Am J Transl Res 2020;12(8):4749-4756
www.ajtr.org /ISSN:1943-8141/AJTR0111217

Original Article

MiRNA-27a-3p induces temozolomide resistance in glioma by inhibiting NF1 level

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Received March 22, 2020; Accepted July 19, 2020; Epub August 15, 2020; Published August 30, 2020

Abstract: To elucidate the role of miRNA-27a-3p in inducing Temozolomide (TMZ) resistance in glioma and the underlying mechanism. Relative levels of miRNA-27a-3p and NF1 in glioma tissues and peritumoral ones were detected. In addition, their levels in TMZ-sensitive and TMZ-resistant glioma tissues were examined as well. TMZ-resistant glioma cell lines SHG44-TG and U87-TR were used for *in vitro* experiments. Relative levels of miRNA-27a-3p and NF1 in TMZ-resistant glioma cells and their parental cells were determined. Subsequently, regulatory effects of miRNA-27a-3p on IC_{50} value of TMZ, viability and migratory ability in SHG44-TG and U87-TR cells were assessed. The interaction between miRNA-27a-3p and NF1 was evaluated by luciferase assay and Western blot. MiRNA-27a-3p was upregulated in glioma tissues than controls, especially TMZ-resistant ones, whereas NF-1 displayed a negative correlation to miRNA-27a-3p as its downstream target. Overexpression of miRNA-27a-3p increased IC_{50} value of TMZ, viability and migratory ability in SHG44-TG and U87-TR cells. Regulatory effects of miRNA-27a-3p on phenotypes of TMZ-resistant glioma cells were abolished by overexpression of NF1. MiRNA-27a-3p induces TMZ resistance in glioma by negatively regulating NF1 level.

Keywords: MiRNA-27a-3p, NF1, temozolomide, glioma, drug resistance

Introduction

Glioma is a common central nervous system tumor with an extremely poor prognosis. Its median survival is only about 15 months. Surgery combined postoperative chemotherapy and/or radiotherapy is the major strategy for glioma treatment [1]. Temozolomide (TMZ) is widely applied for chemotherapy. As a DNA alkylating agent, TMZ mediates destructive modification on DNA fragments and thus triggers apoptosis or necrosis of tumor cells. Nevertheless, a great number of tumor patients develop TMZ resistance during the middle or late course of chemotherapy, resulting in a poor chemotherapy efficacy [2]. Therefore, it is important to effectively maintain TMZ sensitivity in glioma patients.

Enhanced activities of MGMT and hMLH1 are responsible for TMZ resistance. In addition, GST, NER, ACBC1, Bcl-2 and Caspase are also involved in the development of TMZ resistance. However, current evidences have found that an effective regulation on one or more abovementioned genes cannot prevent or block the development of TMZ resistance [3]. It is suggested that TMZ resistance is a complicated process involving multiple targets.

MiRNAs are endogenous, non-coding RNAs with 21-24 nt long. They post-transcriptionally regulate target gene expressions by recognizing and binding the 3'UTR, and thus inhibit translation or degrade mRNA of target genes [4]. By mediating mRNA stability and controlling the translation rate, miRNAs therefore regulate protein synthesis. It is estimated that miRNAs are able to mediate about 30% of human protein-encoding genes. MiRNAs are extensively involved in life activities in mammals, including growth, differentiation, development and metabolism [5].

NF1 locates on human chromosome 17q11.2 [6]. The exon region 21-27 of the NF1 gene is responsible for encoding a protein consisting of 2818 amino acids (NP_000258.1), which is known as Neurofibromin [7]. Neurofibromin is widely expressed in human tissues. The nervous system is the mostly enriched with Neurofibromin, and Neurofibromin is also expressed in neurons, glial cells, and Schwann cells. Serving as a multifunctional protein, Neurofibromin can be used as a scaffold protein that affects the construction of cytoskeleton and the structure of cell membranes. Besides, Neurofibromin can also transmit signals for cell growth, proliferation, adhesion and migration. A relevant study has reported that Neurofibromin exerts an anti-cancer role in promoting apoptosis and inhibiting cell cycle distribution in tumor cells [8]. NF1 has been identified to be a tumor-suppressor gene as well. NF1 and Neurofibromin participate in cell proliferation and differentiation by activating the Ras pathway. The main functional region consisting of the 300-400 amino acid in the center of Neurofibromin is similar to GAP sequence, where is known as the NF1 GAP-related region (GRD). GRD is able to activate Ras GTPase *in vivo* [9]. In this paper, we elucidated the role of miRNA-27a-3p and NF1 in influencing TMZ sensitivity in glioma.

Methods

Subjects

A total of 41 cases of paraffin-embedded glioma tissues were collected from glioma patients treated in Beijing Tiantan Hospital from January 2012 to October 2019. There were 21 male patients and 20 females with 29-69 years. Based on differentiation level, 11 cases were well differentiated, 15 were moderately differentiated and 15 were poorly differentiated. 26/41 glioma patients had lymphatic metastases. Peritumoral tissues in glioma patients were collected as well. This study got approval by Ethics Committee of Beijing Tiantan Hospital and it was conducted after informed consent of each subject. This study was conducted in accordance with the Declaration of Helsinki.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cell lysate using TRIzol (Invitrogen, Carlsbad, CA, USA) reagent and purified. It was reversely transcribed into cDNA using the PrimeScript RT reagent Kit (Takara, Dalian, China). The cDNA was subjected to qRT-PCR using the SYBR Green Master Mix (Applied Biosystems, San Diego, CA, USA). Primer sequences (5'-3'): NF1: Forward: AGATGAAACGATGCTGGTCAAA, Reverse: CCTGTAACCTGGTAGAAATGCGA; miRNA-27a-3p: Forward: ACACTCCAGCTGGGCGCCTTGAATCGGTG, Reverse: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAGTGTCA; GAPDH: Forward: TCCTCCACCTTTGATGCG, Reverse: GTGCCTGGCTCACTCCTT; U6: Forward: CTCGCTTCGGCAGCACA, Reverse: AACGCTTCACGAATTTGCGT.

Cell culture and transfection

Glioma cell lines and TMZ-resistant glioma cell lines were cultured in Dulbecco's modified eagle medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). Cells were cultured to 50-70% density and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 4-6 h. Transfected cells for 48 h were used for following experiments.

Drug resistance model of human glioma cells induced by high dose shock

SHG44 and U87 cells in logarithmic growth phase were seeded in a 250 mL culture flask. When cells reached 70%-80% confluence, the cells were added with TMZ until the final concentration was 5 μg/mL. After 2 hours of treatment, the drug-containing medium was discarded and the living cells were then seeded in a new culture bottle. When the cells reached 70%-80% confluence, the above steps were repeated until the cell death rate was less than 5% at the mass concentration of TMZ of 0.4-0.5 μg/mL. The drug-resistant cell lines were named SHG44-TR and U87-TR, respectively.

Cell proliferation assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the Cell Counting Kit (CCK-8) kit (RIBOBIO, Guangzhou, China) for plotting the viability curves. The inhibited concentration of half of the cells (IC_{50}) of TMZ was also detected.

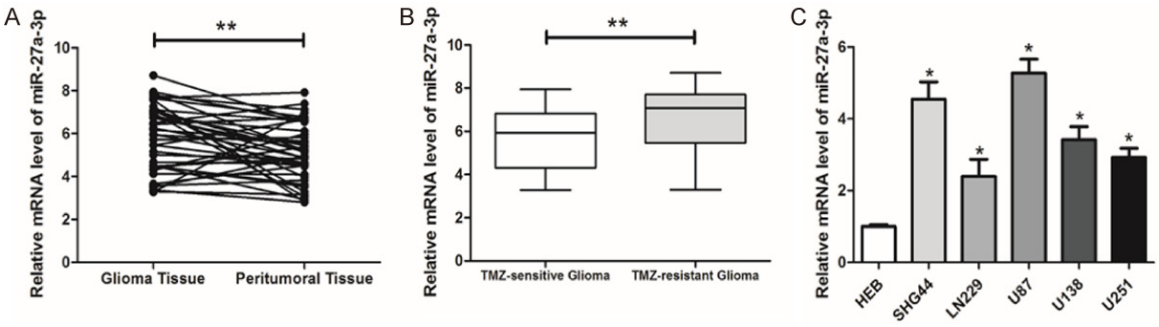

Figure 1. MiRNA-27a-3p was upregulated in glioma samples. A. MiRNA-27a-3p levels in glioma tissues and peritumoral ones. B. MiRNA-27a-3p levels in TMZ-sensitive glioma tissues and TMZ-resistant ones. C. MiRNA-27a-3p levels in glioma cell lines. *P < 0.05, **P < 0.01.

Transwell assay

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate. 200 μl of suspension (3×10^4 cells/ml) was applied in the upper layer of the chamber with 600 μL of medium containing 20% FBS in the bottom. After 48-h incubation, migratory cells in the bottom were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 10 random selected fields per sample.

Western blot

Cells were lysed for isolating proteins and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, nonspecific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses of grey values were finally conducted.

Luciferase assay

Cells were co-transfected with wild-type pGL3-NF1/mutant-type pGL3-NF1 and negative control/miRNA-27a-3p mimics. Cells were lysed in Dual-Glo® Luciferase Reagent for 10 min, followed by luciferase activity measurement.

Statistical analysis

Stata 9.2 software (StataCorp., College Station, Texas) was used for data analysis. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the two-tailed *t*-test. Pearson correlation test was applied for assessing correlation between two genes. $P < 0.05$ was considered as statistically significant.

Results

MiRNA-27a-3p was upregulated in glioma samples

We collected 41 glioma tissues and peritumoral ones, and miRNA-27a-3p was detected to be highly expressed in tumor tissues (**Figure 1A**). In 17 TMZ-resistant glioma tissues, miRNA-27a-3p was markedly upregulated than those of TMZ-sensitive ones (**Figure 1B**). Identically, miRNA-27a-3p was upregulated in glioma cell lines compared with that of human brain glial cell line (**Figure 1C**). Among the 5 detected glioma cells, SHG44 and U87 cells expressed the highest abundance of miRNA-27a-3p.

MiRNA-27a-3p was upregulated in TMZ-resistant glioma cell lines

IC_{50} value of TMZ was detected in 5 glioma cell lines, and it remained the lowest in SHG44 and U87 cells (**Figure 2A**). Compared with parental cells, IC_{50} value of TMZ was remarkably higher in TMZ-resistant SHG44 (SHG44-TR) and U87 (U87-TR) cells, proving the characteristic of TMZ resistance in glioma cell lines (**Figure 2B**). MiRNA-27a-3p level was higher in SHG44-TR and U87-TR cells than their parental cells (**Figure 2C**).

MiRNA-27a-3p increased TMZ resistance in glioma

After intervening miRNA-27a-3p level in TMZ-resistant glioma cells, we evaluated the poten-

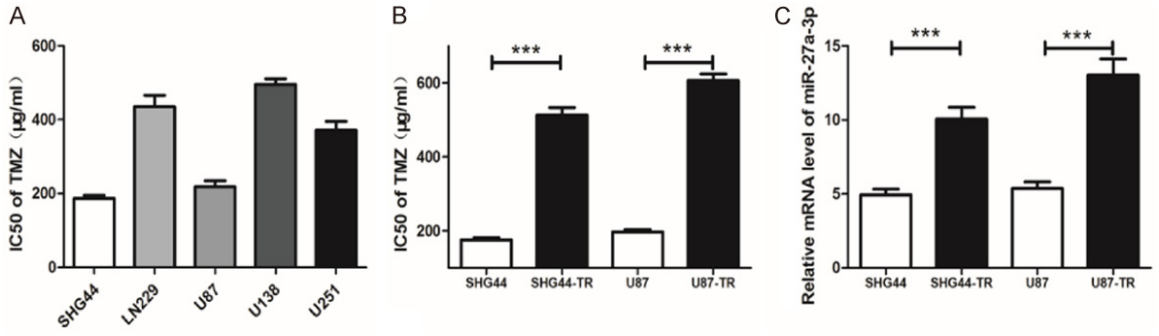

Figure 2. MiRNA-27a-3p was upregulated in TMZ-resistant glioma cell lines. A. IC_{50} value of TMZ in glioma cell lines. B. IC_{50} value of TMZ in SHG44-TR and U87-TR cells, and their parental ones. C. MiRNA-27a-3p levels in SHG44-TR and U87-TR cells, and their parental ones. ***P < 0.001.

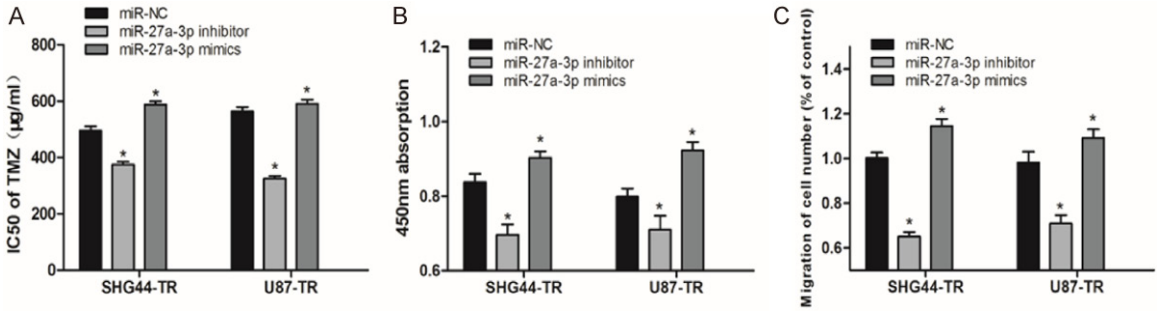

Figure 3. MiRNA-27a-3p increased TMZ resistance in glioma. A. IC_{50} value of TMZ in SHG44-TR and U87-TR cells regulated by miRNA-27a-3p. B. Viability in SHG44-TR and U87-TR cells regulated by miRNA-27a-3p. C. Migration in SHG44-TR and U87-TR cells regulated by miRNA-27a-3p. *P < 0.05.

tial influence of miRNA-27a-3p on drug resistance by measuring IC_{50} value of TMZ. Transfection of miRNA-27a-3p mimics markedly enhanced IC_{50} value of TMZ in SHG44-TR and U87-TR cells, whereas knockdown of miRNA-27a-3p obtained the opposite outcome (**Figure 3A**). Viability and migratory cell number were enhanced in TMZ-resistant glioma cells overexpressing miRNA-27a-3p (**Figure 3B**, **3C**). It is suggested that miRNA-27a-3p stimulated proliferative and migratory potentials in TMZ-resistant glioma cells.

A negative interaction between miRNA-27a-3p and NF1

No significant difference was observed in NF1 level between glioma tissues and peritumoral ones (**Figure 4A**). However, NF1 was downregulated in TMZ-resistant glioma tissues than those sensitive ones (**Figure 4B**). As expected, lower level of NF1 was observed in SHG44-TR and U87-TR cells compared with their parental cells (**Figure 4C**). In TMZ-resistant glioma tissues we collected, a negative correlation was identified between relative levels of miRNA-27a-3p and NF1 (**Figure 4D**). According to the binding sequences in the 3'UTR of NF1 and miRNA-27a-3p (**Figure 4E**), wild-type and mutant-type pGL3-NF1 vectors were constructed for conducting luciferase assay. Overexpression of miRNA-27a-3p decreased luciferase activity in wild-type pGL3-NF1, confirming the binding between miRNA-27a-3p and NF1 (**Figure 4F**).

We next explored the involvement of NF1 in TMZ resistance in glioma regulated by miRNA-27a-3p. Protein level of NF1 was markedly downregulated in TMZ-resistant glioma cells than parental ones. It is found that overexpression of NF1 reduced IC_{50} value of TMZ in TMZ-resistant glioma cells, which was further reversed by overexpressed miRNA-27a-3p (**Figure 5A**). Inhibited viability and migratory ability in SHG44-TR and U87-TR cells overexpressing NF1 were partially reversed by miRNA-27a-3p overexpression (**Figure 5B**, **5C**). Moreover, knockdown of miRNA-27a-3p upregulated NF1 in SHG44-TR and U87-TR cells (**Figure 5D**).

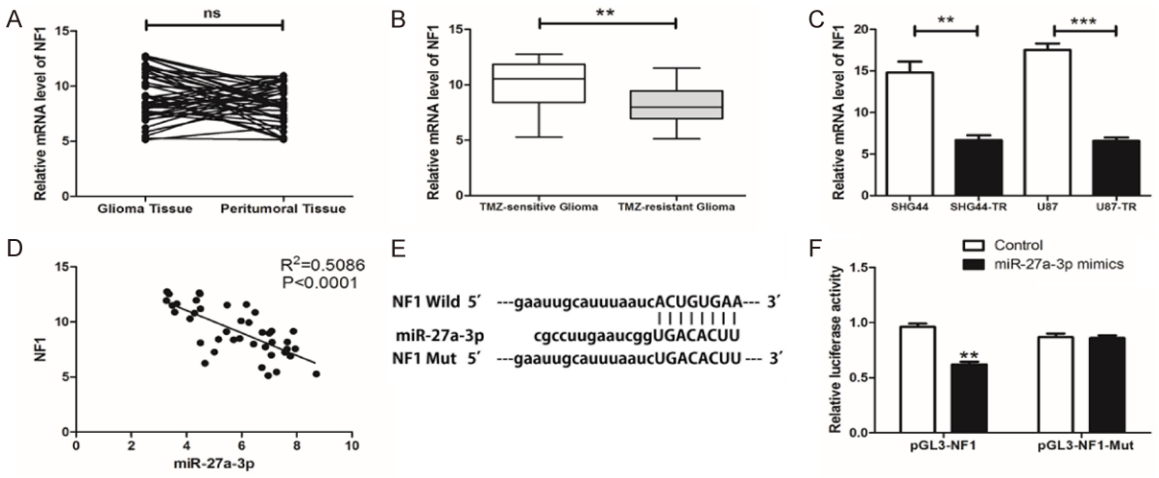

Figure 4. A negative interaction between miRNA-27a-3p and NF1. A. NF1 levels in glioma tissues and peritumoral ones. B. NF1 levels in TMZ-sensitive glioma tissues and TMZ-resistant ones. C. NF1 levels in SHG44-TR and U87-TR cells, and their parental ones. D. A negative correlation between relative levels of miRNA-27a-3p and NF1 in TMZ-resistant glioma tissues. E. Binding sequences in the 3'UTR of NF1 and miRNA-27a-3p. F. Luciferase activity in cells co-transfected with wild-type pGL3-NF1/mutant-type pGL3-NF1 and negative control/miRNA-27a-3p mimics. **P < 0.01, ***P < 0.001.

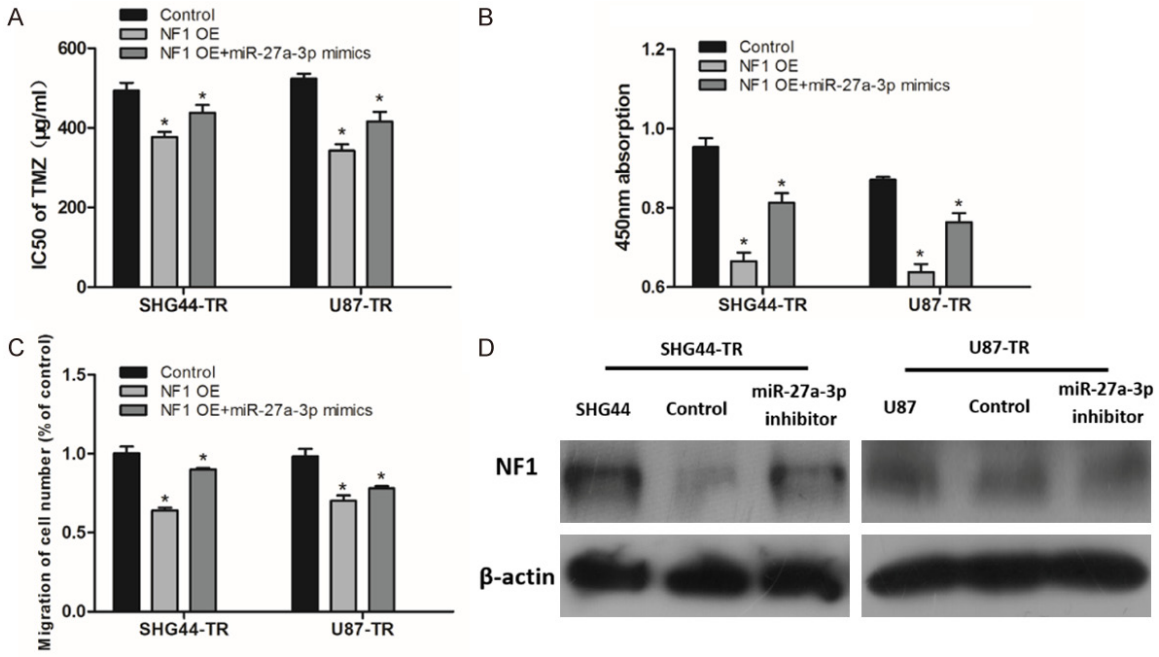

Figure 5. MiRNA-27a-3p regulated TMZ resistance in glioma by targeting NF1. A. IC_{50} value of TMZ in SHG44-TR and U87-TR cells co-regulated by miRNA-27a-3p and NF1. B. Viability in SHG44-TR and U87-TR cells co-regulated by miRNA-27a-3p and NF1. C. Migration in SHG44-TR and U87-TR cells co-regulated by miRNA-27a-3p and NF1. D. Protein level of NF1 in SHG44 and U87 cells, and SHG44-TR and U87-TR cells with miRNA-27a-3p knockdown. *P < 0.05.

Discussion

The miR-23a-27a-24-2 cluster is able to produce mature miR-23a-3p, miRNA-27a-3p and miR-24-3p [10, 11]. MiRNA-27a-3p can regulate adipogenesis, myoblast differentiation [12], skeletal muscle development [13], and osteoblast differentiation [14]. It is reported to be able to enhance expressions of specific protein transcription factors in breast cancer cells, and

these factors promote tumor cell proliferation and angiogenesis *via* mediating angiopoietin levels [15]. As a vital regulator, miRNA-27a-3p participates in mediating FOXO-1 level in breast cancer cells [16], MDR1/P-glycoprotein [17] and RXRα in adipogenesis [18].

The role of miRNAs in influencing chemotherapy sensitivity has been well concerned [19, 20]. Differentially miRNAs in glioma profiling are closely linked to histological subtypes, tumor staging, tumor recurrence and overall survival [21, 22]. They are potential targets for alleviating chemotherapy/radiotherapy resistance [23, 24]. Our results demonstrated that miRNA-27a-3p was upregulated glioma tissues than peritumoral ones. In particular, it was highly expressed in TMZ-resistant glioma tissues. Compared with parental cells, miRNA-27a-3p was identically upregulated in SHG44-TR and U87-TR cells. It is suggested that miRNA-27a-3p was involved in TMZ resistance in glioma. We subsequently demonstrated that overexpression of miRNA-27a-3p enhanced IC_{50} value of TMZ, viability and migratory cell number in TMZ-resistant glioma cells. On the contrary, knockdown of miRNA-27a-3p obtained the opposite results, indicating that miRNA-27a-3p aggravated the malignant phenotypes in TMZ-resistant glioma.

NF1 is the transcriptional factor in the RAS/MAPK pathway that is able to regulate cell phenotypes. Overexpression of NF1 can activate the RAS/MAPK pathway and thus participates in tumor progression [25, 26]. A previous study has shown that NF1 is upregulated in glioma and triggers glioma cell metastasis by targeting MXI1 [27]. The deficiency in NF1 will cause changes in Ras and its downstream pathways, including the Raf/MEK/ERK, PI3K/AKT/mTOR and Wnt/β-catenin pathway. Wnt protein binds to receptors on the cell membrane through autocrine or paracrine effects, thus regulating target gene expressions by activating intracellular pathways. Wnt is of significance in mediating cell growth, differentiation, metastasis, polarization and apoptosis [28]. Our findings revealed that NF1 was downregulated in TMZ-resistant glioma tissues and cell lines. Through bioinformatics analysis and luciferase assay, we have confirmed the binding between NF1 and miRNA-27a-3p. A negative interaction between them was further identified in TMZ-resistant glioma tissues. Notably, overexpression of miRNA-27a-3p abolished the role of NF1 in weakening the cytotoxicity in TMZ-resistant glioma cells. Collectively, miRNA-27a-3p stimulated TMZ resistance in glioma by promoting proliferative and migratory abilities with the negative interaction with NF1. Our conclusion should be further validated in *in vivo* experiments. This study preliminarily proved that MiR-27a-3p can regulate temozolomide resistance by targeting the expression of NF1 in glioma, providing a new perspective for the resistance mechanism of glioma, and also for clinical gliomas treatment provides a theoretical basis.

Conclusions

This study demonstrates the role of miRNA-27a-3p in promoting TMZ resistance in glioma cells through targeting its downstream gene NF1. Knockdown of miRNA-27a-3p may help improve the therapeutic effect of TMZ-based chemotherapy on glioma. Our findings provide novel ideas for developing therapeutic strategies for TMZ-resistant glioma.

Disclosure of conflict of interest

None.

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