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Original Article

MicroRNA-137 inhibits growth of glioblastoma through EGFR suppression

Zhenxing Zhang[1], Xiaofeng Song[2], He Tian[2], Ye Miao[1], Xu Feng[1], Yang Li[1], Honglei Wang[1]

[1]*Department of Neurosurgery, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou 121001, China;*
[2]*Department of Histology and Embryology, Jinzhou Medical University, Jinzhou 121001, China*

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Abstract: Aberrant expression of certain microRNAs (miRNAs) has been shown to contribute to the development of Glioblastoma multiforme (GBM). However, the involvement of miR-137 in the carcinogenesis of GBM has not been reported. Here, we showed that miR-137 levels in GBM tissues were significantly lower than the paired normal brain tissue in patients' specimens. Moreover, low miR-137 levels in GBM tissue were associated with poor prognosis. In vitro, overexpression of miR-137 decreased GBM cell growth and increased cell apoptosis, while depletion of miR-137 enhanced cell growth and decreased cell apoptosis. Combined bioinformatics analysis and dual luciferase reporter assay showed that miR-137 may target the 3'-UTR of the epidermal growth factor receptor (EGFR) to reduce its protein translation, resulting in suppression of EGFR signaling in GBM cells. Together, our data suggest that reduction in miR-137 levels in GBM tissues may increase cell growth and decrease cell apoptosis, possibly through suppression of EGFR.

Keywords: Glioblastoma multiforme (GBM), miR-137, epidermal growth factor receptor (EGFR)

Introduction

Glioblastoma multiforme (GBM) is the most malignant primary cancer in the central neural system. The GFM patients have a very low 5-year survival, since that GBM has rapidly growing manner and the brain is a closed area that cannot bare large tumor [1-4]. Therefore, suppressing of the GBM growth is critical for improving therapeutic outcome [5-9].

Epidermal growth factor receptor (EGFR) signaling has been shown to play a role in the carcinogenesis of GBM. The aberrant expression of EGFR leads to impaired apoptosis, increased proliferation, and neo-angiogenesis in GBM [10-13]. Hence, treatments targeting EGFR to suppress EGFR signaling in GBM are attractive approaches in GBM therapy [14, 15]. However, so far no such therapies obtained affirmatory outcome in the treatment for GBM.

MicroRNAs (miRNAs) are non-coding small RNAs that control some genes post-transcriptionally, through targeting the 3'-untranslated region (3'-UTR) of target mRNA. Importantly, miRNAs have been found to control carcinogenesis and cancer progression [16-18]. Aberrant expression of some microRNAs (miRNAs) was found to augment the tumorigenesis of GBM [19-23]. However, among all miRNAs, the function of miR-137 has been very rarely studied [24-28], and its involvement in the carcinogenesis of GBM is unknown.

Here, we showed that miR-137 levels in GBM tissues were significantly lower than the paired normal brain tissue in patients' specimens. Moreover, low miR-137 levels in GBM tissue are associated with poor prognosis. In vitro, overexpression of miR-137 decreased GBM cell growth and increased cell apoptosis, while depletion of miR-137 enhanced cell growth and decreased cell apoptosis. Bioinformatics analysis and a dual luciferase reporter assay showed that miR-137 may target the 3'-UTR of EGFR to reduce its protein translation, resulting in suppression of EGFR signaling in GBM cells. Together, our data suggest that reduction in miR-137 levels in GBM tissues may increase cell growth and decrease cell apoptosis, possibly through targeting suppression of EGFR.

Table 1. Clinicopathologic parameters of the patients (total)

	Patients (n; %)	*P* values
GBM tissue/Normal tumor-adjacent tissue	35 (100%)/35 (100%)	
Age (<60/≥60 years old)	30 (86%)/5 (14%)	0.25
Gender (male/female)	20 (58%)/15 (42%)	0.73
Tumor site (brain)	35 (100%)	
Tumor grade (well or moderate/poor)	5 (14%)/10 (28%)/20 (58%)	0.03
Tumor stage (I/II/III/IV)	0 (0%)/3 (9%)/15 (42%)/17 (49%)	0.01
Lymph node metastasis (no/yes)	10 (28%)/25 (72%)	0.05
Distal metastasis (no/yes)	30 (86%)/5 (4%)	0.01

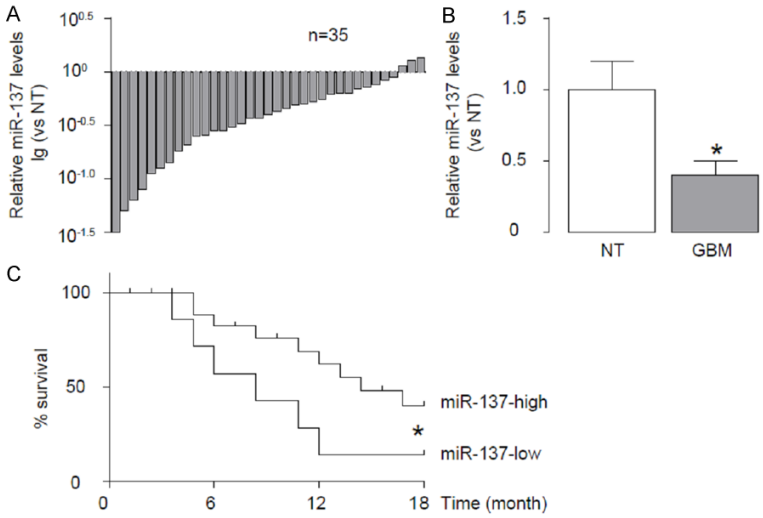

Figure 1. Low miR-137 is correlated with poor prognosis of GBM patients. (A, B) The miR-137 levels in 35 pairs of GBM and in matched tumor-adjacent normal brain tissues (NT) were analyzed by RT-qPCR. The difference in levels of miR-137 between GBM and NT was compared, shown by individual samples (A), and by mean (B). (C) Kaplan-Meier plot of overall survival in GBM patients based on high or low miR-137 levels at time of surgical removal of primary cancers. Data are mean ± S.D. of three independent experiments. *P<0.05.

Table 2. Analysis of the prognostic values of miR-137 in GBM patients by Cox regression model

	HR	95% CI	*P* value
miR-137 (low vs high)	5.1	2.3-11.4	0.003

A subset analysis was performed on patients who had high vs low miR-137 levels in GBM. The findings showed poor prognosis of miR-137-low cases after adjustment by the factors from Heng risk (HR) stratification.

Materials and methods

Patient specimen

Surgical specimens from 35 GBM patients and matched tumor-adjacent normal brain tissues (NT) were obtained postoperatively in 2008 Jinzhou Medical University. Ethical approval for the study was obtained from Jinzhou Medical University. All patients gave signed, informed consent for their tissues to be used for scientific research. All diagnoses were based on pathological evidence. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria. All patients had been followed-up for 18 months.

Cell line culture

A human GBM cell line A172 (GBM) [29] was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). A172 cells were cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA) in a humidified chamber with 5% CO_2 at 37°C.

MicroRNA target prediction

MiRNAs targets were predicted using the algorithms TargetSan (https://www.targetscan.org) as described [30].

Transfections

MiRNAs mimics (miR-137) and miRNAs antisense oligonucleotides (as-miR-137) were obtained from Origene (Beijing, China). As-mi-

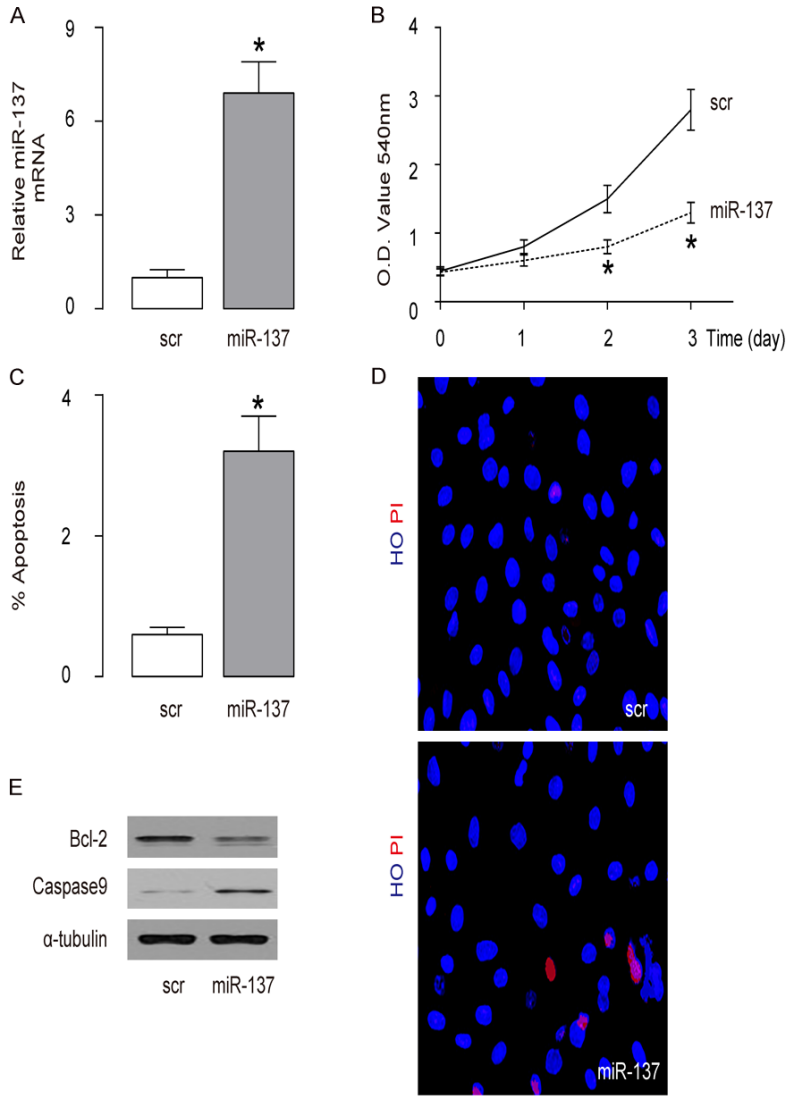

Figure 2. Transfection with miR-137 inhibits GBM cell growth and increases cell apoptosis. (A) The levels of miR-137 cells in A-172 were assayed by RT-qPCR, 72 h after transfection with miR-137. (B) After miR-137 transfection, the cell growth of A-172 cells was examined by MTT. (C, D) Seventy-two hours after miR-137 transfection, the cell apoptosis was assayed by HO and PI staining shown by quantification (C), and representative images (D). (E) Western blot for Bcl-2 and Caspase 9. Data are mean ± S.D. of three independent experiments. *P<0.05.

RNAs, miRNAs, and control with scrambled sequence (scr) were transfected into cells at a concentration of 50 nmol/l using according to the manufacturer's instructions (Invitrogen).

3'UTR luciferase-reporter assay

The EGFR 3'-UTR reporter plasmid (pRL-EGFR) was purchased from Creative Biogene (Shirley, NY, USA). Luciferase reporter assay has been described before [30].

RNA extraction, reverse transcription and quantitative RT-PCR (RT-qPCR)

RNA extraction, reverse transcription and quantitative RT-PCR (RT-qPCR) have been described before [30].

MTT assay

For assay of cell growth, 5×10^3 cell per well were seeded into 96 well-plate and subjected to a Cell Proliferation Kit (MTT, Roche, Indianapolis, IN, USA), according to the instruction of the manufacturer. The quantification was done by checking absorbance value (OD) at 570 nm in a microtiter plate reader (Promega, Fitchburg, WI, USA). Experiments were performed 5 times.

Apoptosis assay

Cells from each group were harvested 48 hours after transfection. Cells were resuspended at a density of 10^6 cells/ml in PBS. After double staining with Hoechst 33342 (HO, Sigma-Aldrich) and propidium iodide (PI, Sigma-Aldrich), the images were taken and quantification was performed. The PI+ cells in the total HO+ cells were used to determine the apoptotic cells.

Western blot

Western blot has been described before [30]. Primary antibodies for Western Blot are rabbit anti-Bcl-2, anti-Caspase 9, anti-EGFR and anti-α-tubulin (all purchased from Cell Signaling, St Jose, LA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson Immuno-Research Labs, West Grove, PA, USA). Images shown in the figure were representatives from 3 repeats.

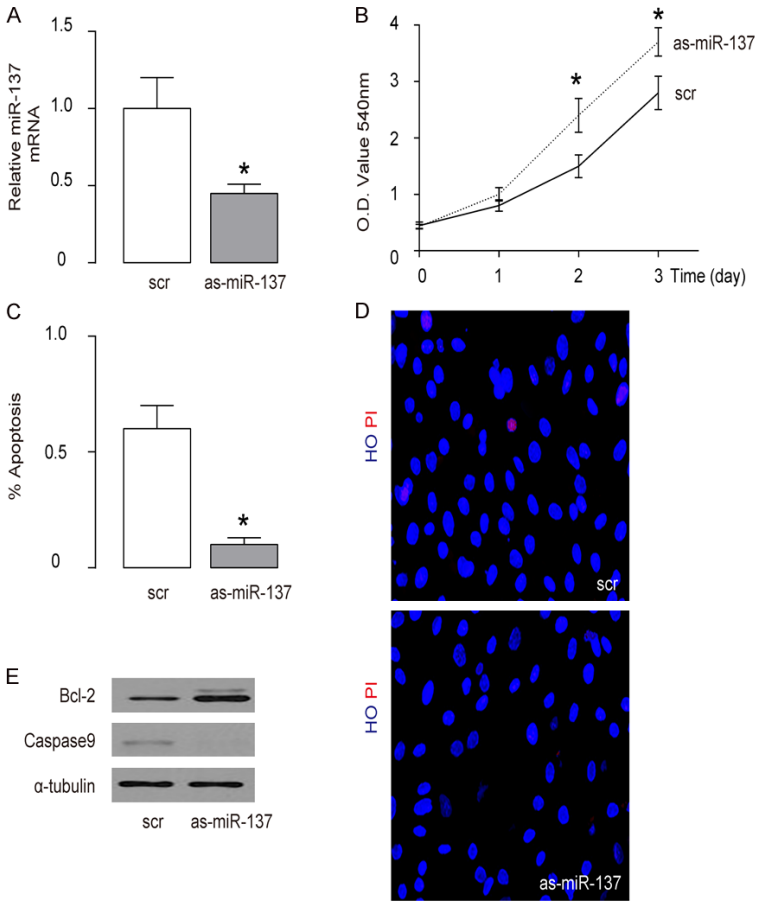

Figure 3. Transfection with as-miR-137 increases GBM cell growth and inhibits cell apoptosis. (A) The levels of miR-137 in A-172 cells were assayed by RT-qPCR, 72 h after transfection with as-miR-137. (B) After as-miR-137 transfection, the cell growth of A-172 cells was examined by MTT assay. (C) Seventy-two hours after as-miR-137 transfection, the cell apoptosis was assayed by HO and PI staining shown by quantification (C), and representative images (D). (E) Western blot for Bcl-2 and Caspase 9. Data are mean ± S.D. of three independent experiments. *P<0.05.

Statistical analysis

All of the statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Statistical analysis of group differences was carried out using a one-way analysis of variance (ANOVA) test followed by Turkey multiple comparison post-hoc analysis. The relationship of miR-137 and clinicopathological characteristics was evaluated using multivariate Cox regression analysis. Patients' survival was determined by Kaplan-Meier analysis. All values represent the mean ± standard deviation (SD). A value of $P<0.05$ was considered statistically significant after Bonferroni correction.

Results

Low miR-137 in GBM is correlated with poor prognosis of GBM patients

We examined the levels of miR-137 in 35 pairs of GBM tissues and matched tumor-adjacent normal brain tissues (NT) by RT-qPCR (**Table 1**). We detected significantly lower miR-137 levels in GBM tissue, compared to the matched NT (**Figure 1A** and **1B**). To evaluate the clinical significance of low miR-137 in GBM, we investigated whether the levels of miR-137 levels may correlate with overall survival in GBM. Overall survival, which was defined as the time from randomization to death, and preset as 18 months here, was evaluated. The relationship of miR-137 expression and clinicopathological characteristics was evaluated using multivariate Cox regression analysis (**Table 2**). The median value of all 35 cases was chosen as the cutoff point for separating miR-137 high-expression cases (n=18) from miR-137 low-expression cases (n=17). Kaplan-Meier curves indicated that GBM patients with low miR-137 levels had a significantly shorter overall survival than those with high miR-137 levels (**Figure 1C**).

Overexpression of miR-137 inhibits cell growth and increases apoptosis in GBM cells

Next we used A-172, a human GBM cell line, to investigate the role of miR-137 in the growth of GBM cells. We firstly transfected A-172 cells with miR-137. The levels of miR-137 in A-172 cells were assayed by RT-qPCR, 72 hours after transfection. We found that the miR-137 levels significantly increased in miR-137-transfected cells (**Figure 2A**). The cell growth was then analyzed by MTT assay. We found that overexpres-

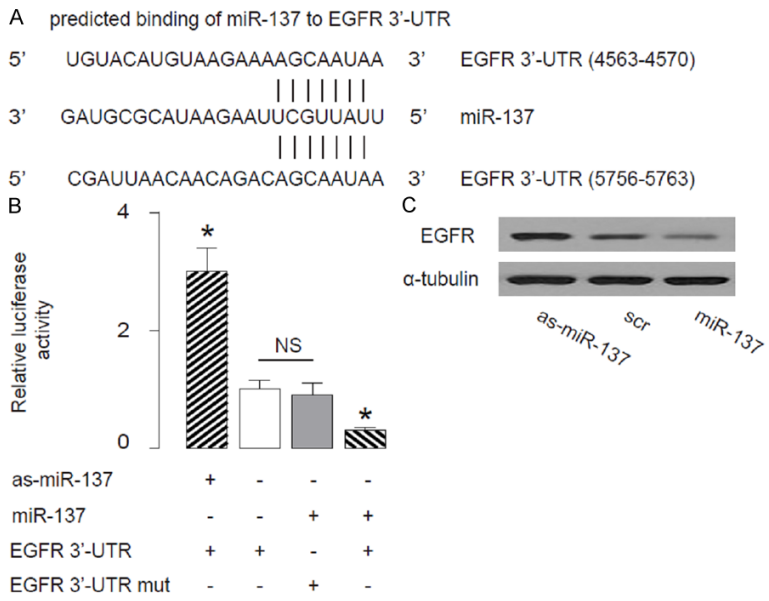

Figure 4. EGFR is a target gene of miR-137. A. Bioinformatics analysis shows that there are 2 binding sites for miR-137 on the 3'-UTR of EGFR mRNA. B. The luciferase reporter plasmids containing either wild-type or mutant 3'-UTRs of EGFR were constructed. The luciferase reporter assay was set up to identify the direct miR-137-EGFR interaction. The relative luciferase activity was significantly lower in cells after 48 hours co-transfection with miR-137-modified plasmids and p3'-UTR-EGFR or miR-137. There was a statistically difference between cells co-transfected with p3'-UTR-mut and cells co-transfected with the miR-137. C. Western blot for EGFR in miR-137-modified A-172 cells. *P<0.05. NS: non-significant. N=5.

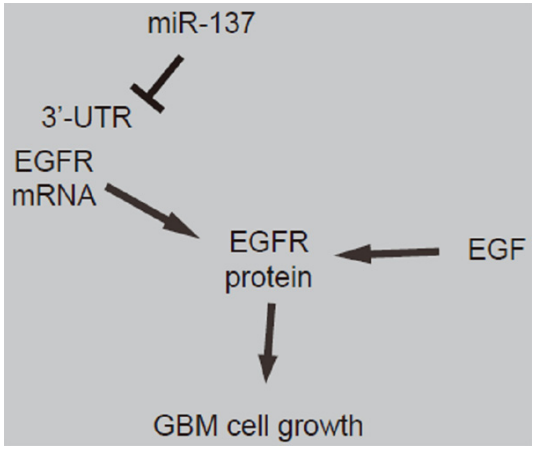

Figure 5. Schematic of the model. MiR-137 targets the 3'-UTR of EGFR to reduce its protein translation, resulting in suppression of EGFR signaling in GBM cells.

sion of miR-137 significantly reduced the proliferation of A-172 cells (**Figure 2B**). The cell apoptosis was analyzed by combined HO and PI staining, showing that overexpression of miR-137 significantly increased cells apoptosis, by quantification (**Figure 2C**), and by representative images (**Figure 2D**). These effects seemed to be mediated through downregulation of cell-cycle inhibitor Bcl-2 and upregulation of apoptotic genes Caspase 9 (**Figure 2E**). Thus, overexpression of miR-137 inhibits cell growth and increases apoptosis in GBM cells.

Depletion of miR-137 increases cell growth and inhibits apoptosis

Similarly, we suppressed the miR-137 in A-172 cells, by transfection of antisense for miR-137 (as-miR-137). The miR-137 levels in A-172 cells were assayed by RT-qPCR (**Figure 3A**). The cell growth was then analyzed by MTT assay. We found that depletion of miR-137 significantly increased the proliferation of A-172 cells (**Figure 3B**). The cell apoptosis was analyzed by combined HO and PI staining, showing that depletion of miR-137 significantly decreased cell apoptosis, by quantification (**Figure 3C**), and by representative images (**Figure 3D**). These effects seemed to be mediated through upregulation of cell-cycle inhibitor Bcl-2 and downregulation of apoptotic genes Caspase 9 (**Figure 3E**). Thus, depletion of miR-137 inhibits cell growth and promotes apoptosis in GBM cells.

MiR-137 targets 3'-UTR of mRNA of EGFR to inhibit its protein translation

To investigate the underlying mechanisms, we performed bioinformatics analysis and detected 2 binding sites for miR-137 on the 3'-UTR of EGFR mRNA (**Figure 4A**). Thus, we used miR-137-modified A-172 cells to examine whether this binding may affect the translation of EGFR mRNA. Therefore, the luciferase reporter plasmids containing either wild-type or mutant 3'-UTRs of EGFR were constructed. The luciferase reporter assay was set up to identify the direct miR-137-EGFR interaction. The relative

luciferase activity was significantly lower in cells after 48 hours co-transfection with miR-137-modified plasmids and p3'-UTR-EGFR or miR-137. There was a statistically difference between cells co-transfected with p3'-UTR-mut and cells co-transfected with the miR-137 (**Figure 4B**). Those consequences indicated that miR-137 could specifically bind to seed zone of EGFR 3'-UTR to inhibit its expression, while mut vector could not combine with miR-137 to decrease the relative luciferase activity. EGFR is thus a specific and direct target gene of miR-137. Moreover, the miR-137-mediated modification of EGFR mRNA were further found to alter protein levels (**Figure 4C**). Together, our data suggest that miR-137 may function at least partially via regulation of translation of EGFR in GBM cells, which is summarize in a schematic (**Figure 5**).

Discussion

There is increasing evidence that support an essential role of a number of miRNAs in the carcinogenesis of GBM [19-23]. Among all miRNAs, miR-137 has been initially found to regulate micropthalmia-associated transcription factor for melanocyte development [31]. Later on, several reports showed different functions of miR-137 in a variety of diseases. For example, bioinformatics analysis predicted that the AMPKalpha1 was a potential target gene of miR-137. Luciferase reporter assay demonstrated that miR-137 could directly target AMPKalpha1. Down-regulation of AMPKalpha1 by miR-137 reversed High-glucose-induced oxidative stress in human umbilical vein endothelial cells [32]. Moreover, miR-137 has been studies and showed to be a tumor suppressor in several cancers. For example, FoxD3-regulated miR-137 is found to inhibit the growth and metastasis of human hepatocellular carcinoma via AKT2 [26]. In colorectal cancer, miR-137 was shown to suppress Musashi-1 to regulate cancer progression [28]. In lung cancer, miR-137 inhibits tumor growth and sensitizes chemosensitivity of cancer cells to paclitaxel and cisplatin [27]. Of note, miR-137 is found to be downregulated in GBM and inhibits the stemness of glioma stem cells by targeting RTVP-1 [25]. These studies encouraged us to study the role of miR-137 in the tumorigenesis of GBM.

Here, we showed that low level of miR-137 in GBM tissues were correlated with poor prognosis of GBM patients. Overexpression of miR-137 inhibited cell growth and increased apoptosis, while depletion of miR-137 increased cell growth and suppressed apoptosis. The 3'-UTR of EGFR was targeted by miR-137, in a functional manner. Accordingly, the miR-137 may inhibit GBM cell growth by targeting oncogene EGFR, which regulates the levels of Bcl-2 and Caspase 9. Previous studies have demonstrated that the receptor tyrosine kinase EGFR is the cell surface receptor for EGF. Moreover, EGFR ligands-mediated activation of EGFR induces the invasive growth of GBM cells. Thus, the reduction of miR-137 allows the activation of the EGF/EGFR axis in GBM, which is associated with an aggressive phenotype and poor prognosis. Here, our study provides new insights into strategies to interfere with this pathway.

In conclusion, our study demonstrates a role of miR-137 as a tumor suppressor in GBM. Our data also provide evidence for the mechanisms underlying the anti-GBM effects of miR-137, which may be important for developing novel therapeutic targets for GBM therapy.

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Disclosure of conflict of interest

None.

Address correspondence to: Xiaofeng Song, Department of Histology and Embryology, Jinzhou Medical University, 3-40 Songpo Road, Jinzhou 121001, China. Tel: +864162629716; E-mail: xiaofeng_song15@163.com

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