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Original Article

Decreased miR-28-5p contributes to glioma progression and promotes glioma cell proliferation, migration and invasion

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Abstract: Background: microRNAs (miRNAs) play crucial roles in tumorigenesis. miR-28-5p was previously reported acting as a tumor suppressor in diverse cancers. However, the function of miR-28-5p in glioma remains unknown. Methods: In the present study, we explored miR-28-5p expression on 43 glioma tissues by qRT-PCR. Then, we performed function analysis of miR-28-5p to investigate its potential roles on glioma cell proliferation, migration and invasion in vitro. Furthermore, the target gene of miR-28-5p in glioma tumorigenesis was was established. Results: Our results showed that miR-28-5p was downregulated in glioma tissues and correlated with tumor grade. Ectopic miR-28-5p expression inhibited glioma cell proliferation, migration and invasion in vitro. Mechanistic investigations showed that RAP1B, a member of the RAS oncogene family, was a direct functional target of miR-28-5p in glioma. RAP1B overexpression reversed the potential influence of miR-28-5p on the proliferation of glioma cells. Furthermore, RAP1B was upregulated expression in glioma tissues and inversely correlated with miR-28-5p expression. Conclusions: Our data demonstrated that miR-28-5p acted as a tumor suppressor in glioma progression by targeting RAP1B. MiR-28-5p could be used as a new therapeutic target for the treatment of glioma.

Keywords: miR-28-5p, glioma, RAP1B, progression

Introduction

Glioma is the most common tumors in the central nervous system, accounting for about 80% of brain tumors and advanced glioma has a high mortality rate [1, 2]. Despite the development of multimodal and aggressive treatments including surgical resection, chemotherapy and radiation therapy in the past decades, the prognosis of glioma remains poor [3, 4]. Therefore, a better understanding of the molecular mechanisms underlying glioma progression will help identify effective therapeutic strategies for the malignancy.

MicroRNAs (miRNAs) are short, non-coding RNA molecules that regulate gene expression by directly binding to the 3'-untranslated region (3'-UTR) of their target gene mRNA [5]. Previous studies found that miRNAs were involved in various cellular processes, including cell proliferation, differentiation and apoptosis [6]. Aberrant expression of miRNAs have been observed in various types of cancers, including glioma. For example, Sasayama et al reported that miR-10b was overexpressed in glioma and associated with tumor invasive factors, uPAR and RhoC [7]. Gabriely et al suggested that miR-21 promoted glioma cells invasion by targeting matrix metalloproteinase regulator [8]. Zhang et al showed that miR-128 inhibited glioma cells proliferation by targeting transcription factor E2F3a [9]. However, the roles of miR-28-5p in glioma progression remains unclear.

In the present study, we showed that the expression of miR-28-5p was significantly lower in glioma tissues than in the adjacent non-tumor tissues, and inversely associated with advanced tumor grade. Overexpression of miR-28-5p suppressed glioma cells proliferation, migration and invasion in vitro. Furthermore, RAP1B, a member of the RAS oncogene family, was identified as a functional target of miR-28-5p.

RAP1B overexpression reversed the potential influence of miR-28-5p on the proliferation of glioma cells. In addition, RAP1B was overexpressed and inversely correlated with miR-28-5p expression in glioma tissues. Therefore, our data provided that miR-28-5p downregulation contributed to glioma progression via regulating RAP1B.

Materials and methods

Tissue samples

43 glioma tissue samples were acquired from patients who underwent radical resection between November 2013 and June 2015 at the Zhumadian Central Hospital. 13 healthy control brain samples were obtained from trauma/epilepsy surgery. All the samples were snap-frozen in liquid nitrogen and then stored at -80°C until use. All patients were not previously treated with chemo- or radiotherapy before operation. The informed consent was provided by all the patients and all the experiments were approved by the Institute Research Ethics Committee according to the Helsinki Declaration.

Cell culture

Human glioma cell lines (U87, U138, LN118 and U251) and primary normal human astrocytes (NHA) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2. MiR-28-5p mimics, pSUPER-RAP1B, mimics negative control (miR-NC) and pSUPER vector were purchased from GenePharma (Shanghai). The cells were transiently transfected with miR-NC, miR-28-5p mimics or miR-28-5p+RAP1B using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissues or cells was using Trizol Reagent (Life Technologies). A Reverse Transcription Kit (Life Technologies) was used to synthesize cDNA. For mRNA and miRNA detection, real-time PCR was conducted using a Q-PCR Detection Kit (Life Technologies) and miRNA Q-PCR Detection Kit (GeneCopoeia) on an ABI 7500 thermocycler (Life Technologies), respectively. GAPDH or U6 gene was used as internal control. The PCR reaction conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The relative expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Cell lysates were prepared with RIPA Lysis buffer (Beyotime) supplemented with the protease inhibitor cocktail. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). Protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% fat-free milk, the membrane was probed with primary anti-RAP1B and anti-β-actin (Abcam) antibody. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. The signal was visualized using the ECL detection system (Amersham Pharmacia Biotech).

CCK-8 assay

Cell growth was measured using Cell Counting Kit-8 (CCK-8, Dojindo). After plating cells in 96-well plates (Corning Costar) at 1×10^3/well, 10 μl of CCK-8 was added to each well at the time of harvest (24, 48, 72 and 96 h) according to the manufacturer's instructions. 1 h after adding CCK-8, cellular viability was measured at 450 nm using an ELISA plate reader.

Cell migration and invasion assays

For the cell migration assay, 1×10^4 cells in 100 μl medium without FBS were seeded on a fibronectin-coated polycarbonate membrane insert in a transwell apparatus (Costar). In the lower chamber, 500 μl medium with 10% FBS was added as chemoattractant. After the cells were incubated for 6 h at 37°C in a 5% CO_2 atmosphere, the insert was washed with PBS, and cells on the top surface of the insert were removed with a cotton swab. Cells adhering to the lower surface were fixed with methanol, stained with crystal violet solution and counted under a microscope in 5 pre-determined fields (×100). All assays were independently repeated at least thrice. The procedure for the cell invasion assay was similar to the cell migration assay, except that the transwell membranes were precoated with 24 μg/μl matrigel (R&D

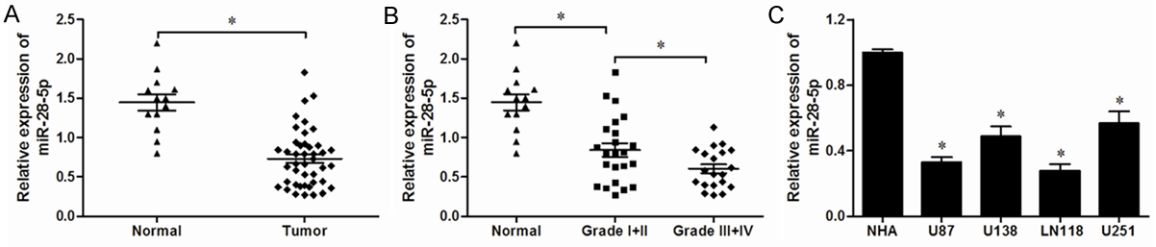

Figure 1. miR-28-5p was downregulated in glioma tissues and cell lines. A. The expression level of miR-28-5p in 43 glioma tissue samples and 13 healthy control brain samples were detected by qRT-PCR. B. miR-28-5p expression was significantly lower in patients with high tumor grade than in patients with low tumor grade. C. The expression level of miR-28-5p in four glioma cell lines (U87, U138, LN118 and U251) and primary normal human astrocytes (NHA) were measured by qRT-PCR. U6 was used as the internal control. *P<0.05.

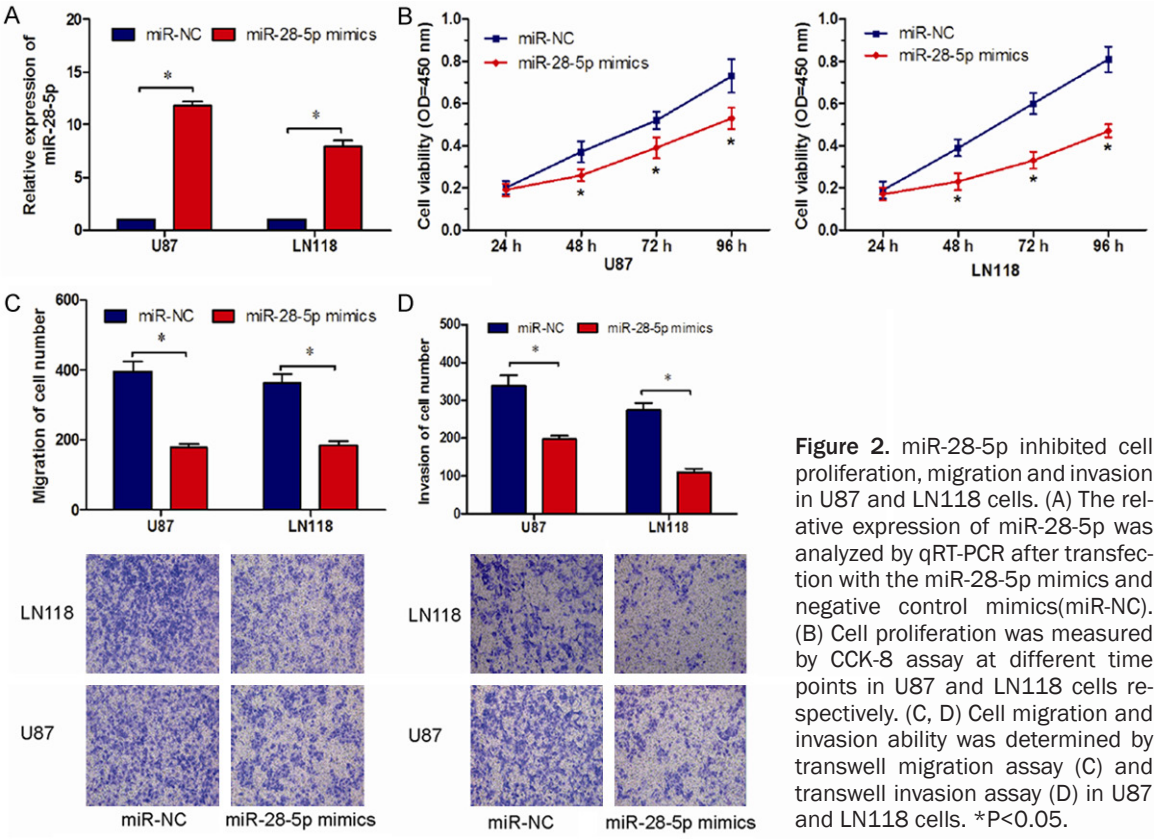

Figure 2. miR-28-5p inhibited cell proliferation, migration and invasion in U87 and LN118 cells. (A) The relative expression of miR-28-5p was analyzed by qRT-PCR after transfection with the miR-28-5p mimics and negative control mimics(miR-NC). (B) Cell proliferation was measured by CCK-8 assay at different time points in U87 and LN118 cells respectively. (C, D) Cell migration and invasion ability was determined by transwell migration assay (C) and transwell invasion assay (D) in U87 and LN118 cells. *P<0.05.

Systems) and the cells were incubated for 48 h at 37°C in a 5% CO_2 atmosphere. Cells adhering to the lower surface were counted the same way as the cell migration assay.

Luciferase reporter assay

The 3'-UTR of the human RAP1B gene which contains a miR-28-5p binding site was cloned downstream of the luciferase reporter gene in a modified pGL3-Control vector (Ambion). The resulting plasmid was labeled pGL3-RAP1B 3'-UTR. The seed regions were mutated from GAGCUCCU to GACGCCUU in order to generate the pGL3-RAP1B 3'-UTR Mut. The cells were seeded in 24-well plates. The cells were co-transfected with the pGL3 vector, which included the 3'-UTR of RAP1B, a Renilla vector and miR-28-5p mimics using lipofectamine 2000 (Invitrogen). Luciferase activity values were established using the Dual-Luciferase Reporter Assay System (Promega).

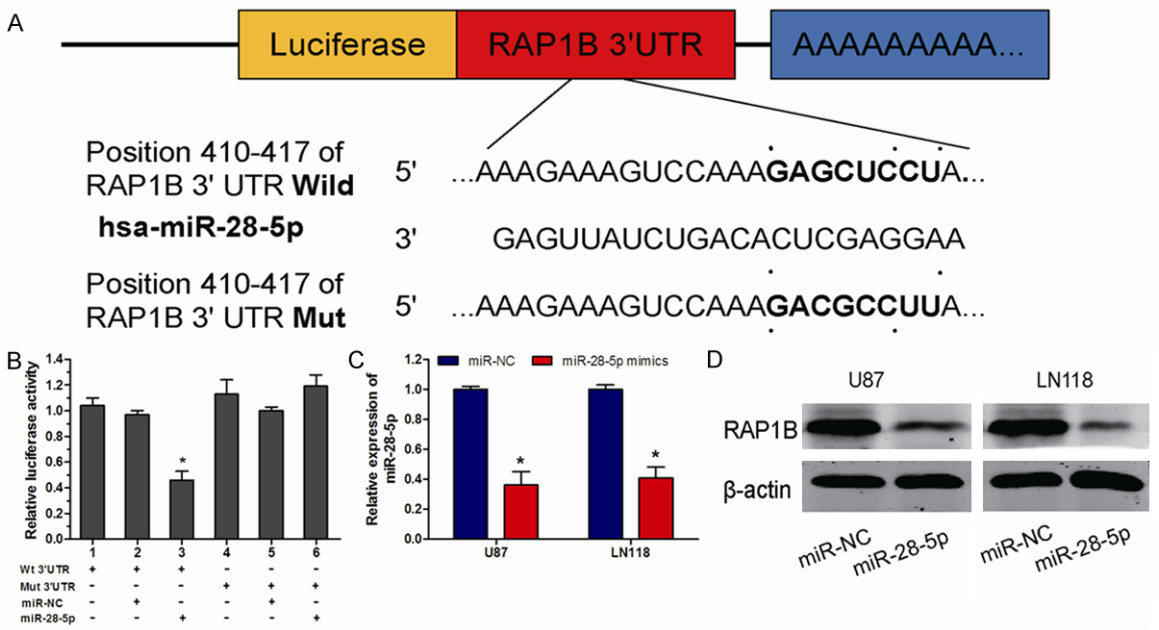

Figure 3. RAP1B is a direct target of miR-28-5p. A. Computer prediction of the miR-28-5p binding site in the 3'-UTR of the human RAP1B gene. B. Relative luciferase activity of reporter plasmids carrying Wild-type or Mut RAP1B 3'-UTR in HEK293T cells co-transfected with miR-28-5p mimics and miR-NC. C. RAP1B expression was detected by qRT-PCR in U87 and LN118 cells transfected with miR-28-5p mimics and miR-NC. D. RAP1B expression was detected by Western blot in U87 and LN118 cells transfected with miR-28-5p mimics and miR-NC. *P<0.05.

Statistical analysis

Statistical analysis was performed suing the SPSS 17.0 software package. Each experiment was conducted at least three times, and all the data are presented as the mean ± SD. Differences between groups were analyzed using Student's t test or one-way ANOVA. Pearson's correlation was used to analyze the relationship between the expressions of miR-28-5p and RAP1B mRNA. P<0.05 was considered statistically significant.

Results

Decreased expression of miR-28-5p was inversely associated with advanced tumor grade of glioma

The expression of miR-28-5p in glioma tissues and normal brain tissues were determined by qRT-PCR. Our data showed that the expression of miR-28-5p was significantly decreased in glioma tissues compared to normal brain tissues (P<0.05, **Figure 1A**). In addition, we found that miR-28-5p expression was markedly lower in glioma tissues that displayed high tumor grade than in glioma tissues with low tumor grade (P<0.05, **Figure 1B**). Furthermore, we explored the expression of miR-28-5p in human glioma cell lines (U87, U138, LN118 and U251) and primary normal human astrocytes (NHA) by qRT-PCR. Our results showed a significantly decreased expression of miR-28-5p in four glioma cell lines compared to NHA cells (P<0.05, **Figure 1C**). These results indicated that miR-28-5p was involved in the progression of human glioma.

miR-28-5p overexpression suppressed glioma cell proliferation, migration and invasion

To determine the biological relevance of miR-28-5p in glioma progression, the miR-28-5p mimics were transfected into the U87 and LN118 cells using lipofectamine 2000. qRT-PCR showed that the expression of miR-28-5p was increased by transfection with miR-28-5p mimics (P<0.05, **Figure 2A**). CCK-8 assay showed that overexpression of miR-28-5p significantly inhibited the proliferation rate of U87 and LN118 cells compared with miR-NC group (P<0.05, **Figure 2B**). As demonstrated by transwell migration and invasion assays, upregulated expression of miR-28-5p significantly suppressed the migration and invasion ability of

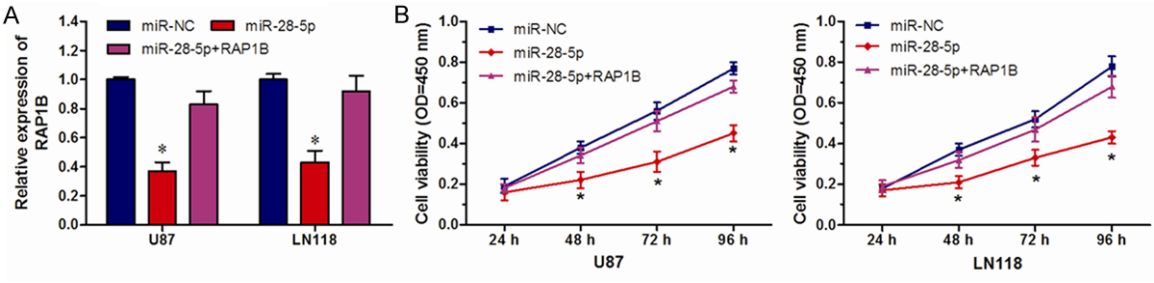

Figure 4. miR-28-5p inhibited glioma cell proliferation through targeting RAP1B. A. qRT-PCR was used to determined RAP1B expression in miR-NC, miR-28-5p mimics and miR-28-5p+RAP1B treated U87 and LN118 cells. B. Cell proliferation was preformed after transfection with miR-NC, miR-28-5p mimics and miR-28-5p+RAP1B in U87 and LN118 cells. *P<0.05.

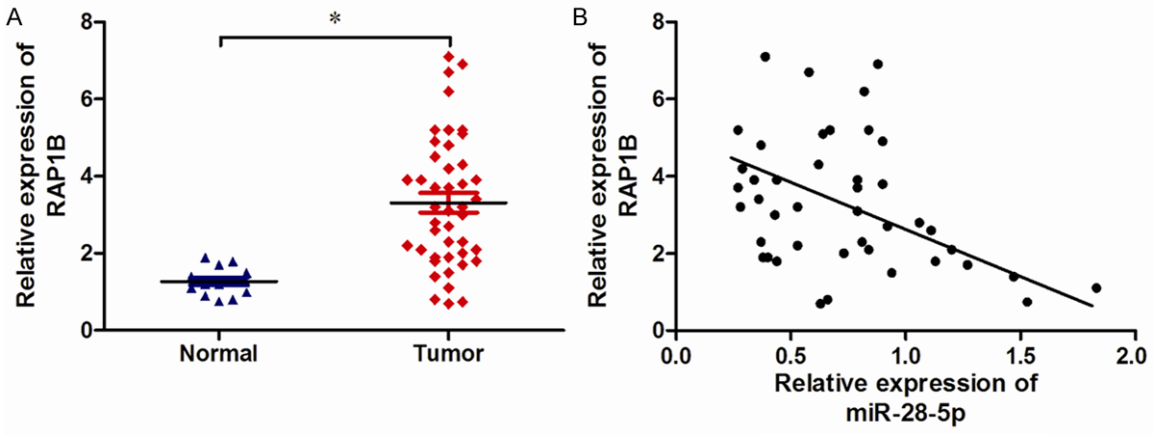

Figure 5. The expression level of RAP1B in glioma tissues. A. The expression level of RAP1B in glioma tissues was analysed by qRT-PCR. B. Analysis of the relationship between miR-28-5p expression and RAP1B levels in glioma. (r^2=0.1375) *P<0.05.

U87 and LN118 cells compared with miR-NC group (P<0.05, **Figure 2C**, **2D**). These data suggested that miR-28-5p could inhibit the aggressive phenotypes of glioma cells.

miR-28-5p directly targeted RAP1B

We identified the molecular targets of miR-28-5p by TargetScan 6.2, among which the putative gene encoding RAP1B harbors a potential miR-28-5p binding site (**Figure 3A**). luciferase assay showed that miR-28-5p mimics caused a decline in luciferase activity when the reporter gene included the RAP1B 3'-UTR in HEK293T. However, there was no obvious difference in luciferase activity when the seed region of RAP1B was mutated (P<0.05, **Figure 3B**). In U87 and LN118 cells, miR-28-5p mimics decreased the endogenous expression of RAP1B compared with miR-NC group (P<0.05, **Figure 3C**, **3D**). These results suggested that RAP1B was a direct target of miR-28-5p in glioma cells.

Overexpression of miR-28-5p inhibited cell proliferation by downregulation of RAP1B in glioma cells

To further investigate the anticancer role of miR-28-5p in glioma, we treated U87 and LN118 cells with miR-NC, miR-28-5p mimics, or miR-28-5p+RAP1B. QRT-PCR showed that miR-28-5p suppressed the expression level of RAP1B in U87 and LN118 cells, while miR-28-5p+RAP1B group showed an increase in the RAP1B mRNA expression than miR-28-5p mimics alone treated U87 and LN118 cells (P<0.05, **Figure 4A**). Then, we explored the role of miR-28-5p on glioma cell proliferation, we found that miR-28-5p overexpression significantly attenuated U87 and LN118 cells vitality, while overexpression of RAP1B reversed the growth-

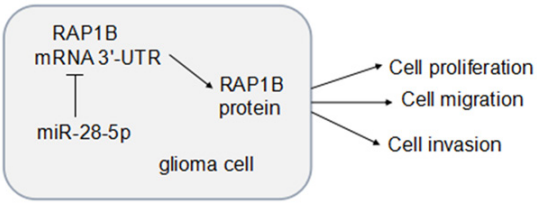

Figure 6. Schematic miR-28-5p suppressed glioma cell progression via RAP1B suppression.

inhibitory role of miR-28-5p on cell proliferation ($P<0.05$, **Figure 4B**). Furthermore, we evaluated the expression of RAP1B in 43 glioma tissues by qRT-PCR. Our results showed that the average level of RAP1B mRNA was significantly increased in glioma tissues compared with that in normal brain tissues ($P<0.05$, **Figure 5A**). Moreover, the expression level of RAP1B mRNA and miR-28-5p exhibited a significant inverse correlation ($r^2=0.1375$, $P<0.05$, **Figure 5B**). These findings demonstrated that the RAP1B is an important target of miR-28-5p involved in regulating the progression and development of glioma (**Figure 6**).

Discussion

Glioma is the most common primary brain tumor and is produced by brain and spinal cord glial cells [10]. Despite the significant advancements in treatment options, improvements in glioma patient survival have been limited owing to lack of early detection and limited capacity for optimal therapeutic decision-making [11]. Glioma is still one of the most common causes of cancer-related mortality [12].

MiRNAs are known to play key roles in tumorigenesis and the dysregulation of miRNAs in cancer has been repeatedly described. For example, Karbasy et al reported that upregulation of miR-300 and downregulation of miR-125b acted as potential predictor biomarkers in progression, metastasis and poor prognosis of osteosarcoma [13]. Yang et al suggested that miR-506 was downregulated in clear cell renal cell carcinoma and inhibited cell growth and metastasis via targeting FLOT1 [14]. Li et al found that miR-205 functioned as a tumor suppressor in colorectal cancer by targeting CREB1 [15]. Thus, it is possible that miRNA, a highly conserved gene regulatory factor, may shed light on the potential mechanism of glioma.

In this study, we demonstrated that miR-28-5p was downregulated in glioma tissues compared with normal brain tissues and associated with advanced tumor stage. Overexpression of miR-28-5p inhibited the proliferation, migration and invasion of glioma cells in vitro. These data indicated that miR-28-5p could act as a tumor suppressor whose downregulation might contribute to the progression of glioma. Data on the involvement of miR-28-5p in other cancers are limited and few potential targets of this miRNA have been identified. For example, in human hepatocellular carcinoma, Shi et al suggested that downregulated miR-28-5p expression was correlated with tumor proliferation and migration by targeting IGF1 [16]. In colorectal cancer (CRC), Almeida et al found that miR-28-5p was downregulated and inhibited CRC cell proliferation, migration and invasion by targeting CCND1 and HOXB3 [17]. These studies together with our results confirmed that miR-28-5p could act as a tumor suppressor in tumor progression.

RAP1B is a member of the RAP1 family, and is a Ras-like GTPase that regulates several basic cellular functions, such as growth, adhesion and migration [18]. Recent studies showed that RAP1B was associated with cancer progression and RAP1B expression was elevated in several cancers. For example, Yang et al suggested that RAP1B was high expressed in the gastric cancer and associated with poor prognosis, furthermore, they indicated that RAP1B could promote an aggressive phenotype in gastric cancer [19]. She et al suggested that miR-128 and miR-149 enhanced the chemosensitivity of temozolomide by RAP1B-mediated cytoskeletal remodeling in glioblastoma [20]. Zhang et al showed that miR-518b inhibited cell proliferation and invasion by targeting RAP1B in esophageal squamous cell carcinoma [21]. In the present study, we identified that miR-28-5p could directly regulate mRNA expression by targeting the 3'-UTR of RAP1B. RAP1B overexpression reversed the potential influence of miR-28-5p on the proliferation of glioma cells. In addition, our observation showed that RAP1B was upregulated expression in glioma tissues and there was an inverse correlation between miR-28-5p expression and RAP1B expression in glioma tissues.

In conclusion, miR-28-5p inhibited the proliferation, migration and invasion of glioma cell

lines by targeting RAP1B. Our study indicated that miR-28-5p played an essential role in regulation of glioma and could serve as a therapeutic target for the treatment of glioma. Understanding the precise role played by miR-28-5p will not only advance our knowledge of glioma biology, but also reveal the extensive influence exerted by miR-28-5p in cancer development.

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Disclosure of conflict of interest

None.

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