MiR-325-3p functions as a suppressor miRNA and inhibits the proliferation and metastasis of glioma through targeting FOXM1

Qijiang Xiong¹, Hai Su¹, ²*

¹Department of Neurosurgery, Yonchuan Hospital of Chongqing Medical University, Yonchuan, 402160 Chongqing, China
²Correspondence: 700222@hospital.cqmu.edu.cn (Hai Su)

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Glioma is a malignant brain tumor exhibiting high levels of proliferation and metastasis, and these have been related to its poor prognosis and high mortality rate. MicroRNA (miRNA)-miR-325-3p exhibits tissue-specific expression profiles and is aberrantly expressed in multiple types of malignant tumors. Our research focuses on determining the function and mechanism of action of miR-325-3p in glioma. The relative expression levels of miR-325-3p in glioma tumor tissues and cell lines were verified by qRT-PCR. The effect of 325-3p on glioma tumor cell behavior was assessed using CCK-8 assays, EDU staining, colony formation assays, flow cytometry, transwell invasion assays, and a xenograft model. In addition, we searched for miR-325-3p targets, and their potential mechanism of action was demonstrated using a reporter assay and rescue experiments. Results showed that the expression levels of miR-325-3p in glioma cancer tissues and tumor cell lines were significantly lower than that of normal paired adjacent tissue or normal cell lines. Functional experiments illustrated that tumor proliferation, migration and invasion were suppressed via upregulation of miR-325-3p. To assess whether FOXM1 is a target of miR-325-3p, we examined this hypothesis using a luciferase reporter assay and then found that miR-325-3p could modulate the expression of FOXM1. Furthermore, the functional role of miR-325-3p was also confirmed in a xenograft model using nude mice. Together, our data demonstrated that in glioma, miR-325-3p may inhibit cancer cell growth through the suppression of FOXM1 and could be a promising new target for treating this type of brain cancer.

Keywords
MiR-325-3p, FOXM1, Proliferation, Metastasis, Glioma, Neuro-oncology

1. Introduction
Glioma is one of the most malignant tumors and accounts for more than 80% of all primary malignant tumors of the central nervous system [1]. The median overall survival rate is constant at 16 months, although huge progress has been made in therapy [2]. At present, surgery, chemotherapy, and radiotherapy are the main strategies for glioma treatment [3]. However, this disease is virtually incurable, and its five-year survival rate remains unsatisfactorily low [4]. Thus, it is key to understand the molecular mechanisms for glioma’s occurrence and progression, which may help uncover new therapeutic targets. Recent studies have revealed many abnormally expressed genes, which may be involved in glioma progression [5]. MiRNAs are endogenous, non-coding RNAs that regulate multiple physiological and pathological processes at the posttranscriptional level [6–8] and mounting evidence suggests the involvement of miRNAs in multiple cancers, including glioma. Here, we specifically focused on miR-325-3p, which was found to be aberrantly expressed in multiple types of malignant tumors. For example, miR-325-3p showed low expression levels in colorectal cancer (CRC), representing a key regulator of bone metastasis [9]. In gastric cancer (GC), the miR-325-3p was decreased, and its low expression level was also associated with metastasis [10]. Furthermore, miR-325-3p has an effective therapeutic function in treating lung and bladder cancer [11, 12]. However, the exact functions of miR-325-3p in glioma have yet to be determined, and thus these data suggest that miR-325-3p could be a potential therapeutic target for malignant glioma (Fig. 1).

2. Materials and methods
2.1 Human tissues
Twenty-four glioma tissue samples and adjacent normal tissue samples were obtained from Yonchuan Hospital of Chongqing Medical University, and all written informed consent was obtained from the patients. At least two pathologists diagnosed and confirmed all tissues had not been treated with radiotherapy or chemotherapy before surgery. The tissues were collected and kept at -80 °C until analysis, and the Ethics Committee of Yonchuan Hospital of Chongqing Medical University approved this research.

2.2 Cell culture and transfection
Normal human astrocytes (NHAs) and glioma cell lines SW1783, U87, and LN229 were purchased from the Chinese Academy of Sciences cell bank. All Cells were cultured using DMEM and RPMI-1640 (Gibco, Carlsbad, CA, USA), 10% fetal bovine serum (Gibco) in an incubator at 37 °C, 5% CO₂, and 100% humidity. MiR-325-3p mimics (miR-325-3p-mimic), miR-325-3p inhibitors (miR-325-3p-inhibitor), miR-325-3p negative controls (miR-325-3p-NC), and lentivirus containing miR-325-3p or its control
were designed and synthesized by Wuhan Genesil Biotechnology Co., Ltd (Wuhan, China) and transfected using Lipo-fectamine transfection reagent (Invitrogen, MA, USA) according to the manufacturer’s instructions.

2.3 qRT-PCR

Cells or tissues were collected, and total RNA was extracted with TRIZOL reagent. Then total miRNAs were isolated using the mirVana™ miRNA Isolation Kit. Samples were reverse transcribed into cDNA using a Revert Aid First Strand cDNA Synthesis Kit (RR047AA, TaKaRa, Japan) and TaqMan™ MicroRNA Reverse Transcription Kit (miRNA) (4366596, Invitrogen, USA). After the reverse transcription, products were diluted two-fold, and qRT-PCR was performed using: 2 µL of cDNA added to 10 µL of SYBR Premix Ex Taq™ (2×) (RR420A, TaKaRa, Japan) and 0.2 mol/L of each primer. Finally, sterile distilled water was used to complete the reaction to 20 µL. The reaction was cycled and analyzed on an ABI 7500 Fast Real-Time PCR system (Biosystems, USA). The reaction conditions were as follows: 95 °C, 3 min; 40 cycles: 95 °C, 30 s; 60 °C, 15 s and the data were analyzed using the 2−ΔΔCt method. The mRNA expressions of FOXM1 were normalized to GAPDH, while miR-325-3p was normalized to U6. The following primers were used:

- miR-325-3p, forward: 5′-GCGCCTAGTGTCACGTC-3′ and reverse: 5′-CTCAACTGTTGCATGAGTC-3′
- FOXM1, forward: 5′-GGTGCTGAGTGGCAGTACCCG-3′ and reverse: 5′-GGTGCTAGTGTCATGAGTC-3′
- U6, forward: 5′-CTCGTCTCCAGCAGACACA-3′ and reverse: 5′-AACGGTTCAAGATTTGGCT-3′
- GAPDH, forward: 5′-GGACGGAGATCCCTCCAAAAT-3′ and reverse: 5′-GGCTGTTGTCATACTTCTCATGG-3′.

2.4 Cell viability assay

For cell viability assays [13], a CCK-8 kit (C0038, Beyotime Biotechnology, China) was used according to the manufacturer’s instructions. Transfected cells were inoculated into 96-well plates and incubated overnight. Add CCK-8 reagent 10 mL to each well, and incubate at 37 °C for 2 hours. The absorption value was measured at 450 nm with a microplate analyzer.

2.5 EDU incorporation assay

Twenty µM of EDU was used to label cells for 15 minutes at 37 °C, and they were then fixed with 4% paraformaldehyde for 10 minutes, followed by denaturation with HCl (pH = 2) at 37 °C and neutralized in 20 mM Na₂CO₃ for 1 hour. Next, the cells were blocked with 5% bovine serum albumin (BSA) for 30 minutes and immunostained with anti-EDU (1:500) primary antibody overnight. Secondary antibodies were then used at 1:1000 dilution, and DAPI (1:1000, Sigma) was added during secondary antibody incubation.

2.6 Colony formation assay

Cells were plated in 6-well plates (500 per/well), and the medium was replaced every 3–4 days. When cell colonies were formed after 2 weeks, they were fixed and stained. Visible colonies were photographed and counted.
2.7 Transwell assays

As previously described, cell invasion assays were performed using the Bio-Coat Cell Migration Chamber (BD Biosciences, MA, USA) [14]. Then a 24-well plate containing an 8 µm-pore filter cell culture insert was used. Before the invasion experiment, 70 µL of matrix glue (diluted with DMEM: 1:4) was spread on the bottom of the upper chamber and placed in an incubator for 3 hours. The invasion assay was as follows: transfected cells (3 × 10^5 cells/500 µL) were re-suspended in DMEM and transferred to the upper chamber of the transwell plate. DMEM containing 20% FBS was placed in the transwell plate chamber (500 µL), and after incubation (5% CO_2, 37 °C) for 24 hours, the non-transfected cells were rubbed with cotton swab. The invasive cells were then fixed with 4% paraformaldehyde (P0099, Beyotime Biotechnology, China) and stained with crystal violet (C0121, Beyotime Biotechnology, China). The invasive cells were then selected from five microscope fields (100×) (NIB620, Boshida, China).

2.8 Immunohistochemistry

After the sections were rehydrated as above [15], heat-induced antigen retrieval was performed in sodium citrate for 15 minutes in a 95 °C water bath, followed by the addition of 3% hydrogen peroxide and 5% BSA to block endogenous peroxidase activity and non-specific antigens. Next, the sections were incubated with primary antibodies targeted against FOXM1, Ki67, and N-cadherin (Abcam) at 4 °C overnight. The sections were incubated in HRP conjugated secondary antibodies the next day, and sections were visualized using DAB and imaged using a bright field microscope.

2.9 Dual-luciferase activity assay

The interactions between miR-325-3p and FOXM1 were assessed using TargetScan tools. The 3’-UTR. FOXM1 fragment was cloned full-length into a pmirGLO expression vector (Promega, WI, USA). The luciferase vector and miR-325-3p mimics were cotransfected into U87 cells and the luciferase activity was measured with a Dual-Luciferase Assay System (Promega, Madison, WI, USA) 48 h later.

2.10 Western blotting

Total protein was extracted with RIPA reagent (P0013B, Beyotime Biotechnology, China), and the protein concentration was measured using a BCA protein detection kit (P0012, Beyotime Biotechnology, China). Equal amounts of total protein 30 µg were then loaded with size markers (PR1910, Solarbio, China), and 10% SDS-PAGE was performed. Then, proteins were transferred to a PVDF membrane and blocked with 5% skimmed milk for 1 hour at room temperature and washed with TBST for 5 minutes. The blocked membrane was incubated overnight at 4 °C with primary antibody (FOXM1, 1:2000; Abcam, MA, USA; E-cadherin, 1:3000; Abcam; N-cadherin, 1:4000; Abcam; vimentin, 1:4000; Abcam; Fibronectin, 1:5000; Abcam; GAPDH, 1:4000, Abcam) and washed four times (5 min each), followed by incubation with secondary antibody (1:5000, Abcam) at room temperature for 1 hour. After further TBST washes, protein bands were detected using an enhanced chemiluminescence kit (WBKLS0500, MILLIPORE, USA) and enhanced Chemiluminescence Advanced System (Bio-Rad, CA, USA) and quantified with Image J software (National Institutes of Health, MD, USA). In addition, GAPDH served as an internal control for quantifying the target proteins.

2.11 Glioma xenograft mouse models

The Animal Care Committee approved the animal experiments of Yongchuan Hospital of Chongqing Medical University. All experiments involving mice were conducted in accordance with the guidelines for animal welfare formulated by the laboratory animal center at Yongchuan Hospital of Chongqing Medical University. The nude mice aged 6 weeks were purchased from Chongqing Medical University Experimental Animal Center. Each mouse was injected subcutaneously with 6 × 10^5 U87 cells transfected with miR-325-3p suspended in 200 µL of Hank's balanced salt solution. The tumor size in each animal was measured every 7 days. On day 21, the mice were sacrificed, and xenografts were excised, weighed, photographed, paraffin-embedded and sectioned for HE and immunohistochemical staining.

2.12 Statistical analysis

Data were assessed using Student’s t-test or one-way ANOVA in GraphPad Prism 7.0 (GraphPad, CA, USA) software and expressed as mean ± standard error of the mean (SEM). p-value < 0.05 was considered statistically significant.

3. Results

3.1 Decreased miR-325-3p expression in glioma tissues and cell lines

To determine the expression profile of miR-325-3p in glioma tissues, more than twenty-paired surgical glioblastoma and RT-qPCR analyzed para-carcinoma tissues. It can be seen that miR-325-3p expression was significantly decreased in glioma tissues compared to the adjacent normal tissues (Fig. 2A, p < 0.01). We further performed RT-qPCR analysis of miR-325-3p expression in three human glioma cell lines SW1783, U87, LN229, and an astrocyte cell line NHA. As expected, miR-325-3p levels were lower in the glioma cell lines when compared to the astrocytes (Fig. 2B). These results suggested that miR-325-3p is a promising prognostic biomarker for glioma and may contribute to malignant progression.

3.2 In vitro upregulation of miR-325-3p inhibited glioma cell viability and proliferation

As an association between miR-325-3p and glioma has been confirmed, we next investigated the functional roles of miR-325-3p in glioma cell growth. MiRNA-mimics were specifically and effectively used to upregulate miR-325-3p expression in SW1783 and U87 glioma cell lines (Fig. 3A). Furthermore, the CCK8 assay showed that upregulation of miR-
325-3p inhibited the proliferation rate of SW1783 and U87 cells (Fig. 3B). Using the EdU and colony formation assays, we also found that upregulation of miR-325-3p inhibited the growth of SW1783 and U87 cells (Fig. 3C,D). These data showed that miR-325-3p upregulation could effectively inhibit glioma cell proliferation.

3.3 Upregulation of miR-325-3p decreased glioma cell migration and invasion

Metastasis is a major risk for patients with glioma, and therefore, to further determine the function of miR-325-3p in glioma cell migration and invasion in vitro, transwell assays were employed. We found that upregulation of miR-325-3p significantly inhibited the migration of SW1783 and U87 cells (Fig. 4A,B). The transwell assay also consistently indicated that overexpression of miR-325-3p also decreased their
invasive capability (Fig. 4C,D). Such findings suggested that miR-325-3p suppressed the malignant behaviors of glioma cells.

3.4 FOXM1 is a direct target for miR-325-3p

Cumulative evidence indicates that FOXM1 serves as a tumor promoter or suppressor in multiple tumors, regulating a wide range of biologic processes [16–18]. Through bioinformatics software TargetScan (http://www.targetscan.org) analysis, we predicted the potential targets of miR-325-3p and found that FOXM1 may represent such a target. FOXM1 does not interact with any known miR-325-3p targets [9–12]. After the histological analysis, immunohistochemistry revealed that FOXM1 expression was increased in glioma tissue (Fig. 5A), and as shown in Fig. 5B, FOXM1 expression was inversely correlated with miR-325-3p. Thus, both wild-type (WT) and mutant (Mut) luciferase reporters for FOXM1 were generated. Results showed that miR-325-3p suppressed activity in the Foxm1-WT group, but the opposite result was seen for U87 cells (Fig. 5C), thus confirming an interaction. Moreover, western blot analysis revealed that transfection with the miR-325-3p mimic or inhibitor significantly increased/decreased FOXM1 protein levels’ expression compared to the control (Fig. 5D,E). These results confirmed a role for miR-325-3p in the regulation of FOXM1 in glioma cell lines.

3.5 FOXM1 is involved in the miR-325-3p induced migration and invasion of glioma cells

A series of rescue assays were performed to determine whether FOXM1 expression accounted for miR-325-3p-mediated migration and invasion in glioma cells. U87 cells were cotransfected with miR-325-3p-NC or miR-325-3p-mimic and pcDNA3.0 plasmid containing control or FOXM1 plasmid, and our western blot results confirmed the effectiveness of this rescue strategy (Fig. 6A). As expected, overex-

Fig. 4. Upregulation of miR-325-3p suppressed glioma cell migration and invasion. (A,B) Transwell assay revealed that upregulation of miR-325-3p decreased the migration capability of SW1783 and U87 cells. (C,D) Transwell assay revealed that upregulation of miR-325-3p decreased the invasion capability of SW1783 and U87 cells. Data are presented as the mean ± SEM, ***p < 0.001, compared to the miR-325-3p-NC group.
expression of FOXM1 attenuated the inhibitory effect of miR-325-3p on migration (Fig. 6B,C) and invasion (Fig. 6D,E). Furthermore, cancer cell invasion and migration are usually associated with epithelial-mesenchymal transition (EMT). For this reason, we further explored proteins associated with the EMT pathway. We found that FOXM1 overexpression reversed the effect of miR-325-3p upregulation, causing a decrease in E-cadherin expressions and an increase in N-cadherin, Vimentin, and Fibronectin in U87 cells (Fig. 6F). Remarkably, FOXM1 overexpression reversed the suppressive effect induced by miR-325-3p upregulation in U87 cells.

3.6 Upregulation of miR-325-3p repressed glioma cell growth in vivo

To validate our results in vivo, nude BALB/c mice were injected in the left flanks with NC or miR-325-3p-overexpressing U87 cells. Tumor volumes and weights in the miR-325-3p-overexpressing group were significantly reduced compared to the NC group (Fig. 7A,B). Furthermore, H&E staining and immunohistochemistry for the proliferation marker, Ki67, indicated that upregulation of miR-325-3p inhibited glioma proliferation in vivo (Fig. 6C). IHC staining showed that the EMT-associated marker N-cadherin was significantly lower in the miR-325-3p-overexpressing group when compared to the NC group, which is consistent with our in vitro results (Fig. 7C). Thus, these results demonstrated that miR-325-3p could inhibit glioma cell growth in vivo.

4. Discussion

Glioma is one of the most aggressive and terminal diseases associated with the central nervous system and is associated with a very poor median survival of fifteen months. Therefore, there is an urgent need to uncover more biomarkers to enable clinicians to predict when and what therapy to deploy and help determine prognosis [19]. A growing number of studies have focused on the role of miRNAs in tumorigenesis, as they are important for many biological processes, such as cell proliferation, differentiation and invasion in a variety of cancer types. The loss of miR-325-3p expression has been reported in CRC and gastric cancer [9, 10].

These results have highlighted miR-325-3p as a potential new tumor regulatory molecule. However, the mechanism involved in the regulation of glioma growth by miR-325-3p remains unknown. We firstly detected the downregulation of miR-325-3p in glioma tissues and cells. We then confirmed the inhibitory effect of miR-325-3p on proliferation...
Fig. 6. MiR-325-3p regulates migration and invasion in U87 cells by targeting FOXM1 expression. (A) Western blot analysis showed that upregulation of miR-325-3p decreased the expression of FOXM1, and this effect was reversed by transfection with pcDNA3.0-FOXM1 in U87 cells. (B–E) Functional rescue experiments with Transwell assays showed that pcDNA3.0-FOXM1 transfection restored the migration and invasion abilities of U87 cells transfected with miR-325-3p-mimic. (F) The expressions of EMT-related genes were detected after FOXM1 was overexpressed in U87 cells via western blot. Data are presented as the mean ± SEM, ***p < 0.001, compared to the miR-325-3p-NC + pcDNA-Con group; ##p < 0.01, ###p < 0.001, compared to the miR-325-3p-mimic + pcDNA-FOXM1 group.
Fig. 7. MiR-325-3p repressed glioma growth in vivo. (A) Tumor volumes from the U87 cell xenograft model from the miR-325-3p-overexpressing group were significantly reduced. (B) Representative tumor images from the xenograft mice. (C) As indicated, representative H&E staining and IHC images of Ki67 and N-cadherin in subcutaneous xenografts derived from cells. Scale bar: 100 µm. Data are presented as the mean ± SEM, ***p < 0.001, compared to the LV-NC group.

and invasion in glioma U87 and SW1783 cells using in vitro assays. These results provided elementary evidence for a role for miR-325-3p as a tumor-suppressor in glioma.

Similarly, it has been demonstrated that overexpression of miR-325-3p inhibits proliferation and metastasis of bladder cancer cells [11] and miR-325 can also inhibit proliferation but induce apoptosis of T cells in acute lymphoblastic leukemia [20]. In hepatocellular carcinoma cells, miR-325-3p can inhibit cell proliferation and induce apoptosis in hepatitis B virus-related hepatocellular carcinoma [21]. The performance of miR-325-3p in regulating glioma cells migration and invasion implied an essential role for this miRNA in the mediation of glioma oncogenesis and tumor behavior, which is similar to previous studies mentioned above.

We further explored the potential mechanism by which miR-325-3p inhibited the invasion and migration in glioma and found FOXM1 as a potential target for miR-325-3p according to microRNA target databases. We found that FOXM1 was upregulated in glioma tissues and glioma cell lines compared to the control tissues and cells. Furthermore, statistical analysis showed a clear negative correlation between miR-325-3p and FOXM1. Our western blot data showed that miR-325-3p could reduce FOXM1 protein expression in vitro, and our dual-luciferase reporter assay confirmed an interaction between miR-325-3p and FOXM1 mRNA by directly targeting its 3'-UTR. Furthermore, in our rescue experiments, overexpression of FOXM1 countered the miR-325-3p-induced inhibitory effect on invasion and migration and metastasis-associated EMT pro-
tein expression, further supporting an association between miR-325-3p and FOXM1. Functionally FOXM1 as a typical proliferation-associated transcription factor is required to execute the mitotic program and chromosome stability [16]. It is also significantly elevated in most human tumors and promotes tumorigenesis in many tissues, including pancreatic, esophageal, gastric, ovarian, and breast cancers [17, 18, 22, 23]. Tissue microarray analysis revealed that FOXM1 expression was significantly higher in high-grade gliomas than low-grade astrocytomas. The expression level of FOXM1 protein is directly related to the advanced grade, metastasis, and is negatively correlated with patient survival [24–26].

Several studies have also demonstrated that overexpression of FOXM1 promotes tumorigenicity, invasion, and angiogenesis of glioma cells [27–29]. However, in FOXM1 transgenic mice, no spontaneous brain tumors were observed [30], suggesting that FOXM1 overexpression alone does not induce gliomas or the presence of upstream regulatory mechanisms. Thus, it was important to determine the upstream miRNAs involved in the direct regulation of FOXM1, as these could represent potential biomarkers or therapeutical targets. We first confirmed the downregulation of miR-325-3p in glioma tissue and glioma cell lines. Therefore, upregulation of miR-325-3p could potently inhibit glioma cell proliferation and metastasis both in vitro and in vivo. Mechanistically, we found that the miR-325-3p/FOXM1 signaling pathway revealed a novel molecular mechanism for glioma progression suggesting that miR-325-3p could be a potential therapeutic target for malignant glioma. Finally, in our in vivo xenografted study, we confirmed again that miR-325-3p negatively inhibited tumor growth in mice.

5. Conclusions

MiR-325-3p was demonstrated to function as a tumor suppressor in glioma, at least in part, by targeting FOXM1. These findings may further elucidate the molecular mechanisms underlying glioma progression and provide a novel target and a better theoretical basis for new potential mechanisms involving the pathogenesis and molecular therapeutic strategy for glioma.

Abbreviations

miRNA, MicroRNA; CRC, colorectal cancer; GC, gastric cancer; EMT, epithelialmesenchymal transition.

Author contributions

HS conceived and designed the present study. HS and QJX performed the experiments and analyzed the data. HS and QJX interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animals were raised and handled at the QJX at the Laboratory Animal Center of the Chongqing Medical University. All animal experiments were carried out in accordance with the guidelines of the current institutional guidelines for the care and use of experimental animals and approved by the Animal Care Committee of Yongchuan Hospital of Chongqing Medical University. The glioma tissues were obtained with all participants’ informed consent, and the research conducted was approved by the Ethics Committee of Yongchuan Hospital of Chongqing Medical University (YJZQN201525).

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Conflict of interest

The authors declare no conflict of interest.

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