MiR-592 functions as a tumor suppressor in glioma by targeting IGFBP2

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Abstract
A growing body of evidence suggests that microRNA-592 is involved in tumor initiation and development in several types of human cancers. However, the biological functions and molecular mechanism of microRNA-592 in glioma remain unclear. In this study, we explored the potential role of microRNA-592 in glioma as well as the possible molecular mechanisms. Our results proved that microRNA-592 expression was significantly downregulated in glioma tissues and cell lines (p < 0.01). Functional assays revealed that overexpression of microRNA-592 dramatically reduced the cell proliferation, migration, and invasion and induced cell arrest at G1/G0 phase in vitro. Mechanistic investigations defined insulin-like growth factor binding protein 2 as a direct and functional downstream target of microRNA-592, which was involved in the microRNA-592-mediated tumor-suppressive effects in glioma cells. Moreover, the in vivo study showed that microRNA-592 overexpression produced the smaller tumor volume and weight in nude mice. In summary, these results elucidated the function of microRNA-592 in glioma progression and suggested a promising application of it in glioma treatment.

Keywords
Glioma, microRNA-592, proliferation, invasion, insulin-like growth factor binding protein 2

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Introduction
Malignant gliomas are the most common and the most aggressive tumors in the central nervous system (CNS) with increasing relapse and mortality.1,2 The occurrence and progression of glioma is a multi-step process involved in the deregulation of multiple oncopgenes and tumor suppressors.3 Although great efforts have been made to understand the complicated pathogenesis of glioma and to improve its treatment, the molecular mechanism driving its development is still largely unknown, limiting the treatment of glioma. Thus, it is urgent to understand the molecular mechanisms underlying glioma development for diagnosis and treatment of this disease.

MicroRNAs (miRNAs) are a group of small non-coding RNA molecules that regulate gene expression at post-transcriptional level by binding to the 3'-untranslated region (UTR) of messenger RNA (mRNA).4 Through controlling the expression of their target genes, miRNAs were involved in various biological processes, such as cell proliferation, differentiation, migration, apoptosis, angiogenesis, and tumorigenesis.5,6 Numerous studies have shown that miRNAs play crucial roles in various physiological and pathological processes during cancer development.7,8 For glioma, multiple alterations of miRNAs' expression have been

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implicated in glioma procession and function as oncogene or tumor suppressor.9,10
There is particularly a growing interest toward microRNA-592 (miR-592), in the context of numerous cancers. MiR-592 has been reported to function as a tumor suppressor or oncogene in a variety of human cancers.11–16 Previous study has shown that miR-592 was dysregulated in glioma.17 Nevertheless, whether miR-592 was involved in regulating biological behaviors of glioma has not been well elucidated. Therefore, the aim of this study is to investigate the biological function and the potential mechanisms of miR-592 in glioma.

Materials and methods
Clinical samples
Glioma samples and adjacent normal brain tissues were collected from 44 patients undergoing complete or partial surgical resection at the Department of Neurosurgery, the First Hospital of Jilin University in accordance with the national regulation of clinical sampling in China. The diagnosis was established histologically by two experienced clinical pathologists. The samples were obtained from those without necrosis and coagulation parts according to the World Health Organization (WHO) classification. All tissues specimens were immediately sectioned and frozen in liquid nitrogen and stored at −80°C until use. All participants provided their written informed consent, and the Medical Ethics Committee of Jilin University approved the experiments.

Cell lines and transfection
The glioma cell lines U251, U87, U118, and LN18 and primary normal human astrocytes (NHA) were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were routinely incubated at 37°C in MEM-EBSS (Minimum Essential Medium Eagles with Earle’s Balanced Salts Solution; HyClone, Logan, Utah, USA) medium with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) without phenol red in a humidified atmosphere containing 5% CO₂. The medium was changed every 48 h.

MiR-592 mimic (miR-592; UGUAGUAGCGUA UAACUGUGUU) and corresponding miRNA negative control (miR-NC; UUCUCGAACGUGUCACGUUUU) were brought form RiboBio Co. (Guangzhou, China). The IGFBP2 overexpression vector was constructed by introducing the IGFBP2 gene (exclusion of 3’-UTR) into the pCDNA3.1 vector (GenePharma Co., Ltd, Shanghai, China), was brought from GenePharma Co., Ltd, and was designated as pCDNA3.1-IGFBP2. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per manufacturer’s instructions. Transfection efficiencies were determined in every experiment at 48 h after transfection. The time after transfection 24–72 h was considered as the collected time in the subsequent experiments.

Quantitative reverse transcription polymerase chain reaction
Total RNA from cultured cells or tissues was isolated using TRizol reagent (Invitrogen) following the manufacturer’s instructions. Total RNA was reverse-transcribed into complementary DNA (cDNA) using the Takara PrimeScript™ First-Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer’s instructions. The levels of miR-592 were determined using a TaqMan MicroRNA Assays kit (Foster City, CA, USA) with specific primers for miR-592 and U6 (Applied Biosystems) under an ABI PRISM7900 Sequence Detection System (Applied Biosystems). The U6 was used as an internal control. To detect IGFBP2 mRNA expression, quantitative PCR was performed using Real-time PCR Mixture Reagent (Takara) under an ABI PRISM7900 Sequence Detection System. The primers for IGFBP2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used in this study were described as previously.18 The GAPDH was used as an internal control. The relative miRNA and mRNA expression levels were calculated utilizing the 2–ΔΔCt method.

Cell proliferation, colony formation, and flow cytometry assays
Cell proliferation was determined by Cell Counting Kit 8 (CCK-8; Dojindo, Tokyo, Japan) following the manufacturer’s instructions. For the colony formation assay, 1000 transfected cells were seeded in each well of a six-well plate and maintained in a medium containing 10% FBS at 37°C under 5% CO₂ for 14 days. The colonies were fixed with methanol and stained with 0.1% crystal violet for 10 min. The colonies were counted and taken pictures using an inverted microscope (Olympus, Tokyo, Japan). The medium was replaced every 3 days. For cell-cycle arrest assay, the cells were harvested using trypsinization, washed in ice-cold phosphate-buffered saline (PBS), and fixed in ice-cold ethanol in PBS 48 h after transfection. Then, cell-cycle distribution was determined by FACScalibur flow cytometer (BD Biosciences, Mansfield, MA, USA) according to the manufacturer’s instructions.

Scratch wound-healing assay and cell invasion assay
To examine the migration ability of cells in vitro, a wound-healing assay was performed. In briefly, the transfected
cells were seeded into six-well plates at the density of $1 \times 10^4$ cells/well until they grew to full confluence. Monolayer cells were wounded by scratching with a sterilized tip, ensuring the width of each line was same. The wells were rinsed with PBS three times to remove floating cells and debris. Then, medium without serum were added and cells were cultured for 24 h at 37°C. Images were taken with an inverted phase-contrast light microscope (Olympus) at indicated time (0 and 24 h).

Cell invasion in vitro was assessed by Transwell inserts (6.5 mm in diameter, 8 μm pore size; Costar, Corning, NY, USA). In brief, transfected cells ($5 \times 10^4$) were seeded per well in the upper well of the Matrigel-coated invasion chamber in Dulbecco’s Modified Eagle’s Medium (DMEM) without serum. The lower chamber was filled with medium containing 10% FBS to attract cells. After incubation at 37°C for 24 h, the noninvading cells were removed with cotton swabs, whereas invasive cells in the lower surface of the membrane were fixed with 90% alcohol and stained with 0.1% crystal violet for 5 min. Then, the picture of stained cells was taken with an inverted microscope (Olympus). And the cell number was counted in five random fields and the average number was calculated.

**Luciferase assay**

The wild-type 3′-UTR of IGFBP2 fragment containing the predicted potential miR-592 binding sites were cloned into the luciferase reporter vector pGL3-control vector (Ambion, Austin, TX, USA) at the NheI and XhoI restriction sites; the mutant-type 3′-UTR of IGFBP2 carrying the mutated sequence in the complementary site for the seed region of miR-592 was constructed by overlap extension PCR. For luciferase assays, the $1 \times 10^5$ cells were plated in 24-well plates and cultured overnight. Then, cells were cotransfected with 100 ng wild-type or mutant-type reporter constructs and 50 nM miR-592 mimic or the miR-NC with Lipofectamine 2000. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) 48 h after transfection. Renilla values were normalized to Firefly luciferase.

**Western blot assay**

The levels of IGFBP2 were measured using a rabbit polyclonal anti-human IGFBP2 antibody at a dilution of 1:1000 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) by western blotting as previously described. Normalization was performed by blotting the same samples with an antibody against GAPDH (Santa Cruz Biotechnology Inc.).

**Tumor formation in nude mice**

All animal experiments were conducted strictly in accordance with a protocol approved by the Administrative Panel on Laboratory Animal Care of the First of Hospital of Jilin University. The 4-week-old BALB/C athymic nude mice were obtained from Experimental Animal Center of Changchun Biological Institute (Changchun, China). To establish the glioma xenografts, U87 cells stably expressing miR-592 or miR-NC were subcutaneously injected into right flank of BALB/c nude mouse (n = 5). The tumor growth was measured every 5 days, until 30 days post-injection. Tumor volume was calculated according to the formula: volume (mm$^3$) = length × width$^2$/2; 30 days after inoculation, the mice were euthanized with sodium pentobarbital (50 mg/kg) by subcutaneous injection, and the tumor tissues were stripped and weighted and stored at −80°C until use.

**Statistical analysis**

Data from at least three independent experiments are expressed as mean ± standard deviation (SD). The SPSS software package (version 19.0; SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis. Student’s t-test was used to compare differences between two groups. One-way analysis of variance (ANOVA) with Bonferroni post hoc tests was performed to compare differences between three or more groups. Associations of miR-592 expression and IGFBP2 expression were estimated using Spearman’s correlation analysis. A p value of less than 0.05 was considered to be statistically significant.

**Results**

**miR-592 is downregulated in human glioma cell lines and tissue specimens**

A panel of human glioma cancer cell lines was first analyzed to detect the expression level of miR-592. The result of quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed that the expression level of miR-592 was downregulated in glioma cell lines compared with the NHA (Figure 1(a)). Additionally, the expression level of miR-592 in U87 line was lowest compared with other cell lines; thus, U87 cells were selected for the rest of the study. The expression levels of miR-592 were also examined in the 44 glioma tissues and the matched normal specimens by qRT-PCR. It was found that the expression of miR-592 was downregulated in glioma tissues compared with the adjacent normal tissues (Figure 1(b)).

**Overexpression of miR-592 inhibits cell proliferation and colony formation, but induces cell-cycle arrest at G1/G0 phase in glioma cells**

To investigate the biological function of miR-592 in glioma cells, U87 cells were transfected with miR-592
Figure 1. miR-592 expression was regulated in glioma cell lines and tissues. (a) MiR-592 expression was detected in four glioma cell lines U251, U87, U118, and LN18 and primary normal human astrocytes (NHA) by qRT-PCR. U6 was used as loading control. (b) MiR-592 expression was detected in 44 pairs of glioma tissues and adjacent normal tissues (ANT) by qRT-PCR. U6 was used as loading control (**p < 0.01).

Figure 2. MiR-592 inhibits proliferation and colony formation and induces cell-cycle arrest at G1/G0 phase of glioma cells. (a) Expression of miR-592 was detected in U87 cells transfected with miR-592 mimic or miR-NC by qRT-PCR. (b–d) Cell proliferation, colony formation, and cell cycle stage were assessed in U87 cells after being transfected with miR-592 mimic or miR-NC (*p < 0.05 and **p < 0.01).
mimic or miR-NC at a final concentration of 100 nM, and the efficacy of transfection was assessed by qRT-PCR 48 h after transfection. It was found that miR-592 expression was significantly increased in U87 cells transfected with miR-592 mimic compared with cells transfected with miR-NC (Figure 2(a)), suggesting that transfection with miR-592 mimic could gain high transfection efficiencies. The Cell Counting Kit-8 (CCK-8) assay showed that transfection with miR-592 mimic significantly inhibited cell proliferation compared with transfection with miR-NC (Figure 2(b)). Consistent with this result, overexpression of miR-592 inhibited colony formation in glioma cells (Figure 2(c)). Flow cytometry was then used to test the role of miR-592 in cell cycle. The results showed that upregulated miR-592 induced a significant increase in the percentage of cells in the G1/G0 peak and a decrease in the percentage of cells in the S peak (Figure 2(d)). These results suggest that miR-592 inhibits glioma growth by regulating cell-cycle arrest.

miR-592 inhibited cell migration and invasion in glioma

We next assessed the effect of miR-592 on the migration and invasion of glioma cells by wound-healing and invasion chamber assays, respectively. It was found that overexpression of miR-592 in U87 cells markedly decreased the migratory and invasive capabilities (p < 0.05; Figure 3(a) and (b)).

IGFBP2 is a direct target of miR-592

The potential targets of miR-592 were investigated with the help of target prediction algorithms: TargetScan Human Release 7.1 (http://www.targetscan.org/). A putative binding site was found at 106–112 positions in 3′-UTR of IGFBP2 mRNA, which is a complete complementary sequence of the seed region of miR-592 (Figure 4(a)). To assess whether miR-592 directly targets IGFBP2, luciferase reporter assay was performed in U87 cells cotransfected with wild-type 3′-UTR IGFBP2 reporter plasmid or mutant-type 3′-UTR IGF2BP2, along with miR-592 mimic or miR-NC. It was found that restoration of miR-592 significantly decreased the luciferase activity of the wide-type IGFBP2 3′-UTR in U87 cells (Figure 4(b)), suggesting that miR-592 was able to bind with IGFBP2 3′-UTR (Figure 4(b)). In addition, overexpression of miR-592 in U87 cells markedly inhibited the expression of IGFBP2 at both mRNA and protein levels (Figure 4(c) and (d)). Meanwhile, we also found that the expression of IGF2BP1 mRNA expression was upregulated (Figure 4(e)) and was inversely correlated with miR-592 mRNA expression in glioma tissues (Figure 4(f); r = −0.522, p < 0.05). These data suggest that IGFBP2 is a target of miR-592 in glioma.
IGFBP2 overexpression reverses the inhibitory effects of miR-592 in glioma

To clarify whether IGFBP2 was involved in the miR-592-mediated tumor-suppressive effects in glioma cells, the combinations of transfection were conducted prior to the assessment of cell proliferation, cell-cycle arrest, migration, and invasion. As shown in Figure 5(a), the IGFBP2 protein expression was decreased in cells transfected with miR-592 mimic, while its expression was restored in cells cotransfected with miR-592 mimic and IGFBP2 overexpression vector. In addition, IGFBP2 overexpression effectively reversed the inhibition effect on cell proliferation, colony formation, cell-cycle arrest, migration, and invasion of U87 cells induced by miR-592 (Figure 5(b)–(f)). These findings demonstrated that miR-592 exerted suppressive role in glioma cells, at least in part, by targeting IGFBP2.

MiR-592 suppressed tumor growth in vivo

To further confirm the effect of miR-592 on glioma tumor growth in vivo, U87 cells stable expression of miR-592 or miR-NC were subcutaneously inoculated into nude mice. It was found that tumor growth was slower in U87/miR-592 group (Figure 6(a)). At the end of the treatment, a significant decrease in the size and weight of tumor was observed in U87/miR-592 group (Figure 6(b) and (c)).
Figure 5. IGFBP2 overexpression reverses the inhibitory effects of miR-592 in glioma. (a) IGFBP2 protein expression was measured in U87 cells transfected with miR-592 mimic with/without IGFBP2 overexpression plasmid. GAPDH was used as an internal control. (b–f) Cell proliferation, colony formation, cell-cycle stage, migration, and invasion were determined in U87 cells transfected with miR-592 mimic with/without IGFBP2 overexpression plasmid (*p < 0.05 and **p < 0.01).

Figure 6. MiR-592 inhibits tumor growth in vivo. (a) Tumor growth curve in xenograft mouse model. (b) Photographs of tumor tissues. (c) The weight of tumor tissues. (d) MiR-592 expression in tumor tissues was detected by qRT-PCR. (e) IGFBP2 protein expression in tumor tissues was measured by western blot. GAPDH was used as an internal control (**p < 0.01).
Furthermore, miR-592 and IGFBP2 expression was also determined in tumor tissues by qRT-PCR and western blot analysis, respectively. We found that the miR-592 expression was upregulated (Figure 6(d)), whereas IGFBP2 expression was downregulated in U87/miR-592 group (Figure 6(e)). These findings suggest that miR-592 can suppress glioma growth in vivo by targeting IGFBP2.

Discussion

In this study, we found that miR-592 was downregulated in glioma cell lines and tissues. Further investigations showed that the ectopic expression of miR-592 by transfection with miR-592 mimic inhibited glioma proliferation, migration, and invasion in vitro, as well as suppressed tumor growth in nude mice. IGFBP2 was identified and confirmed to be a direct and functional target of miR-592 in glioma. Previous studies have indicated that the downregulation of miR-592 is frequently observed in non-small-cell lung cancer\(^1\) and hepatocellular carcinoma,\(^13,16\) suggesting that miR-592 functions as tumor suppressor in two types of cancer. On the contrary, miR-592 expression was upregulated and functioned as oncogene in colorectal cancer\(^1,2,15\) and prostate cancer.\(^14\) These studies suggested that miR-592 functions as oncogene and tumor suppressor depending on the cancer type. However, until now, no systematic study was carried out on the expression and role of miR-592 in glioma. Our present data indicated that miR-592 expression was decreased in glioma tissues and cell lines, suggesting that miR-592 was involved in glioma development. Furthermore, we identified the effects of miR-592 on the biological behaviors of glioma, showing that miR-592 overexpression significantly inhibited glioma cell proliferation, migration, and invasion in vitro, as well as impaired tumor growth in vivo. These results indicated that miR-592 functioned as a tumor suppressor in glioma.

It was well known that miRNAs exert their biological function by suppressing their specific target genes at a post-transcriptional level.\(^20\) To investigate the possible molecular mechanism of miR-592 suppressive growth in glioma, we used TargetScan7.1 algorithm to predict gene targets for miR-592. Insulin-like growth factor binding protein 2 (IGFBP2) was selected as a potential target of miR-592 since IGFBP2 had been reported to be involved in cancer progression and development.\(^21,22\) IGFBP2 is one of the six proteins in the insulin-like growth factor binding protein (IGFBP) family and appears to play a governing role in insulin-like growth factor (IGF) regulation in the CNS.\(^23\) Previous studies demonstrated that IGFBP2 expression was a poor prognostic marker in a mixed cohort ranging from grade-II glioma to glioblastoma\(^24\) and in glioblastoma alone\(^25\) and that IGFBP2 promoted glioma progression by regulating glioma cell proliferation, migration, invasion, and apoptosis.\(^25-28\) In addition, IGFBP2 was verified to be a new target of miR-204-3p in glioma.\(^29\) In this study, we identified IGFBP2 as a direct and functional target of miR-592 by luciferase reporter assay, qRT-PCR, and western blot and found that its expression was negatively correlated with miR-592 expression in glioma tissues. These results facilitated our understanding of the mechanisms underlying glioma progression. Further studies were performed to confirm whether IGFBP2 was also involved in the regulation of biological behaviors of glioma. Our present data indicated that IGFBP2 overexpression reversed the inhibition effect of miR-592-induced glioma cell proliferation, colony formation, migration, and invasion. The in vivo studies also confirmed that miR-592 overexpression inhibits tumor growth in nude mice by downregulating IGFBP2. These data suggested that miR-592 exerted its suppressive role in glioma by repressing IGFBP2.

In summary, to our knowledge, our study first provides evidence that miR-592 was frequently downregulated in glioma cell lines and tissues and that miR-592 plays a suppressive role in glioma tumorigenesis and progression by inhibiting cell proliferation, migration, and invasion and inducing cell arrest at G1/G0 phase through targeting IGFBP2. These data suggested that miR-592 might serve as a promising new target for glioma treatment.

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Declaration of conflicting interests

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