RESEARCH ARTICLE

AntagomiR-27a Targets FOXO3a in Glioblastoma and Suppresses U87 Cell Growth *in Vitro* and *in Vivo*

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Abstract

Objective: To study the effect of the antagomiR-27a inhibitor on glioblastoma cells. Methods: The miR-27a expression level in specimens of human glioblastoma and normal human brain tissues excised during decompression for traumatic brain injury was assessed using qRT-PCR; The predicted target gene of miR-27a was screened out through bioinformatics databases, and the predicted gene was verified using genetic report assays; the effect of antagomiR-27a on the invasion and proliferation of glioma cells was analyzed using MTT assays and 5-ethynyl-2'-deoxyuridine (EdU) labeling. A xenograft glioblastoma model in BALB-c nude mice was established to detect the effect of antagomiR-27a on tumour growth. Results: qRT-PCR results showed that miR-27a significantly increased in specimens from glioblastoma comparing with normal human brain tissues. Th miR-27a inhibitor significantly suppressed invasion and proliferation of glioblastoma cells. FOXO3a was verified as a new target of miR-27a by Western blotting and reporter analyzes. Tumor growth *in vivo* was suppressed by administration of the miR-27a inhibitor. Conclusion: MiR-27a may be up-regulated in human glioblastoma, and antagomiR-27a could inhibit the proliferation and invasion ability of glioblastoma cells.

Keywords: MiR-27a - antagomiR-27a - glioblastoma - target gene FOXO3a

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Introduction

Glioblastoma (GBM) is one of the most common intracranial tumors, about 60% of caes being malignant. As there is no effective treatment for glioblastoma, even combined surgery, radiotherapy and chemotherapy, the median survival period of glioblastoma patients is about 9-12 months. With the development of molecular biology a few genes, including oncogene and tumor suppressor gene, were found to be associated with the development and progression of glioblastoma, however, the underlying mechanism remains unclear (Burgess et al., 2008). With the improvement of studies on miRNA, more and more evidences indicate that miRNA might play a great role in the development and progression of glioblastoma , thus providing new insights by developing miRNA-based methods to diagnose and treat glioblastoma (Sevignani et al., 2006; Papagiannakopoulos et al., 2008).

MicroRNAs (miRNAs) are endogenous non-coding short chain RNAs with a length of about 22nt and they are highly conserved and widely expressed in the plant and animal cell. By binding to target sites in the 3'-UTR of target mRNAs, miRNAs inhibit the expression of target genes (Cummins et al., 2006). MiR-27, including miR-27a and miR-27b, was firstly cloned form Hela cells by Mourelatos in 2002 (Mourelatos et al., 2002). It has been reported that miR-27a played as oncogene in pancrease cancer, promoting the colony formation, proliferation and invasion via inhibiting the expression of sprouty2 (Ma et al., 2010). MiR-27a also has been found to serve as an oncogene in gastric cancer, promoting the growth of gastric cells via inhibiting the expression of prihibitin (Liu et al., 2009). In addition, it has also been reported in studies of lung and breast cancers (Li et al., 2010; Wang et al., 2011) Although it has been nearly 10 years since miR-27 was firstly found, there are few research articels on miR-27 and its function in glioblastoma.

FOXO3a is a downstream effector of PI3K/AKT transduction signal pathway, belonging to Forkhead boxO family (Castrillon et al., 2003). Paik et al reported in Cell that the shift mutation of FOXO3a gene led to development of tumor (Paik et al., 2007). Animal experiments confirmed that high expression of FOXO3a protein inhibited tumor formation (Hu et al., 2004) FOXO3a is a tumor suppressor (Arden et al., 2007). In vivo and in vitro experiments showed that interaction of FOXO3a and some other proteins such as Bim could inhibit tumor growth (Greer et al., 2005), In gastric cancer, FOXO3a/FKHRL1 could interact with RUNX3 which induces cellular apoptosis and inhibit proliferation, thus stimulating the expression of pro-apoptotic protein Bim and inhibiting the development and progression of gastric cancer (Yamamura et al., 2006). It has been reported that FOXO family could regulate the cell cycle transformation,

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differentiation and metabolism of epithelial cells via mediating the downstream target genes such as Sprouty2 and TRAIL (Arden et al., 2007; Paik et al., 2007). So far, the miRNAs which target FOXO3a gene have barely been reported. This study found that the protein expression level of FOXO3a and the activity of its report gene were inhibited after overexpression of miR-27a and up-regulated after inhibition of miR-27a, indicating FOXO3a might be a target gene of miR-27a .

We interfered U87 glioblastoma cells with antagomiRs to knock down the expression of miR-27a, analyzed the effect of miR-27a on U87 cells and explored the mechanism of regulation of its predicted target gene FOXO3a, meanwhile established subcutaneous xenograft glioblastoma tumor model to verify the in vitro results and confirmed that the miR-27a could promote the proliferation and invasion of glioblastoma cells, thus providing foundation for further study of glioblastoma gene therapy targeting miR-27a.

Materials and Methods

General information

The specimens were obtained from Neurosurgery of Wuxi People's Hospital from May 2008 to May 2010. The types of all tumors were confirmed to be glioblastoma by the pathologic analysis, including 15 males and 12 females, aged form 24 to 65, the average age is 44. According to the classification standard of tumors of central nervous system (2000), there are 16 low-grade specimens and 11 high-grade specimens. All the specimens had never been treated with radiotherapy, chemotherapy or other therapies. 6 specimens of normal forehead or temporal lobe brain tissues were during decompression for traumatic brain injury. All the human materials were used in accordance with the policies of the Institutional Review Board.

Construction of report gene vector

The reporter constructs containing pLUC-FOXO3a and pLUC-mutFOXO3a of target sequence were generated by RiboBIO corporation.

Total RNA extraction

Total RNA extraction was performed with miRNA extraction kit (TIANGEN corporation) according to the manufacturer's instruction. Briefly, unified specimens were placed in the EP tube, adding appropriate volume of lysis buffer as required. After stratification, elution, drying, and finally, RNAs were dissolved with 30 μl Rnase free ddH2O. MiRNA reverse transcription was performed using All-in-One cDNA Synthesis Kit (qcbio .China).

qRT-PCR assay

qRT-PCR was performed with All-in-OneTM qPCR Mix kit (GeneCopoeia). The above cDNA solution was diluted by 10 times. 5.6 μl of the diluted solution was used to make 20 μl reaction system according to the manufacturer's instruction. Real-time PCR was performed using an Applied Biosystems 7500HT Sequence Detection system by standardized protocol.

Cell culture of U87 cells

Human U87 glioblastoma cell lines were purchased from Chinese Academy of Sciences Cell Bank. U87 cells were maintained in a 37˚C, 5% CO_2 incubator in DMEM (Gbico) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Gibco), which were digested and reproduced with 0.25% trypsin (Hyclone).

Transfection antagomiR-27a

miR-27a inhibitor (antagomiR-27a) was perchased from RiboBIO conporation, stock solution was made by adding 1nmol antagomiR-27a into 50 μl Rnase free H_2O, whose concentration was 20 μmol/L. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. Briefly, cells were plated 5×10^5 cells per well in a 6-well plate, maintained in a 37˚C, 5% CO_2 incubator overnight, when cells were grown into 80%-90% confluent, (1) transfect cells with pEGFPR-antagomiR-27a (experimental group): 4μg recombinant plamid and 10 μl lipofectamine 2000 were added into 500μl serum free DMEM per well. Serum free DMEM was changed into DMEM with 10% FBS 4 hours later; (2) transfect with vector (negative control group): U87 cell was transfected with pEGFPR as in (1); (3) blank control group: normally cultured U87 cells as blank control. Fluorescence microscope was used to observe the transfection efficiency after transfection for 48 hours.

Cellular proliferation assay

U87 cells in logarithmic growth phase were plated into 96-well plates with 5 replicate wells for each group. 5g/L MTT (20 μl)) was added into each well at each day of consecutive 6 days and the cells were incubated for additional 4 h, the supernatant was then discarded. DMSO (200 μl) was added to each well to dissolve the precipitate. Optical density (OD) was measured at wavelength of 490 nm. Cell growth inhibition rate formula is (1- AG)/Abx100% (AB, absorbance value of the blank control group; AG, absorbance value of the experimental group). Repeat the experiment 3 times and calculate the average value to draw the growth curve.

5-ethynyl-2'-deoxyuridine (EdU) (RiboBIO .China) labeling

Sterilized coverslips was put into 24-well plate, which was covered by U87 cells. Cells were transfected with pEGFPR-antagomiR-27a, pEGFPR-NC, BLANK reagent, respectively. Every well was added 200 μl EdU after transfected for 24 hours. Cells were incubated in 37˚C, 5% CO_2 incubator for 2 hours. The nuclei was stained by Hoechst33342. Cells were then detected with a fluorescence microscope.

Western blotting analysis

Proteins (5 mg/lane) were resolved in 10% SDS–PAGE and electrotransferred to a PVDF membrane using standard procedures. After blocking for 1 h with 5% nonfat dry milk in TBS-T buffer (20 mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween-20), the blots were probed with primary antibodies at 4˚C overngiht, then reacted with a peroxidase-conjugated secondary antibody for 1 hour at

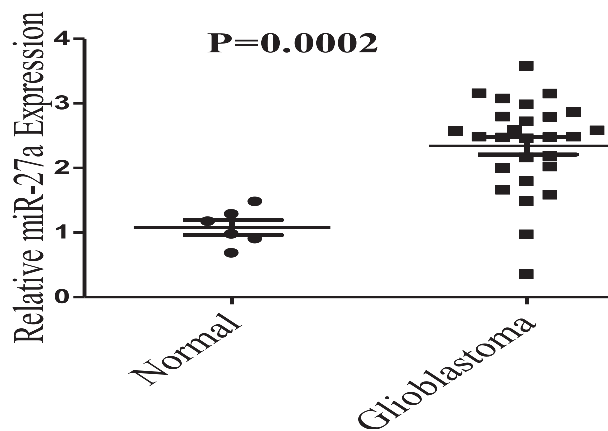

Figure 1. MiR-27a Expression in GBM Tissues. MiR-27a expression in GBM specimens and normal brain tissues was measured using qRT-PCR and normalized to that of GAPDH

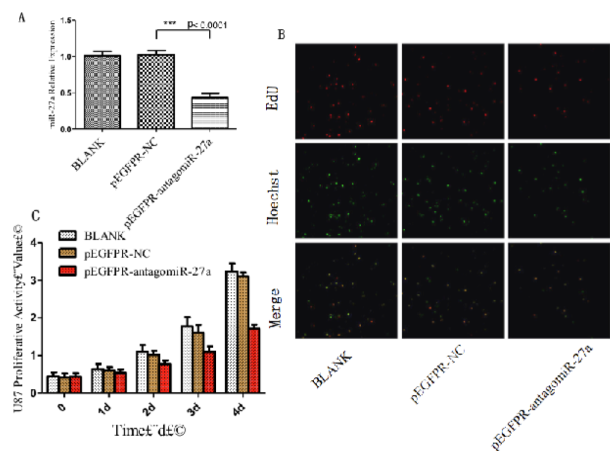

Figure 2. MiR-27a Inhibitor (antagomiR-27a) Suppressed Cell Proliferation. (A) U87 cells were transfected with pEGFPR-NC, or pEGFPR-antagomiR-27a, total RNA was extracted and qRT-PCR was performed to determine expression of miR-27a 48 h after transfection. *** $P < 0.0001$. (B) EdU assay of cell proliferation. EdU (100 mM) was added to cells and cultured for 2h. EdU and Hoechst staining were performed as described in 'Materials and Methods' section. (C) Cell viability was detected by MTT assay

room temperature, followed by detection of the proteins with ECL reagents and then exposure to X-ray films. Primary antibodies: mouse-anti-FOXO3a(BioVision) (1/1000), mouse-anti-AKT1(Santa Cruz)(1/1000), mouse-anti-Sprouty2(Santa Cruz)(1/1000), mouse-anti-β-actin(Santa Cruz)(1/1000). Secondary antibody: goat-anti-mouse (Santa Cruz)(1/1000).

Xenograft tumor assay

5×10^6 U87 cells were subcutaneously implanted in the backs of nude mice, when the subcutaneous tumor reached 200 mm3 in size, 24 mice were randomly divided into 3 groups. Respectively, pEGFPR-antagomiR-27a, pEGFPR-NC or PBS was injected into the xenograft tumor model in a multi-site injection manner. The tumor volume was measured with a caliper every 2 days. Tumor volumes were calculated as follows: volume $=(D \times d^2)/2$, where D meant the longest diameter and d meant the shortest diameter.

Results

Expression of miR-27a is frequently up-regulated and inversely correlated with FOXO3a expression in GBM

Our previous miRNA expression profiling study indicated that miR-27a was commonly up-regulated in GBM and that overexpression of miR-27a was associated with poor prognosis. However, the functional role of miR-27a in GBM remains elusive. In this study, we sought to confirm the miR-27a up-regulation in an independent primary GBM cohort. The expression level of mature miR-27a in 27 pairs of snap-frozen GBM and their corresponding Non-GBM brain tissues specimens was examined by qRT-PCR and normalized against an endogenous control. As shown in Figure1, the expression of miR-27a was significantly overexpressed in glioblastoma specimens ($p = 0.0002$).

AntagomiR-27a inhibits cell proliferation in U87 cells

To explore the role of miR-27a in GBM, U87 cells were transfected with the recombinant plasmid pEGFPR-antagomiR-27a as the experimental group, empty plasmid pEGFPR-NC (negative control), and U87 cells as blank control. To detect whether miR-27a was suppressed by antagomiR-27a, qRT-PCR was used to determine the expression of miR-27a U87 cell lines transfected with pEGFPR-antagomiR-27a. As shown in Figure 2A, miR-27a expression was significantly downregulated by antagomiR-27a ($p<0.0001$). To analyze the effect of miR-27a inhibitor on cell proliferation, we employed EdU and MTT analysis. The percentage of EdU positive cell in cells transfected with of antagomiR-27a was much more lower than negative control or blank control ($P<0.05$); while there is no significant difference between negative control and blank control ($P>0.05$) (Figure 2B); MTT results showed that proliferation of cells transfected with of antagomiR-27a was significantly inhibited compared with negative control and blank control ($P<0.05$), while there is no significant difference between begative control and blank control ($P>0.05$), indicating the cellular proliferation was inhibited after transfection with antagomiR-27a (Figure 2C).

FOXO3a is one of the direct targets of miR-27a

To elucidate the tumor effects of miR-27a in GBM, we further exploited the predicted target gene of miR-27a through database log TargetScan Human 6.2(http://www.targetscan.org/vert 42/). Interestingly, FOXO3a is a putative target of miR-27a. Luciferase assay indicated that miR-27a could directly aim at its predicted binding site of FOXO3a and lead to the suppression of luciferase activity of pLUC-FOXO3a (Figure 3A), whereas inhibition of miR-27a significantly induced luciferase activity of pLUC-FOXO3a; in other hands, neither miR-27a mimics or anti-miR-7a had obvious effects on pLUC-mutFOXO3a (Figure 3B). Western blotting showed that miR-27a mimics suppressed expression of FOXO3a, while anti-miR-27a upregulated it (Figure 3C). Furthermore, p-AKT and sprouty2, the FOXO3a related signaling molecules, were both upregulated by miR-27a mimics (Figure 3D). More interestingly, we found that

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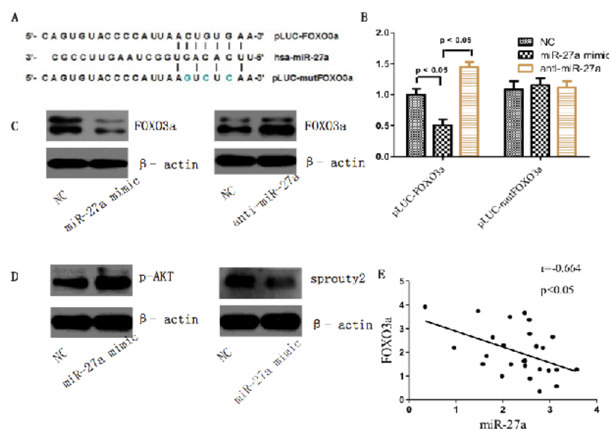

Figure 3. FOXO3a is One of the Direct Targets of miR-27a. (A) Putative miR-27a binding site of FOXO3a 3'-UTR. (B) Luciferase reporter assay. U87 cells were transfected with Notarget control, miR-27a mimics or anti-miR-27a together with pLUC-FOXO3a or pLUC-mutFOXO3a, respectively. Luciferase activity was detected at 48 h after transfection. (C) U87 cells were transfected with miR-27a mimics, anti-miR-27a, or negative control for 48h. Western blot analysis was used to detect FOXO3a expression. β- actin was used as an internal control. (D) Western blotting analysis of FOXO3a related signal molecules p-AKT and sprouty2. (E) Inverse correlation of miR-27a and FOXO3a expression in GBM specimens. In order to confirm the correlation between miR-27a and its target gene FOXO3a, the GBM specimens were subjected to western blotting to measure FOXO3a expression, or qRT-PCR to measure miR-27a expression, respectively. Pearson's correlation was curved (r=-0.664, $p < 0.05$)

expression of miR-27a was conversely correlated with expression of FOXO3a in human glioblastoma specimens (Figure 3 E).

AntagomiR-27a inhibits tumor growth of glioblastoma xenografts in nude mice

Considering the important roles of miR-27a in GBM, potentially therapeutic use of antagomiR-27a attracted our attention. Subcutaneous xenograft glioblastoma model were established in BALB-c nude mice using U87 cells. When tumors reached palpable, the mice were adminstered by pEGPR-antagomiR-27a, pEGPR-NC and PBS, respectively. The growth curves of pEGPR-antagomiR-27a and pEGPR-NC treated tumors became divergent on day 11 and the average fold increase of tumor volumes at the sacrifice with respect to the first measurements in pEGPR-antagomiR-27a treated tumors was much smaller than that in control tumors ($P<0.05$) (Figure 4A). There was a significant difference between the size and weights of glioblastoma treated with pEGPR-antagomiR-27a and those treated with pEGPR-NC (negative control) or blank control ($P<0.01$), moreover, this difference became more and more significant with time and reached the maximum at the end of the observation period (Figure 4B and C). The significant decreased in miR-27a expression could be verified by qRT-PCR treated tumors with pEGFPR-antagomiR-27a ($P<0.0001$), while there is no significant difference between pEGPR-NC(negative control) and blank control ($P>0.05$) (Figure 4D).

To clarify the cellular mechanisms underlying antagomiR-27a-mediated tumor suppression, resected

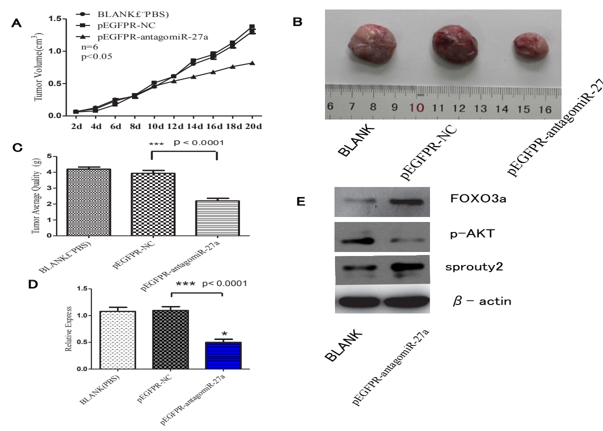

Figure 4. Analysis of Subcutaneous Xenograft Glioblastoma Model: (A)Tumor growth curve. U87 cells were subcutaneously injected into blanks of mice. When tumor reached palpable, pEGPR-antagomiR-27a, pEGFPR-NC, or PBS (blank control) were respectively injected into tumors in multiple sites every 2 days. Tumor sizes were measured using caliper every time after injection. (B) Representative picture of tumors. (C) Tumor weight. Tumors were weighed after sacrificing mice. (D) miR-27a expression in tumors. Total RNA was extracted from tumors and qRT-PCR was performed to detect expression of miR-27a.*** $P < 0.0001$. (E) Western blotting analysis of the FOXO3a, *p*-AKT and sprouty2 in tumors

tissues from those treated xenograft tumors were analyzed to detect miR-27a and FOXO3a expression. Western blotting analysis showed that FOXO3a, the target of miR-27a, was induced in tumors treated with pEGFPR-antagomiR-27a, p-AKT was decreased, Sprouty2 was increased (Figure 4E).

Discussion

MicroRNAs (miRNAs), as a new family of regulatory factors, mainly regulate gene expression at the posttranscriptional level by binding to their target message RNAs (mRNAs) completely or incompletely. A lot of researches indicate that miRNAs are involved in various biological processes, including cell proliferation, differentiation, apoptosis and metabolism. Accumulating evidences suggest that alterations of miRNAs expression may play a role in the development of human cancers. Therefore, miRNAs have potential to become an important tool for the study of occurrence, development, diagnosis and treatment of human tumors. MiRNAs interfere method is an efficient, specific gene silencing technique with small side effects, however, its efficiency must rely on its effective target genes. In this study, (1) qRT-PCR analysis showed miR-27a was upregulated in GBM specimens, indicating that miR-27a maybe correlated GBM progression. (2)We firstly screened out FOXO3a as the potential target gene of miR-27a through TargetScan database. Then, we verified that FOXO3a is a target gene of miRNA-27a: the protein expression level of FOXO3a and the activity of its report gene were significantly inhibited after overexpression of miR-27a, while they were significantly up-regulated after inhibition of miR-27a, indicating that FOXO3a is a direct target gene of miR-27a. More interestingly, we found that expression

of miR-27a was conversely correlated with expression of FOXO3a in human glioblastoma specimens. (3) MiRNA-27a mimics could induce multiple biological phenotypes of U87 malignant human glioblastoma cells, such as growth, proliferation. Western blotting analysis showed that miRNA-27a mimics decreased expression of FOXO3a, but increased AKT, Sprouty2 was decreased. (4) In vivo, we found that tumor size of those treated with recombinant pEGPR-antagomiR-27a was significantly smaller than those treated with pEGFR-NC (negative control) or PBS (blank control). Furthermore, H&E staining showed that the blood vessels were significantly reduced in tumors treated with pEGPR-antagomiR-27a compared with negative control or blank control, suggesting that inhibition of miR-27a suppressed tumor growth. Western blotting analysis showed that the protein expression level of FOXO3a was increased, while AKT was decreased, Sprouty2 was increased, in tumors treated with pEGPR-antagomiR-27a compared with negative control or blank control, demonstrating miR-27a inhibitor antagomiR-27a might mediate the downstream tumor growth associated genes via up-regulating its target gene FOXO3a expression.

Mertens Talcott et al found that, miR-27a is highly expressed in breast cancer cells, besides, anti-miR-27a could inhibit proliferation of breast cancer cells and reduce the percentage of cells in S stage (Li et al., 2010). It has been reported that miR-27a is also highly expressed in lung cancer, gastric cancer and kidney cancer. In the upstream of FOXO, the over activation of oncogene signal transduction PI3K/AKT pathway is identical biological phenotypes in many kinds of human cancers, however, AKT inhibits the transcriptional activity of FOXO by up-regulation the phosphorylation level of T32 in FOXO; in the downstream of FOXO, FOXO family could regulate the cell cycle transformation, differentiation and metabolism of epithelial cells via mediating its target genes, such as Sprouty2 and TRAIL (Arden et al., 2007; Paik et al., 2007). According to our results and literature search, we presumed that: (1) MiR-27a inhibitor antagomiR-27a inhibited the growth of glioblastoma via target gene FOXO3a, FOXO3a might be able to inhibit the activity of PI3K/AKT signal pathway and bind to promoter enhancer of gene p21 to regulate tumor suppressor gene FOXG (c-Qin), thus inhibiting the proliferation of glioma cells (Seoane et al., 2004). (2) In its downstream, FOXO3a mediates Srpouty2 to up-regulate the intracellular content of protein tyrosine phosphatase IB (PTPIB), thus ultimately inhibiting the pro-migration and pro-invasion activity of G protein Racl and reducing the invasion ability of tumor cells (Lim et al., 2000; Yigzaw et al., 2001; Yigzaw et al., 2003; Lee et al., 2004).

Accroding to our results, we consider that, miR-27a may plays as an oncogene in glioblastoma and could promote the proliferation and invasion of glioblastoma cells. At the same time, we believe that anti-miR-27a could inhibit the growth of glioblastoma and has potential of clinical application. There are many difficulties in the study of miRNAs, especially studies about the clinical application of miRNAs, presently, most of study on miRNAs has been confined to vitro or animal experiments. There are a few problems regarding its clinical application, such as the dose of clinical application, duration of action, toxicity of its degradation, the choice of target gene, all of which are need to be further studied, need our further study. Nonetheless, miRNAs have broad prospects in the study of glioblastoma.

In conclusion, currently, the common neurosurgical treatment of glioblastoma is combination of surgical resection with postoperative chemotherapy and radiation therapy. However, a more desirable way is gene-targeted treatment. The studies of glioblastoma associated with miRNAs and regulation of its target genes are still preliminary. Changing one miRNA could change the expression of its target genes, thus making multi-gene treatment possible. With the development of molecular biology, people have a better understanding of the molecular pathological basis of malignant glioblastoma, making it possible to cure glioblastoma using gene treatment methods at DNA or RNA levels.

Acknowledgements

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