MiR-124 inhibits malignant biological behaviors of glioma cells by targeting SDCBP

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Abstract: As the most common endocranial tumor, human brain glioma leads to high mortality and morbidity. In this study, we aimed to determine whether miR-124 regulates the malignant phenotype of glioma cells by targeted silencing of SDCBP. From the results of quantitative real-time polymerase chain reaction, miR-124 was expressed at low levels in glioma tissues and cell lines compared with normal brain tissues and cells, and down-regulation of miR-124 was correlated with a worse histological tumor grade. Overexpression of miR-124 promoted apoptosis and inhibited cell proliferation. Consistent with these in vitro results, increased miR-124 expression inhibited tumor growth in vivo. Luciferase activity assay, biotin-avidin pull-down assay, and western blot analysis verified that SDCBP is a specific target gene of miR-124. Subsequent experiments showed that up-regulation of SDCBP significantly inhibited apoptosis and increased cell proliferation of U251 and U87 cells. The regulatory effects of miR-124 on the malignant phenotype of glioma cells was partly reversed by up-regulation of SDCBP. Taken together, the results showed down-regulation of miR-124 to be an independent prognostic biomarker associated with a poor prognosis in glioma patients, and SDCBP was a specific target gene of miR-124, which regulated the malignant phenotype of glioma cells partly through direct silencing of SDCBP expression.

Keywords: Apoptosis, cell proliferation, glioma, MicroRNAs

Introduction

Glioma is a tumor originating from glial cells and is the most common endocranial tumor. Glioma is associated with very high mortality and morbidity rates, accounting for 46.1%, of approximately 3.2/10 million [1]. According to the WHO Central Nervous System Tumors classification, glioma is classified as WHO grade I-IV, with grades III and IV being high-grade gliomas [2]. Based on the statistics of the Central Brain Tumor Registry of the United States, gliomas account for approximately 27% of all central nervous system tumors and approximately 80% of malignant tumors [3]. The 1-year and 5-year survival rates of adult patients with high-grade gliomas are 30% and 13%, respectively, while the median survival times of patients with anaplastic gliomas and glioblastomas are approximately 2-3 years and 1 year, respectively [4]. The prognosis of gliomas is poor, because these tumors show significant malignant proliferation and invasion, which leads to their migration and recurrence [5].

MicroRNAs (miRNAs) are endogenous non-coding RNAs approximately 18-22 nucleotides in length. miRNAs suppress gene expression at the post-transcriptional level by targeting the 3'-untranslated region of specific mRNAs, resulting in mRNA degradation or translational repression [6]. Importantly, recent studies reported that miRNAs function as oncogenes or tumor suppressors in many malignant cancers, in which the such miRNAs exhibit anomalous expression levels and functions [7]. MiR-124, a tumor suppressor expressed at low levels in some types of malignant tumors, has been reported to play an important role in lung cancer [8], gastric cancer [9], and esophageal squamous cell carcinoma [10]. Regarding the potential regulatory mechanisms, bioinformatic analyses have predicted that SDCBP is a potential target gene of miR-124. However, the cause
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of SDCBP overexpression and its regulation of miR-124 to induce a malignant phenotype of glioma cells are still unclear. In this study, we characterized the function of SDCBP in this regulation and investigated the molecular mechanism involving how miR-124 causes a malignant phenotype of gliomas.

Material and methods

Patients and tissues

A total of 32 resected glioma specimens and 15 adjacent normal brain tissues were collected at Qinghai Provincial People’s Hospital (Xining, China) from January 2015 to May 2017. This project was approved with the Institutional Ethics Committee of Qinghai Provincial People’s Hospital and written informed consent was get from each patient. After resection, tissues were immediately snap-frozen in liquid nitrogen and then maintained at -80°C for subsequent analysis. Among the glioma patients, there were 6 cases of grade I, 8 cases of grade II, 8 cases of grade III, and 10 cases of grade IV according to the histology.

Cell culture

Human brain glioma cell lines (U251, U87 and SHG44) and primary normal human astrocytes (NHA) were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences and cultured in DMEM medium containing 10% FBS (GIBCO, USA), and maintained at 37°C in a humidified 5% CO₂ incubator.

Quantitative real-time PCR (qRT-PCR)

Total RNA of samples was extracted with TRIzol according to the manufacturer’s instructions (Invitrogen). Complementary DNA was synthesized by the Superscript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturer’s protocol. Expression of miR-124 and SDCBP was detected with SYBR Green qPCR SuperMix-UDG (Invitrogen). GAPDH and U6 were used as endogenous controls. The Ct value of detected genes was obtained from 3 independent experiments and quantified with the 2^-ΔΔCt method.

Transfection

The precursor of miR-124 (pre-miR-124), pre-NC, inhibitor of miR-124 (anti-miR-124), and anti-NC were synthesized from RiboBio (Guangzhou, China). Cells at 70-80% concentration were seeded into 6-well plates before transfection. The miRNAs were transfected into cells using Lipofectamine™ 3000 Reagent (Invitrogen, USA) following the introduction recommended by the manufacture.

Biological behavior assay in glioma cells

MTT assay was performed to evaluate cell viability. Cells were seeded in 96-well plates at 2 × 10^4 cells/mL and cultured for the indicated times. After treatment, cells were incubated with 10 μL of 0.5% MTT solution for 4 h at 37°C. The supernatant was discarded, and 150 μL of dimethyl sulfoxide was added to each well. The 96-well plates were shaken for 5 min until the crystals dissolved completely. The absorbance measured at a wavelength of 490 nm using a microplate reader. Apoptosis rate was determined with dual-color flow cytometric method. Cells were harvested, and the apoptosis level was detected by Flow cytometry. Then the results were analyzed using a FACScan flow cytometry (BD Biosciences) equipped with CellQuest software (Becton Dickinson, Mountain view, CA, US). Results were represented as percentage of apoptotic cells relative to total cells.

MicroRNA targets prediction and dual-luciferase reporter assay

The target gene of miR-124 was predicted by TargetScan (http://www.targetscan.org). The 3'-UTR of SDCBP and a mutant reporter (mut-SDCBP), in which the predicted miR-124 binding site on SDCBP was mutated, was cloned into luciferase reporters and co-transfected with either a miR-124 mimic or a control. After 24 h of transfection, the luciferase activity was determined using a dual-luciferase reporter assay system (Promega Corporation, Fitchburg, WI, USA) and normalized to its corresponding Renilla activity.

Pull-down assay

MiR-124, miR-124-Mut, and miR-124-NC were biotinylated to be bio-miR-124-wt, bio-miR-124-mut, and bio-NC, respectively, by GenePharma Company (Shanghai, China), and transfected into glioma cells. At 48 hours, the cells were harvested and lysed. Samples were then
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Figure 1. miR-124 was down-regulated in the glioma clinical specimens and human glioma cell lines. A. miR-124 expression was determined by qRT-PCR in glioma tissue (Tumor) and the normal brain tissues (Normal). B. Assessment of miR-124 levels from the total RNA derived from glioma tissues according to the tumor stage. C. The relative expression of miR-124 in the glioma cell lines compared with the normal human astrocytes (NHA). *P<0.05, **P<0.01, student t-test.

Figure 2. A. Compared with control groups, pre-miR-124 increased the expression of miR-124 in U251 and U87 cells, and anti-miR-124 can inhibit miR-124 expression. B, C. MiR-124 up-regulation inhibited cell viability and advanced apoptosis of U251 and U87 cells. *P<0.05, **P<0.01, one-way ANOVA.

Cells were collected and lysed in 1 × RIPA buffer (Beyotime, Shanghai, China) and then subjected to western blot. A total of 20 mg of protein were separated by 10% SDS-PAGE gels and transferred to PVDF membranes. The immunoreactive bands firstly were blocked with 5% non-fat dried milk for 2 h and then incubated with the primary antibodies, including SDCBP (1:1000 dilution, Abcam), and β-actin (1:3000 dilution, Boster, BA0410) at 4°C overnight. And the immunoreactive bands were incubated with the secondary antibody and visualized using ECL-PLUS Kit (Beyotime Institute of Biotechnology, Haimen, China). Each experiment was performed 3 times.

In vivo tumor growth model

All animal experiments were approved by National Institutes of Health Animal Care and the Use Committee Guidelines of the Nanjing Medical University (Nanjing, China). For the in vivo tumor assay, U87 cells (2 × 10^6) transfected with pre-miR-124 mimic or pre-NC were harvested and were washed and re-suspended in serum-free RPMI-1640 medium, and were injected into left side of the posterior flank of nude mouse (n = 6 in each group). Tumor growth were measured using a vernier caliper every 4 days until 28 days, and tumor volume (mm^3) was calculated by the formula: volume = (length × width^2)/2. All mice were killed after 28 days.
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inoculation. And parts of tumours were evaluated by immunohistochemistry with indicated antibodies (Ki67).

Statistical analysis

All statistical analyses were carried out using the SPSS 19 statistical software. The values are presented as mean ± SD and are considered significant if P<0.05. Variances between two or more groups were analysed using two-tailed student's t test or one-way ANOVA.

Results

MiR-124 was up-regulated in glioma tissues and cells

The mRNA expressions of miR-124 in glioma tissues and adjacent normal tissues were examined by qRT-PCR. Our results show that miR-124 expression in glioma samples was much lower than in normal brain samples (P<0.01, Figure 1A), the correlation between miR-124 expression and specific clinical characteristics of glioma was studied. Glioma patients with lower miR-124 expression tended to be correlated with worse histological grade (Figure 1B). And the expression of miR-124 in U251, U87 and SHG44 cells was also much lower than in NHA cells (Figure 1C), which provides initial evidence that the depletion of miR-124 may play a role in glioma tumorigenesis.

Up-regulation of miR-124 inhibits the malignant biological behaviors of glioma cells

We investigated whether miR-124 influences the malignant biological behaviors using glioma cells. Successful increase of miR-124 expression in U251 and U87 cells was confirmed by qRT-PCR (Figure 2A). In comparison to control cells, the cell viability was markedly decreased by MTT assay in U251 and U87 cells with miR-124 up-regulation (Figure 2B), but the apoptosis rate increased significantly in U251 and U87 cells with miR-124 up-regulation (Figure 2C).

Increased miR-124 expression inhibits tumor growth in vivo

To further evaluate the potential effect of miR-124 on glioma growth in vivo, U87 cells transfected with Pre-miR-124 or Pre-NC were inject-
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ed into mouse, and tumor volume was measured every three days until mice was killed. As shown in Figure 3A, the tumors growth of the miR-124 overexpressing group was smaller than that of the miR-NC overexpressing group. At the end of the experimental period, it was found that the tumor size of miR-124 overexpression group was significantly smaller than that of miR-NC overexpressing group (Figure 3B). The tumor weight was shown the same result (Figure 3C). In addition, we also detected the expression of Ki67 in the tumor tissues. Compared with the NC group, miR-124 group showed a significant decrease of Ki67 (Figure 3D).

SDCBP is a specific target gene of miR-124 in glioma

In order to explore the underlying mechanism the growth inhibition by miR-124 in glioma cells, we employed publicly available miRNA-target prediction websites (Targetscan and miRanda), and found that miR-124 could target SDCBP in 3'-UTR (Figure 4A). To confirm whether SDCBP was a direct target of miR-124 in glioma cells, we conducted luciferase reporter assays in U251 and U87 cells. The relative quantitative analysis of SDCBP protein normalized to β-actin protein. IDV is the abbreviation for “integrated density values” *P<0.05, **P<0.01, one-way ANOVA.
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that pre-miR-124 specifically binds to the seed zone of SDCBP 3'-UTR to inhibit luciferase expression, but had no effect on luciferase activity in cells transfected with Mut-SDCBP 3'-UTR, indicating that SDCBP is a specific target gene of miR-124.

In addition, we used biotin-avidin pull-down assay to discover that SDCBP mRNA could be pulled down by bio-miR-124-wt (Figure 4C). However, SDCBP mRNA could not be pulled down by bio-miR-124-mut, which was introduced with mutations in the putative recognition site between SDCBP mRNA and miR-124. Those findings showed that miR-124 can sequence-specifically combine with SDCBP mRNA.

Compared with control, miR-124 enhancement remarkably inhibited the expression of SDCBP protein, and miR-124 silencing induced by the inhibitor of miR-124 (anti-miR-124) caused the up-regulation of SDCBP protein in U251 and U87 cells (Figure 4D, 4E). However, there was no significant alteration of SDCBP mRNA. These results show that miR-124 can silence SDCBP expression at the post-translational level.

**MiR-124 inhibits malignant biological behaviors of glioma cells by targeting SDCBP gene**

Because miR-124 can inhibit malignant biological behaviors of glioma cells, and because SDCBP is a miR-124 targeting gene, we next studied the physiological role of miR-124-target SDCBP in glioma cells after up-regulation of SDCBP. As shown in Figure 5A, 5B, expression vector of SDCBP (pc-SDCBP) significantly increased the expression of SDCBP protein in U251 and U87 cells transfected with pre-miR-124.

Compared to the glioma cells transfected with pre-miR-124, the cell viability in glioma cells co-transfected with pre-miR-124 and pc-SDCBP was significantly upgraded (Figure 5C), and the cell apoptosis was markedly inhibited (Figure 5D). The regulation effects of miR-124 on malignant biological behaviors of glioma cells can be reversed to a certain degree by up-regulation of SDCBP.

In summary, miR-124 was confirmed to control malignant biological behaviors of U251 and U87 cells, partly by targeting SDCBP gene.

**Discussion**

In the past 10 years, the incidences of primary brain tumors have increased rapidly, and gliomas remain the most common central nervous system malignancy [11, 12]. Glioma cells exhibit high proliferation and low apoptosis rates and strong resistance to chemotherapy and radiotherapy, which contribute to their malignancy and recurrence [13, 14].

Accumulating evidence has also suggested that miRNAs are diagnostic and prognostic molecular biomarkers as well as therapeutic targets in cancer [15]. Accumulating studies have identified many miRNAs with aberrant expression in glioma tissues or cells and with tumor suppressor or oncogene functions in gliomas [16]. Our study found that miR-124 was down-regulated in glioma tissues and cells, and its expression level was negatively correlated with the glioma histological grade. We found that miR-124 up-regulation inhibited the proliferation and increased apoptosis of U251 and U87 cells. Accordingly, miR-124 plays impor-
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Important roles in the tumorigenesis of gliomas and is a good prognostic biomarker for gliomas.

SDCBP, comprised of nine exons and spanning 2.96 kb on chromosome 8q12, encodes a scaffolding protein possessing several biological activities involved in cancer metastasis [17, 18]. Studies have reported that SDCBP might represent an important target for inhibition of exosome-mediated tumor development [19, 20]. Previous studies reported that the SDCBP expression level correlates well with invasion and metastasis of BCa cells [17]. SDCBP overexpression also enhanced glioma cell migration by activating p38, JNK, and AKT signaling [18]. Nevertheless, only a few studies on the role of SDCBP in the progression of gliomas have been reported, and the cause of SDCBP up-regulation in gliomas and its effect on the regulation of miR-124 to control the malignant phenotype of glioma cells are still unclear. The conclusion that SDCBP is a target gene of miR-124 was confirmed by a series of gain-of-function experiments. First, the luciferase reporter assay showed that miR-124 specifically binds to the 3'-untranslated regions of SDCBP, thereby silencing the expression of luciferase. Second, the biotin-avidin pull-down assay confirmed the specific and direct binding of miR-124 to SDCBP mRNA. Third, anti-miR-124 enhancement negatively regulated SDCBP expression at the post-translational level, whereas miR-124 silencing displayed the opposite regulatory activities. The targeted modulation of SDCBP by miR-124 was also reported in hepatoblastoma cancer [21].

Because miR-124 overexpression inhibits the malignant phenotype of glioma cells, and SDCBP is an important target gene of miR-124, we hypothesized that miR-124 regulates the malignant phenotype of gliomas by directly down-regulating SDCBP. To test this hypothesis, pc-SDCBP was transfected to up-regulate the expression of SDCBP in U251 and U87 cells by up-regulation of miR-124. The results showed that up-regulation of SDCBP promoted the malignant phenotype of glioma cells. The regulatory effects of pre-miR-124 on the malignant phenotype were partly reversed by SDCBP up-regulation. Accordingly, validation of SDCBP as a target gene for miR-124 may provide a possible explanation for how low expression of miR-124 plays a role as a tumor suppressor in gliomas.

In conclusion, miR-124 acts as a tumor suppressor, and its silencing inhibited the malignant phenotype of glioma cells. SDCBP is a target gene of miR-124, and miR-124 regulates the malignant phenotype of glioma cells, partly via direct silencing of SDCBP expression. Overall, the results of this study increased our understanding of the molecular mechanism of glioma development and progression. In the future, miR-124 may provide a novel therapeutic target in the management of gliomas.

Disclosure of conflict of interest

None.

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