MicroRNA-342 inhibits the progression of glioma by directly targeting PAK4

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Abstract. Glioma is an extremely aggressive and lethal type of brain tumour that originates from glial cells. MicroRNA (miRNA) dysregulation has been implicated in the occurrence and progression of many human cancers, including glioma. Thus, some specific miRNAs are potential therapeutic targets for glioma diagnosis, therapy and prognosis. MicroRNA-342 (miR-342) has been reported to be abnormally expressed in various types of cancer. However, the precise roles of miR-342 in glioma remain unknown. The present study showed that miR-342 is relatively downregulated in glioma tissues and cell lines compared with that in adjacent normal tissues and normal human astrocytes. We observed that low miR-342 expression levels are correlated with advanced WHO grades and low KPS scores of glioma patients. In addition, the results of the functional assays demonstrated that miR-342 overexpression inhibits the proliferation and invasion of glioma cells and induces apoptosis. Further investigation revealed that P21 activated kinases 4 (PAK4) is a direct target of miR-342 in glioma. PAK4 was significantly upregulated in glioma tissues and inversely correlated with miR-342 expression. Moreover, PAK4 knockdown can mimic the effects of miR-342 on glioma cell proliferation, invasion and apoptosis. Notably, restoration of expression of PAK4 reversed the suppressive effects induced by the miR-342 in the glioma cells. The upregulation of miR-342 inactivated the AKT and ERK pathways in glioma. These findings may contribute to the understanding of the molecular mechanism underlying the carcinogenesis and progression of glioma, and to provide novel therapeutic target for the treatment of glioma patients.

Introduction

Glioma, the most common type of malignant tumour in the brain, is an extremely aggressive and lethal type of brain tumour that originates from glial cells. In China, glioma has an annual incidence of 5.26 per 100,000 individuals (1). Gliomas are divided into two groups according to the 2007 World Health Organization (WHO) classification: low-grade (grades I and II) and high-grade (grades III and IV) (2). Despite the advances in radiotherapy and chemotherapy treatments following surgical resection, the prognosis of glioma patients still remains poor (3,4). The median survival of the glioma patients who received comprehensive therapy is <15 months (5). Poor glioma prognosis is partially due to the oncogenic nature and rapid growth of glioma cell and local invasion of tumour into normal brain tissues (6,7). Therefore, improving understanding regarding the underlying mechanisms involved in the formation and progression of glioma is essential to the identification of more effective therapeutic strategies for this disease.

MicroRNA (miRNA) is a group of endogenous, single-stranded and non-protein-coding small RNAs (20-23 nucleotides) without open reading frames (8). miRNAs negatively regulate the expression of their target genes by undergoing base pairing with the 3'-untranslated regions (3'-UTRs) of their target messenger RNAs (mRNAs). The base pairing inhibits mRNA translation or promote mRNA degradation (9). Notably, a single miRNA can modulate several targets simultaneously, whereas a single gene may be regulated by multiple miRNAs (10). Thus, miRNAs are involved in complex regulatory networks that regulate a multitude of biological processes, such as cell proliferation, apoptosis, differentiation, autophagy, angiogenesis, invasion, migration and stem cell renewal (11-13). Moreover, miRNAs are abnormally expressed in various cancers and closely related with cancer initiation and progression (14-16). They also act as tumour suppressors or promoters by directly targeting known oncogenes or tumour suppressor genes (17-19). Therefore, identifying specific miRNAs that play important roles in tumourigenesis and tumour development might provide therapeutic biomarkers for cancer diagnosis, prognosis and therapy.

MicroRNA-342 (miR-342) plays key roles in the development, progression and metastasis of several human cancers.
(20-23). However, the precise roles of miR-342 in glioma remain unknown. Therefore, the aim of our study was to investigate the expression pattern and functions of miR-342 in glioma. Additionally, we examined the molecular mechanisms involved in the association of miR-342 with the proliferation, invasion and apoptosis of glioma cells.

Materials and methods

Clinical samples. Glioma tissues (49 paired) and corresponding normal adjacent tissues (NATs) were collected from glioma patients undergoing surgical resection at the Department of Neurosurgery, The First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) from August 2014 to January 2016. None of these glioma patients received prior radiotherapy or chemotherapy. Tissues were frozen in liquid nitrogen immediately after collection and stored at -80˚C until further use. This study was approved by the Ethics Committee of The First Affiliated Hospital of Wenzhou Medical University. Written informed consent was also obtained from each patient.

Cell lines, oligonucleotides and cell transfection. Glioma cell lines U251, U87, A172, and LN229 were purchased from American Type Culture Collection (Manassas, VA, USA), and routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen) and 10% fetal bovine serum (FBS; Invitrogen). Normal human astrocytes (NHAs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and grown in astrocyte medium (ScienCell Research Laboratories). All cells were maintained at 37˚C in a humidified atmosphere containing 5% CO₂.

miR-342 mimics, corresponding miRNA negative control (miR-NC), PAK4-targeted small interfering RNA (PAK4 siRNA) and siRNA negative control (NC siRNA) were chemically synthesized by GenePharma Co., Ltd. (Shanghai, China). PAK4 overexpressed plasmid (pcDNA3.1-PAK4) and blank plasmid (pcDNA3.1) were purchased from Chinese Academy of Sciences (Changchun, China). For in vitro function assays, cells were seeded in 6-well plates at 50-70% confluence. Transfection and co-transfection was performed through the use of Opti-MEM and Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 6-h transfection, the culture medium was removed and replaced by DMEM containing 10% FBS.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from tissues or cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The quality and concentration of total RNA was examined using a NanoDrop® ND-1000 spectrophotometer. TaqMan® microRNA assays (Applied Biosystems, Foster City, CA, USA) was used to detect miR-342 expression, with RUN6B as an internal control. To quantify PAK4 mRNA, M-MLV Reverse Transcription system (Promega Corp., Madison, WI, USA) was used to synthesize single-stranded cDNA and qPCR was conducted using SYBR Premix Ex Taq (Takara, Dalian, China), with β-actin as an internal control. The primers used in this study were as follows: miR-342 forward, 5'-GTGCTATCTGTAAGTTGAGGA-3' and reverse, 5'-GGGGCGCTACTTGCCCATG-3'; RUN6B forward, 5'-CTCCTGGCCCCAGAAGCTCTTGGC-3' and reverse, 5'-GGGCGCTACTTGCCCATG-3'; β-actin forward, 5'-ATGGGTCGGAAGATTCTATGTGGTG-3', and reverse, 5'-CTTGCAGGAAAACTCTCAGGTC-3'

Bioinformatic analysis and luciferase reporter assay. TargetScan (http://www.Targetscan.org/) and PicTar (http://pic.tar.mdcberlin.de/) were utilized to predicate the potential target genes of miR-342. The wild-type 3'-UTR segment of the...
PAK4 mRNA containing miR-342 binding sites was amplified and inserted into pMIR Reporter (Ambion, Austin, TX, USA) and named pMIR-PAK4-3'-UTR-Wt. To mutate the binding site of miR-342, its complementary sequence in the 3'UTR of PAK4 (GUGUGAU) was replaced by CACACUC, inserted into pMIR Reporter and named as pMIR-PAK4-3'-UTR-Mut. For the luciferase reporter assay, miR-342 mimics or miR-NC was transfected into cells, along with pMIR-PAK4-3'-UTR-Wt or pMIR-PAK4-3'-UTR-Mut, using Lipofectamine 2000 according to the manufacturer's protocol. Luciferase activity was determined at 48-h post-transfection using a Dual-Luciferase Reporter assay system (Promega), according to the manufacturer's instructions. Renilla luciferase activity was used for normalization.

Western blot analysis. Total proteins were extracted from tissues and cells using the RIPA lysis buffer with protease inhibitors (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors (Merck KGaA, Darmstadt, Germany). The concentration of total proteins was quantified using a BCA Protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The same amount of protein was separated by 10% SDS-PAGE gel electrophoresis, transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) and blocked with 5% fat-free milk. The membranes were then incubated overnight at 4°C with primary antibodies: mouse anti-human monoclonal PAK4 (sc-390507; 1:1,000 dilution; Santa Cruz Biotechnology), mouse anti-human monoclonal p-ERK (sc-81492; 1:1,000 dilution; Santa Cruz Biotechnology), mouse anti-human monoclonal ERK (sc-514302; 1:1,000 dilution; Santa Cruz Biotechnology), and mouse anti-human monoclonal GAPDH antibody (sc-47724; 1:1,000 dilution; Santa Cruz Biotechnology). After washing three times with Tris-buffered saline with 0.5% Tween-20 (TBST; Beyotime Institute of Biotechnology, Haimen, China), the membranes were probed with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (sc-2005; 1:5,000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h. The protein blots were visualized using the ECL Protein Detection kit (Pierce Biotechnology). GAPDH was used as a loading control.

Statistical analysis. The data in this study are presented as mean ± SD. Data were compared using Student's t-test or one-way ANOVA with SPSS 16.0 software (SPSS, Chicago, IL, USA). The association between miR-342 and clinicopathologic features of glioma was evaluated using Pearson's χ² test. Differences were considered significant at P<0.05.

Results

miR-342 is downregulated in glioma tissues and cell lines. To investigate the role of miR-342 in glioma, we measured miR-342 expression levels in 49 paired glioma tissues and NATs. The RT-qPCR results showed that miR-342 expression was significantly lower in glioma tissues than in NATs (Fig. 1A, P<0.05). We further evaluated the association between
miR-342 expression and clinicopathological factors of glioma patients. We found that the expression levels of miR-342 were strongly correlated with advanced WHO grades (P=0.001) and low KPS scores (P=0.035). No significant association was observed between miR-342 and each of the following parameters: sex, age and tumour size (all P>0.05; Table I). In addition, the expression levels of miR-342 in four glioma cell lines (U251, U87, A172 and LN229) and normal human astrocytes (NHAs) were examined. As shown in Fig. 1B, expression level of miR-342 decreased in the glioma cell lines in comparison with that in NHAs (P<0.05). These results suggested that low miR-342 expression is correlated with advanced malignancy of gliomas, and miR-342 may serve as a tumour suppressor in glioma occurrence and progression.

Figure 2. Upregulation of miR-342 inhibits cell proliferation and invasion and induces apoptosis in U251 and U87 cells. (A and B) U251 and U87 cells were treated with miR-342 mimics or miR-NC. RT-qPCR was performed to assess its transfection efficiency. *P<0.05 compared with miR-NC. (C and D) Cell proliferation in the miR-342 mimics and miR-NC group was evaluated by MTT assay. *P<0.05 compared with miR-NC. (E) Cell invasion abilities of U251 and U87 cells transfected with miR-342 mimics were detected by cell invasion assay. *P<0.05 compared with miR-NC. (F) Apoptosis rate of the U251 and U87 cells transfected with miR-342 mimics or miR-NC was assessed by flow cytometry analysis. *P<0.05 compared with miR-NC.
miR-342 overexpression inhibits cell proliferation and invasion and activates cell apoptosis in glioma. To investigate miR-342 functions in glioma, we transfected miR-342 mimics into the U251 and U87 cells, which have the lowest miR-342 expression level, to increase its endogenous expression level (Fig. 2A and B, \(P<0.05\)). MTT assay was then used to determine the effect of miR-342 overexpression on the cell proliferation of glioma. As shown in Fig. 2C and D, the proliferation of the U251 and U87 cells was suppressed in the group transfected with the miR-342 mimics compared with that in the miR-NC group (\(P<0.05\)).

We then utilized cell invasion assay to examine the effect of miR-342 on glioma cell invasion. The results indicated that the U251 and U87 cells with high miR-342 level showed lower invasion capacities than the cells with miR-NC (Fig. 2E, \(P<0.05\)). Next, flow cytometry analysis was performed to explore the role of miR-342 in glioma cell apoptosis. As shown in Fig. 2F, the upregulation of miR-342 improved apoptosis in the U251 and U87 cells considerably (\(P<0.05\)). These results highlighted that miR-342 plays tumour-suppressive roles in glioma by inhibiting cell proliferation and invasion and inducing cell apoptosis. Overall, these results indicated that miR-342 affects the growth, metastasis and apoptosis of glioma cells.

miR-342 directly targets and inhibits PAK4 expression in glioma. After observing the tumour-suppressing roles of miR-342 in glioma, we explored its potential targets using bioinformatics analysis. The results indicated a large number of candidate targets of miR-342. Of these targets, P21 activated kinases 4 (PAK4) was selected for further target identification (Fig. 3A), because it was upregulated in glioma tissues and involved in glioma formation and progression (25). To test this hypothesis, we conducted luciferase reporter assay to validate the interaction between miR-342 and the predicted binding site in the 3'-UTR of the PAK4 gene. As illustrated in Fig. 3B and C, the restoration of miR-342 expression inhibited the
miR-342 is a post-transcriptional regulator of PAK4 and binds directly to the 3'UTR of the PAK4 gene.

**PAK4 expression is upregulated and inversely correlates with miR-342 expression in glioma tissues.** To confirm the association between miR-342 and PAK4, we calculated PAK4 mRNA and protein expression levels in glioma tissues and NATs. The data obtained from RT-qPCR and western blot analysis showed that PAK4 mRNA and protein were significantly upregulated in glioma tissues relative to those with NATs (Fig. 4A and B, P<0.05). Additionally, Spearman's correlation analysis results confirmed that the negative association between PAK4 mRNA expression and miR-342 in glioma tissues (Fig. 4C; r=-0.5261, P=0.0001).

**PAK4 downregulation imitates the roles of miR-342 in glioma.** PAK4 was identified as a direct target of miR-342 in glioma. We hypothesized that the roles of PAK4 knockdown on glioma cells are similar with those induced by miR-342 overexpression. To confirm this hypothesis, we transfected the U251 and U87 cells with PAK4 siRNA or NC siRNA. As expected, the PAK4 expression decreased in the U251 and U87 cells after transfection with PAK4 siRNA (Fig. 5A, P<0.05). The results of the subsequent functional assays showed that downregulation of PAK4 repressed the proliferation (Fig. 5B and C, P<0.05) and invasion (Fig. 5D, P<0.05) and induced apoptosis (Fig. 5E, P<0.05) in the U251 and U87 cells. This result supported the hypothesis that PAK4 underexpression plays the same role as that of miR-342 mimics in glioma cells. These results further demonstrated that PAK4 may be a functional target of miR-342 in glioma.

**PAK4 reverses the tumour-suppressing effects of miR-342 on glioma cells.** Rescue experiments were performed to evaluate the contribution of PAK4 to the roles of miR-342 in glioma. After introducing pcDNA3.1-PAK4 or pcDNA3.1 into the U251 and U87 cells, we performed western blot analysis 72 h post-transfection. The results showed that PAK4 was upregulated in the pcDNA3.1-PAK4-transfected U251 and U87 cells. In addition, PAK4 expression was recovered in the miR-342 mimic-transfected cells after being transfected with pcDNA3.1-PAK4 (Fig. 6A, P<0.05). The results of the functional rescue experiments showed that reintroduction of PAK4 effectively rescued the effects of miR-342 overexpression on the proliferation (Fig. 6B and C, P<0.05), invasion (Fig. 6D, P<0.05) and apoptosis (Fig. 6E, P<0.05) of the U251 and U87 cells. These data showed clearly that miR-342 exerted tumour-suppressive roles in glioma, at least in part, by suppressing PAK4.

**miR-342 is associated with the AKT and ERK pathway in glioma.** Previous studies reported that PAK4 plays essential roles in the AKT and ERK pathways (26,27). Thus, to assess the function of miR-342 on these pathways, we transfected miR-342 mimics or miR-NC into the U251 and U87 cells. As shown in Fig. 7, miR-342 overexpression reduced both the p-AKT and p-ERK expression levels in the U251 and U87 cells (P<0.05). However, it did not affect the entire AKT and ERK expression. These results illustrated that miR-342 can inactivate the AKT and ERK pathways in glioma.
Glioma has high mortality, high recurrence rate, and low cure rate, because of its rapid growth and metastasis behaviour (6,7). miRNA dysregulation has been implicated in the occurrence and progression of many human cancers, such as bladder cancer (28), gastric cancer (29), hepatocellular carcinoma (30), colorectal cancer (31) and glioma (32). Thus, some specific miRNAs are potential therapeutic targets for cancer diagnosis, therapy and prognosis (33,34). The present study indicated that miR-342 expression was low in both glioma tissues and cell lines. Additionally, reduced expression of miR-342 was...
significantly correlated with advanced WHO grades and low KPS scores of glioma patients. According to these results, we supposed that miR-342 may act as a tumour suppressor in glioma. To confirm this hypothesis, MTT assay, cell invasion
assay and flow cytometry analysis were performed to examine the effects of miR-342 on cell proliferation and invasion and apoptosis of glioma. We found that the restoration of miR-342 expression suppresses the proliferation and invasion and enhances the apoptotic abilities of glioma cells. Moreover, PAK4 was identified as a direct and functional target of miR-342 in glioma. The upregulation of miR-342 expression inactivated the AKT and ERK pathways in glioma.

miR-342 was previously found to be aberrantly expressed and plays important roles in various human cancers. For example, miR-342 was observed to be downregulated in ERα-positive breast cancer tissues and cell lines and significantly correlated with HER2 and VEGF expression status (20,21). miR-342 was found to be decreased in tamoxifen-resistant tumour cells. Enforced expression of miR-342 improved the chemosensitivity of breast cancer cells to tamoxifen (22). Wang et al reported that miR-342 overexpression inhibits colorectal cancer cell growth and metastasis both in vitro and in vivo (23). Zhao and Zhang (35) then revealed that miR-342 upregulation suppresses cell proliferation in hepatocellular carcinoma by regulating the NF-κB pathway. A study by Xie et al (36) found that resumption of miR-342 expression can reduce cell proliferation and invasion in vitro and cell growth in vivo in non-small cell lung cancer. Li et al (37) demonstrated that miR-342 re-expression attenuated cervical cancer cell proliferation, migration and invasion. These findings indicated that miR-342 has fundamental roles in carcinogenesis and progression of malignant tumours and illustrated potential of miR-342 as a therapeutic target for various cancers.

Exploring the downstream targets of miR-342 can improve our understanding regarding the underlying molecular mechanisms involved in the tumour-suppressing roles of miR-342 on glioma cells. Notably, several tumour suppressors have been identified as direct targets of miR-342 for example, DNMT1 (23), FOXM1 (38) and FOXQ1 (38) in colorectal cancer; IKK-γ (35), TAB2 (35) and TAB3 (35) in hepatocellular carcinoma; RAP2B (36) in non-small cell lung cancer; and FOXM1 (37) in cervical cancer. Through online bioinformatics analysis, PKA4 was predicted to contain a miR-342 seed match at position 488-494 of the PKA4-3'UTR. To test whether miR-342 directly targets 3'UTR of PKA4, we performed luciferase report assays. By measuring changes in the luciferase activity, we demonstrated that miR-342 directly targets 3'UTR of PKA4. Additionally, ectopic expression of miR-342 reduced the PKA4 expression level in glioma cells at the mRNA and protein levels. The expression level of PKA4 was upregulated and negatively correlated with miR-342 expression in glioma tissues. PKA4 knockdown have roles similar to those of miR-342 overexpression in glioma cells, and the upregulation of PKA4 reverses the effects of miR-342 in glioma cells. Moreover, miR-342 can inactivate the AKT and ERK pathways in glioma cells. These results suggested that miR-342 served as a tumour suppressor in glioma, at least in part, by directly targeting PKA4 and indirectly regulating the AKT and ERK pathways.

PAKs belong to a family of serine or threonine kinases, which are best characterized as downstream effectors of Rac and Cdc42 (39). This family comprise 6 mammalian isoforms, and can be divided into two groups: group A, which includes PAKs 1, 2 and 3 and group B, which contains PAKs 4, 5 and 6 (40,41). PAK4, located at 19q13.2, was reported to be upregulated in multiple human cancers, such as pancreatic cancer (42), colorectal cancer (43), gastric cancer (44) and renal cell carcinoma (45). Increasing evidence indicated that PAK4 plays a significant role in a variety of cellular functions, such as cell proliferation, migration, invasion, apoptosis, actin cytoskeletal changes and cytoskeletal organisation (46). Furthermore, PAK4 was identified to serve important functions in tumourigenesis and tumour development (47-49). In glioma, PAK4 is overexpressed and significantly correlated with pathological grades. PKA4 downregulation repressed glioma cell proliferation, motility and adhesion (25). These data indicate a central role of PAK4 in glioma pathogenesis.
The findings of the present study identified PAK4 as a direct gene target of miR-342 and suggested that the miR-342-PAK4 pathway is a promising therapeutic target for the treatment of gliomas.

In conclusion, this study showed a significantly low expression level of miR-342 in glioma tissues and cell lines. Our results indicated that miR-342 suppresses tumour in glioma by directly targeting PAK4 and indirectly regulating the AKT and ERK pathways. Thus, miR-342 may be a candidate diagnostic marker of glioma and a potential therapeutic target for the treatment of patients with this disease.

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