5'-GGCTGTTGTCATACTTCTCATGG-3' (reverse).

Oligonucleotides and Cell Transfection

Small interfering RNAs (siRNAs) and scrambled negative control siRNA (si-NC) that purchased from Tianjin Technology (Kunming, Yunnan, China) were used for ZEB1-AS1 inhibition. The siRNA targeting sequences of ZEB1-AS1 were 5'-UCAAUGAGAUUGAACUUCAGCUGGA-3'. MiR-577 inhibitors, which were synthesized by Tianjin Technology (Kunming, Yunnan, China), were transfected into U87 to decrease miR-577 expression. Transient transfection was conducted using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA).

Cell Viability Assay

Cells were seeded on a 96-well plate (2 x 10^4 cells per well) and incubated at 37°C after transfection. Next, 100 µl MTT solution were added to each well, and the cells were incubated for 4 h at 37°C. After that, 150 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) were added. Absorbance readings at 490 nm were obtained using a spectrophotometric plate reader (Thermo Scientific, Waltham, MA, USA). MTT assay was repeated three times.

Migration and Invasion Assays

For the migration assays, $3x10^4$ transfected U87 cells suspended in serum-free Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) were added into the upper chamber. For the invasion assays, $3x10^4$ transfected U87 cells suspended in serum-free Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) were added into the upper chamber pre-coated with Matrigel (Biosystems, Foster City, CA, USA). For both assays, the bottom chambers of the transwell were filled with cell growth medium containing 20% fetal bovine serum (FBS). After cells had been cultured for 48 h at 37°C, non-invading cells were removed from the top well. The invaded cells were counted and photographed under an optical microscope.

Luciferase Reporter Assay

U87 cells were seeded at 3×10^4 cells per well into 24-well plates and allowed to settle overnight. ZEB1-AS1 full fragments or its mutant containing the putative miR-577-binding sites in ZEB1-AS1 was purchased from Invitrogen (Carlsbad, CA, USA) cloned into pGL3 luciferase promoter plasmid (Biosystems, Foster City, CA, USA). U87 cells were transfected by Lipofectamine 2000 in 24-well plates followed by dual luciferase reporter assays (Promega, Madison, WI, USA). Transfection was performed with at least three different batches of each reporter plasmid. Firefly luciferase units were normalized against Renilla luciferase units to control for transfection efficiency.

Statistical Analysis

Statistical Product and Service Solution 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical treatment of all data. Paired Student's *t*-test was used to compare the genes levels between tumor and adjacent non-tumor tissues. The association between ZEB1-AS1 expression and clinicopathological variables was assessed by x^2-tests. The overall survival was analyzed by log-rank test, and survival curves were plotted according to Kaplan-Meier. Univariate and multivariate Cox regression analyses were performed to analyze the survival data. Differences were con-

sidered statistically significant when the p-value < 0.05.

Results

ZEB1-AS1 Expression was Up-Regulated in Human Glioma Tissues and Associated with Clinical Features

It has been reported that ZEB1-AS1 expression was significantly up-regulated in glioma tissues[18]. In order to demonstrate those results, ZEB1-AS1 expression levels in 65 paired glioma tissues and adjacent nontumor bone tissues from our hospital were measured. The relative expression of ZEB1-AS1 was significantly elevated in tumorous tissue compared with adjacent normal tissues ($p < 0.01$) (Figure 1A). Similarly, we further verified ZEB1-AS1 was differentially increased in 4 glioma cell lines compared with that in the normal HEB ($p < 0.01$, Figure 1B). We further explored the association between ZEB1-AS1 expression and clinical features of glioma patients, which were divided into two groups (High group and Low group) according to the median expression level of ZEB1-AS1. We observed that high ZEB1-AS1 expression correlated with large tumor size ($p = 0.014$), KPS ($p = 0.004$) and advanced WHO grade ($p = 0.001$) (Table I). However, no significant difference was observed between ZEB1-AS1 expression and other clinical features.

Relationship of ZEB1-AS1 to Overall Survival of Glioma Patients

In this study, no patients were lost to follow-up. At the end of the follow-up, 44 (67.7%) patients were deceased and 21 (32.3%) patients were still alive. Then, we performed Kaplan-Meier analysis to explore the prognostic value of ZEB1-AS1 expression in glioma patients. We found that the 5-year overall survival of high ZEB1-AS1 expression group was significantly shorter than that of low ZEB1-AS1 expression group ($p = 0.0055$) (Figure 1C). Then, univariate analysis revealed that ZEB1-AS1 expression ($p = 0.001$), tumor size, KPS and WHO grade were correlated with overall survival of patients with glioma (Table II). Further multivariate Cox analysis confirmed that ZEB1-AS1 was an independent predictor of overall survival for glioma patients [HR (95 % CI): 2.983(1.189-4.739), $p = 0.005$), Table II].

ZEB1-AS1 Promoted Glioma Cell Proliferation and Metastasis in Vitro

To discover the effect of ZEB1-AS1 on glioma cell proliferation, si-ZEB1-AS1 and si-NC were transfected into the human glioma cell line U87, and MTT was performed. The RT-PCR showed that ZEB1-AS1 was down-regulated in U87 cells transfected with si-ZEB1-AS1 as compared with the control cells ($p < 0.01$) (Figure 1A). The results of MTT indicated that when transfected with si-ZEB1-AS1, the proliferation ability of U87 cells was downregulated when compared with the control (Figure 2B). The poor prognosis of glioma patients is mainly due to the cancer cell metastasis. To investigate whether ZEB1-AS1 plays a critical role in glioma cell metastasis, we performed migration and invasion assays. As shown in Figure 2C and 2D, the migration and invasion capacity of U87 cells was significantly reduced after down-regulation of ZEB1-AS1 ($p < 0.01$).

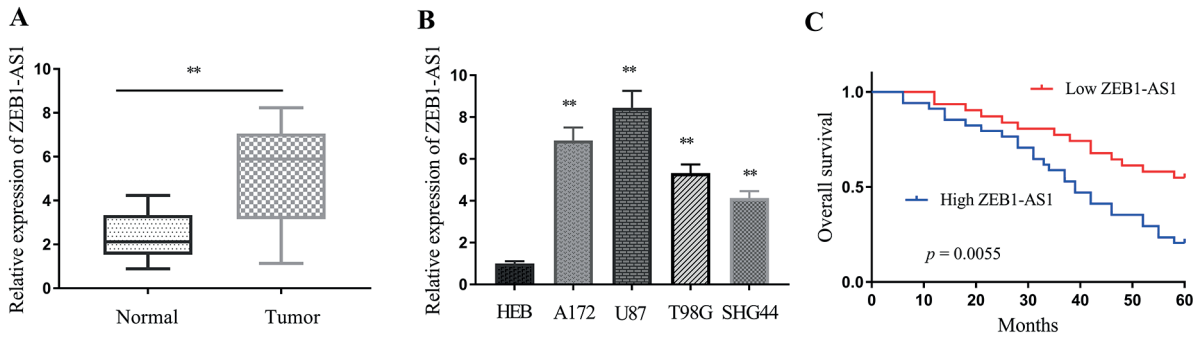

Figure 1. Relative ZEB1-AS1 expression in glioma and its clinical significance. ***(A)*** ZEB1-AS1 expression was analyzed by qRT-PCR in human glioma tissues and pair-matched adjacent normal tissues. ***(B)*** Relative expression levels of ZEB1-AS1 in normal cells HEB and glioma cells (HEB, A172, U87, T98G and SHG44). ***(C)*** Kaplan-Meier curves for overall survival of two groups defined by low and high expression of NEAT1 in patients with glioma ($p = 0.0055$). $^{**}p < 0.01$.

Table II. Prognostic factors for overall survival by univariate and multivariate analysis.

Variables	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*p*-value	HR (95 % CI)	*p*-value
Age	1.562 (0.672-2.213)	0.177	-	-
Gender	1.683 (0.892-2.449)	0.193	-	-
Family history of cancer	1.412 (0.498-2.199)	0.358	-	-
Tumor location	1.883 (0.689-2.774)	0.163	-	-
Tumor size	2.573 (0.893-4.283)	0.014	2.263 (0.699-3.673)	0.035
KPS	3.237 (1.183-5.738)	0.005	2.895 (1.032-4.356)	0.009
WHO grade	3.763 (1.574-6.893)	0.001	3.192 (1.283-5.251)	0.003
ZEB1-AS1 expression	3.442 (1.482-6.213)	0.001	2.983 (1.189-4.739)	0.005

All these observations suggested that silencing of ZEB1-AS1 inhibited cell proliferation, migration and invasion abilities in glioma cells.

MiR-577 Directly Targeted ZEB1-AS1 3'-UTR

To clarify the tumor-promotive function of ZEB1-AS1, we aimed at identifing its regulated targets through bioinformatics software (DIANA TOOLS). We found that miR-577 contained the complementary sequence of ZEB1-AS1. MiR-577 has been reported to be a tumor suppressor in glioma tissues[19] (Figure 3A). To validate the direct binding between ZEB1-AS1 and miR-577, we constructed luciferase reporter vectors containing ZEB1-AS1 or its mutated form and performed dual luciferase reporter assay U87 cells. We found that miR-577 mimic reduced the luciferase activity of the ZEB1-AS1-wt reporter vector but not mutant reporter vector (Figure 2A). Moreover, we detected the expression of miR-577 in glioma tissues, and the results indicated that the relative expression of miR-577 was significantly decreased in tumorous tissue compared with adjacent normal tissues ($p < 0.01$). In addition, the results from Pearson's association revealed that ZEB1-AS1 was negatively correlated to miR-577 expression in glioma patients (Figure 3D). Taken together, our findings indicated that ZEB1-AS1 acted as a molecular sponge for miR-577.

MiR-577 Reverses the Promoting Effect of ZEB1-AS1 on the Development of Glioma Cells

In order to further confirm whether ZEB1-AS1 affected cell proliferation, migration and invasion by regulating miR-577 expression, a series of experiments was performed. RT-PCR results showed that miR-577 expression was significantly down-regulated in glioma tissues (Figure 4A). U87 cells were cotransfected with si-ZEB1-AS1 and/or miR-577 inhibitor. The results from qRT-PCR showed that down-regulation of ZEB1-AS1 inhibited miR-577 expression, and miR-577 inhibitor suppressed miR-577 expression (Figure 4B). Furthermore, MTT assay suggested that miR-577 inhibitor could reverse the inhibition of proliferation caused by ZEB1-AS1 silencing. Besides, down-regulation of ZEB1-AS1 significantly suppressed the migration and invasion of U87 cells, and miR-577 inhibitors could rescue this ZEB1-AS1-silencing induced suppression (Figure 4D and 4E). In summary, our results revealed that miR-577 could rescue both the effect on cell proliferation and metastasis induced by ZEB1-AS1 in U87 cells.

Discussion

Various lncRNAs have been found to become potential prognostic biomarkers for glioma[20,21]. Although previous investigations reported ZEB1-AS1 expression levels and its prognostic value, the evidence is insufficient. In the present research, the overexpression of ZEB1-AS1 was observed in both glioma cell lines and clinical specimens. Moreover, elevated ZEB1-AS1 expression was associated with tumor size, KPS and WHO grade. Survival analysis suggested that high expression of ZEB1-AS1 can be related to shorter overall survival than low expression. In a Cox model, we found that ZEB1-AS1 expression was an independent poor prognostic factor for glioma patients. Findings of ZEB1-AS1 as a potential prognostic biomarker was consistent with previous findings.

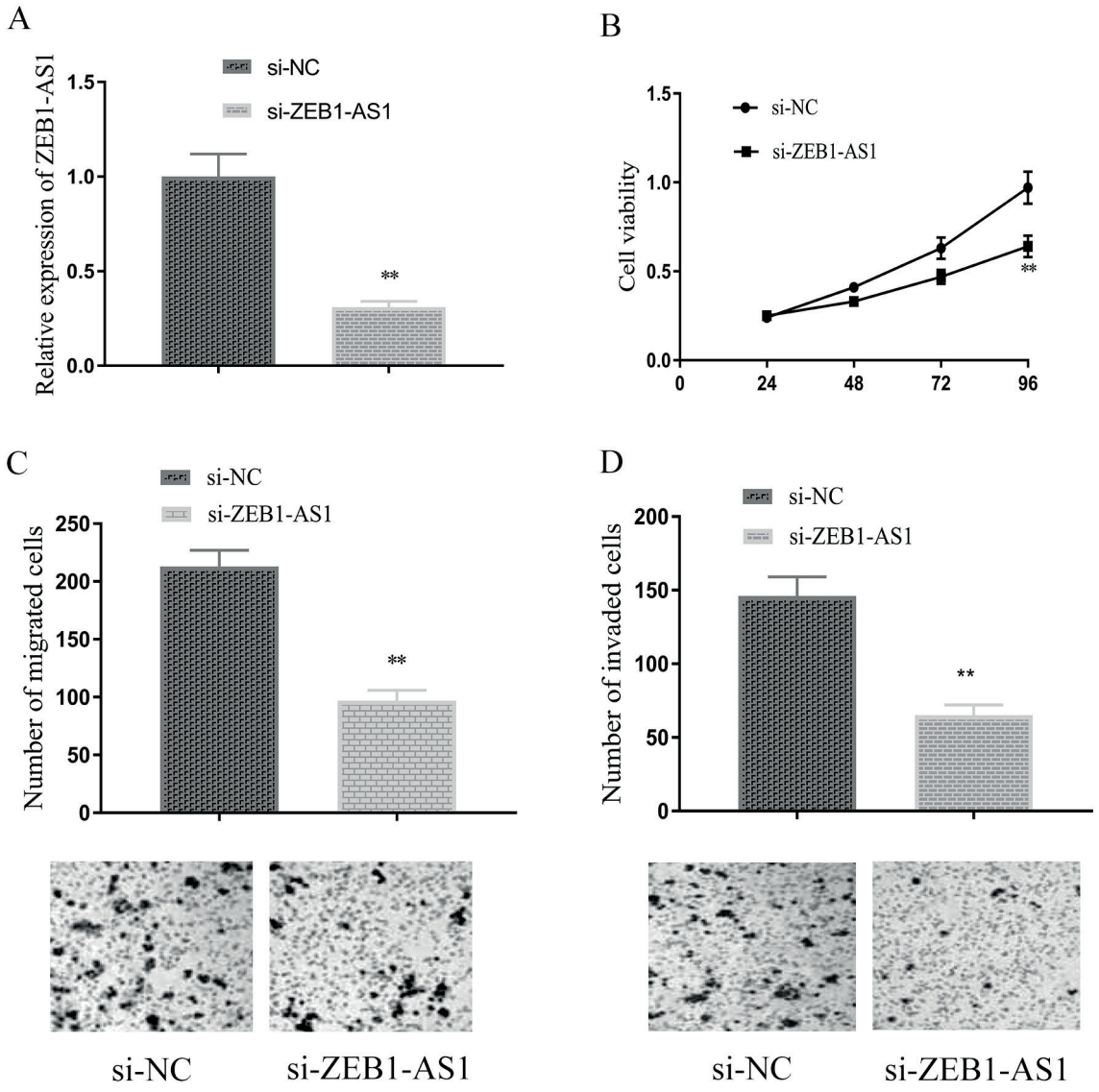

Figure 2. Effects of knockdown of ZEB1-AS1 on glioma cell viability *in vitro*. (A) qRT-PCR analysis of ZEB1-AS1 expression following treatment of U87 cells with si-ZEB1-AS1 or si-NC. (B) MTT assays were performed to determine the proliferation of U87 cells. (C, D) Transwell assays were employed to assess the changes in migratory and invasive capabilities of U87 cells transfected with si-NC or si-ZEB1-AS1. $^{**}p < 0.01$.

ZEB1-AS1 has been implicated in various types of cancers. For instance, Gong et al[22] found that up-regulation of ZEB1-AS1 was highly associated with shorter overall survival of patients with colorectal cancer. Loss-of-function studies suggested that ZEB1-AS1 efficiently promoted colorectal cancer cell proliferation partially by suppressing p15 expression. Zhang et al[23] found that ZEB1-AS1 was highly expressed in gastric cancer and its suppression inhibited gastric cancer cell proliferation and invasion by modulating miR-335-5p expression. In addition, survival assay revealed that patients with high ZEB1-AS1 expression had a poor overall survival. Besides, the tumor-promotive role of ZEB1-AS1 was also observed in osteosarcoma[24], prostate cancer[25] and hepatocellular carcinoma[16]. More importantly, Lv et al[18] showed ZEB1-AS1 as a tumor promoter in glioma. In line with their results, our functional assay also indicated that knockdown of ZEB1-AS1 significantly suppressed glioma cells proliferation, migration and invasion. Thus, our results, together with those reported previously, indicate that ZEB1-AS1 plays a critical role as oncogene in tumors.

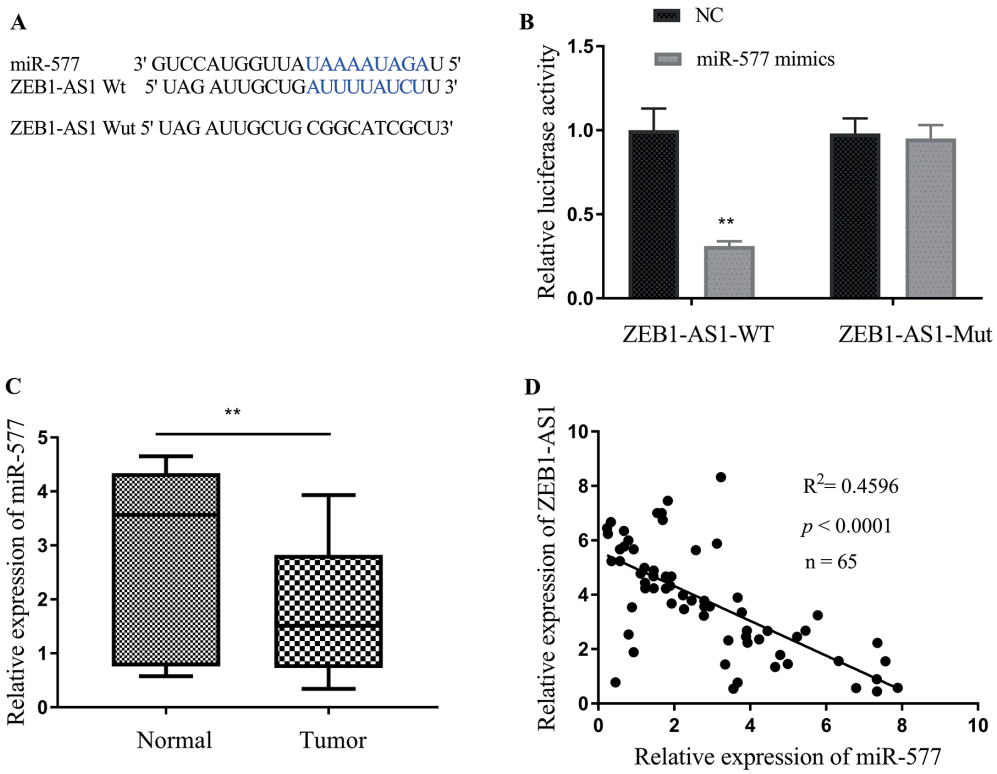

Figure 3. Regulation relationship between ZEB1-AS1 and miR-577. ***(A)*** The binding sites of miR-577 on ZEB1-AS1. ***(B)*** The luciferase assay showed that cells transfected with miR-577 mimics had less luciferase activity than those transfected with NC. ***(C)*** miR-577 expression was detected by RT-PCR in the collected 65 pairs of glioma tissue. ***(D)*** Correlation analysis between ZEB1-AS1 and miR-577 expression in glioma tissues. $^{**}p < 0.01$.

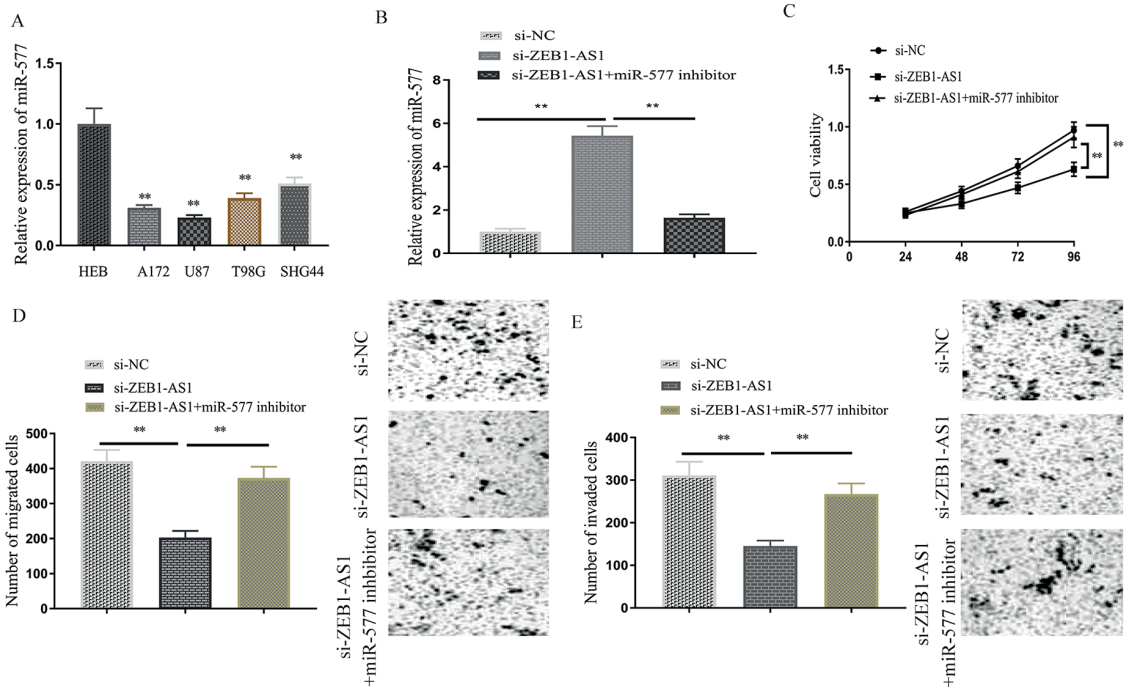

Figure 4. MiR-577 rescued the role of ZEB1-AS1 in U87 cells. ***(A)*** The expression of miR-577 was determined by RT-PCR. ***(B)*** RT-PCR was used to determine the levels of miR-577 in U87 cells cotransfected with si-ZEB1-AS1 and/or miR-577 inhibitor. ***(C)*** The growth curve of ZEB1-AS1 cells after transfection compared to controls by MTT. ***(D-E)*** migration and invasion of glioma cells transfected with miR-577 inhibitor or si-ZEB1-AS1 were determined by transwell assay. $^{**}p < 0.01$.

In order to explore the potential mechanism by which ZEB1-AS1 promoted glioma cells proliferation and metastasis, our attention focused on a novel interaction that lncRNAs could function as miRNA sponges to modulating the levels of miRNAs. MiRNA-577 has been reported to be down-regulated in several tumors[26-28]. Zhang et al[19] reported that miR-577 served as a tumor suppressor by inhibiting glioblastoma tumor growth via the Wnt signaling pathway. In our present study, we found that miR-577 was significantly down-regulated in both glioma tissues and cell lines. Using online software, we identified ZEB1-AS1 as a possible target of miR-577. Then, we verified that miR-577 could directly inhibit ZEB1-AS1 expression by dual luciferase reporter gene detection system. Furthermore, we also found that ZEB1-AS1 was negatively correlated to miR-577 expression in our glioma samples. Moreover, down-regulation of miR-577 expression could reverse the promotive effect of ZEB1-AS1 on the growth and metastasis of glioma cells *in vitro*. Taken together our findings for the first time indicated that ZEB1-AS1 exhibits its tumor-promotive role in glioma by modulating miR-577.

Conclusions

We showed that the oncogene ZEB1-AS1 promoted glioma progression by negatively regulating miR-577. Thus, our results indicated that ZEB1-AS1/ miR-577 regulatory axis may be a novel therapeutic target for glioma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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