Int J Clin Exp Pathol 2016;9(3):2896-2902
www.ijcep.com /ISSN:1936-2625/IJCEP0018740

Original Article

Effect of miR-146a on glioma cell line proliferation in targeted-regulating MIF gene

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Received October 29, 2015; Accepted December 25, 2015; Epub March 1, 2016; Published March 15, 2016

Abstract: It is reported that miR-146a is associated with various tumors. However, its specific effect on glioma has not yet been studied. This paper is to explore the effect of miR-146a on glioma. RT-PCR method was used to detect the expression of miR-146a in neurogliocytoma tissue and corresponding non-tumor normal tissue, as well as in neurogliocytoma cell strain and neurogliocyte in normal people. IHC was used to detect the expression of MIF in neurogliocytoma tissue and corresponding non-tumor normal tissue. Relationship between MIF and miR-146a was detected with the luciferase reporter gene. Cell jamming technology was adopted, by which, miR-146a mimics and miR-146a negative control (NC) were transferred into cell line of neurogliocytoma for detecting the expressions of MIF mRNA and proteins. MTT method was used to detect cell viability and cloning experiments to detect the proliferative potential of cells. The expression quantity of miR-146a in neurogliocytoma tissue was lower than that in corresponding non-tumor normal tissue. The miR-146a in cell line of neurogliocytoma was lower than that in normal neuroglia tissue and the expression quantity in U87 was appropriate. The expression of MIF proteins in neurogliocytoma tissue was higher than that in corresponding non-tumor normal tissue. And luciferase reporter gene had verified that MIF was the downstream target gene of miR-146a and transferred to U87 cell by liposome. miR-146a shows low expressions in neurogliocytoma tissue and cell line, while MIF shows over expressions in neurogliocytoma tissue. The miR-146a expression can be up-regulated by targeted inhibiting the MIF expression, accordingly, to inhibit the proliferation of neurogliocytoma.

Keywords: miR-146a, MIF, glioma

Introduction

Glioma, a kind of malignant tumor, is one of the most harmful cancers to human health in 21st century. Amount of studies have confirmed that MicroRNA (miRNAs) shows abnormal expression in kinds of cancers and is closely related to the development and progression of multiple tumor, in which the glioma is included [1]. miRNAs is an endogenous small-molecule non-encoding RNA, which is highly conserved during evolution. Thereinto, miR-146a is located in chromosome 5 LOC285628 gene with its mature sequences at exon 2. Studies have confirmed that miR-146a content in patients with glioma is lower than that in benign neuroglia tissue and the expression quantity in glioma cells is lower than that in normal nerve cells with low miR-146a expression and high TNM-staging. Low survival rate is closely related to lymph node metastasis [2-4]. It has shown that miR-146a has close relation with the development and progression of the gloma, but the specific mechanism is unknown. Macrophage migration inhibitory factor (MIF) is a kind of highly conserved protein, derived from T cell or can by secreted by monocyte/macrophage and anterior pituitary cells. Several studies have found that MIF is involved into the development and progression of gloma. Kamimura *et al.* [5] have researched the MIF over expression in gloma tissue of gloma patients by IHC and shown the close relation with prognosis. Several research findings show that the expression degree of MIF in different patients with gloma was different [6]. It shows that both miR-146a and MIF can show abnormal expressions in gloma and speculates that MIF may be one the downstream target proteins of miR-146a. Therefore, on the above basis, this paper is respectively detecting the expressions of miR-146a and MIF in gloma by RT-PCR method and

Table 1. RT-PCR primer

Gene	Primer (5'-3')	bp
miR-146a	For: CAGTGCGTGTCGTGGAGT	158
	Rev: GGGTGAGAACTGAATTCCA	
MIF	For: ACTAAGAAAGACCCGAGGC	366
	Rev: GGGGCACGTTGGTGTTTAC	
GADPH	For: AGCCACATCGCTCAGACA	314
	Rev: TGGACTCCACGACGTACT	

IHC, verifying if MIF is the downstream target proteins of miR-146a by luciferase reporter gene analysis and transferring the miR-146a mimics into glioma cell with the liposome transfection method. This paper also aims at exploring the effect of MIF expression on cell proliferation in gloma cells.

Materials and methods

Study subjects

Twenty cases of glioma tissue samples and 20 cases of corresponding non-tumor normal tissue samples which were removed surgically were collected between July 2014 to June 2015 in Laiwu City People Hospital. Among the cases of glioma tissue samples, there were 12 cases that had suffered lymphatic metastasis, while other 8 cases didn't. Cases were divided into different clinical stages in accordance with the TNM-staging standard of Union for International Cancer Control (UICC) in 2009. The TNM-staging was shown below: 14 cases in Stage I-II, 6 cases in Stage III-IV. The cases above had received chemoradiotherapy before operation and were pathologically confirmed.

Inclusion criteria: 1. Patients and family members must be agree to sign; 2. Cases report in detail; 3. Pathological diagnosis result in detail (Observed by microscope, tumor cells consist of more mature astrocytes. Cells which are rich in gelatinous fibre are fibrous astrocytes and cells which are rich in cytoplasm are protoplasmic cells).

Regents and instrument

Glioma cell line of U251, U87 and C6 and normal astrocyte (RA) were purchased from the Cell Institute of China Science Academy in Shanghai, miR-146a monoclonal antibody (Epitmics Company); rabbit-anti MIF antibody (Cell Signaling Technology); methyl thiazolyl tetrazolium (MTT) (Gibco Company); fetal calf serum, RPMI-1640 base (Hyclone Company); Caspase 3 active detection kit (Nanjing KeyGEN Biotech. Co., Ltd). Wild type MIF 3'untranslated region (3'UTR) luciferase vector pGL3-MIF 3'UTR-Wt and mutant type MIF 3'UTR luciferase vector pGL3-MIF 3'UTR-Mut (Invitrogen Company); and the synthetic reagent being ordered and compounded by RiboBio Co. Ltd. ChemiDocTM XRS Gel imaging system (Bio-Rad Company); Luciferase activity detection system purchased from Promega Company and MTT powder purchased from Sangon Biotech.

Detecting U87 cell viability with MTT method

Glioma cell was inoculated into 96-well plates. Transfection on miR-146a mimics and NC was implemented with the Lipofectamine™ 2000 when cell confluence degree reached 50% and the transfection concentration was 50 nmol and 50 nmol respectively. After 48 h, 20 μl 5 mg/ml MTT was added and kept on cultivating for 4 h, after then culture medium was abandoned. 150 μl DMSO was added into each well. Then we shocked to make crystal substance dissolved sufficiently, measured the OD value at 560 nm of microplate reader and calculated relative proliferative effect with the 630 nm wavelength as a reference [7].

Colony forming experiment

Glioma cell was inoculated into 6-well plates. Transfection on miR-146a mimics and NC was implemented with the Lipofectamine™ 2000 when cell confluence degree reached 50% and the transfection concentration was 50 nmol and 50 nmol respectively. After 48 h, containing 10% formaldehyde and 0.1% crystal violet-solution were used to stain fixedly and placed for 30 min in the room temperature. Swing to remove the staining fluid slightly, wash every well with distilled water, invert the culture plate on absorbent paper for drying and finally take photo and analyze.

RT-PCR

Total RNA was extracted referring to the operation instruction of trizol kit (Invitrogen). The whole extraction process was under an environment without RNAase. Primers see **Table 1**. RNA was reversely transcribed into cDNA and PCR amplified by One-step RT-PCR kit and got 5 uL PCR product, which could be used in next

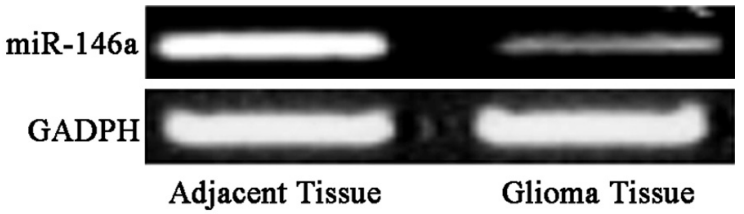

Figure 1. Expressions of miR-146a in glioma tissue and adjacent tissue.

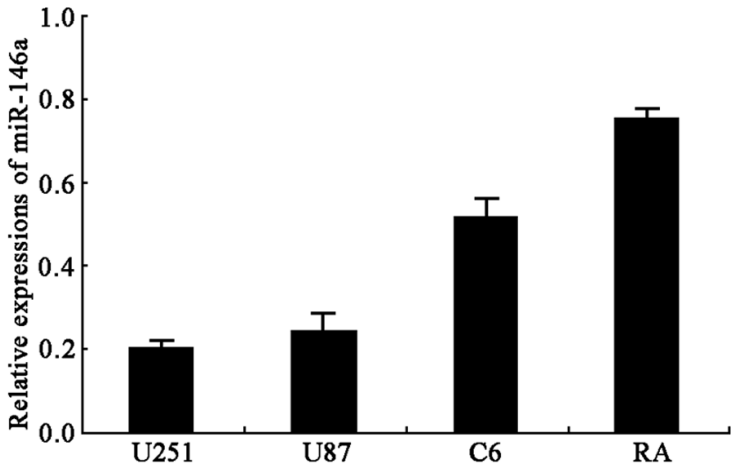

Figure 2. Expressions of miR-146a in glioblastoma cell lines and normal astrocyte cell lines.

step, 2% agarose gel, for detecting and taking photos. Primers were respectively added into 25 μL PCR reaction system with reaction conditions were: 94°C degeneration 45 s, 59°C renaturation 45 s, 72°C extension 60 s, 35 cycles in total.

Immunohistochemical staining to detect the expression of MIF protein in lung tissue

Samples were fixed by 10% formalin, embedded, sliced, dewaxed by xylene, dehydrated by anhydrous, 95% and 80% ethanol, swashed by running water, antigen repaired, antigen blocked by horse serum, primary antibody blocked, second antibody blocked, soaked by hematoxylin and hydrochloric-alcohol solution, swashed by running water to blue, dehydrated by gradient ethanol, with hyaline xylene. After blow-dried, perform neutral balsam mounting and microscopic examination. MIF positive staining was faint yellow and claybank, positioned on cytoplasm.

Luciferase reporter gene detection: plasmid co-transfection in glioma cells. Grouping as follow: miR-146a mimics + Wt MIF, miR-146a mimics NC + Wt MIF, miR-146a mimics + Mut MIF, miR-146a mimics NC + Mut MIF. Dual-luciferase detection system was used to detect the transfected luciferase activity. Steps as shown below: after PBS washed, lysate was added into. After fully lysed, LAR solution was added into. Then, read fluorescence value on enzyme detector, add stop buffer, read fluorescence value once more, calculate relative fluorescence value. The computational formula was: relative fluorescence value = firefly luciferase value/ranilla luciferase value.

Statistical analysis

RT-PCR results and Q-PCR, western-blot results were counted by Image-J software and all the data were counted and analyzed with SPSS 17.0, repeated three times or more for each experiment. It was shown through t-test as significant difference at $P < 0.05$.

Result

Expressions of miR-146a and MIF in glioma tissues and glioma cells

RT-PCR results showed that the expression of miR-146a in glioma tissues was significantly lower than that in corresponding non-tumor normal tissues with the ratio of (0.68 ± 0.05) vs. (0.23 ± 0.00), which showed significant difference ($P < 0.05$) (see **Figure 1**). As shown in **Figure 2**, Q-PCR detection results showed that the relative expressions of miR-146a in glioblastoma cell lines U251, U87 and C6 were significantly lower than those in normal astrocytes (RA), 0.20 ± 0.02, 0.24 ± 0.04, 0.51 ± 0.05, 0.52 ± 0.02) vs. (0.75 ± 0.02). Expression in U87 was moderate. Therefore, U87 was selected to be the study object. As shown in **Figure 3**, immunohistochemical results showed that when compared positive staining of golden area with corresponding non-tumor normal tissues, MIF positive expression in glioma tissues was higher (0.89 ± 0.02) vs. (5.49 ± 0.48), which showed significant difference ($P < 0.01$).

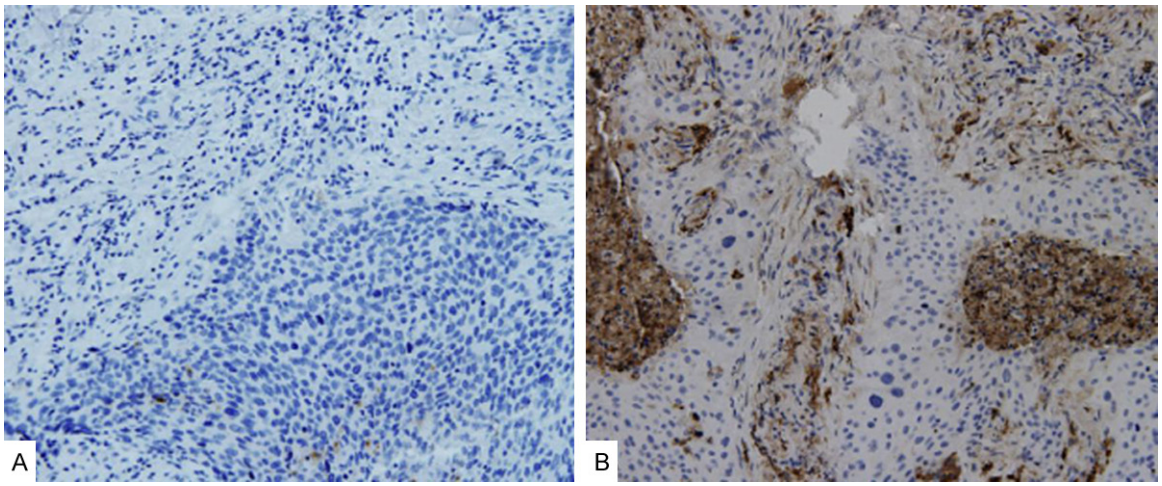

Figure 3. Expressions of MIF in glioma tissue and adjacent tissue under the microscope.

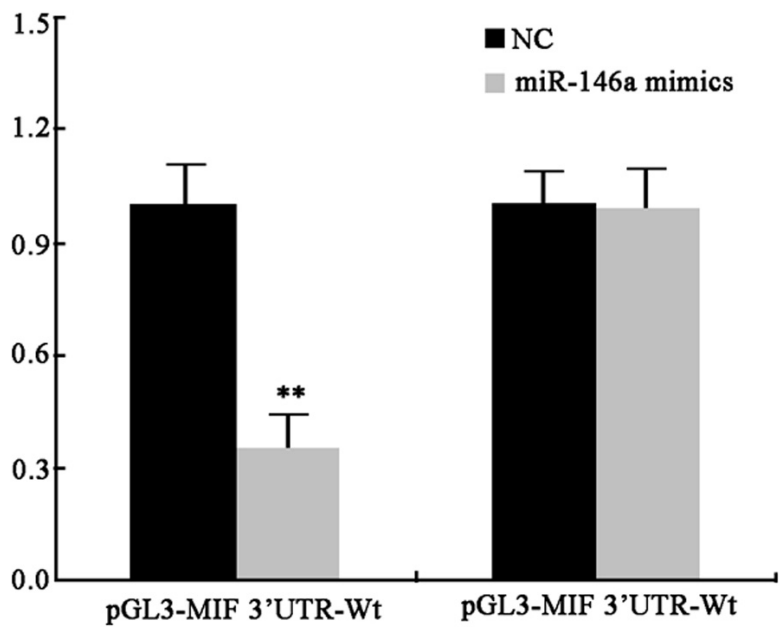

Figure 4. Luciferase reporter gene expression analysis. Compared with that in NC group, **P < 0.01.

Luciferase reporter gene detection

MiR-146a mimics, NC and wild-type vector pGL3-MIF 3'UTR-Wt, as well as mutant-type vector pGL3-MIF 3'UTR-Mut were transfected into U87 cell. The result showed that the fluorescence signal intensity in cotransfection group of miR-146a mimics and wild-type vector pGL3-MIF 3'UTR-Wt was obviously weaker than that in other transfection group, which showed significant difference ($P < 0.01$). Whereas for mutant-type vector pGL3-MIF 3'UTR-Mut, fluorescence intensity in different groups had no any difference ($P > 0.05$), see **Figure 4**.

Effect of miR-146a mimics on MIF protein and mRNA expression in U87 cell

As shown in **Figure 5**, after miR-146a mimics and NC were transfected into U87 cell, MIF protein and mRNA expression quantity were significantly decreased ($P = 0.0045$, $P = 0.0041$).

Effect of miR-146a mimics on U87 cell viability

As shown in **Figure 6**, after transfecting the miR-146a mimics with U87 cell, MTT experiment found that cell viability in transfection group was significantly decreased when compared with that in control group ($P = 0.0074$). As shown in **Figure 7**, after transfecting the miR-146a mimics with U87 cell, cloning experiment found that cell cloning ability in transfection group was significantly decreased when compared with that in control group (68.34 ± 8.23) vs. (35.49 ± 3.65) ($P = 0.00032$).

Discussion

The occurrence of tumor can be caused by many reasons. Plenty of evidence confirmed that miRNAs plays the role of a tumor suppres-

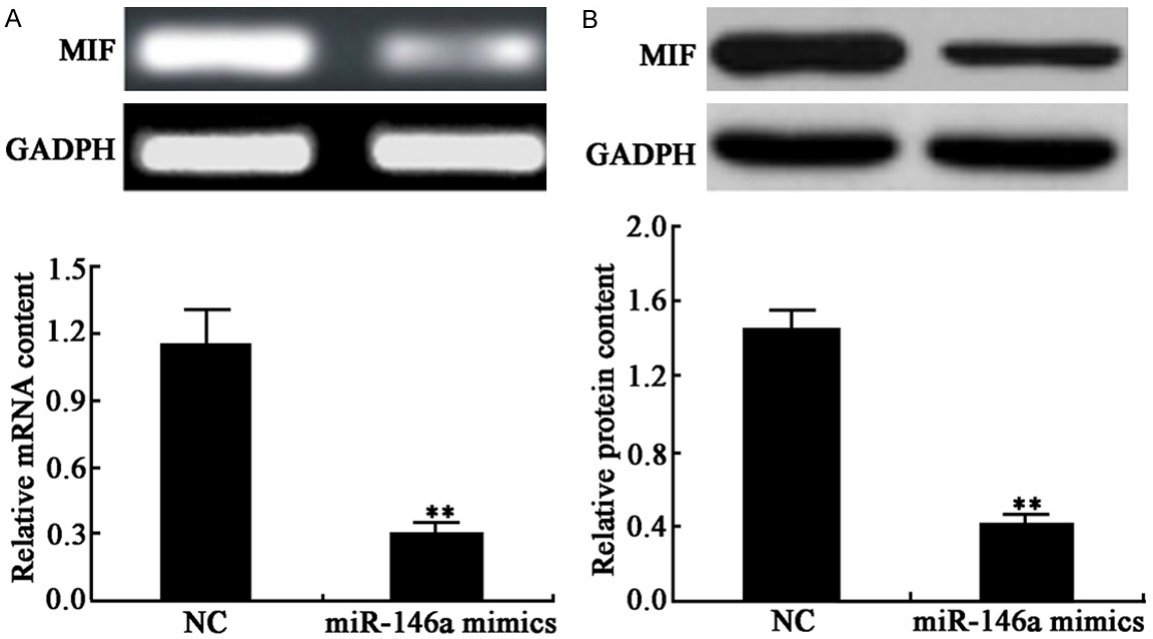

Figure 5. Effect of miR-146a mimics on MIF protein and mRNA expression in U87 cell Compared with that in NC group, **P < 0.01. A: Effect of miR-146a mimics on MIF mRNA expression in U87 cell. B: Effect of miR-146a mimics on protein expression in U87 cell.

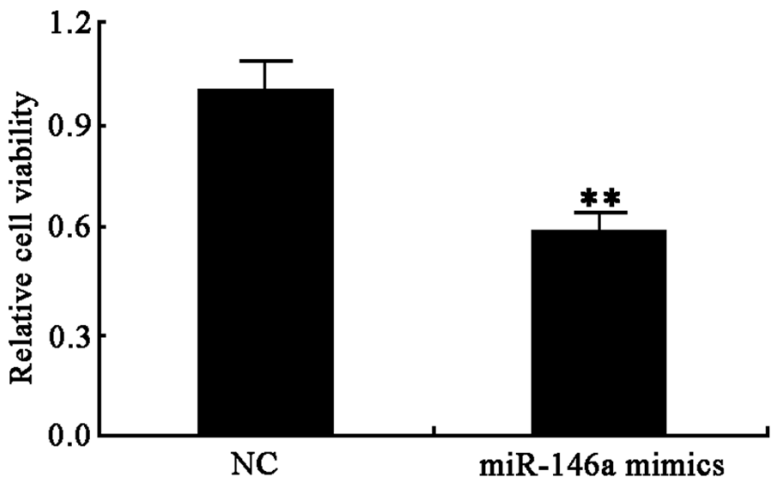

Figure 6. Effect of miR-146a mimics on U87 cell viability. Compared with that in NC group, **P < 0.01.

sor or a tumor promoter in different tumors and also plays an important role in the proliferation, apoptosis and signal transduction of tumor cells, as well as in regulating the pathogenesis of tumors [8]. As a kind of tumor suppressor gene, miR-146a expression is down-regulated in many types of cancer cells [9, 10]. Some researches [11] verified that the expression quantity of miR-146a in lung carcinoma cell was lower than that in normal lung cell, which was fully illustrated that miR-146a low expression occurred in NSCLC and had close relation with the development and progression of NSCLC. The results revealed for the first time that miR-146a showed low expression in glioma. The miR-146a expression in glioma was lower than that in adjacent tissue. And the expressions in glioma cell line U251, U87 and C6 were lower than that of normal astrocyte. Thought the reason why the expression of miR-146a in cell lines differed was not discussed in this research, we suspected that it might be caused by invasive range among cell lines, and the miR-146a expression in U87 was appropriate. In order to show no difference caused by expression quantity of too high and too low, therefore, miR-146a was selected as the follow-up experiments cell line.

The infinite proliferating tumor can lead the tumor cell to division and proliferation continually, which makes the anabolism of protein in

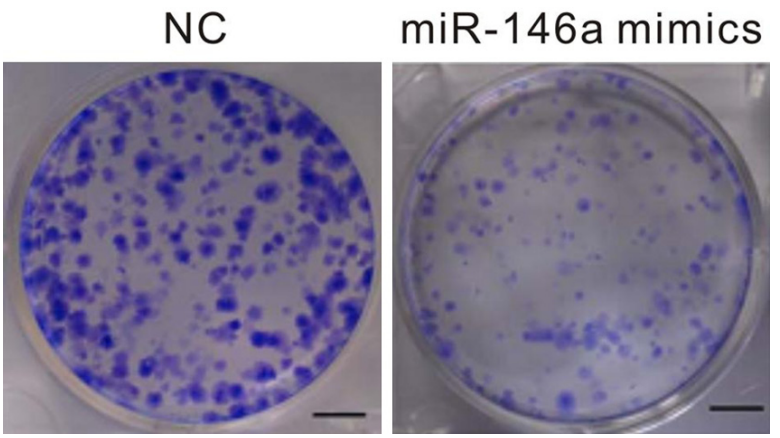

Figure 7. Effect of miR-146a mimics on U87 cell colony-forming ability.

tumor cell be higher than catabolism and despoil products of normal protein metabolism to make the body in a state of cachexia and lead to the further deterioration. And research has identified that miRNAs can regulate the biological behavior of tumor cell by targeted-regulating some molecules [12, 13]. Therefore, it is confirmed by luciferase reporter gene analysis that MIF is the downstream target gene of miR-146a. MIF is a kind of cytokine derived from T lymphocyte, which can inhibit the macrophage migration and give rise to the aggregation and infiltration of macrophage in delayed-onset hypersensitivity to participate in the inflammatory response. The expressions of MIF in melanoma, NSCLC, prostatic carcinoma and colorectal cancer, etc. are higher [14]. This research has verified by immunohistochemistry that MIF protein expression in glioma is higher than that in adjacent tissue. And another research [15] shows that MIF over expression can be achieved by immunohistochemistry of lung tissue of glioma and be related to prognosis. Research has found that overall survival of high expression MIF is lower than that of low expression [16]. Some studies indicate that high expression MIF in glioma tissue is positively correlated with VEGF high expression in blood, and is significantly and positively correlated with the microvessal density [17], which is fully illustrated that MIF is over-expressed in glioma, so does in glioma as the downstream of miR-146a. Therefore, the over expression of MIF may be closely related with the development and progression of glioma [18-20]. The subsequent experiment results show that the over expression of miR-146a has affected the cell proliferation, which has been confirmed by our subsequent colony forming experiment. At the same time, MIF protein and mRNA expressions in U87 cell are significantly inhibited by up-regulating the miR-146a expression, which declares that MIF is the downstream target molecule of miR-146a protein. So with the above basis, we can further probe the action mechanism of miR-146a in regulating the proliferation of U87 cell by targeted-regulating the MIF molecule. However, this research does not confirm whether the miR-146a regulates the multiplication capacity of cell by its downstream MIF protein or not. And no further exposition shows regulation is made by which signal molecular mechanism, which still needs to research further.

In conclusion, miR-146a shows low expression in glioma tissue and in cell line, while MIF shows high expression in glioma tissue. After up-regulated, miR-146a expression can inhibit MIF expression targeted to further inhibit the U87 tumor cell proliferation. Therefore, future experiment can probe the effect of miR-146a on biological behavior, such as glioma cell invasion and metastasis, on a specific signaling pathway.

Disclosure of conflict of interest

None.

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