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MicroRNA-28-5p regulates glioma cell proliferation, invasion and migration by targeting SphK1

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) are a conserved class of endogenous and short non-coding RNAs that post-transcriptionally regulate the expression of genes involved in diverse cellular processes. MiR-28-5p has been reported to be associated with several cancers, including human glioma. However, the roles of miR-28-5p in glioma development are poorly understood.

MATERIALS AND METHODS: Sixteen human glioma tissues and paired adjacent normal tissues were acquired through the Gansu Provincial Hospital. We performed quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) to detect the miR-28-5p expression between 16 paired adjacent normal and glioma tissues, as well as the miR-28-5p expression between normal human astrocytes cells and five glioma cell lines. To examine the functional roles of the downregulated miR-28-5p in glioma, cell viability and colony formation assays were performed for the analysis of cell growth. We overexpressed miR-28-5p by transient transfection of miRNAs mimics and performed the transwell Matrigel invasion assay and transwell migration (without Matrigel) assay. To investigate the roles of miR-28-5p in SphK1 expression, Western blot and Real Time-Polymerase Chain Reaction assays were performed.

RESULTS: In this work, we demonstrated that miR-28-5p is downregulated in glioma tissues compared to the adjacent normal tissues. Functional studies showed that miR-28-5p overexpression inhibited the cell viability, colony formation and proliferation; meanwhile, it induced the cell apoptosis. The transwell invasion assay indicated that miR-28-5p blocked the invasion and migration of glioma cells. SphK1 (Sphingosine kinase 1 antibody) is predicted as a targeted candidate of miR-28-5p. Then, the Luciferase reporter assay, Western blot and Real Time-Polymerase Chain Reaction (PCR) validated that miR-28-5p negatively regulated SphK1 expression by directly targeting its 3'untranslated regions (3'UTR) in U87 cells. Furthermore, rescue assay suggested that overexpression of SphK1 without its 3'UTR could prevent the miR-28-5p from inducing the inhibition of glioma tumor cells.

CONCLUSIONS: Our findings showed that miR-28-5p could suppress the growth, invasion and migration of glioma cells by suppressing the SphK1 expression. The results demonstrated that miR-28-5p might serve as an important potential therapeutic target for glioma.

Key Words:
MiR-28-5p, Glioma, SphK1, Proliferation, Invasion and migration.

Introduction

MicroRNAs (miRNAs) are a conserved family of small non-coding RNA molecules that are recognized as key regulators of gene expression. They regulate the expression of the target gene through the degradation of the target gene or post-transcriptional translation inhibition caused by binding to the 3'-UTR region of the target gene[1,2]. A great amount of evidence[3] showed that miRNAs participate in diverse cellular processes, such as cell growth, development, apoptosis, and even in cancers. Calin et al[4] reported that approximately 50% of miRNAs were located in tumor-related or fragile regions and validated that abnormal miRNA expression was closely related to cancer initiation and progression. Zhang et al[5] have shown that about 60% of protein-coding genes are regulated by miRNAs. Depending on the potential roles of their targets in the tumor,

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miRNAs function as either an oncogene[6] or a tumor suppressor. For instance, miR-125b inhibits liver cancer cell growth and metastasis by targeting LIN28B, functioning as a tumor suppressor. These data implied that miRNAs played crucial roles in the processes of cancers and might serve as novel biomarkers for cancer diagnosis and progression.

Human glioma is one of the most common malignancies and is the third leading cause of cancer-related death worldwide[7]. However, the molecular mechanism accounting for glioma growth and progression is less understood; therefore, it is crucial to explore and identify novel molecules responsible for glioma development. Recent studies demonstrated that abnormal expression of miRNAs is involved in glioma, such as miR-519a[8], miR-608[9], and miR-137[10]. However, the roles of miR-28-5p in glioma have not been elucidated. In this work, we showed that miR-28-5p was downregulated in glioma tissues, and miR-28-5p overexpression led to inhibit the cell growth and induce the cell apoptosis. Furthermore, we validated the direct target gene of miR-28-5p in glioma cells.

Patients and Methods

Tissue Samples, Cell Culture and Transfection

Sixteen human glioma tissues and paired adjacent normal tissues were acquired by the Department of Neurosurgery of the Gansu Provincial Hospital. We obtained informed consent by glioma patients and tissues were confirmed by immunohistochemical staining and pathological diagnosis. This study was approved by the Ethics Committee of the Gansu Provincial Hospital. The tissues were stored at -80°C. The human glioma cells, U87, were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA). U251 cells were cultured in L-15 medium supplemented with 10% FBS. All the cells were maintained in a humidified incubator with 5% CO_2 at 37°C. MiR-28-5p mimics and the scramble controls were purchased from Genepharma (Shanghai, China). The cells were transfected by Lipofectamine TM 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

RNA Isolation and Real Time-Polymerase Chain Reaction (RT-PCR)

The total RNA was isolated using the TRIzol reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Then, 500 ng of RNA was used for the reverse transcription (RT) reaction, and special RT primers were used for complementary deoxyribose nucleic acid (cDNA) synthesis of miR-28-5p. RNU6B (U6 small nuclear B non-coding RNA) was used as an internal control for the normalization of miR-28-5p. For cDNA synthesis of large oligonucleotides, oligo (dT) was used as a common primer. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for the normalization of SphK1 expression. The Real Time-Polymerase Chain Reaction (RT-PCR) was performed by the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the following conditions: 95°C for 5 min followed by 40 cycles of application at 95°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. Primer sequences used in this study were as follows: miR-28-5p, F: 5'-CGATGTGTCCGAAGGCCTCCTTACTC-3', R: 5'-GGCTAGGCAGGAACCA-3'; SphK1, F: 5'-GGATTGCGTGCTCGACTAGTCG-3', R: 5'-GGTGTAAACATCTCGTGGG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

The transfected cells were collected at 48 h after transfection and lysed by radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) buffer (50 mM Tris-HCl, pH 8.8, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) for 30 min at 4°C. The protein concentration was measured using the bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA). 50 μg of protein was used for the analysis of SphK1 expression and actin was used as a loading control. Rabbit monoclonal anti-SphK1 antibody (Abcam, Cambridge, MA, USA, 1:200 dilutions) and anti-actin antibody (Abcam, Cambridge, MA, USA, 1:1000 dilutions) were used as the primary antibodies. The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP; 1:1000 dilution).

WST-1 Assay

The transfected cells were plated with a density of 4×10^3 cells/well into 96-well plates. When transfected for 12 h, 24 h and 48 h, the cells were incubated with WST-1 reagent for about 1 h at 37°C, which is similar to the MTT reagent (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA). The absorbance at 490 nm (OD 490 nm) was measured with a spectrophotometer.

Colony Formation Assay

The transfected cells were seeded with a density of 200 cells/well into 12-well plates. The medium was refreshed every three days until most of the colonies compose of more than 50 cells. The colonies were washed, fixed and stained by crystal violet (Sigma-Aldrich, St. Louis, MO, USA). Finally, the stained colonies were imaged and counted.

Annexin V-FITC/PI Apoptosis Assay

Camptothecin (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium of transfected cells for induction of cell apoptosis. At 24 h after incubation, the cells were collected and detected with an Annexin V-fluorescein isothiocyanate/Propidium Iodide (FITC/PI; Beyotime, Shanghai, China) double staining kit on a BD FACS CaliburTM system (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instruction as described previously[11].

Transwell Migration and Invasion Assays

The migratory and invasive abilities were measured using transwell migration and invasion (with Matrigel-coated membrane) chambers (BD Biosciences, Franklin Lakes, NJ, USA). The transfected cells were plated in a serum-free medium ($2.5*10^4$ cells/well) in the upper layer of the chamber in 24-well plates for migration and invasion (with Matrigel). The lower chamber was incubated with a medium containing 10% of fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). After migration or invasion for approximately 20 h, the cells that migrated or invaded into the chamber membrane were fixed followed by being stained with 1% crystal violet and counted under a microscope.

Luciferase Reporter Assay

The 3'untranslated region (3'UTR) of SphK1 was amplified and inserted into the downstream of the Luciferase reporter gene. The mutant 3'UTR of SphK1 (GCUCC into CAAGG) was amplified using wild-type SphK1 3'UTR as the template. The cells were co-transfected with miRNA mimics and wild-type or mutant SphK1 3'UTR. After transfection for 48 h, the cells were collected and lysed by RIPA buffer (Beyotime, Shanghai, China). The Luciferase intensity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) following the manufacturer's protocols.

Statistical Analysis

All the data were represented as mean ± SD (Standard Deviation) and acquired from three independent experiments. The difference between groups was analyzed by paired Students' *t*-test, and $p<0.05$ was considered statistically significant.

Results

MiR-28-5p Is Downregulated in Human Glioma

To investigate the roles of miR-28-5p in human cancer development, miRNAMap2.0[12] was used for the analysis of miR-28-5p in diverse normal tissues and tumor tissues. As shown in Figure 1A, miR-28-5p was predicted to be downregulated in various tumor tissues compared to normal tissues, which implied it might serve as a tumor suppressor. Then, we performed Real Time-PCR to detect miR-28-5p expression between 16 paired adjacent normal and glioma tissues, and between normal human astrocytes cells and five glioma cell lines (Figure 1B, 1C). We found that miR-28-5p is downregulated in glioma tissues and cell lines compared to the control. These data implied that the abnormal expression of miR-28-5p might play an important role in glioma.

MiR-28-5p Overexpression Inhibits Glioma Cell Growth

To examine the functional roles of the downregulated miR-28-5p in glioma, cell viability and colony formation assays were performed for analysis of cell growth. The abundance of miR-28-5p in U87 and U251 glioma cells treated with miR-28-5p mimics was confirmed (Figure 2A). The results from WST-1 assay showed that miR-28-5p led to the inhibition of U87 cell viability by about 20-30% at different time points, compared to the cells with scramble control (Figure 2B). Accordingly, miR-28-5p inhibited the cell viability of

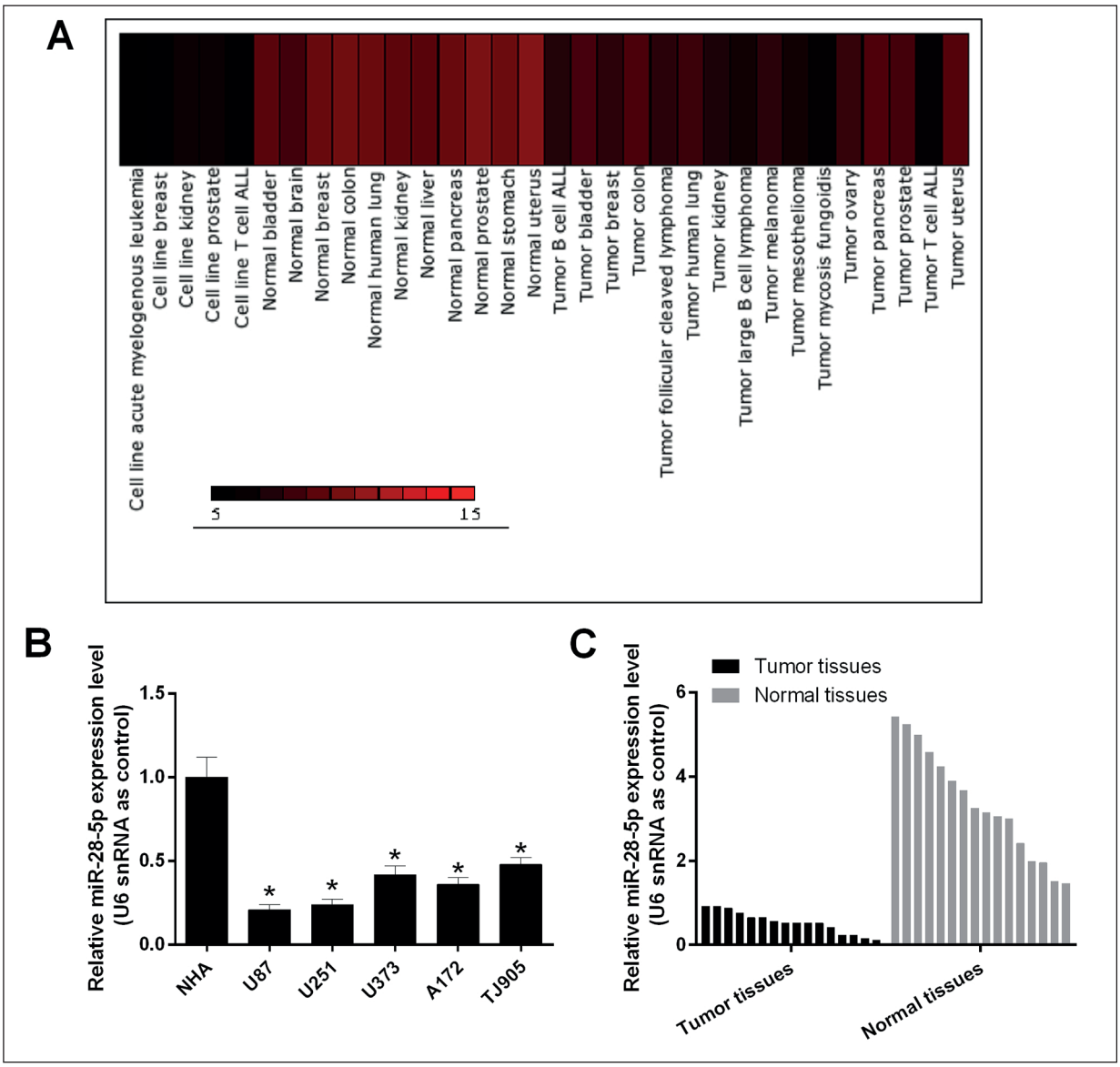

Figure 1. MiR-28-5p is downregulated in human glioma tissues. ***A,*** The expression of miR-28-5p was analyzed by miRNAMap-2.0 among diverse normal tissues and cancer tissues. The color represented the relative expression level of miR-28-5p. ***B,*** MiR-28-5p expression was quantified by Real Time-PCR in normal human astrocytes cells and five glioma cells. U6 was used as an internal control. ***C,*** MiR-28-5p expression was quantified by Real Time-PCR in 16 paired glioma tissues and normal tissues. U6 was used as an internal control. *$p<0.05$.

U251 (Figure 2C). Consistent with the roles of miR-28-5p in cell viability, miR-28-5p inhibited the number of U87 and U251 cell colonies by about 73% and 72%, respectively (Figure 2D, E). Then, the apoptosis experiment of glioma cells was performed using the Annexin V-FITC/PI double staining method. As shown in Figure 2F, the cells treated with miR-28-5p mimics had a higher apoptotic rate than the cells with scramble control. These data suggested that miR-28-5p might play a key role in glioma growth.

MiR-28-5p Suppresses Cell Invasion and Migration in Glioma Cells

To investigate the biological roles of miR-28-5p, we determined their effects on cell invasion and migration in U87. We overexpressed miR-28-5p by transient transfection of miRNAs mimics, respectively, and performed the transwell Matrigel invasion assay and transwell migration (without Matrigel) assay. The results showed that the number of cells invaded or migrated into the membrane was smaller upon miR-28-5p over-

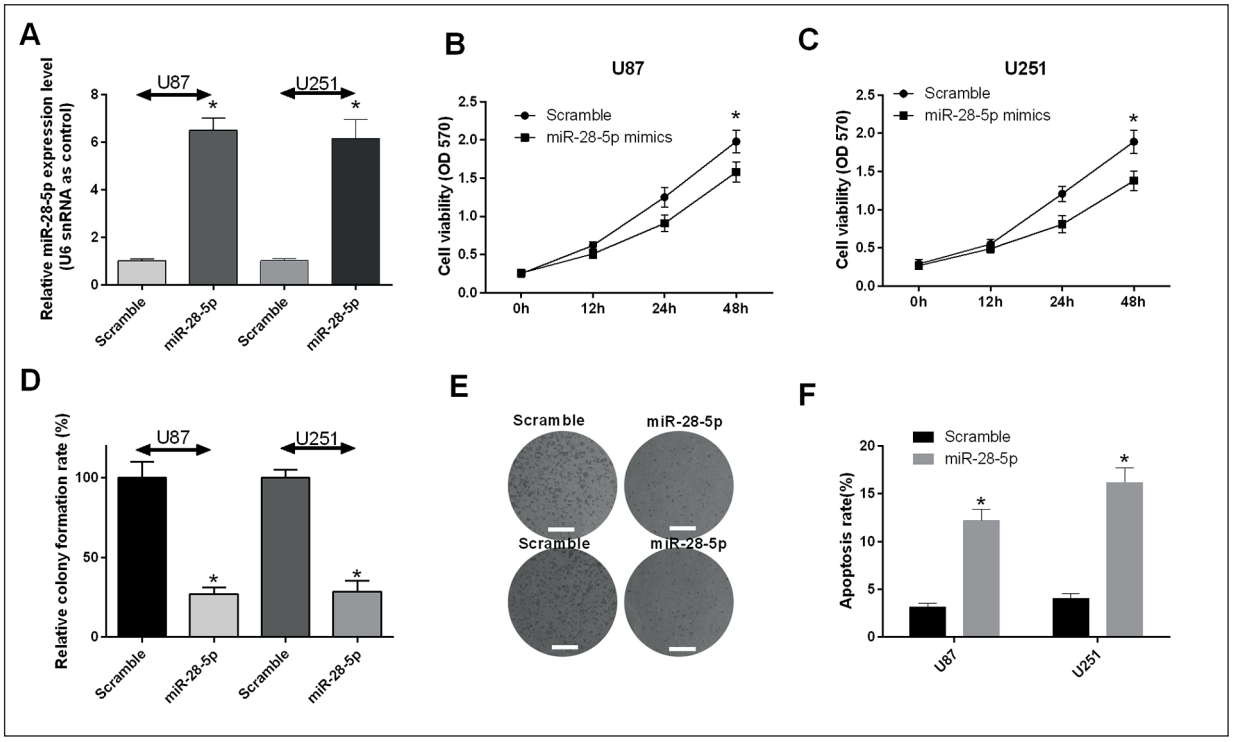

Figure 2. MiR-28-5p inhibits the growth of glioma cells. The cells were transfected with miR-28-5p mimics or scramble controls and Real Time-PCR. ***A,*** The experiment was performed for the validation of the overexpression of miR-28-5p. ***B, C,*** The transfected cells with miR-28-5p mimics or scramble controls were subjected to WST-1 assay for the analysis of cell viability at certain time point, including 0 h, 12 h, 24 h and 48 h. ***D, E,*** The transfected cells were subjected to colony formation assay for the analysis of cell growth. The images below represented the stained colonies (Magnification × 40). ***F,*** The cell apoptosis was performed using the Annexin V-FITC/PI double staining method. The results showed that the apoptotic rate of U87 and U251 cells with miR-28-5p mimics was 14.8% and 17.2%, while that of scramble controls was 4.18% and 4.98%. *$p<0.05$.

expression, compared with cells with scrambled control (Figure 3A, 3C). Similar results were observed in U251 cell (Figure 3B, 3D).

SphK1 Is a Direct Target Gene of MiR-28-5p in Glioma

To explore the molecular mechanism of the regulation of miR-28-5p in glioma growth, invasion and migration, Targetscan software was used for the prediction of the target of miR-28-5p. Among the candidates, SphK1 was selected for further researches. Figure 4A showed that there was a binding site of miR-28-5p in the 3'UTR of SphK1. To validate whether SphK1 is a direct target of miR-28-5p, a point mutation was generated with binding sites and cloned into the downstream of the Luciferase reporter gene (Figure 4A). Then, we co-transfected the U87 cells with mR-28-5p mimics and wild-type or mutant SphK1 3'UTR. The results from the Luciferase assay indicated that miR-28-5p led to the inhibition of the Luciferase intensity of SphK1 3'UTR, while this inhibition was abolished in the mutant SphK1 3'UTR (Figure 4B). To investigate the roles of miR-28-5p in SphK1 expression, Western blot and Real Time-PCR assays were performed. We discovered that miR-28-5p inhibited the expression of SphK1 protein and mRNA in U87 cells (Figure 4C). These results indicated that miR-28-5p negatively regulated SphK1 expression by directly binding to its 3'UTR in glioma cells.

SphK1 Overexpression Ameliorates the Inhibitory Effects of MiR-28-5p in Glioma Cells

Considering that SphK1 is a direct target of miR-28-5p, we determined whether SphK1 mediated the roles of miR-28-5p in cell proliferation, migration and invasion. We constructed SphK1 overexpressing plasmid (without its 3'UTR) and performed rescue experiment. The cells were co-transfected with miR-28-5p and SphK1 plasmid, in parallel with controls. We found that the overexpression of SphK1 restored several cell changes regulated by miR-28-5p, including the

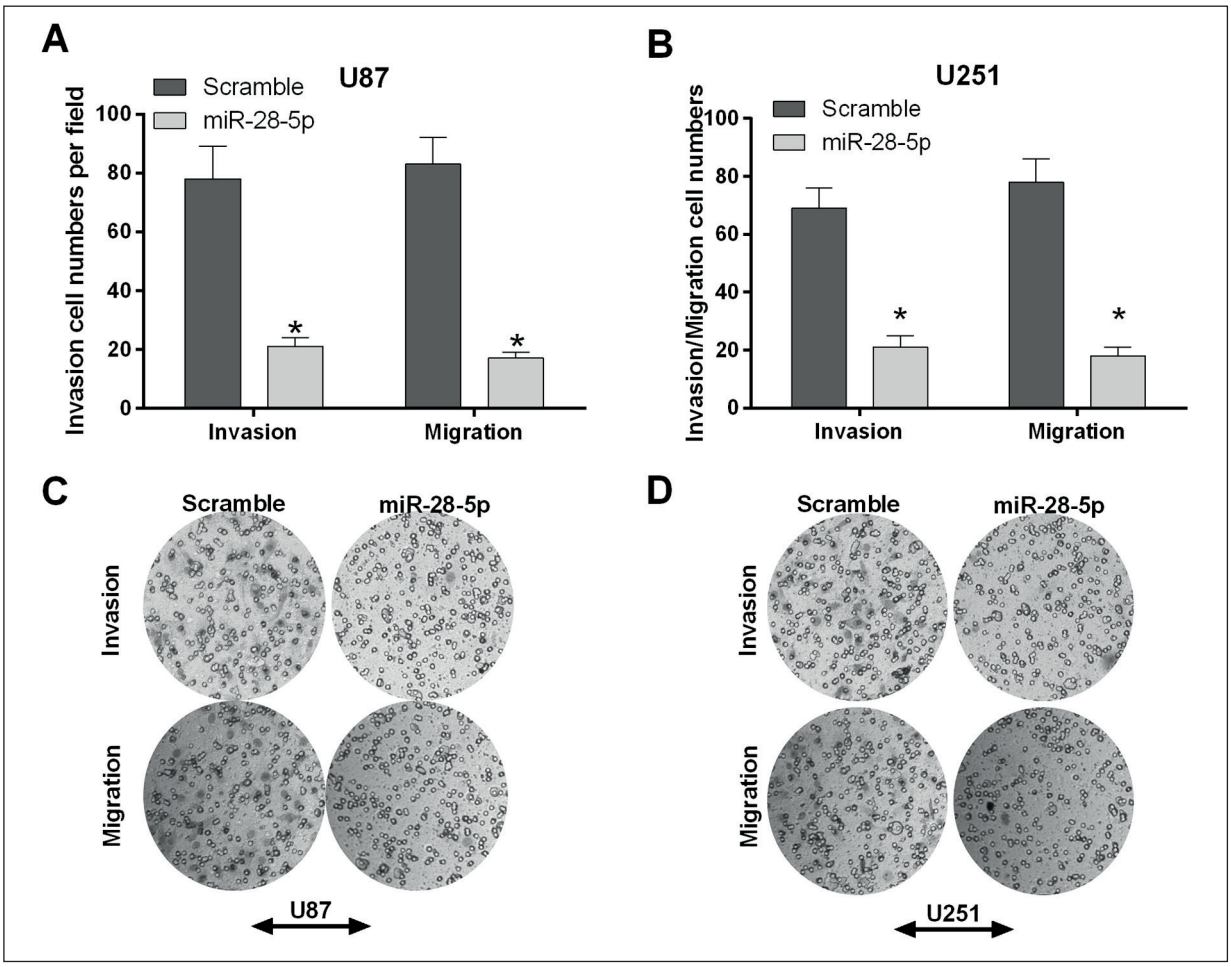

Figure 3. MiR-28-5p inhibits glioma cell invasion and migration. ***A, B,*** Transwell migration and Matrigel invasion assays indicated that miR-28-5p inhibited glioma cell migration and invasion abilities. ***C, D,*** The image represented the cells that invaded or migrated into the membrane (Magnification × 40). Each experiment was repeated in triplicate. *$p<0.05$.

cell viability (Figure 4D), cell apoptosis (Figure 4E) and cell invasion (Figure 4F) . These data indicated that miR-28-5p exerted the inhibitory effect by downregulating SphK1 in glioma cell proliferation and invasion.

Discussion

Recently[11], miRNAs have been investigated to be closely involved in the regulation of cellular functions through post-transcriptional regulation of gene expression *via* translational repression and/or targeted degradation caused by binding to 3'-UTRs in mRNA. In this work, we demonstrated that miR-28-5p acted as a tumor suppressor in glioma, and that its expression was frequently downregulated in glioma specimens and cell lines. Of course, we will perform further analysis to evaluate the prognostic value of miR-28-5p in clinical diagnose. Li et al[12] have been reported that many miRNAs, including miR-222, miR-370, miR-34a, miR-145, and miR-182 have predicted prognosis value in glioma. We speculated that miR-28-5p would be a new ideal biomarker as glioma.

MicroRNA-28 (miR-28), which was encoded by the sixth intron of the LIM domain lipoma-preferred partner (LPP) gene located on chromosome 3, is an intronic miRNA. Girardot et al[13] reported that the expression of miR-28 was controlled by LPP transcription regulation. Other research reports[14,15] also investigated the coordinated role of miR-28 with its host gene LPP in cell migration and adhesion, proliferation and apoptosis. However, the precise mech-

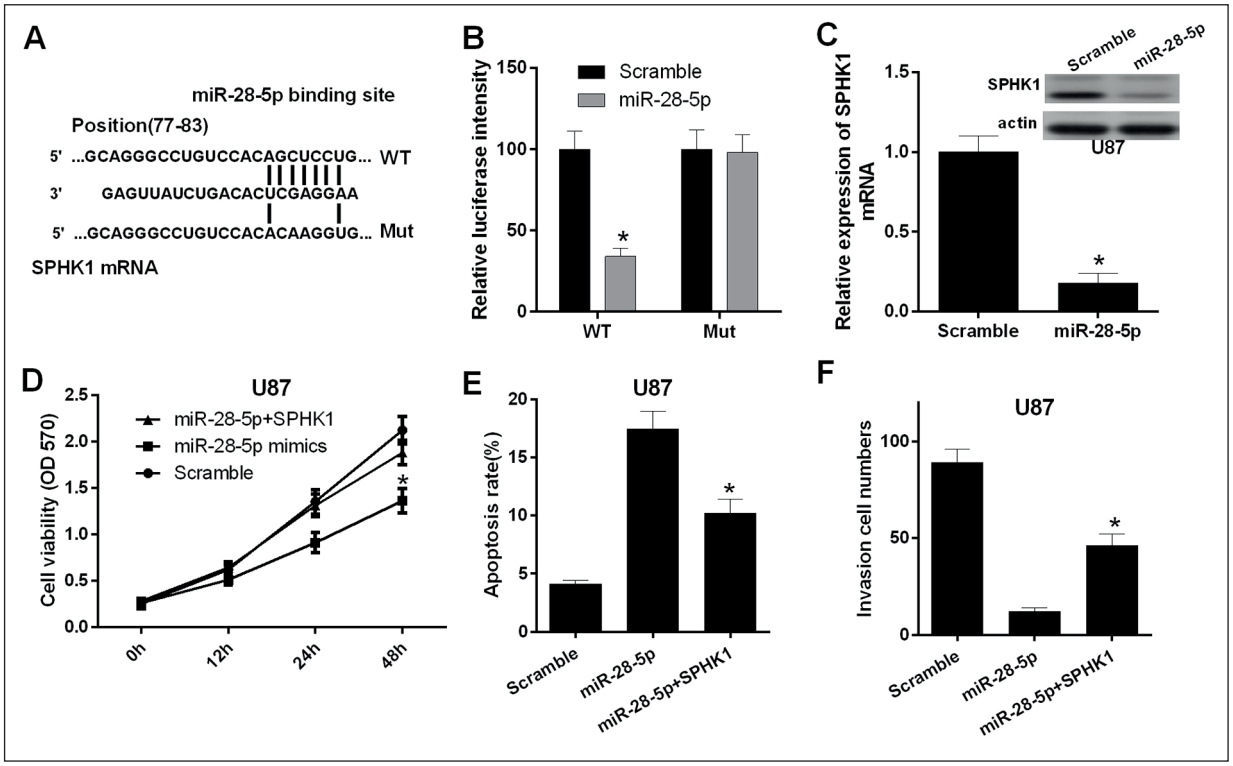

Figure 4. SphK1 is a direct target gene of miR-28-5p. ***A,*** Sequence alignment of SphK1 3'UTR potential binding sites and miR-28-5p seed sequence. A point mutation was generated within the binding sites. The seed sequence was AGCUCCU to muted ACAAGGU. ***B,*** The cells were co-transfected miR-28-5p mimics and wild-type or mutant SphK1 3'UTR, and the Luciferase intensity was examined by the Luciferase reporter assay. ***C,*** Real Time-PCR and Western blot assay showed that miR-28-5p inhibited SphK1 mRNA and protein levels. Actin was used as a loading control. ***D,*** WTS-1 assays were performed to analyze the cell proliferation. ***E,*** The cell apoptosis was performed using the Annexin V-FITC/PI double staining method. ***F,*** The transwell invasion (Matrigel) assay were performed to analyze the cell invasion abilities. Each experiment was repeated in triplicate. *$p<0.05$.

anism of miR-28 in glioma was still largely unknown. In the present work, we demonstrated that miR-28 was downregulated in glioma tissues and cell lines. Meanwhile, it inhibited glioma cell proliferation, invasion and migration. These findings strongly supported that miR-28 might serve as a tumor suppressor in glioma, which is corrected with other tumors. Furthermore, we identified the SphK1 as a downstream target of miR-28-5p.

Sphingosine kinase 1 (SphK1), has been reported to act as an oncogene in tumorigenesis in various types of cancers, by phosphorylating sphingosine to sphingosine-1-phosphate (S1P)[16-18]. Recent studies[19-23] have suggested that SphK1 plays an important role in ER-positive breast cancer, prostate cancer, primary colon cancer, lung cancer and adrenocortical carcinoma. However, relatively little is known about the events involving SphK1 in glioma progression, especially regarding the regulation mechanism of SphK1 in glioma. In our work, we first found that SphK1 was involved in glioma cell proliferation, invasion and migration inhibition phenotype which were induced by miR-28-5p. Our result showed that restoring the expression of SphK1 could significantly block the cell ability and invasion of U87 cells *in vitro.*

Conclusions

We found that miR-28-5p expression was downregulated in the TNBC (triple negative breast cancer) clinical tissue specimens and cell lines. Cell culture studies confirmed that miR-28-5p could suppress cell proliferation, invasion and migration of glioma *in vitro.* SphK1 was a direct target of miR-28-5p in glioma and proved that miR-28-5p/SphK1 axis might be a candidate target for new therapies.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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