

MicroRNA-129-3p suppresses tumor growth by targeting E2F5 in glioblastoma

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Abstract. – **OBJECTIVE:** Neuroma is the most common intracranial tumor. The mechanism of miRNA in glioma has gradually been understood. The purpose of this study was to investigate the role of MicroRNA-129-3p (miR-129-3p) in the pathogenesis of glioblastoma (GBM).

PATIENTS AND METHODS: Differential expression of miR-129-3p in samples was analyzed by bioinformatics. PCR was used to detect the expression of miR-129-3p in samples. CCK8 assay was used to detect the cell viability. Transfection of mimic and inhibitor altered the expression of miR-129-3p, and the biological function of miRNA was explored. Luciferase reporter gene was used to detect target genes of miRNA. E2F5 expression was inhibited by transfection of small interfering RNAs. Western blotting was used to detect protein expressions of cells.

RESULTS: miR-129-3p was low-expressed in the tissue samples. By transfecting mimic and the inhibitor, we found that increasing the expression of miR-129-3p can inhibit the cell viability. In contrast, inhibition of miR-129-3p promoted cell growth. Luciferase reporter gene and Western blot results suggested that E2F5 can be used as the target gene of miR-129-3p. Knock-down the target gene of the miR-129-3p, E2F5, also inhibited proliferation of glioblastoma.

CONCLUSIONS: miR-129-3p can inhibit the growth of glioblastoma by down-regulating the expression of E2F5. miR-129-3p can be a new target for the treatment of glioblastoma. Our research provides new ideas for the target therapy of glioma.

Key Words:

miR-129-3p, microRNA, Glioblastoma.

Introduction

Glioma is the most common intracranial tumor, accounting for about 40-50% of brain tu-

mors. As glioma has characteristics of rapid proliferation, easy metastasis and recurrence, and high degree of malignancy, which can't be completely removed by surgical treatment, even if combined with radiotherapy and chemotherapy, the effect was still not ideal¹. The median survival of untreated GBM patients was only about 4 months², even after the standard concurrent chemoradiation combined with temozolomide adjuvant chemotherapy; the median survival was only 15 months³. Therefore, exploring new treatment methods is imminent since gene therapy has become the current focus of glioma research⁴. MicroRNAs (miRNAs) are a family of endogenous small RNAs with approximately 20-24 nucleotides in length and have multiple important regulatory roles in cells. Each miRNA can have multiple target genes, and several miRNAs can also regulate one gene. This complex regulatory network can not only regulate the same gene but also regulate expressions of multiple genes either through a single miRNA or the combination of several miRNAs⁵. In recent years, the mechanism of miRNA in glioma has gradually been understood. There are 23 oncogenes (miR-21, miR-221/222 and miR-25) which are up-regulated in glioma cells by microarray to analyze expressions of miRNAs in glioma tissues and their cell lines, and there are another 34 tumor suppressor genes (such as miR-125b and miR-251) that are downregulated in miRNA expressions⁶. Related research found that miR-129-3p acted as a tumor suppressor molecule that could inhibit tumor development. Silencing of miR-129-3p promoted tumor growth, for example renal cancer⁷ and prostate cancer⁸. E2F transcription factor 5 (E2F5), an E2F family of proteins, can bind directly to promoters of cell cycle-related genes and regulate cell cycle progression⁹. Knockout of E2F5 can inhibit glioblastoma amplification¹⁰. In this study,

we found that miR-129-3p was low expressed in glioblastoma. However, the effect of miR-129-3p on the development of glioblastoma and its regulatory mechanism were not clear. Therefore, we investigated the role of miR-129-3p in the pathogenesis of glioblastoma.

Patients and Methods

Patients and Samples

The microRNA expression file of GBM was downloaded from GEO datasets and the dysregulated microRNAs were calculated with Limma R package. A total of 30 glioblastomas and 10 paracancerous specimens were obtained from the Department of Neurosurgery of our Hospital. Received date of samples was from July 2013 to July 2016. All glioblastoma samples were diagnosed by WHO as the standard. All samples were taken immediately after the liquid nitrogen, and stored at -8°C. This study was approved by the Ethics Committee of Huai'an First People's Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture and Transfection

Human glioblastoma cell lines, U87, U251, U373, LN229 and T98 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All glioblastoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS) and placed at 37°C, 5% CO₂ incubator. Normal human astrocytes (NHAs) were purchased from Lonza (Walkersville, MD, USA) and the culture conditions were in compliance with the manufacturer's instructions.

Quantitative Real-time PCR (qRT-PCR)

RNA was extracted from tissues and cells using TRIzol (Invitrogen, Carlsbad, CA, USA). The cell RNA concentration was measured using a Nanodrop Spectrophotometer (IMPLEN GmbH, Munich, Germany). MiR-129-3p and U6 RNA were purchased from GeneCopoeia (Guangzhou, China). The extracted RNA was inverted into cDNA using a PrimeScript RT reagent kit (TaKaRa, Otsu, Shiga, Japan). qRT-PCR was performed on a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with mixed primers and RNA mixed with SYBR Primix Ex Taq II kit (TaKaRa, Otsu, Shiga, Japan). Each experiment was performed in triplicate.

RNA Transfection of Cells

The mimic, inhibitor, negative control (NC), si-E2F5, and si-NC of miR-129-2-3p were synthesized by Genepharma (Shanghai, China). Mimic, inhibitor, negative control (NC), si-E2F5 and si-NC of miR-129-3p were transfected into 6-well plate with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were placed at 37°C, 5% CO₂ incubator; after culturing for 6 h, fresh medium was replaced. 24 hours later, cells were used for the further experiments.

Cell Viability Assay

Viability of glioma cells was detected by Cell Counting Kit-8 (CCK8, Beyotime Institute of Biotechnology, Shanghai, China). 2,000 cells were planted into each well of the plate. After 72 h, 100 uL of media in each well were remained and 10 ul of CCK8 reagent were also added. Cells were placed at 37°C, 5% CO₂ incubator for 2 h. Absorbance value at 450 nm wavelength was detected by microplate reader. Each experiment was performed in triplicate.

Luciferase Reporter Assay

The miR-129-3p target gene was predicted by the miRDB database. The wild-type and mutant E2F5 3'-UTR sequence-bound miR-129-3p regions were cloned into the downstream of the luciferase reporter in the pGL3-REPORT luciferase vector (Invitrogen, Carlsbad, CA, USA). After 24 h, fluorescence was measured using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's protocols.

Western Blot Analysis

Cells were lysed in a solution containing 20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na₃VO₄, 0.5 µg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride. Cell protein concentrations were determined using a Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amount of protein from each sample was added to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfected into polyvinylidene difluoride membranes (PVDF) (Millipore, Billerica, MA, USA). Primary antibodies were incubated overnight at 4°C. Primary antibodies were: E2F5 (Abcam; ab59769, Cambridge, MA, USA), GAPDH (Beyotime; AF0009, Shanghai, China).

After incubation, the membrane and the second anti-incubation solution were incubated for 1 h at room temperature. Tris-buffered saline and Tween 20 (TBST) (phosphate-buffered solution with 0.05% Tween 20) was used and the membrane was washed 6 times; protein bands were analyzed by Find-do \times 6 Tanon (Tanon, Shanghai, China).

Statistical Analysis

Statistical analysis was performed with Prism GraphPad version 6.0 (GraphPad Software Inc., La Jolla, CA, USA) software presented as mean \pm SD. Student's *t*-test was used to access the statistical significance of the data. $p < 0.05$ was considered statistically significant.

Results

miR-129-3p is Low Expressed in Glioblastoma

To investigate the expression of miR-129-3p in glioblastoma, differential miRNA expressions in Gene Expression Omnibus (GEO) database

GSE25631 were analyzed (Figure 1A). The results showed that miR-129-3p was low expressed in glioblastoma (Figure 1B). To further investigate the expression of miR-129-3p in glioblastoma, 10 cases of glioblastoma adjacent tissues and 30 cases of glioblastoma samples were collected. PCR was used to analyze differential expressions of miR-129-3p. Figure 1C showed that miR-129-3p was also low-expressed in glioma samples. This indicated that miR-129-3p was low expressed in glioblastoma.

Overexpression of miR-129-3p Inhibits the Growth of Glioblastoma

We also examined the expression of miR-129-3p in glioblastoma. MiR-129-3p was low expressed in glioma cell lines U87, U251, U373, LN229, and T98 (Figure 2A). NHAs was served as the control group. In order to investigate the biological function of miR-129-3p in glioblastoma, mimic and inhibitors of miR-129-3p were transfected with the glioblastoma cell lines U251 and U373. As shown in Figure 2B, miR-129-3p was highly expressed in glioblastoma cells after

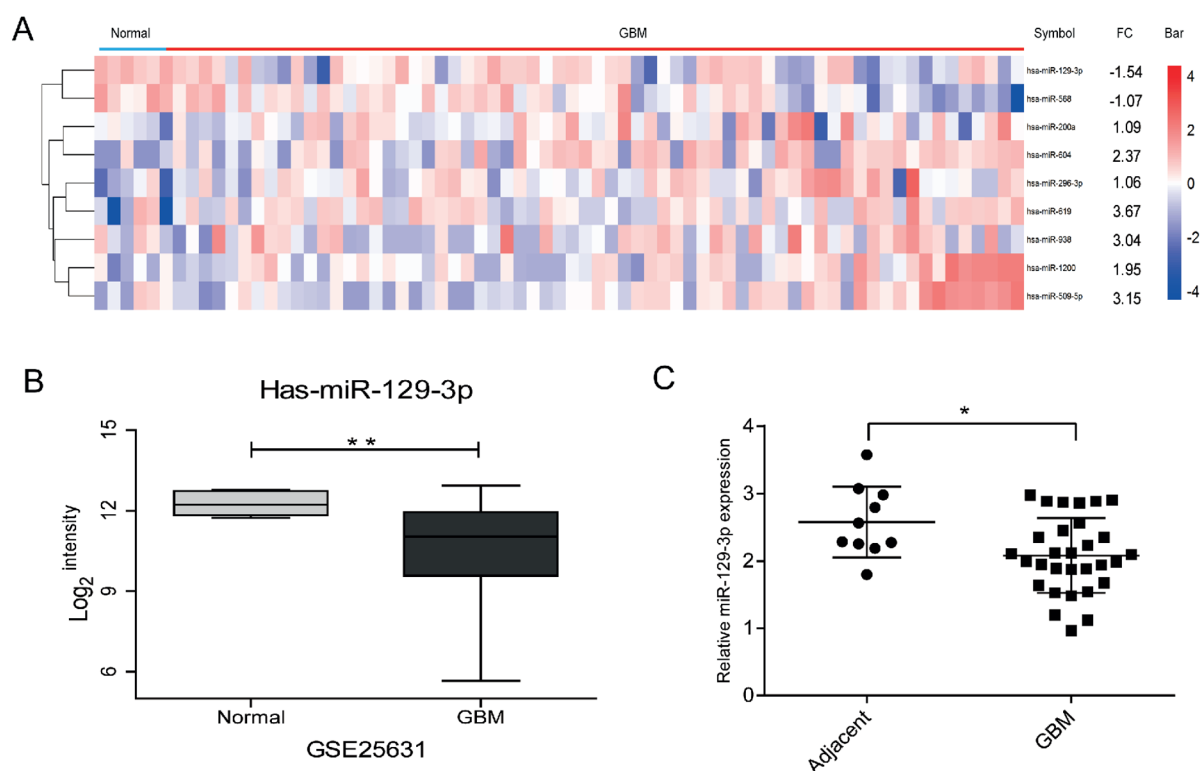


Figure 1. miR-129-3p is highly expressed in glioblastoma samples. **A**, Thermogram analysis showed that the expression of has-miR-129-3p in the Gene Expression Omnibus (GEO) database was highly expressed. Correlation analysis was shown in **B**, $**p < 0.01$ vs. Normal. **C**, The expression of miR-129-3p in glioma ($n = 30$) and adjacent tissues ($n = 10$) was detected by PCR, $*p < 0.05$ vs. Adjacent.

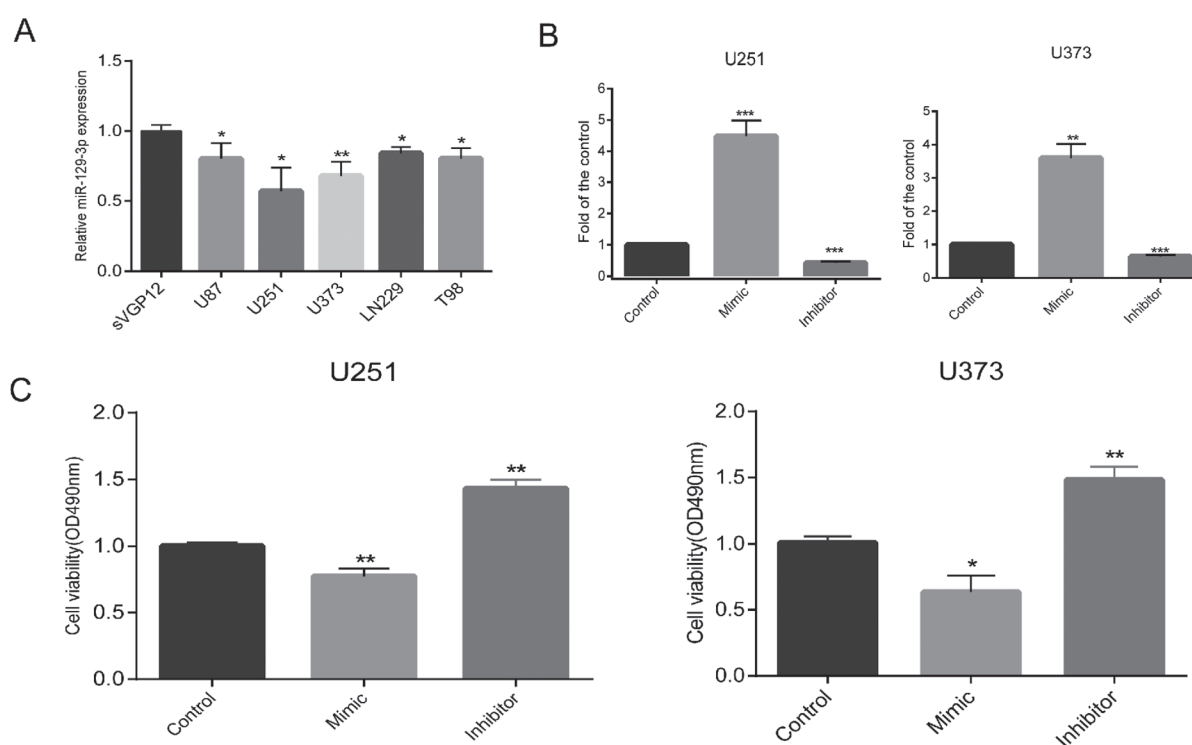


Figure 2. miR-129-3p inhibits the growth of glioblastoma cells. **A**, Detection of miR-129-3p expression in NHAs, U87, U251, U373, LN229 and T98 cells by PCR. $*p < 0.05$ and $**p < 0.01$ vs. NHAs. **B**, Mimic and inhibitor of miR-129-3p were transfected into U251 and U373 cells to detect the expression of miR-129-2-3p. $**p < 0.01$ and $***p < 0.001$. **C**, CCK8 assay is used to detect the viability of U251 and U373 cells after transfecting mimic and inhibitor of miR-129-2-3p. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs. Control.

transfected with miR-129-3p mimic. After transfected with miR-129-3p inhibitor, miR-129-3p expression was significantly down-regulated. Effect of miR-129-3p on proliferation of glioblastoma cells was detected by CCK8 assay. Treatment of mimic of miR-129-3p increased the expression of miR-129-3p but decreased cell viability (Figure 2C). Similarly, the addition of miR-129-3p inhibitor reduced the expression of miR-129-3p and the corresponding cell viability increased (Figure 2C). The above results indicated that miR-129-3p inhibited the growth of glioblastoma as a tumor suppressor gene.

MiR-129-2-3p Targets E2F5 in Glioblastoma

To further explore the underlying mechanism of miR-129-3p in regulating glioblastoma, miRDB webpage program was used to search for target genes of miR-129-3p. We found that E2F5 3'UTR bound directly to miR-129-3p (Figure 3A). In glioblastoma samples, the expression of miR-129-3p was negatively correlated with E2F5 (Figure 2B). To further confirm the regulation of miR-129-3p

on the expression of E2F5, dual-luciferase reporter was used. Consistent with the prediction, 3'UTR luciferase activity of the wild type E2F5 was significantly inhibited by miR-129-3p; however, the activity of mutant E2F5 did not alter significantly (Figure 3C). Transfection of mimic of miR-129-3p down-regulated E2F5 protein in glioblastoma, whereas its inhibitor increased E2F5 expression (Figure 3D). The above results confirmed that miR-129-3p targeted E2F5 in glioblastoma.

Down-regulation of E2F5 Inhibits Glioblastoma Growth

We further verified whether E2F5 was the target gene miR-129-3p. After transfected with small interfering RNA, the corresponding E2F5 protein expression was significantly reduced (Figure 4A). CCK8 assay showed that downregulation of E2F5 significantly inhibited glioblastoma growth (Figure 4B). It was suggested that E2F5 played an important role in promoting the growth of glioblastoma. Overexpression of miR-129-3p downregulated E2F5 in glioblastoma, thereby inhibiting the growth of glioblastoma.

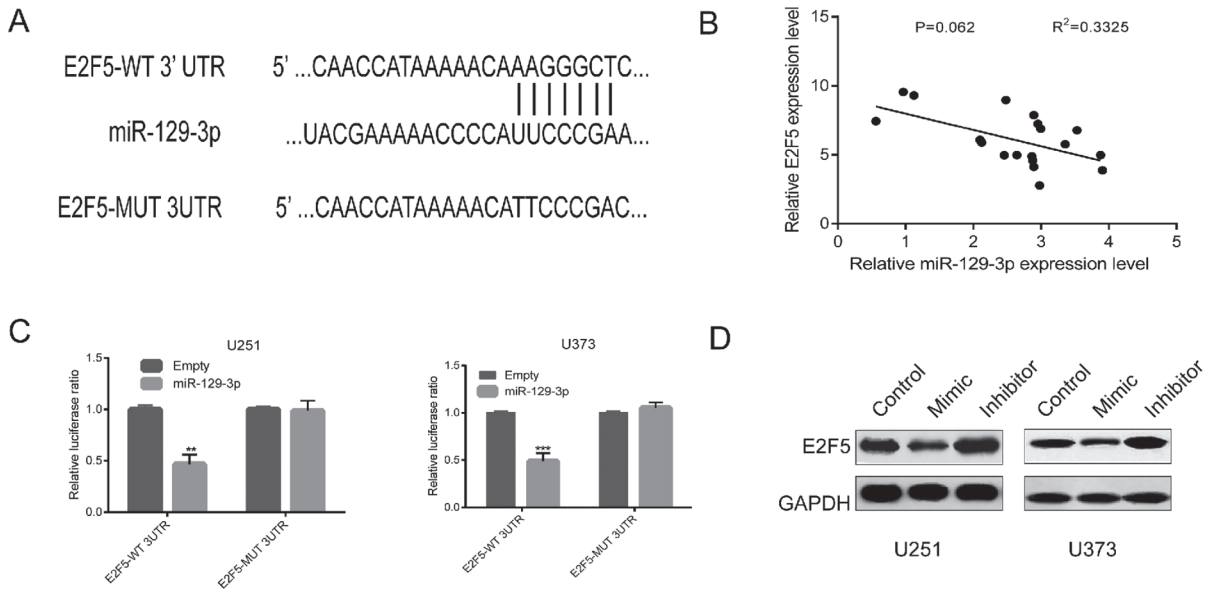


Figure 3. E2F5 is predicted to be the target gene of miR-129-3p. **A**, Bioinformatics predicts E2F5 as a potential target gene for miR-129-3p. Sequence binding details are presented. **B**, Pearson correlation analyzes negative correlation between miR-129-3p and E2F5 in clinical samples. **C**, Fluorescein reporter assay detects the activity of WT E2F5 3'UTR and MUT E2F5 3'UTR after co-transfecting mimic and inhibitor in U251 and U373 cells. (** $p < 0.01$, *** $p < 0.001$ vs. empty group). **D**, Western blotting to detect E2F5 protein expression level after transfected with mimic and inhibitor of miR-129-3p

Discussion

MiRNAs can regulate glioma cell proliferation, cell cycle and apoptosis, invasion ability, angiogenesis and chemosensitivity of temozolomide by inhibiting the expression of downstream target genes¹¹⁻¹³. In this work, the expression of miR-129-3p in 30 glioblastoma tissues was significantly lower than that in peritumoral tissues. MiR-129-3p can inhibit the proliferation of glioma cells and the biological function of miR-129-3p was mediated by binding to the 3-UTR region of E2F5 gene. MiR-129-3p gene was located at chromosome 11p11.2, indicating that the miRNA gene was located near or on the island of cytosine-phosphate-guanine

(CpG). By methylation, miR-129-3p can be silenced and play a biological function; this mechanism has frequently been reported in a variety of tumors. Upregulation of miR-129-3p in prostate cancer was able to reduce the CP110 expression and prevented cancer cell expansion and invasion⁸. In renal cell carcinoma, miR-129-3p can reduce the expression of invasion-related genes, including SOX4, MMP-2/9⁷. In the study of glioma, Qing et al¹⁴ found that the activation of NTS/NTSR1 inhibited the proliferation of glioma cells by NTS/NTSR1, which can improve the methylation of CpG island of miR-129-3p, thus inhibiting its expression. However, the hypothesis still needs to be further confirmed. In this study, we demon-

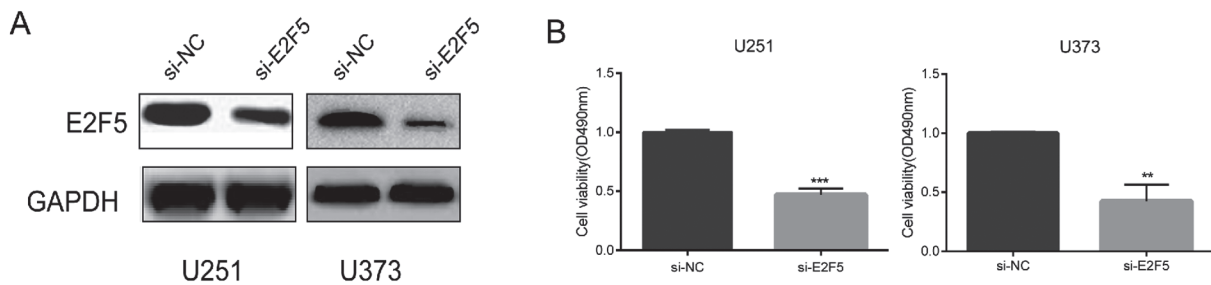


Figure 4. Downregulation of E2F5 reduces cell proliferation. **A**, Detection of E2F5 protein expression in U251 and U373 cells after transfected with small interfering RNAE2F5h and si-NC. **B**, CCK8 assay to detect cell proliferation of U251 and U373 after transfected with small interfering RNAE2F5h and si-NC. (** $p < 0.01$ and *** $p < 0.001$ vs. si-NC.

strated that MiR-129-3p was low expressed in glioblastoma. In addition, miR-129-3p expression was lower in glioblastoma than in normal glial cells. Overexpression of miR-129-3p significantly inhibited the growth of glioblastoma.

Recently, many researches have confirmed that E2F5 played an important biological role in the development of tumors. E2F5 regulated cell proliferation and cell cycle of G1-related proteins^{9,15-17}. E2F5 has been reported to be regulated by many miRNAs such as miR-154-5p¹⁸, miR-106¹⁹, miR-98²⁰, miR-128-2²¹ and miR-34a. In our study, we found that overexpression of miR-129-3p can down-regulate E2F5 and inhibit the growth of glioblastoma.

Conclusions

We showed that miR-129-3p inhibited glioblastoma by down-regulating E2F5 expression. Our research attempts to find targets for the regulation of glioma formation and development and also to provide new ideas for the target therapy of glioma.

Acknowledgements

The study was supported by the “533 Talent Project” research foundation of Huai’an to Dr. L.S. Ding (China, No. HAA201741)

Conflict of Interest

The Authors declare that they have no conflict of interests.

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