Loss of miR-124 and miR-137 Expression in Glioblastoma Multiforme and Their Roles in Glioma Cell Proliferation and Differentation

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ABSTRACT

Background: Glioblastoma Multiforme (GBM) is a common malignant tumor of the brain and is characterized by rapid growth and high tumor heterogeneity. However, the molecular mechanisms driving GBM tumorigenesis and progression remain to be fully elucidated.

Methods: miR-124 and miR-137 expression in 43 high-grade GBM tumor tissues was measured by real-time PCR and compared with that in 24 normal brain tissues. The effects of miR-124 and miR-137 overexpression on the proliferation and differentiation of glioma U251 cells were analyzed. The expression of Tuj1 and GFAP was detected by immunofluorescence staining. Cell proliferation was detected with flow cytometry.

Results: The expression of miR-124 and miR-137 was significantly decreased in GBM tumor tissues compared to that in the normal brain tissues ($P < 0.01$). Immunofluorescence showed, after miR-124 and miR-137 transfection, structure of normal synapse grew in GBM, and expression of neuronal differentiation factors significantly increased, including Tuj1 and GFAP. Flow cytometry analysis showed GBM cell cycle extended and differentiation was repressed.

Conclusion: miR-124 and miR-137 function as tumor suppressors to inhibit cell proliferation and differentiation and their expression was significantly decreased in GBM.

Keywords: Glioblastoma Multiforme; miR-124; miR-137; proliferation, Differentiation

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INTRODUCTION

Glioblastoma Multiforme (GBM) is a common malignant tumor, accounting for 80% of adult primary malignant tumors in the brain with unknown etiology [1]. According to its pathology and clinical features, GBM is divided into astrocytoma, oligodendroglioma, oligoastrocytomas, and ependymoma [2]. GBM is characterized by rapid growth, high tumor heterogeneity, and diffuse infiltrative growth, which makes treatment difficult and results in high recurrence rate and

poor survival rate [3]. Current therapy for GBM treatment is based on surgery combined with radiotherapy, chemotherapy, immunotherapy, and molecular targeting treatment. Such therapy regimen can improve GBM patient prognosis, but fail to provide a radical cure of GBM [4]. Studying regulatory mechanisms of GBM cell proliferation and differentiation is of great significance to prolonging patients' survival and improving prognosis.

A microRNA (miRNA) is a conserved small non-coding RNA molecule (~18-25 nucleotides long), which functions in post-transcriptional regulation of gene expression through binding to the 3'-UTR of a target mRNA and causing subsequent cleavage, destabilization, and less efficient translation of the mRNA [5,6]. Currently, over 1000 miRNAs have been found in humans, which was proposed to target about 60% of total genes [7,8]. Neural differentiation is characterized with expression of specific proteins, including Tuj1 and GFAP, which are essential for cytoskeleton and associated with cellular morphology [9, 10]. Overexpression of miR-124 and miR-137 resulted in changes in cell proliferation and differentiation [11]. Human β-Tubulin 3 is a structural protein (450 amino acid) expressed in neurons and is thought to be a neuron specific class III tubulin (Tuj1), which contributes to microtubule stability in neuronal cell bodies and axons [12]. Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is expressed by numerous cell types of the central nervous system, including astrocytes and ependymal cells [13]. GFAP is involved in many important CNS processes, including astrocyte-neuron interactions, cell-cell communication, maintaining the functioning of the blood brain barrier, and repair of CNS injury [14,15]. This study investigated miR-124 and miR-137 expression in GBM and their effects on proliferation and differentiation and then examined Tuj1 and GFAP expression in GBM cells.

MATERIALS AND METHODS

Sample Collection

43 high grade GBM tumor tissues were collected from patients before chemotherapy, radiotherapy, and other treatments from December 2012 to December 2014. GBM was diagnosed with head MRI and pathological examination. Of the 43 cases, 23 were male and 20 were female with an average age of 39.1±6.5 years old. 24 relative normal brain tissues were collected around the trauma of cerebral internal injuries as controls. Of the 24 controls, 13 were male and 11 were female with an average age of 41.3±8.2 years. Tumor and control brain tissues were acquired during surgery and immediately placed in liquid nitrogen. This study was approved by ethics committee and signed consent forms were collected from all study subjects.

Cell Culture

The U251 glioma cell line was provided by the Chinese Academy Sciences Cell Bank (China) and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FCS), 4× nonessential amino acids, 3 mM L-Glutamine, Penicillin (100 U/ml) and Streptomycin (100 mg/ml) at 37°C, 5% CO2.

Real-time RT-PCR

Expression levels of miR-124 and miR-137 in GBM tumor tissues and normal brain tissues were detected by Real-time RT-PCR. First, primers were designed based on sequence of miR-124 and miR-137 (GeneBank accession number were NR_0296 68, NR_029679, respectively) (Table 1). RNA was extracted from tissues with RNAprep Pure Tissue Kit. Real-time RT-PCR was performed in a Real-time PCR Amplifier (Bio-Rad) using mirVanat qRT-PCR miRNA kit (Ambion) with the following reaction conditions: 95°C, 3min, followed by 95°C, 15s, 60°C, 30s for 40 cycles. U6 was used as a reference gene. Results were analyzed by 2-ΔΔCt [16].

Cell Transfection

The sequences of matured miR-124 and miR-137 were used for establishing the miR-124 and miR-137 overexpression system. The corresponding sequences with the same composition of oligonucleotide sequences were assigned as negative control. miR-124 and miR-137 sequences were synthesized by Nanjing Shengxing company. After cloning the synthesized miRNA sequences into the pMIR-REPORT expression system (Ambion), the obtained plasmid DNA with correct insertion were transfected into U251 glioma cells using INTERFERinTM Polyplus transfection reagents. GBM cell line U251 was purchased from cell bank of Chinese Academy of Science. Cells were cultured in RPMI-1640 medium (HyClone), 37°C, 5% CO2.24 hrs after transfection, cells were subjected to further analysis.

Imunofluorescence

Expression level of Tuji and GFAP in miRNA-expression-vector-transfected U251 cells was detected by immunofluorescence. Briefly, transfected cells in the logarithmic growth phase were fixed with paraffin on to slides. After blocking, cells were incubated with mouse anti-human Tuji and GFAP(1:2000) antibody for 2 hrs

at 37°C, followed by incubation with fluorescence marked goat anti-mouse secondary antibody (1:1000) for 1 hr at 37°C. After washing with 1 × PBS and attaching cover slips to the slices, the fluorescent staining was observed under a fluorescence microscope.

Flow Cytometry Assay

The miR-124 and miR-137 transfected U251 glioma cells were grown to the logarithmic phase (24 hrs) and fixed with 90% ethanol overnight at 4°C. After removing the ethanol, cells were incubated with RNase for 30 min at 37°C and the cells' nuclei were stained with PI. The stained cells were subjected to flow cytometry assay (Becton Dickinson) with an excitation wavelength of 488nm and an emission wavelength of 630nm. The FL-2 area and DNA histogram were analyzed with Modifit software. All experiments were repeated for 3 times.

Statistical Analysis

Results were analyzed using SPSS 20.0 and presented with mean ± standard deviation. T test was used for comparative analysis. $P < 0.05$ was considered to be statistically significant.

RESULTS

qRT-PCR of miR-124 and miR-137 Expression in GBM Tissues

Expression of miR-124 and miR-137 in GBM was examined with RT-PCR (Fig.1). The expression of miR-124 and miR-137 in GBM tissues was significantly decreased compared to that in normal brain tissue ($P<0.01$). Expression of miR-124 and miR-137 in normal brain tissue is 12 and 15 times higher than that in GBM tumor tissues, respectively.

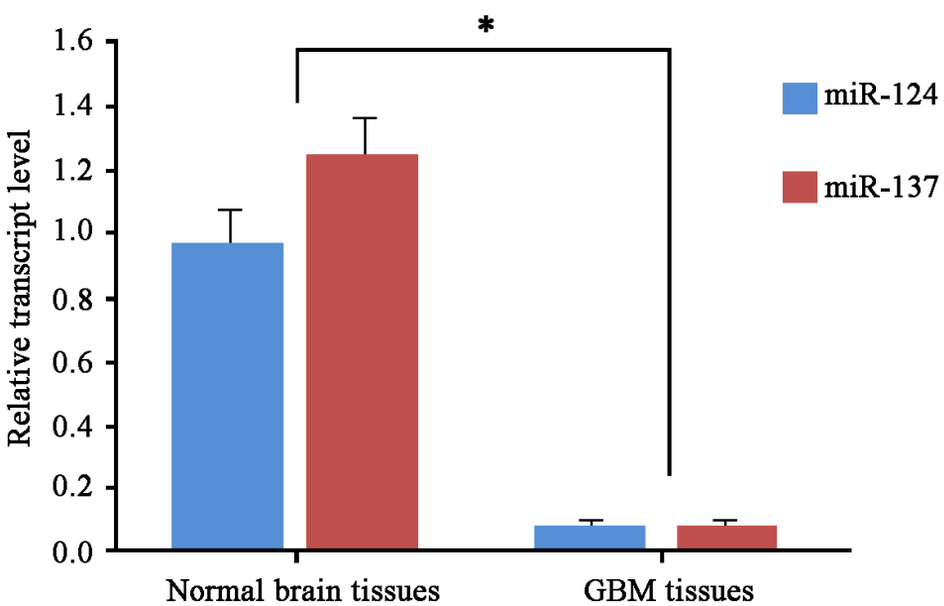

Fig.1 Relative expression of miR-124 and miR-137. miR-124 and miR-137 expression in normal brain tissues and GBM tumor tissues was measured by real-time PCR. *$P<0.01$ between normal brain tissues and GBM tumor tissues.

Overexpression of miR-124 and miR-137

The pMIR-REPORT expression system was used to establish overexpression of miR-124 and miR-137 in U251 glioma cells. Total RNA was extracted and miR-124 and miR-137 expression was measured by real-time PCR. The relative expression of miR-124 and miR-137 was shown in Figure.2. Cell transfection significantly increased the expression of miR-124 and miR-137 in U251 glioma cells compared to control cells ($P<0.05$).

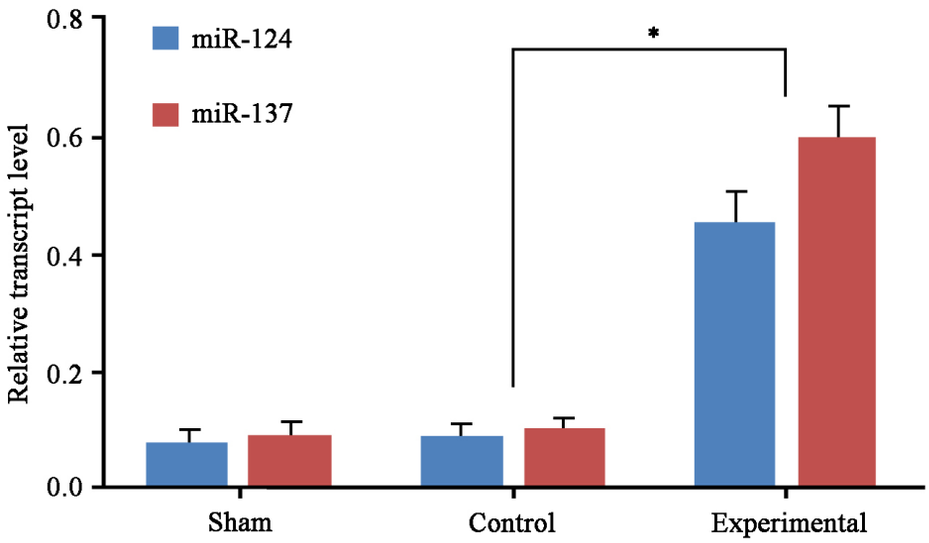

Fig.2 Overexpression of miR-124 and miR-137 in U251 cells. Glioma U251 cells were trransfected with miR-124 and miR-137 expression vector, negative control vector (control), and without vector (sham). 24 hrs after the transfection, miR-124 and miR-137 expression in transfected cells were measured by real-time PCR. P<0.05 between miR-124 or miR-137 trransfected cells and control cells.

Immunofluorescence of Tuj1 and GFAP Expression

The miR-124 and miR-137 transfected U251 cells were harvested every other day. Tuj1 and GFAP expression was detected by immunofluorescence staining (Fig.3). Tuj1 was shown with GFP (a green fluorescent marker), GFAP with rhodamine (a red fluorescent marker). Cellular expression level of Tuj1 and GFAP represent the degree of cell differentiation as specific protein of neuron system. With progression of cell culture, expression of Tuj1 and GFAP gradually increased after miR-124 and miR-137 transfection. Phase contrast microscope showed neuron-specific synapse structure in U251 cells after miR-124 and miR-137 overexpression, suggesting that the U251 cells differentiated into normal neurons.

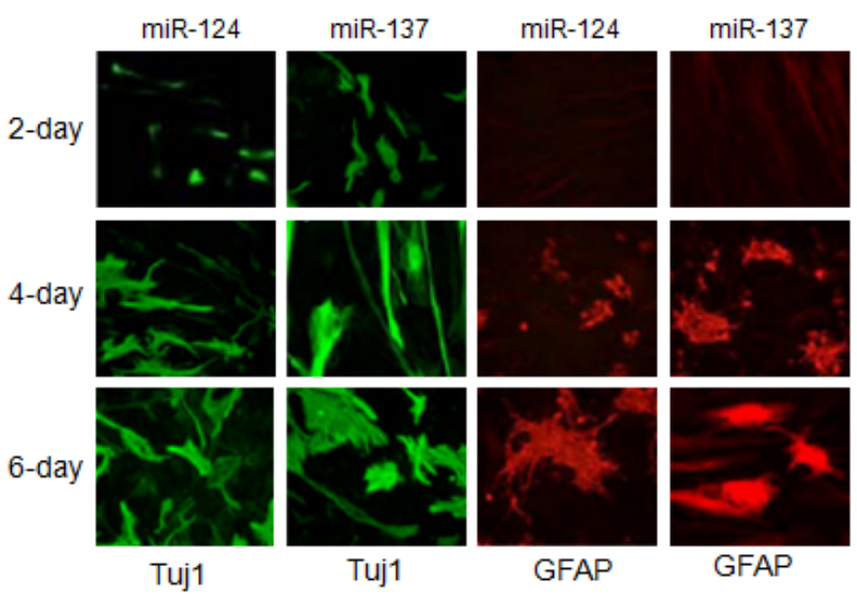

Fig. 3

Fig.3 Immunofluorescence of Tuj1 and GFAP expression. U251 cells were transfected with miR-124 or miR-137 expression vectors. Tuj1 (green) and GFAP (red) expression was detected by immunofluorescence staining (400×). A time-dependent increase in fluorescence intensity (expression level) was observed.

Flow Cytometry Analysis of the Cell Cycle

The cell growth cycle was examined using flow cytometry in miR-124 and miR-137 transfected, blank-control, and negative-control U251 cells. FL-2 area was analyzed by Modifit software (Fig.4A), and the distribution of cells in different cell cycle stages was analyzed by SPSS 20.0 software (Fig. 4B, 4C). No difference was observed between blank control and negative control cells in G0 / G1 phase, S phase, and G2 / M phase ($P > 0.05$). miR-124 and miR-137 transfection significantly increased the number of U251 cells in the G0 / G1 phase and decreased the number of U251 cells in the S phase and G2 / M phase ($P < 0.05$), suggesting that cell cycle was prolonged and mitosis was attenuated.

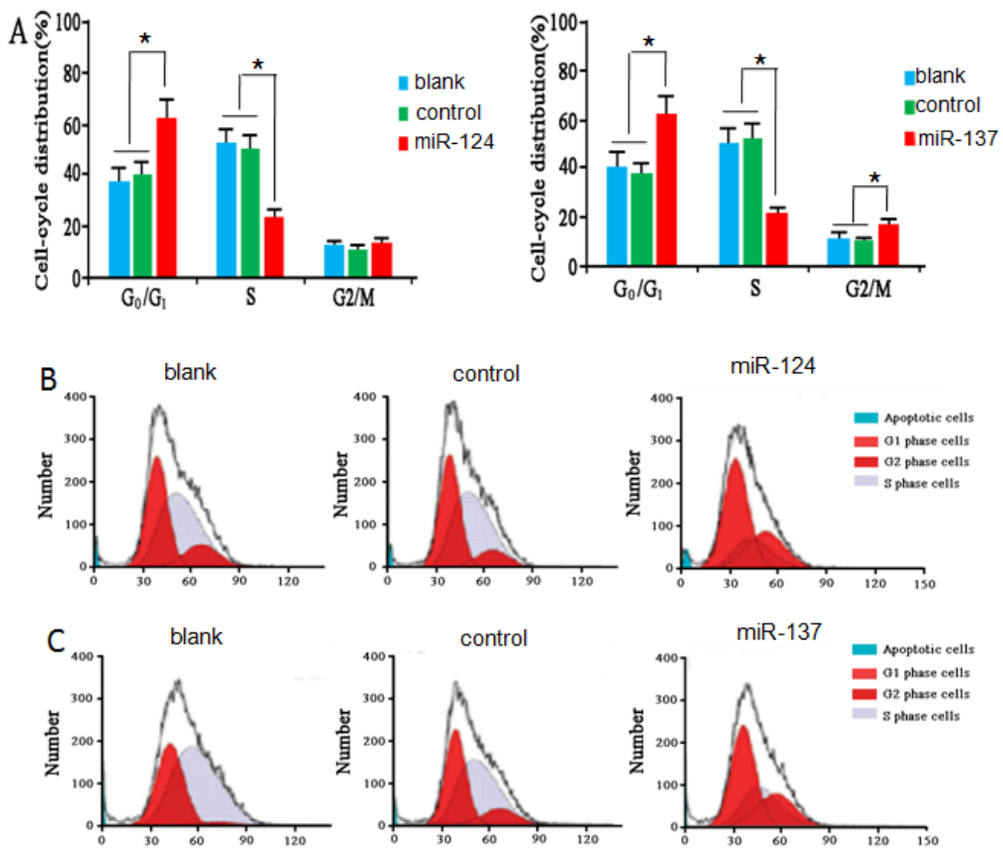

Fig. 4

Fig.4 Flow cytometry assay of cell cycle of miR-124 and miR-137 overexpressed U251 cells. (A) Cell cycle distribution. *P<0.05 between miR-124 or miR-137 trransfected cells and control cells. (B) Representative photogram of the number of cells in each cell cycle in U251 cells transfected with miR-124 vector, negative vector, and without vector. (C) Representative photogram of cell cycle distribution in U251 cells transfected with miR-137 vector, negative vector, and without vector.

DISCUSSION

This study first found significantly decreased expression of miR-124 and miR-137 in high grade GBM tumor tissues. Further investigation in glioma U251 cells using an established overexpression model validated the effects of miR124 and miR-137 in cell proliferation. This study also revealed that miR124 and miR-137 overexpression stimulated neuronal differentiation using immunofluorescence staining. Although abnormalities in miR-124 and miR-137 have been previously reported in GBM, their biological functions have not been fully elucidated. Santos et al study demonstrated that low miR-124 and miR-137 expression is associated with GBM cell proliferation through maintaining the activation of growth factors, such as EGF and FGF[17]. The decreased miR-124 and miR-137 expression in GBM is proposed to result from irreversible modification on transcription regulation. For example, Chakrabarti et al study showed that the expression

level of miR-124 was elevated in GBM after treatment with DNA demethylation (hypomethylation) modifier 5-Aza-2-deoxycytidine [18]. Although this study provided no evidence for the cause of low expression of miR-124 and miR-137 in GBM tumor tissues, we demonstrated that the expression of miR-124 and miR-137 was significantly lowered in high grade GBM tumor tissues. Cell culture experiments with artificial expression of these two miRNAs significantly inhibited glioma U251 cell proliferation. Our study suggests that both miR-124 and miR-137 are tumor suppressors in GBM. The roles of miR-124 and miR-137 in neuronal differentiation have been previously observed. For example, overexpression of miR-124 can enhance the differentiation of embryonic stem cells into neurons [19]. miR-124 was found to regulate the expression of PTBP1, which plays a role in inhibiting alternative splicing patterns of mRNA precursors in non-neuronal cells [20]. Research has also found that miR-124 promotes neuronal differentiation through inhibiting the expression of SCP1 [21]. miR-137 was reported to inhibit the expression of CDK6 and subsequently take part in regulation of the cell cycle and mitosis [22]. In this study, miR-124 and miR-137 overexpression was found to promote the differentiation of U251 glioma cells into normal neurons accompanied by elevation of Tuj1 and GFAP expression.

As a severe brain tumor, GBM puts a threat on human health, thus, diagnosis and treatment for GBM is an issues of common concern in clinical scenario [1]. Current study proved expression of miR-124 and miR-137 significant decreased in GBM cells, providing a data evidence for clinical diagnosis. We also found miR-124 and miR-137 overexpression induced GBM differentiation.Given that differentiation therapy is a hot spot in tumor field, which induces cancer cell to transform with normal morphology, gene expression and function [22], our finding will contribute to improvement of differentiation therapy.

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