miR-454-3p promotes of human glioma cell growth by targeting EGR3

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Abstract. Gliomas are the most common primary brain tumors in adults and are associated with high mortality rates. In the present study, the aim was to evaluate the role of miR-454-3p in the pathogenesis of human glioma and to explore the underlying mechanism. Reverse transcription-quantitative PCR was performed to compare the expression levels of miR-454-3p in glioma and adjacent normal tissue. The effects of miR-454-3p on cell proliferation was tested by combining MTT and colony formation assays. Dual-luciferase assay was used to identify the target gene of miR-454-3p. The results showed that miR-454-3p was upregulated in glioma tissues where it exerts a positively regulatory role on cell growth. Dual-luciferase assay confirmed Early Growth Response 3 (EGR3) to be a target for miR-454-3p. Overexpression of EGR3 in glioma cells was found to impair miR-454-3p mimic-induced cell proliferation. These results suggested that upregulated miR-454-3p served an important role in glioma tumorigenesis by targeting EGR3, which provided valuable insights into the underlying mechanism of the disease that may lead to possible novel therapeutic strategies.

Introduction

Glioma is a primary brain tumor in the central nervous system that is highly aggressive. Although the morbidity of glioma is relatively low (accounting for ~2% in all adult malignancies), the number of newly diagnosed patients is increasing (1). Since gliomas have tendencies to invade surrounding tissues, current treatment strategies are ineffective and consequently the prognosis for patients remains poor (2). Therefore, there is currently an urgent need to explore the pathological mechanism of this disease to devise new therapeutic interventions.

With the advancing knowledge and understanding of microRNAs (miRNAs), miRNAs are now known as an important class of post-transcriptional regulators of gene expression by translational repression and mRNA breakdown (3-5). It has been shown to participate in a variety of biological processes, including cell proliferation, differentiation, migration, apoptosis, cell-cycle regulation and invasion, development and metabolism by targeting the 3′-untranslated regions (3′UTR) of target genes (6). Accumulating evidence have also indicated aberrant expression of miRNAs were found in a number of diseases, particularly in cancers (7). Indeed, a wide range of miRNAs have been reported to function as biomarkers for diagnosis and prognosis of human glioma (8). Decreased miR-374a expression was associated with glioma progression (9), whereas increased expressions of miRNA-21, miR-128 or miR-342-3p were positively correlated with histopathological grades of glioma (10,11). In fact, >97 miRNAs were found to be aberrantly expressed through miRNA microarrays and involved in the pathogenesis of glioma through major signaling pathways (12). However, the role of miR-454-3p and underlying mechanism in glioma remains poorly understood.

Early growth response 3 (EGR3) is a member of the EGR family of C2H2-type zinc-finger proteins (13). It regulates gene transcription and has been found to be involved in multiple biological processes, including the development of muscle, lymphocyte and neurons, in addition to the growth, migration, invasion and metastasis of cancer cells (14-16). Therefore, associations between abnormal EGR3 expression and schizoaffective disorder, schizophrenia, systemic autoimmunity, and a variety of cancers has been previously demonstrated (14-16). In hepatocellular carcinoma, downregulation of EGR3 expression of promoted cell proliferation by upregulating Fas ligand expression (17). In contrast, reduction in EGR3 expression by KH-type splicing regulatory protein suppressed cell invasion and metastasis in non-small cell lung cancer (NSCLC) (14). These previous findings suggest that the function of EGR3 may differ depending on the type of cancer in question.

In the present study, the aim is to elucidate the potential role of miR-454-3p in glioma and explore the potential mechanism. The results can deepen the understanding on the pathogenesis of glioma which may provide novel molecular targets for treating this disease.

Materials and methods

Ethics statement. The present study was approved by the Research Ethics Committee of The First People's Hospital of
Taizhou. All patients provided written informed consent prior to enrollment in the study.

**Tissue specimens.** Clinical samples were obtained from 40 patients (sex, 20 males and 20 females; age range, 28-71 years; mean age, 58.5±14.1 years) who were diagnosed with glioma according to World Health Organization (WHO) classification without preoperative radiation or chemotherapy, by surgery at the The First People's Hospital of Taizhou from January 2015 to December 2016 (Taizhou, China). Patients who had received neoadjuvant or adjuvant therapy were excluded from the present study. Tumors and adjacent non-tumor intracranial tissue samples were collected and immediately frozen in liquid nitrogen.

**Cell line and culture.** Human glioma cell lines U138MG (cat.no. ATCC® HTB-16™) and U251 (cat.no. ATCC® HTB-17), together with 293T cells (cat. no. ATCC® CRL-11268™) were purchased from the American Type Culture Collection. And normal human astrocytes (NHA; cat. no. BNCCC41796), A172 (cat. no. BNCCC41782) cells were purchased from BeNa culture collection. U138MG, NHA, A172 and 293T cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and penicillin (100 IU/ml)/streptomycin (100 µg/ml), whilst U251 cells were cultured in RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and penicillin (100 IU/ml)/streptomycin (100 µg/ml) in a humidified incubator containing 5% CO₂ at 37°C.

**Cell transfection with mimics, inhibitor, siRNA and plasmids.** miR-454-3p mimic and its negative control (NC), inhibitor of miR-454-3p and its negative control (NC-in), siRNA for EGR3 (siEGR3) and its negative control (siNC) were synthesized by Shanghai GenePharma Co., Ltd., with their respective sequences listed in Table II. For the overexpression of EGR3, the coding sequence for EGR3 was amplified from NHA cells using KOD-Plus-Neo Polymerase (cat. no. KOD-401; Toyobo Life Science) using the forward primer containing a restriction site, 5' -GGG AAT TCT CAG GCG CAG GTG GTG CCG AGA AGC-3' and the reverse primer, containing a restriction site, 5' -GGG GAT CCA TGA CCG GCA AAC TCG ACCAGG-3' and then subcloned into the pcDNA3.1 plasmid restriction site. 

**Western blot analysis.** For western blotting, tissues were homogenized in RIPA buffer (Cell Signaling Technology, Inc.) from which protein samples were isolated. After quantification using Pierce® Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific, Inc.), 40 µg total protein samples per lane were subjected to 10% SDS-PAGE and were subsequently transferred to a PVDF membrane. The membranes were then blocked with 5% skimmed milk dissolved in PBS for 1 h at room temperature followed by incubation with primary antibodies against EGR3 (1:100; cat. no. sc-390967; Santa Cruz Biotechnology, Inc.) and GAPDH (1:500; cat. no. D16H11; Cell Signaling Technology, Inc.) according to manufacturers' protocols. Firefly luciferase activities were assayed 24 h after transfection using Dual-Luciferase® Reporter Assay System (Promega Corporation) according to manufacturer's protocols. Firefly luciferase activities were normalized to Renilla luciferase activities. Each experiment was repeated three times independently.

**MTT assay.** Cell viability assay. Cell growth was measured using MTT (Sigma-Aldrich; Merck KGaA) and colony formation assays. For MTT assays, approximately 3,000 cells/well were seeded into 96-well plates, following which 20 µl MTT dissolved in PBS (5 mg/ml) was added to each well at indicated times and incubated for a further 4 h. The supernatant was then carefully removed and 150 µl DMSO was added to each well and plates were subsequently shaken for 10 min to dissolve the formazan crystals. Optical density at 490 nm was then measured per well using a Thermo Scientific Multiskan (Thermo Fisher Scientific, Inc.). For colony formation assays, 1,000 cells were plated into each well of a 6-well plate and cultured for 14 days. Colonies were subsequently fixed using 10% formaldehyde for 5 min at room temperature (RT) and stained using 0.1% crystal violet for 30 sec at RT. The numbers of colonies were counted for each condition using light microscopy. Each experiment was repeated three times independently.

**Prediction targets of miR-454-3p.** The targets of miR-454-3p were predicted using the TargetScan website (Version 7.1; http://www.targetscan.org/vert_71/) (19).

**Dual-luciferase reporter assay.** For dual-luciferase reporter assays, 4,000 293T cells were seeded into 96-well plates and pMIRGLO plasmids (1 ng/µl) (Promega Corporation) encoding wild-type or mutant 3’UTR of EGR3 were co-transfected into cells with 20 nM either miR-454-3p mimics or NC using Lipofectamine 3000. Firefly and Renilla luciferase activities were assayed 24 h after transfection using Dual-Luciferase® Reporter Assay System (Promega Corporation) according to manufacturer’s protocols. Firefly luciferase activities were normalized to Renilla luciferase activities. Each experiment was repeated three times independently.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from the cells or tissues using mirVana™ miRNA Isolation Kit (Ambion; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. To measure the levels of miR-454-3p expression, TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used to perform reverse transcription according to manufacturer's protocol. To determine the mRNA levels of EGR3, total RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd.). qPCR was performed using SYBR® PrimeScript™ RT-PCR kit (Takara Biotechnology Co., Ltd.) according to manufacturers’ protocols using Applied Biosystems® 7500 Real-Time PCR Systems (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following thermocycling conditions: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. U6 snRNA and GAPDH were used as reference genes for miR-454-3p and EGR3 respectively. Relative expression levels were calculated using the 2^ΔΔCq method (18). All PCRs were performed in triplicate for analysis and the sequences of primers used were listed in Table III.

**Conclusion.** This study demonstrates that miR-454-3p may function as a tumor suppressor in glioma through targeting EGR3. The miR-454-3p/EGR3 axis may provide a potential therapeutic target for glioma treatment.
miR-454-3p is upregulated in human glioma tissues. In order to investigate the role of miR-454-3p in human glioma pathogenesis, expression levels of miR-454-3p were compared between glioma and adjacent non-tumor samples in intracranial tissues from 40 patients using RT-qPCR. The baseline patient and tumor characteristics were summarized in Table I. The expression levels of miR-454-3p were associated with the tumor size (P=0.024) and WHO stage (P=0.011). As shown in Fig. 1A, miR-454-3p expression in glioma tissues (0.54±0.03, n=40) was significantly higher compared with that in adjacent normal tissues (1.10±0.04, n=40). Overall survival analysis revealed that patients with higher expression levels of miR-454-3p were associated with significantly shorter overall survival (median survival: 687 vs. 1,143 days, n=40, Fig. 1B). The results indicated that miR-453-3p may be involved in glioma tumorigenesis. To determine the function of miR-454-3p further in glioma cells, the expression levels of miR-454-3p were measured in U251, U138MG and A172 glioma cell lines and NHA cells. When compared with NHA cells, miR-454-3p expression was found to be significantly increased in glioma cells, with the magnitude the greatest in U251 cells (Fig. 1C).

miR-454-3p promotes human glioma cell proliferation. MTT and colony formation assay were performed to determine the effects of miR-454-3p on cell proliferation. Considering the different expression levels of miR-454-3p in U138MG and U251 cells, U138MG and U251 cells were first transfected with miR-454-3p mimics or inhibitor, respectively. The expression levels of miR-454-3p were significantly increased in U138MG cells following transfection with miR-454-3p mimics compared with its corresponding negative control (NC); in contrast, miR-454-3p expression was significantly reduced in U251 cells following transfection with the miR-454-3p inhibitor compared with its corresponding NC-in (n=3; Fig. 2A). MTT assay showed that miR-454-3p overexpression significantly increased U138MG cell viability, whereas miR-454-3p knockdown significantly reduced U251 cell viability (n=5; Fig. 2B). Colony formation assay showed consistent results (Fig. 2C) U138MG cells transfected with miR-454-3p mimics exhibited significantly more colonies compared with those transfected with NC (26±3 vs. 117±9, n=3), whilst U251 cells transfected with miR-454-3p inhibitor resulted in significantly decreased colony numbers (11±9 vs. 28±8, n=3). These results suggested that the overexpression of miR-454-3p enhanced human glioma cell growth in vitro.

miR-454-3p directly targets the 3’-UTR of the EGR3 gene. Potential target genes of miR-454-3p were next predicted using the TargetScan Database. As shown in Fig. 3A, a potential binding site on the 3’UTR of EGR3 was detected using the TargetScan Database. As shown in Fig. 3A, a potential binding site on the 3’UTR of EGR3 was detected using the TargetScan Database. As shown in Fig. 3A, a potential binding site on the 3’UTR of EGR3 was detected using the TargetScan Database.

**Table I. Association between clinicopathological features and expression of miR-454-3p.**

| Characteristic       | n  | Low | High | P-value |
|----------------------|----|-----|------|---------|
| Age                  |    |     |      |         |
| <45                  | 16 | 6   | 10   | 0.301   |
| ≥45                  | 24 | 13  | 11   |         |
| Sex                  |    |     |      |         |
| Female               | 20 | 12  | 8    | 0.113   |
| Male                 | 20 | 7   | 13   |         |
| Tumor size           |    |     |      |         |
| <5 cm                | 22 | 14  | 8    | 0.024   |
| ≥5 cm                | 18 | 5   | 13   |         |
| Peritumoral brain edema | 20 | 8   | 12   | 0.342   |
| Tumor size           |    |     |      |         |
| <1 cm                | 20 | 8   | 12   | 0.011   |
| ≥1 cm                | 20 | 11  | 9    |         |
| WHO stage            |    |     |      |         |
| I                    | 9  | 8   | 1    |         |
| II                   | 8  | 5   | 3    |         |
| III                  | 8  | 2   | 6    |         |
| IV                   | 15 | 4   | 11   |         |

miR, microRNA; WHO, world health organization. Results

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| I                    | 9  | 8   | 1    |         |
| II                   | 8  | 5   | 3    |         |
| III                  | 8  | 2   | 6    |         |
| IV                   | 15 | 4   | 11   |         |

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n=3; Fig. 3A). According to RT-qPCR analysis, the expression levels of EGR3 mRNA in U138MG cells transfected with miR-454-3p mimics was significantly decreased compared with cells transfected with NC. On the contrary, transfection with the miR-454-3p inhibitor significantly reduced EGR3 expression in U251 cells compared with cells transfected with NC-in (Fig. 3B). Transfection with miR-454-3p mimics significantly reduced the expression level of EGR3 protein compared with those transfected with NC (0.13±0.01 vs. 0.92±0.04, n=3), whilst transfection with the miR-454-3p inhibitor significantly increased EGR3 protein expression in U251 cells (0.79±0.02 vs. 0.20±0.01, n=3) compared with those transfected with NC-in (Fig. 3C). These results suggest EGR3 to be a target gene of miR-454-3p.

**EGR3 is downregulated in glioma tissues and its overexpression inhibits glioma cell proliferation.** To reveal the function and significance of EGR3 in glioma tumors, the expression levels of EGR3 in glioma and adjacent normal tissues were tested using RT-qPCR and western blot analysis. The expression of EGR3 mRNA (0.67±0.03 vs. 0.35±0.02, n=40) and protein (0.75±0.04 vs. 0.27±0.02, n=3) in glioma tissues were significantly decreased compared with adjacent normal tissues (Fig. 4A and B). The effects of EGR3 on glioma cell proliferation were investigated further using MTT and colony formation assays. EGR3 was first overexpressed in U138MG cells by transfection with plasmids expressing EGR3 and knocked down in U251 cells by siRNA transfection. Transfection efficiency was confirmed using RT-qPCR and western blot analyses (n=3, Fig. 4C and D). EGR3 overexpression significantly reduced the viability of U138MG cells (n=5, Fig. 4E), in addition to significantly reducing the colony number of U138MG cells compared with cells transfected with negative control vector (85±3 vs. 36±3; n=3; Fig. 4F). In contrast, following transfection with siEGR3, U251 cell viability was significantly increased (n=5) and colony number was also significantly higher compared with those transfected with siNC (81±4 vs. 151±7, n=3; Fig. 4E and F).

**EGR3 is involved in miR-454-3p-induced elevation of glioma cell proliferation.** Based on the findings already obtained from the present study, it was hypothesized that miR-454-3p may promote glioma cell proliferation of by targeting and regulating EGR3 expression. U138MG and U251 cells were either transfected with miR-454-3p mimics only or co-transfected with miR-454-3p mimics and EGR3 plasmids. Transfection with miR-454-3p mimics significantly reduced EGR3 expression, which was reversed by co-transfection with EGR3 plasmids (n=3, Fig. 5A and B). Besides, the results of MTT and colony formation assays showed that transfection with
Figure 1. Expression levels of miR-454-3p was upregulated in glioma. (A) Relative expression levels of miR-454-3p in glioma and adjacent normal tissues were determined using RT-qPCR. ***P<0.001. (B) Comparison of overall patient survival between the high miR-454-3p expression and low miR-454-3p expression groups. The high miR-454-3p expression group exhibited significantly shorter survival compared with that of the low miR-454-3p expression group. P=0.0304. (C) Relative miR-454-3p expression in cell lines were determined using RT-qPCR. **P<0.01 and ***P<0.001 vs. NHA cells. RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA; NHA, normal human astrocyte cells.

Figure 2. miR-454-3p promoted cell proliferation in glioma cell lines. (A) Expression levels of miR-454-3p were determined using reverse transcription-quantitative PCR in U138MG cells transfected with miR-454-3p mimics and in U251 cells transfected with miR-454-3p inhibitor. (B) MTT assay were performed to assess viability in U138MG cells transfected with miR-454-3p mimics and U251 cells transfected with miR-454-3p inhibitor. (C) Colony formation assay was performed to measure the growth of U138MG cells transfected with miR-454-3p mimics and U251 cells transfected with miR-454-3p inhibitor. Representative images of crystal violet-stained cell colonies are shown. *P<0.01, **P<0.05 and ***P<0.001 vs. NC or NC-in. NC, negative control; miR, microRNA; OD, optical density.
miR-454-3p mimics increased cell viability (n=5) and colony formation (n=3), but was reversed by co-transfection with EGR3 plasmids in U138MG and U251 cells (Fig. 5C and D). These results indicated that miR-454-3p promote glioma cell proliferation in by negatively regulating EGR3 expression.

**Discussion**

Glioma is a common malignancy in the central nervous system with the overall prognosis of ~1 year for most patients from the time of diagnosis (20). Combined therapies currently available for glioma have limited benefits due to the high rates of invasion (21). It is therefore imperative to explore the pathological mechanism and new therapeutic strategies for the diagnosis and treatment of this disease.

A previous study have demonstrated that plasma levels of miR-454-3p were upregulated in patients with glioma compared with healthy controls, where the prognosis of glioma with high miR-454-3p expression was significantly worse compared with that of glioma with low miR-454-3p expression (22). Indeed, there is accumulating evidence that aberrant expression of miRNA is involved in the pathogenesis of many diseases including cancers (23). A number of studies have previously found that miR-454-3p was downregulated in gastric cancer and osteosarcomas (23,24), whereas it was upregulated in prostate cancer, hepatocellular carcinoma and non-small cell lung cancer (25-27). These results suggest that expression profiles of miR-454-3p are distinct in different types of cancers. In the present study, it was found that miR-454-3p expression was upregulated in glioma tissues compared with adjacent normal tissues, which was concordant with previous results found in the plasma (22). In addition, association between increased miR-454-3p expression with poor prognosis were also previously found in hepatocellular carcinoma (26) and triple-negative breast cancer (28). Taken together, these results suggest that miR-454-3p may serve as a potential prognostic biomarker for glioma, though the role of miR-454-3p in glioma remains unknown.

Since cell proliferation is one of the most important hallmarks of cancer pathophysiology (11), the present study evaluated the effect of miR-454-3p on glioma cell growth and found that miR-454-3p overexpression could significantly promote cell growth. EGR3 was subsequently identified as a direct target of miR-454-3p, where the overexpression of miR-454-3p inhibited EGR3 mRNA and protein expression. Overexpression of EGR3 was also found to reverse miR-454-3p mimic-induced cell proliferation in vitro. Previous studies reported that EGR3 was ubiquitously expressed in the cerebral cortex, substrate ganglion and neuromuscular spindle (13). Increased EGR3 expression induced expression of IL-6 and IL-8 has been previously demonstrated in prostate cancer (29), suggesting that EGR3 also serves an important role in tumorigenesis. Of note, EGR3 were...
also shown to be downregulated in glioma tissues in comparison with adjacent normal tissues, and EGR3 knockdown in vitro promoted glioma cell proliferation.

It was previously found that overexpression of miR-454-3p in hepatic stellate cells by transfection with miR-454-3p mimics could deactivate the hepatic stellate cells by targeting SMAD4, which is involved in transforming growth factor-β signaling (30). Overexpression of miR-454-3p could also promote prostate cancer cell growth by targeting N-myc downstream-regulated gene 2 (25), whereas the upregulation miR-454-3p expression promoted the progression of NSCLCs by directly targeting PTEN (27). These results suggest that
miR-454-3p is involved in the regulation of a number of signaling pathways by binding to multiple targets. Therefore, the role of miR-454-3p in glioma may also be through other signaling pathway, which require further study.

To conclude, the present study was the first to demonstrate that miR-454-3p is involved in the cell proliferation of glioma, by negatively regulating EGR3 expression. These findings provide valuable insights into the underlying biological features of this disease and may provide an avenue for the development of possible novel therapeutic targets.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
GS and JW designed all the experiments and revised the paper. ZM, DC, LL and XL performed the experiments and wrote the paper.

Ethics approval and consent to participate
The present study was approved by the Research Ethics Committee of The First People's Hospital of Taizhou. All patients provided written informed consent prior to enrollment in the study.

Patient consent for publication
All patients provided written informed consent prior to enrollment in the study.

Competing interests
The authors declare that they have no competing interests.

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