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Regular paper

miR-342-3p Suppresses glioblastoma development *via* targeting CDK6

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Background: Glioblastoma is the most malignant primary brain tumor with dysregulated microRNAs affecting development and malignant transformation. Methods: Gene Expression Omnibus (GEO) dataset (GSE165937) was retrieved, and the differential expressed microRNAs were screened and testified by quantitative real-time PCR (qRT-PCR) in glioblastoma cells. miR-342-3p mimic was transfected into U87MG and U251MG cells. EdU staining, cell counting kit-8, and transwell assay were utilized to evaluate the proliferation, migration, and invasion of glioblastoma. The potential binding sequences between miR-342-3p and CDK6 were predicted and testified by TargetScan and luciferase reporter assay. Relative CDK6 and miR-342-3p expression were detected with qRT-PCR and Western blot. Results: Down-regulated miR-342-3p was observed in both glioblastoma tissues and cell lines. Over-expressed miR-342-3p prohibited glioblastoma cells proliferation, migration, and invasion, which could be rescued by further CDK6 transfection. Mechanically, miR-342-3p could directly bind with CDK6 as testified with luciferase analysis and down-regulated CDK6 expression. Conclusion: Down-regulated miR-342-3p may promote glioblastoma cells proliferation, migration, and invasion with up-regulated CDK6, which indicates that miR-342-3p/CDK6 might be a treatment target in glioblastoma development.

Keywords: miR-342-3p, CDK6, glioblastoma, cancer

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Abbreviations: ATCC, American Type Culture Collection; CEMEM, Eagle's Minimum Essential Medium; CNS, central nervous system; DK6, cell division protein kinase 6; GEO, Gene Expression Omnibus; miRNAs, microRNAs; miRNAs, microRNAs; NEAA, Non-Essential Amino Acids; PFA, paraformaldehyde; qRT-PCR, quantitative real-time PCR; WHO, World Health Organization; xCCK-8, cell counting kit-8

INTRODUCTION

As the most aggressive primary central nervous system (CNS) neoplasms, glioma accounts for about eighty percent of all tumors in the CNS with the character of malignant transformation and recurrence (Jung *et al*., 2019; Lim *et al*., 2018; Tan *et al*., 2020). According to the pathologic growth and diffusion velocity features, glioma has been categorized into I-IV grades by the World Health Organization (WHO). Maximal surgical resection, temozolomide chemotherapy, and fractionated radiation therapy are traditional treatments for glioma (Chen *et al*., 2017; Mesfin & Al-Dhahir, 2021). For the most aggressive subtype of glioma (grade IV, glioblastoma), the median survival is less than 15 months due to a diffuse infiltration at presentation and relentless progression even after the aggressive treatment (Franceschi *et al*., 2019; Jackson *et al*., 2019). Hence, understanding the molecular mechanism contributing to glioblastoma progression is urgent to develop a novel therapeutic strategy (Lu *et al*., 2020).

Some oncogenic and tumor-suppressing microRNAs (miRNAs) have been demonstrated to directly target candidate genes and pathways for glioblastoma, contributing to cell proliferation, migration, invasion, metastasis, angiogenesis, and temozolomide resistance (Banelli *et al*., 2017; Pottoo *et al*., 2021). What makes it more complicated is that miRNAs may utilize complex regulatory circuitry involving genetic and epigenetic machinery to promote glioblastoma malignant transformation and progression (de Menezes *et al*., 2021; Uddin *et al*., 2020; Westphal & Lamszus, 2015). Therefore, miRNAs-mediated dysregulation of tumor development is a promising target in glioblastoma therapy.

In this investigation, the Gene Expression Omnibus (GEO) dataset (GSE165937) is screened, and miR-342-3p is identified as a significantly down-regulated miRNA in glioblastoma tissue, which is positively correlated with histopathological grades of glioma (Wang *et al*., 2012) and can directly target cell division protein kinase 6 (CDK6) to mediate the proliferation, migration, and invasion process. Therefore, we demonstrate that miR-342-3p/CDK6 mediates the development and progression of glioblastoma, which may be considered as a future treatment target.

METHODS AND MATERIALS

Bioinformatics analysis

GSE165937 dataset (nine glioblastoma tissue samples and four normal brain tissue samples) was retrieved from GEO (Yeh *et al*., 2021), and a GEO2R analyzer was adopted to screen out differentially expressed miRNAs in glioblastoma tissues compared with normal tissues. Volcano plots were utilized to represent the differentially expressed miRNAs in glioblastoma. The raw miR-342-3p expression data were extracted and averaged to indicate the relative content. The target-binding sequences between miR-342-3p and CDK6 were predicted by TargetScan (http://www.targetscan.org/) (Agarwal *et al*., 2015).

Cell lines

The human fetal astrocyte HA1800 and glioblastoma cell lines U87MG, U251MG, SHG-44, and LN229 cell

lines were ordered from American Type Culture Collection (ATCC). HA1800 cells were maintained in Dulbecco's Modified Eagle Medium: F-12 (DMEM/F12, GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, GIBCO). U-87MG cells and U251MG cells were cultured in Eagle's Minimum Essential Medium (EMEM, GIBCO) with 10% FBS, which was further supplemented with 2 mM Glutamine, 1% Non-Essential Amino Acids (NEAA), 1 mM sodium pyruvate (NaP). SHG-44 cells were cultured in RPMI1640 medium (GIBCO) supplemented with 10% FBS, as indicated in the previous report (Zhou *et al.*, 2010). LN229 cells were cultured in DMEM medium with 10% FBS.

Cell transfection

miR-342-3p mimic, Pcdna3.1-CDK6, and negative controls were ordered from Ribobio Co., LTD (Guangzhou, China). miR-342-3p mimic was transfected into U87MG and U251MG cells with Lipo293™ Transfection Reagent (Beyotime, Shanghai, China) for 24 hours, which was further transfected with Pcdna3.1-CDK6 to decipher the regulatory relationships between miR-342-3p and CDK6.

Cell proliferation assay

2×10^3 glioblastoma cells were seeded into 96-well plates and further cultured for the indicated time (0, 24, 48, and 72 hours), and 10 μL cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was added and incubated for an additional 1 hour at 37°C. A SpectraMax M5 plate reader was utilized to measure the optical density at 450 nm.

5-Ethynyl-2′-deoxyuridine (EdU) assay

Glioblastoma cells (2×10^4) were plated and further cultured in 24-well plates for twenty-four hours, then 50 μmol/L EdU was utilized to incubate glioblastoma cells for another two hours at 37°C, which were then fixed with 4% paraformaldehyde (PFA, Beyotime) for thirty minutes and permeabilized with 0.5% Triton X-100 for ten minutes at room temperature. After adding 400 μL ApolloR reaction cocktail (RiboBio) to interact with EdU for thirty minutes, 400 μL Hoechst 33342 (RiboBio) was added to stain the nuclei for thirty minutes. A Nikon 80*i* microscope was utilized to capture the image, and the proliferation was quantified by counting the mean cells in the three fields of each sample.

Transwell assay

Transwell assay was utilized to detect cell migration and invasion. For migration assay, glioblastoma cells were inoculated into the upper chamber of Transwell (Becton Dickinson, San Jose, CA) with the serum-free medium. Glioblastoma cells were placed into a Matrigel® pre-coated (Sigma-Aldrich, St. Louis, MO) upper chamber for invasion assay. The lower chamber was inoculated with 600 μl DMEM medium containing 10% FBS. The migrating or invading glioblastoma cells on the back of the upper chamber were fixed with 4% PFA and stained with 0.5% crystal violet (Beyotime) after being cultured for twenty-four hours. The cells number was counted with an Olympus IXplore Standard system.

Luciferase reporter assay

First, wild or mutant CDK6 plasmids were cloned into the pmirGLO vector (Promega, Madison, WI) to construct pmirGLO-CDK6-mut or pmirGLO-CDK6-wt vectors. Then, miR-342-3p mimic, pmirGLO-CDK6-mut, or pmirGLO-CDK6-wt were co-transfected into U87MG cells and U251MG cells with Lipo293™ Transfection Kit (Beyotime) for 24 hours. Finally, the Dual-Luciferase Reporter assay (ThermoFisher, Waltham, MA) was utilized to indicate luciferase under the manufacturer's instruction.

qRT-PCR

TRIzol (ThermoFisher) was utilized to extract total RNA, which was further reverse-transcribed with PrimeScript® RT Master Mix and One Step PrimeScript® miRNA cDNA Synthesis Kit (TAKARA, Beijing, China) to generate the interest mRNAs and miRNAs. SYBR Green master mix (Roche, Penzberg, Upper Bavaria, Germany) was used to assay the amplification according to the following procedures: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The relative expression was normalized with *β-actin* or *U6* using the comparative $2^{-\Delta\Delta CT}$ method. The primers were listed in Table 1.

Western blot

The glioblastoma cell lysate was loaded and separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was further transferred onto polyvinylidene fluoride (PVDF) membranes (Beyotime). A 1:1000 diluted rabbit CDK6 primary antibody (ab151247, Abcam, Cambridge, MA) was utilized to incubate the membranes at 4°C overnight, which were further incubated with a peroxidase-conjugated secondary antibody (Sigma-Aldrich) for two hours at room temperature and further developed with an ECL system (GE Healthcare Life Sciences, Chalfont, UK). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Santa Cruz, Dallas, TX) was utilized as the internal control.

Statistical Analysis

Data were shown as the means ± standard deviation (SD). The Student's *t*-test or one-way ANOVA with a post hoc test was utilized to estimate the significance level. $P<0.05$ was considered to be statistically significant.

RESULTS

Down-regulated miR-342-3p in glioblastoma tissues

Ninty six miRNAs were significantly down-regulated in glioblastoma tissues compared with normal tissues

Table 1. The sequence of primers used for quantitative real-time PCR

Gene	Forward primer (5′-3′)	Reverse Primer (5′-3′)
miR-342-3p	TCCTCGCTCTCACACAGAAATC	TATGGTTGTTCACGACTCCTTCAC
U6	ATTGGAACGATACAGAGAAGATT	GGAACGCTTCACGAATTTG
CDK6	GGATAAAGTTCCAGAGCCTGGAG	GCGATGCACTACTCGGTGTGAA
β-actin	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT

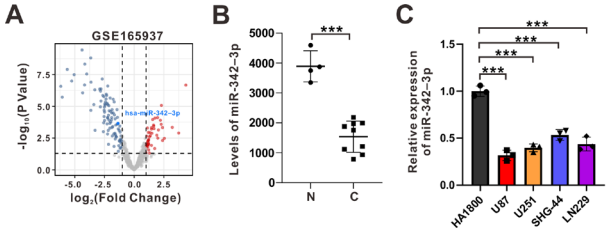

Figure 1. Down-regulated miR-342-3p in glioblastoma tissues.
(**A**) GSE165937 dataset was selected, and a GEO2R analyzer was utilized to identify the differential expressed miRNAs in glioblastoma tissues compared with normal tissues. A volcano plot was utilized to represent the differential expressed miRNAs (blue section, down-regulated; red section, up-regulated) and miR-342-3p (log2FC=–1.4141, *P*-value=0.000252) was marked. (**B**) Raw miR-342-3p expression data were extracted in glioblastoma samples and normal samples of the GSE165937 dataset. (**C**) The relative expression of miR-342-3p in glioblastoma cell lines was detected with qRT-PCR. The data were represented as the means ± S.D. of n = 3 independent experiments. ***P<0.001.

(GSE165937, fold change >1, *P*-value <0.05, Fig. 1A). As one of the most critical miRNAs found in glioblastoma, the raw data of miR-342-3p was retrieved and averaged to show the significant down-regulated expression in the glioblastoma (Fig. 1B). Compared with primary human fetal astrocyte Ha1800 cells, the glioblastoma cell lines (U87MG, U251MG, SHG-44, and LN229) showed a diminished miR-342-3p expression (Fig. 1C). All of these data indicated that down-regulated miR-342-3p was universally observed in glioblastoma tissue and cell lines.

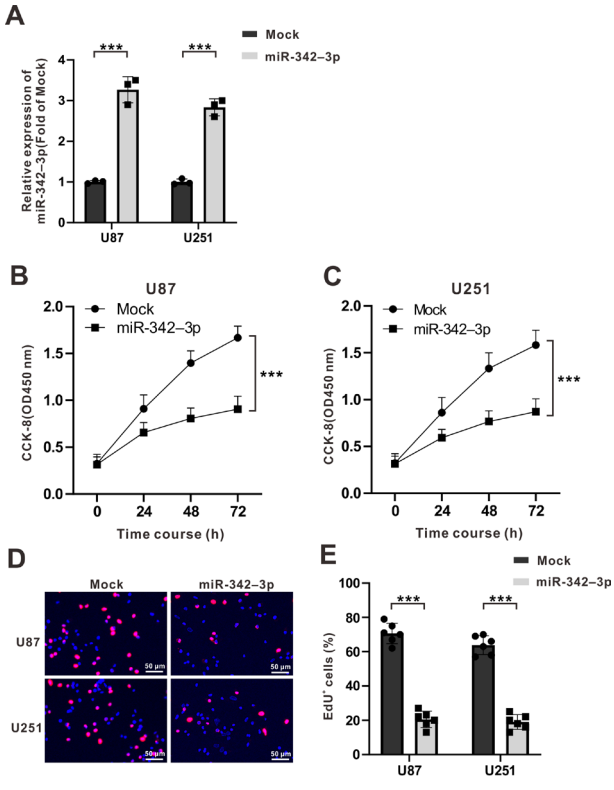

Figure 2. miR-342-3p overexpression inhibits the proliferation of glioblastoma cells.
miR-342-3p overexpressed U251MG and U87MG cells were constructed, and the relative miR-342-3p expression was measured with qRT-PCR (**A**) n = 3 independent experiments. CCK-8 assay demonstrated that miR-342-3p overexpression attenuated U87MG (**B**) and U251MG cells (**C**) proliferation. EdU staining for proliferation assessment (**D and E**) n = 6 independent experiments. Data were represented as the means ± S.D., ***P<0.001.

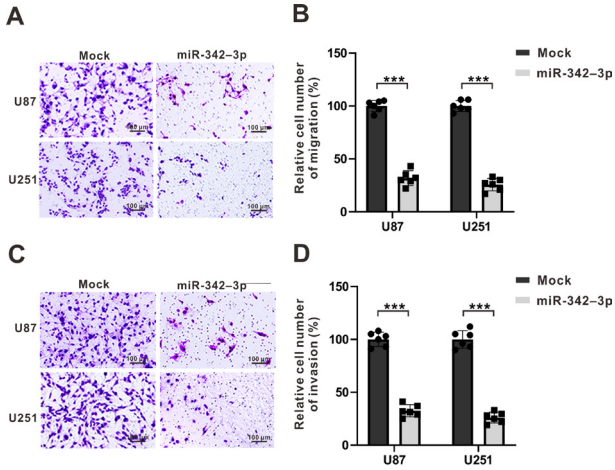

Figure 3. Overexpressed miR-342-3p inhibits the migration and invasion progress of glioblastoma cells.
The effect of over-expressed miR-342-3p on the migration (**A–B**) and invasion (**C–D**) of glioblastoma cells. The data were represented as the means ± S.D., n = 6 independent experiments. ***P<0.001.

miR-342-3p inhibits glioblastoma cell proliferation

miR-342-3p Overexpressed U251MG and U87MG cells were constructed to decipher the role of miR-342-3p in glioblastoma (Fig. 2A). It was further testified that overexpressed miR-342-3p could diminish the proliferation of U87MG (Fig. 2B, *P*<0.001) and U251MG cells (Fig. 2C, *P*<0.001) indicated by CCK-8 assays. Such inhibition of proliferation was also testified with EdU staining, which showed decreasing numbers of cells staining in miR-342-3p over-expressed U87MG and U251MG cells (Fig. 2D and Fig. 2E, *P*<0.001). Transwell assay proved that miR-342-3p over-expression inhibited the migration (Fig. 3A and 3B, *P*<0.001) and invasion (Fig. 3C and 3D, *P*<0.001) of glioblastoma cells. All of these data indicated that miR-342-3p could prevent the proliferation, migration, and invasion of glioblastoma cells.

miR-342-3p targets CDK6

The TargetScan tool was used to predict miR-342-3p targeted mRNAs. CDK6 ranked top with the highest score, and the predicted binding sites in CDK6 with miR-342-3p were demonstrated in Fig. 4A. Luciferase reporter analysis confirmed the direct binding of miR-342-3p and CDK6 in both U87MG cells (Fig. 4B) and U251MG cells (Fig. 4C). Overexpressed miR-342-3p resulted in decreased CDK6 mRNA expression (Fig. 4D) and protein expression (Fig. 4E), which indicated that miR-342-3p could directly target CDK6.

miR-342-3p modulates glioblastoma progression *via* targeting CDK6

In order to decipher the role of CDK6 in glioblastoma, CDK6 plasmid was transfected into miR-342-3p over-expressed U251MG and U87MG cells, which was testified by the up-regulated CDK6 expression (Fig. 5A, *P*<0.001). The further CDK6 transfection could rescue the inhibited cell growth (Fig. 5B), migration (Fig. 5C), and invasion (Fig. 5D) induced by miR-342-3p over-

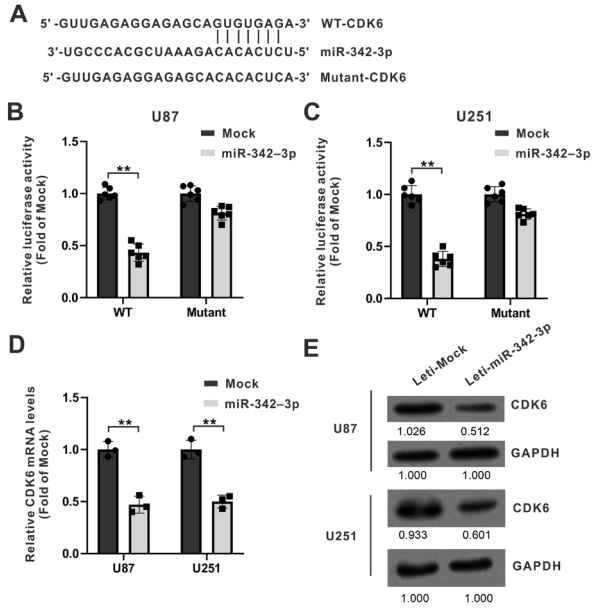

Figure 4. miR-342-3p directly targets CDK6.
The predicted miR-342-3p and CDK6 binding sequence through TargetScan software and the CDK6 mutant sequence were also indicated (**A**). The luciferase activity of the mutant-type CDK6 and wild-type CDK6 in miR-342-3p overexpressed U87MG (**B**) and U251MG cells (**C**). n=3 independent experiments. Over-expressed miR-342-3p could decrease CDK6 expression in both U87MG and U251MG cells indicated by qRT-PCR (**D**). n=3 independent experiments. Western blot assayed the relative CDK6 expression in lenti-control and Lenti-miR-342-3p over-expressed U251MG and U87MG cells (E). The data were represented as the means ± S.D., $^{**}P<0.01$.

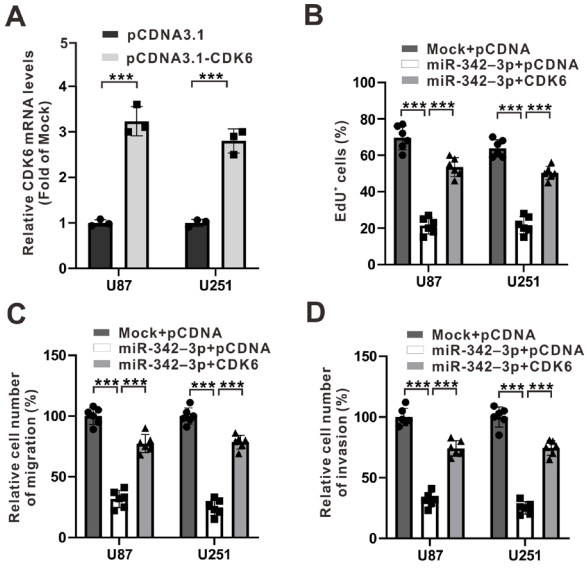

Figure 5. miR-342-3p modulates glioblastoma progression via targeting CDK6.
CDK6 vector was transfected into U251MG and U87MG cells, and the relative CDK6 expression was detected with qRT-PCR (**A**). n=3 independent experiments. EdU staining showed that the further CDK6 transfection could rescue the inhibited cell growth in miR-342-3p overexpressed U251MG and U87MG cells (**B**). Transwell assays showed that the further CDK6 transfection could rescue the inhibited cell migration (**C**) and invasion (**D**) in miR-342-3p overexpressed U251MG and U87MG cells. n=6 independent experiments. The data were represented as the means ± S.D., $^{***}P<0.001$.

expression in both U87MG and U251MG cells. All of these results indicated that miR-342-3p/CDK6 was vital for glioblastoma development.

DISCUSSION

In this investigation, miR-342-3p is found to be down-regulated in glioblastoma, where it can regulate the proliferation, migration, and invasion process. Mechanistically, for the first time, miR-342-3p is identified by the luciferase activity analysis to bind to CDK6 directly. Up-regulated miR-342-3p could down-regulate Cdk6 protein expression to inhibit glioblastoma development. These results indicate the possibility of targeting miR-342-3p/CDK6 in the development and progression of glioblastoma.

Aside from the diminished miR-342-3p expression in tumor tissue, down-regulated expression was also reported in peripheral blood (Roth *et al.*, 2011) and plasma of glioblastoma patients (Wang *et al.*, 2012). Mechanistically, miR-342-3p can be regulated by long noncoding RNA (LncRNA) SNHG7 (Cheng *et al.*, 2020) or LncRNA FTX (Zhang *et al.*, 2017) in glioma cells to improve the growth and invasion. In the glioma stem cells, circRNA ARF1/miR-342-3p/ISL2 feedback loop is reported to promote the angiogenesis process (Jiang *et al.*, 2020). All of these studies indicate the importance of miR-342-3p in glioblastoma development, while little analysis is performed to decipher the relevant downstream regulation.

As a cell cycle regulator, up-regulated CDK6 expression is frequently observed in glioma tissues, and increased CDK6 expression correlates well with the progress of malignancy (Li *et al.*, 2012). Earlier investigations also indicate that Yes-associated Protein 1 (YAP1)-CDK6 signaling may mediate the senescence of glioma cells (Yang *et al.*, 2021), and CDK6 may promote temozolomide resistance (Li *et al.*, 2012). Palbociclib, a highly specific inhibitor of CDK6, can modulate the LncRNA SNHG15/CDK6/miR-627 circuit to inhibit tumorigenesis and overcome temozolomide resistance (Li *et al.*, 2019). PD-0332991, a CDK4/6-specific inhibitor, can arrest glioblastoma growth in the intracranial xenografts model (Michaud *et al.*, 2010). All of these investigations indicate that as an oncogenic gene, *CDK6* is a suitable target and warrants further exploration.

Some limitations should be noted here. The progression of glioblastoma is mainly attributed to the invasion and metastasis process (Revilla-Pacheco *et al.*, 2021; Schritz *et al.*, 2021), while the association of miR-342-3p/CDK6 with the metastasis process is not deciphered in this investigation. As an intronic microRNA of the Enah/Vasp-Like (EVL) host gene, diminished miR-342-3p expression is observed in glioblastoma, while the relevant epigenetic regulation mechanism is not indicated in our investigation.

In conclusion, this investigation confirms the down-regulated miR-342-3p expression in both glioblastoma tissues and cell lines. Our results testify that miR-342-3p suppresses glioblastoma proliferation, migration, and invasion by directly targeting CDK6. Thus, miR-342-3p/CDK6 might be a candidate treatment target for glioblastoma patients.

CONCLUSIONS

Dysregulated miR-342-3p/CDK6 promotes the development of glioblastoma, which can be utilized as a treatment option.

Funding

None.

Competing Interests

None to declare.

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