miR-126 Suppresses Invasion and Migration of Malignant Glioma by Targeting Mature T Cell Proliferation 1 (MTCP1)

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Background: The aim of this study was to assess the utility of miR-126 in promoting malignant glioma progression and determine if miR-126 might be a target for malignant glioma treatment.

Material/Methods: The expression of miR-126 in malignant glioma tissues and cells was detected by reverse transcription polymerase chain reaction (RT-PCR). Western blot analysis was used to detect changes in protein levels. Transwell assay was applied to assess the migration and invasion in vitro. Luciferase reporter assay was used to confirm the binding of miR-126 and mature T cell proliferation 1 (MTCP1). A nude mouse tumor model was used to assess the molecular mechanism in vivo.

Results: The expression level of miR-126 in patients with stage III~IV malignant glioma was significant lower than that in patients with stage I~II. In different malignant glioma cell lines, the expression was significantly reduced in U87MG. Compared with the control mimics group, the expression of MTCP1 was significantly decreased. The results of Transwell assay showed that the invasiveness and migration in the miR-126 and mature T cell proliferation 1 (MTCP1) group was significantly lower than in the control mimics groups. miR-126 mimics did not affect luciferase activity in the Mut-miR-126/MTCP1 group, while miR-126 mimics reduced luciferase activity by 54% in the Wt-miR-126/MTCP1 group. The results of invasion showed that the invasion ability in the miR-126 inhibitor group was significantly increased compared with that in the normal control (NC) group, while the invasion and migration abilities in the MTCP1 siRNA group were significantly increased. After 6 weeks, the tumor volume in the miR-126 inhibitor group was significantly increased, while that in the MTCP1 siRNA group was significantly decreased.

Conclusions: miR-126 inhibits the migration of malignant glioma cells by inhibiting MTCP1.

MeSH Keywords: Adenocarcinoma • Astrocytoma • Neoplasm Invasiveness

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Background

Malignant glioma is a type of tumor that always starts in the brain and spine and arises from glial cells [1]. It causes various symptoms based on location, including headaches, vomiting, seizures, visual loss, pain, and weakness [2]. The disease does not metastasize by blood but spreads via the cerebrospinal route, causing "drop metastases" to the spinal cord and leading to poor prognosis [3]. Treatment for malignant glioma is often a combined approach using surgery, chemotherapy, and radiation therapy, but with higher recurrence rates [4]. Gene targeting treatment is new malignant glioma treatment. Studies have confirmed that microRNAs (miRNAs) play key roles in development of malignant glioma, such as proliferation, AKT signaling pathway, cell growth, and expression of key kinases [5]. Therefore, targeting miRNA genes relevant in pathogenesis of malignant glioma might be a promising strategy for the therapy of this disease.

miR-126 has been confirmed to be a key factor in various cancers by participating in different pathways and mechanisms. In 2012, Chen et al. [6] found that miR-126 could decrease the expression of Matrix Metallopeptidase 2 (MMP-2) and inhibit invasion of brain glioma cells. In addition, downregulation of miR-126 in glioma patients was confirmed to be associated with methylation phenomenon [7]. miR-126 was also found to target KRAS Proto-Oncogene, GTPase (KRAS), regulate extracellular regulated protein kinases (ERK) pathway, and inhibit cell proliferation and invasion of glioma cells [8]. However, the detailed mechanism of miR-126 in invasion and migration of malignant glioma is unknown.

In this study, we examined the expression of miR-126 in tissues from malignant glioma patients compared to normal tissue. Moreover, the target gene Mature T Cell Proliferation 1 (MTCP1) was illustrated to be involved in invasion and migration mechanisms. The findings of this study demonstrate the miR-126 promotes malignant glioma progression and indicate that miR-126 might be a target for malignant glioma treatment.

Material and Methods

Patients and tissue

Malignant glioma tissue and corresponding adjacent tissues were obtained from 89 malignant glioma patients undergoing resection of malignant glioma from 2014 to 2017 in our hospital. The study was explained to patients and their family by doctors. The study was approved by the Ethics Committee of our hospital. All patients signed informed consent.

Cell lines and culture

Normal glial cells line NHA and malignant glioma cell lines HS683, SW173, LN299, and U87MG were purchased from the American Type Culture Collection (Manassas, USA). All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C, 5% CO₂, and 95% humidity. Cell culture passage numbers less than 5 were used in this study.

RNA oligoribonucleotides construction and lipofection transfection

All miRNA and siRNA for transfection were purchased from Gemma Biology (Shanghai, China). In the process of lipofection transfection, the experiments were divided into 5 groups: NHA group, miRNA-126 mimics group (miRNA-126 mimics transfection for U87MG), control mimics group (miRNA-negative control of mimics transfection for U87MG), MTCP1 siRNA group (MTCP1 siRNA transfection for U87MG), and miRNA-126 inhibitor + MTCP1 siRNA (miR-126 inhibitor and MTCP1 siRNA transfection for U87MG). For lipofection transfection, cells were plated in the complete medium in a 50-ml culture flask until the cell density reached 30% to 50%. Lipofectamine 2000 and DNA were put in sterile EP tubes: 5 µl lipofectamine 2000 was added in 100 µl serum-free medium and kept at room temperature for 5 min; 0.5 nmol transfection was added in 100 µl serum-free medium, then mixed and kept at room temperature for 20 min to form the complex. The cultured cells were washed by serum-free medium. The complex was mixed in serum-free medium and added to cells. Then, cells were cultured and incubated at 37°C, 5% CO₂, and 95% humidity for 8 h. The culture medium was changed to complete medium, and cells were continually cultured for 24 to 48 h. Then, cells were collected and total proteins were extracted.

RT-PCR and Western blot detected the expression of miR-126 and MTCP1

The detailed procedure of RNA isolation, RT-PCR, and Western blot for tissues and cells were performed as described before. Genomic DNA was extracted from cells using the QIAGEN DNA extraction kit (Qiagen, Chengdu, China) according to the instructions of the manufacturer. If DNA samples were not to be processed immediately, they were kept at -20°C. Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH) level was used as an internal control for protein loading. The primer sequences are shown in Table 1. After 35 rounds of amplification (95°C for 1 min, 62°C for 1 min, 72°C for 1.5 min), 10 µl of the reaction were resolved on a 1% agarose gel and the amplification product was visualized under UV light after staining the gel with ethidium bromide. Western blot analysis was carried out according to standard methodology. We added 1 ml RIPA buffer to cells and centrifuged them at 12 000×g...
for 5 min at 4°C. Supernatants were collected and either used immediately or stored frozen. The protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After being blocked with 5% nonfat milk in Tris-buffered solution containing 0.1% Tween 20 for 1 h, the membranes were incubated overnight at 4°C with antibody. The primary antibodies used in Western blot analysis were MTCP1 (Abcam, ab128358, 1: 1000) and GADPH (Abcam, ab131385, 1: 1000). The immunoreactive bands were visualized by enhanced chemiluminescence (Western Biotechnology Corporation).

**Migration and Invasion**

The Transwell chamber had a 24-well insert with pore size of 8 µm (BD biosciences). For in vitro assays of migration and invasion, 2×10⁵ cells of miR-126 mimics group, control mimics group, and NHA group were re-suspended in serum-free medium and then added into the upper chamber, while 500 µL complete medium was placed in the lower chamber. For invasion assay, 30 µL matrigel was used to form a matrix barrier in the membrane of the upper chamber. The Transwell chambers were incubated at 37°C, 5% CO₂, and 95% humidity for 48 h. The cells on the surface were fixed by 70% ethanol and dyed by crystal violet. All cells were scanned and counted at 400× magnification.

**Luciferase reporter assay**

Based on the operation manual of the TIANamp Genomic DNA Kit (TIANGEN Biotech, China), DNA was extracted for building luciferase reporter vector. At 48 h after transfection, cells were collected and the Dual-Luciferase Reporter Assay System (E1910) (Promega, China) was used to detect the luciferase activation of samples. The tests were repeated 3 times.

**The xenograft assay of malignant glioma cells in nude mice**

A total of 24 nude mice were purchased from the Experimental Animal Center of the Chinese Academy of Sciences in Beijing. All mice were randomly divided into 4 groups: MTCP1 siRNA group, miR-126 group, MTCP1 siRNA+miR-126 group, and normal control group. All procedures met the ethical standards for animal experiments. The nude mice were kept on aseptic laminar at room temperature (25°C). The feed and water were processed by sterile disinfection treatment. Cells in the transfected group and control group were suspended in PBS and subcutaneously injected into nude mice to construct the malignant glioma nude mouse model. Tumor volume of each nude mouse was detected each week until death, observing continuously for 6 months and taking the average value of each group. U87MG cells (1×10⁷), which were stably expressing mir-126 or NC, were suspended in 100 μl phosphate-buffered saline (PBS) and then injected subcutaneously into the posterior flank of female BALB/c athymic nude mice. Tumor volumes in mice were measured with a slide caliper every week until sacrifice. Twelve nude mice were included and they were sacrificed 6 weeks after injection. The computational formula of tumor volume was: V=0.52d³ (d: diameter of tumor). Results are presented as mean ± standard error of the mean.

**Statistical analysis**

The association between miR-126 expression and clinicopathological variables was assessed by chi-square tests. Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). and GraphPad Prism 5.0. P<0.05 was regarded as statistically significant.

**Results**

Expression level of miR-126 in malignant glioma and its related clinicopathological features

The relationship between expression level of miR-126 and clinicopathological features is shown in Table 2. Malignant glioma always occurred in patients more than 50 years old. Moreover, the expression of miR-126 did not significantly differ with age, sex, or sizes and numbers of tumors (P>0.05). However,
the expression level of miR-126 in patients at stage III–IV was significantly lower than in patients at stage I–II (P<0.05).

The expression of miR-126 in malignant glioma tissue and cells

RT-PCR was used to assess the expression of miR-126 in malignant glioma tissue and para-carcinoma tissue, normal glial cells (NHA), and malignant glioma cells (HS683, SW173, LN229, and U87MG). The results showed that the expression of miR-126 was not significantly different between tissue from I–II stage patients and normal tissue, while the expression was significantly reduced in III–IV stage patients (P<0.01) (Figure 1A). The expression level was highest in NHA cell lines. In different malignant glioma cell lines, the expression was significantly decreased in U87MG and LN299 cell lines, and it was lowest in U87MG (Figure 1B). Therefore, the expression of miR-126 was significantly decreased in malignant glioma cell lines, especially in highly different malignant glioma cell lines.

Table 2. The expression level of miR-126 in malignant glioma and its related clinicopathological features.

| Variables        | All patients (n=89) | MiR-126 expression | P value |
|------------------|---------------------|--------------------|---------|
|                  |                     | Low                | High    |         |
| Age (years)      |                     |                    |         |
| ≤50              | 31                  | 16                 | 15      | 1       |
| >50              | 68                  | 34                 | 34      |         |
| Gender           |                     |                    |         |
| Male             | 43                  | 21                 | 22      | 1       |
| Female           | 46                  | 23                 | 23      |         |
| Size of tumor    |                     |                    | 0.383   |
| ≤3 cm            | 38                  | 15                 | 23      |         |
| >3 cm            | 61                  | 33                 | 28      |         |
| TNM staging      |                     |                    | 0.031   |
| I–II             | 47                  | 23                 | 24      |         |
| III–IV           | 42                  | 11                 | 31      |         |

The median expression level was used as the cut-off. Low expression of miR-126 was classified as values below the 50th percentile. High miR-126 expression was classified as values at or above the 50th percentile. For analysis of correlation between miR-126 expressions and clinical features, chi-square tests were used. Results were considered statistically significant at P<0.05. TNM – tumor node metastasis.

Figure 1. RT-PCR detect the expression level of miR-126 in tissue and cells of malignant glioma. (A) The relative expression of miR-126 in malignant glioma tissue and para-carcinoma tissue. (B) The relative expression of miR-126 in normal NHA cell line and malignant glioma cell lines (HS683, SW173, LN229, and U87MG). ** Compared with para-carcinoma tissue, P<0.01; * compared with NH cell lines, P<0.05.
Overexpression of miR-126 inhibits expression of MTCP1

RT-PCR and Western blot analysis were used to detect the mRNA and protein level of MTCP1 in control mimics group, miR-126 mimics group, and NHA group. The results showed that compared with control mimics group, the expression of MTCP1 was significantly decreased. There was no difference in MTCP1 expression between the miR-126 mimics group and NHA group. Therefore, overexpression of miR-126 inhibited expression of MTCP1 (P<0.05) (Figure 2).

Overexpression of miR-126 inhibits motility and invasiveness of malignant glioma

The results of Transwell assay showed that after 48 h of transfection, invasiveness in the miR-126 mimics group was significantly lower than in the control mimics groups (P<0.05), while invasiveness in the miR-126 mimics group was not significantly different from that in the NHA groups (P>0.05) (Figure 3A). The migration in the miR-126 mimics group was significantly lower than in the control mimics groups (P<0.01) (Figure 3B). Therefore, overexpression of miR-126 inhibited motility and invasiveness of malignant glioma.

Figure 2. RT-PCR and Western blot detected the effects of upregulated miR-126 in expression of MTCP1. (A) RT-PCR detecting the effects of upregulated miR-126 in expression of MTCP1. (B) Western blot detected the effects of upregulated miR-126 in expression of MTCP1. * P<0.05.

Figure 3. miR-126 inhibited the migration and invasion of U87MG (×400). (A) Transwell method was used to detect the invasion of U87MG. (B) Transwell method was used to detect the migration of U87MG. * P<0.05.
miR-126 target MTCP1

Online prediction software Target Scan was used to ensure the binding site of MTCP1 and miR-126. The sequence in the 3'-UTR region of MTCP1 mRNA and miR-126 binding site is shown in Figure 4. Luciferase activity was assessed in the Wt-miR-126/MTCP1 group and Mut-miR-126/MTCP1 group. The results showed that miR-126 mimics did not affect luciferase activity in the Mut-miR-126/MTCP1 group, while miR-126 mimics reduced luciferase activity by 54% in the Wt-miR-126/MTCP1 group.

Migration and invasion

The results of invasion showed that the invasion ability in the miR-126 inhibitor group was significantly increased compared

Figure 4. miR-126 directly targets MTCP1. (A) Target scan was used to predict the binding site of MTCP1 and miR-126. (B) Luciferase activity was used in the Wt-miR-126/MTCP1 group and Mut-miR-126/MTCP1 group. ** P<0.01; * P<0.05.

Figure 5. (A, B) The expression of MTCP1 affected the migration and invasion of U87MG (×400). * P<0.05
with that in the NC group, while the invasion ability in the MTCP1 siRNA group was significantly increased (P<0.05). The invasion ability in the MTCP1 inhibitor+miR-126 group was between that of the miR-126 inhibitor group and the MTCP1 siRNA group but was not significantly different from that in the NC group (P>0.05) (Figure 5A). Similarly, with the tendency of invasion, the migration ability of the miR-126 inhibitor group was significantly increased compared with that in the NC group, while the migration ability in the MTCP1 siRNA group was significantly increased (P<0.05). The migration ability in the MTCP1 inhibitor+miR-126 group was between that of the miR-126 inhibitor group and the MTCP1 siRNA group but was not significantly different from that in the NC group (P>0.05) (Figure 5B). Therefore, we concluded that downregulation of miR-126 increased the migration and invasion ability, while MTCP1 siRNA decreased the migration and invasion ability of malignant glioma cells.

The xenograft assay of malignant glioma cells in nude mice

After 4 weeks of transfections, tumor volume in the miR-126 inhibitor group and MTCP1 siRNA group was significantly changed compared with that in the NC group. After 6 weeks of transfections, the tumor volume in the miR-126 inhibitor group was significantly increased, while that in the MTCP1 siRNA group was significantly decreased. The tumor volume in the MTCP1 inhibitor + miR-126 group was between that of the miR-126 inhibitor group and the MTCP1 siRNA group but was not significantly different from that in the NC group (P>0.05) (Figure 6).

Discussion

It is well known that malignant glioma always starts from the brain or spine, and further deteriorates with symptoms of invasion and migration. Many miRNAs related to malignant development have been reported, and various miRNAs pro- or anti-invasion and migration remain highlights, such as miR-622 [9], miR-548b [10], miR-21 [11], and miR-1224-5p [12]. miRNAs pro- or anti-invasion and migration might be a crucial anti-malignant glioma target. However, these miRNAs and their related molecular mechanisms are unclear. In this study, we demonstrate for the first time that miR-126 inhibits the migration of malignant glioma cells by inhibiting MTCP1. In addition, the expression of miR-126 in patients at different stages was assessed.

In previous studies, miR-126 has to be reported to be related with migration and invasion in various cancers, such as colorectal cancer cells, osteosarcoma cells, and endometrial cancer cells [13–15]. Here, we found that the expression of miR-126 was not significantly different between tissue of I–II stage patients and normal tissue, while the expression was significantly reduced in III–IV stage patients. Expression was significantly reduced in U87MG and LN299 cell lines. Therefore, the expression of miR-126 was significantly decreased in malignant glioma cell lines, especially in highly different malignant glioma cell lines.

Importantly, we identified a novel target of miR-126 in malignant glioma cells. miR-126 always regulates its associated genes, such as Vascular Cell Adhesion Molecule 1 (VCAM-1) [16], CRK Proto-Oncogene, Adaptor Protein (Crk) [17], and CRK Like Proto-Oncogene Adaptor Protein (CRKL) [18]. In this study, miR-126 was found to inhibit the expression of MTCP1 and decreased the migration and invasion ability of malignant glioma cells. MTCP1 has been confirmed to interact with AKT1 [19]. Moreover, Akt/PKB might be responsible for mediating vanadate’s protective effect on death and proliferation of glioma cells [20]. By knocking down AKT1 by siRNA, the invasion of malignant glioma U251 was suppressed [21]. However, the direct effect of MTCP1 has not been confirmed. In this
study, we found that downregulated miR-126 increased the migration and invasion ability, while MTCP1 siRNA decreased the migration and invasion ability of malignant glioma cells. Moreover, after 6 weeks of transfections, the tumor volume in the miR-126 inhibitor group was significantly increased, while that in the MTCP1 siRNA group was significantly decreased.

Conclusions

We demonstrate that miR-126 inhibits the migration of malignant glioma cells by inhibiting MTCP1. The study may provide a new target for controlling the invasion and metastasis of malignant glioma.

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