miR-454-3p suppresses cell migration and invasion by targeting CPEB1 in human glioblastoma

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Abstract. MicroRNAs (miRNA/miRs) serve crucial roles in the progression of human glioblastoma (GBM); however, the exact regulatory mechanisms of miRNAs in human GBM remain unclear. The present study aimed to investigate the roles of miR-454-3p in human GBM. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was performed to examine the expression of miR-454-3p in glioma tissues and adjacent tissues. Human GBM cell lines (LN-229, A172 and GL15) and a normal human astrocyte cells (HA1800) were used for analysis. In addition, RT-qPCR and western blotting were applied for mRNA and protein expression analysis, respectively. The cell proliferation was measured using a Cell Counting kit-8 assay. Furthermore, scratch and Transwell assays were employed for the analysis of cell migration and invasion. A luciferase reporter assay was used to verify the target of miR-454-3p. The results revealed that miR-454-3p was downregulated in the glioma tissues and GBM cell lines, including LN-229, A172 and GL15. Additionally, the overexpression of miR-454-3p significantly suppressed the proliferation, migration and invasion of LN-229 cells. Furthermore, cytoplasmic polyadenylation element-binding protein 1 (CPEB1) was confirmed as a direct target of miR-454-3p. These findings indicated that the overexpression of miR-454-3p inhibited cell proliferation, migration and invasion by downregulating CPEB1. Therefore, miR-454-3p may act as a tumor suppressor and represent an effective therapeutic strategy in GBM.

Introduction

Glioblastoma (GBM) is one of the most common types of malignant tumor and is a prevalent type of primary cancer of the central nervous system in adults (1-4). Developments in the treatments for GBM, including chemotherapy with temozolomide, radiotherapy, and combination therapy with tumor resection have been made; in patients with newly diagnosed malignant glioma, improvements in the 2-year survival rate (from 11 to 27%), the 3-year survival rate (from 4 to 16%) and the 5-year survival rate (from 2 to 10%) were observed in 2014 (5,6). However, these survival rates remain relatively low; the underlying molecular mechanisms of GBM-associated tumorigenesis, chemotherapy resistance, progression and metastasis require further investigation. Therefore, it is of clinical importance that novel therapeutic targets and biomarkers associated with GBM are identified.

MicroRNAs (miRNA/miRs) are a class of small non-coding RNAs of 19-24 nucleotides in length that serve important regulatory roles in post-transcriptional gene expression (7,8). Additionally, downregulation of miR-454-3p has been identified in certain types of cancer in humans, including pancreatic ductal adenocarcinoma (9) and breast cancer (10), while upregulation of miR-454 has been observed in other types of cancer, including hepatocellular carcinoma, non-small cell lung cancer (11) and colorectal cancer (12). In pancreatic ductal adenocarcinoma, it has been reported that miR-454 is able to regulate stromal cell proliferation, thereby controlling the growth of pancreatic ductal adenocarcinoma (13). Additionally, in osteosarcoma, miR-454 functions as a tumor suppressor gene that suppresses cell proliferation and invasion by directly targeting c-Met (14). In hepatocellular carcinoma, miR-454 functions as an oncogene by inhibiting chromodomain-helicase-DNA-binding protein 5, resulting in the inhibition of tumor cell proliferation and invasion (15). Additionally, in uveal melanoma, miR-454 functions as an oncogene by inhibiting chromodomain-helicase-DNA-binding protein 5, resulting in the inhibition of tumor cell proliferation and invasion (15). Additionally, in uveal melanoma, miR-454 functions as an oncogene by inhibiting chromodomain-helicase-DNA-binding protein 5, resulting in the inhibition of tumor cell proliferation and invasion (15). Additionally, in uveal melanoma, miR-454 functions as an oncogene by inhibiting chromodomain-helicase-DNA-binding protein 5, resulting in the inhibition of tumor cell proliferation and invasion (15). Additionally, in uveal melanoma, miR-454 functions as an oncogene by inhibiting chromodomain-helicase-DNA-binding protein 5, resulting in the inhibition of tumor cell proliferation and invasion (15). Additionally, in uveal melanoma, miR-454 functions as an oncogene by inhibiting chromodomain-helicase-DNA-binding protein 5, resulting in the inhibition of tumor cell proliferation and invasion (15).
the control of cell cycle progression, cellular senescence and inflammation (19-21). Additionally, CPEB1 has been considered to serve as a tumor suppressor gene. For instance, a recent study reported that CPEB1 mediated epithelial-to-mesenchymal transition (EMT) and breast cancer metastasis by regulating the apical localization of tight junction protein ZO-1 mRNA (22). In melanoma, miR-455-5p has been reported to promote melanoma metastasis via inhibition of CPEB1 (23); in glioma, knockdown of CPEB1 reduces cell senescence by regulating the expression and distribution of cellular tumor antigen p53, and CPEB1 was also demonstrated to be directly regulated by the tumor suppressor miR-101 (24).

The results of the present study revealed that the over-expression of miR-454 in GBM cells in vitro inhibited cell proliferation, migration and invasion. Importantly, the present study also observed that miR-454-3p negatively regulated the target gene CPEB1. Thus, the present study investigated whether miR-454-3p and CPEB1 may be a novel therapeutic target for the treatment of patients with GBM.

Patients and methods

Patient and tissue samples. A total of 30 human glioma and matched adjacent tissues were collected between January 2015 and December 2016 at Huai'an First People's Hospital (Huai'an, China). The median age of the patients was 51 years, and the age ranged from 29-73 years and there were 19 male and 11 female. All tissue samples were collected for analysis upon obtaining informed consent from all patients. In addition, patients did not receive chemotherapy, radiotherapy or any other treatments prior to surgery. The present study was approved by the Research Ethics Committee of Huai'an First People's Hospital (Huai'an, China).

Cell culture. Human GBM cell lines (LN-229, A172 and GL15) and normal human astrocyte cells (HA1800) were obtained from the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). Adherent cultures of LN-229 and HA1800 cell lines were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China). All cells were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO₂.

Cell transfection. The human GBM cells (LN-229) were seeded in 6-well plates (2x10⁵ cells/well) 1 day prior to transfection, which was conducted when cells reached 60-70% confluence. The LN-229 cells were untransfected or transfected with mimic control or mimc-454-3p mimic (Shanghai GenePharma Co., Ltd., Shanghai, China) at a concentration of 40 nM, and the untreated cells served as the control. The successful transfection was determined by RT-qPCR. The sequences of miR-454-3p was: 5' -UAGUGCAAAUAUGCUAUAGG GU-3'; miR-454-3p mimic was: 5' -UAGUGCAAAUAUGCUAUAGGGU-3' and mimic control was: 5'-UCACACACCUCCUAGAAAAGAGUAGA-3'. Then the cells were incubated for 24 h to continue the further analysis. Transfection with the miRNAs and with the plasmids for the luciferase assay (described below) was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Human GBM cell lines (LN-229, A172 and GL15) and normal human astrocyte cells (HA1800) were lysed in TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for total RNA extraction; a total of 1 µg total RNA was used for cDNA synthesis with an RNA PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). For cDNA synthesis, samples were incubated at 42°C for 30 min, 99°C for 5 min and 5°C for 5 min. qPCR was conducted using an ABI7500 Real-Time PCR Instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR conditions were as follows: Pre-denaturation at 95°C for 5 min, followed by initiation at 94°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 75°C for 1.5 min for 32 cycles, following which samples were stored at 4°C. Hairpin-it™ of miR-454-3p and U6 RT-qPCR Primer Set (Shanghai GenePharma Co., Ltd.) were used to measure the relative quantity of miR-454-3p; the expression of miR-454 was normalized to the endogenous expression of U6. The primers used were as follows: miR-454-3p forward, 5'-CTC AACTGGTGTGGAGTCCGGAATTCGTTAGACCCTATA-3' and reverse, 5'-ACACTCCACGTGGTAGTCAAATTTGCTTA-3'; U6 forward, 5'-CTCGTCCGCGAGCA CA-3' and reverse, 5'-ACACGTCTACAGAATTCGCCTG-3'. Additionally, metadherin, astrocyte elevated gene-1 (AEG-1) and matrix metalloproteinase-9 (MMP-9) RT-qPCR Primer Sets (Shanghai GenePharma Co., Ltd.) were used to measure the relative quantities of metadherin, AEG-1 and MMP-9, with normalization of the mRNA expression levels to the endogenous expression levels of GAPDH. The primers used were as follows: Metadherin forward, 5'-AAATGGGCCGAC TGTGTAAGT-3' and reverse, 5'-CTTGGTTGCACTGCTT TTAGCAGT-3'; AEG-1 forward, 5'-AAGATGGCCGAGCT TGAAGT-3' and reverse, 5'-CTGTTGTCGACTGCTT TTA-3'; MMP-9 forward, 5'-GGGAGGCGAGCACGTC GTA-3' and reverse, 5'-CTACTCATGCTCGAGGAGG-3'; and GAPDH forward, 5'-TGTTGGGCCATCAATGGATTG TTA-3' and reverse, 5'-ACACCATGTATTCCGGGTCAAT-3'. All reactions were performed in triplicate. The relative expression of genes was normalized to GAPDH. Data were analyzed using the 2⁻ΔΔCq method (25).

Cell Counting kit-8 (CCK-8) assay. Cellular proliferation was measured with a CCK-8 kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. LN-229 cells were assigned with untreated (control), mimic control and miR-454-3p mimic groups. At 24 h post-transfection, 100 µl cells suspension were seeded into 96-well plates at a density of 8x10³ cells/well and incubated at 37°C for 6 h. At the indicated time points (12, 24 and 48 h), 10 µl CCK-8 solution was added to each well. Following incubation at 37°C for 1 h, the absorbance was measured with a plate reader at 450 nm.

Transwell and scratch assays of LN-229 cell migration and invasion. Transwell culture inserts (8-mm pore size; Falcon; BD Biosciences, Franklin Lakes, NJ, USA) were placed into
the wells of 24-well culture plates, separating the upper and lower chambers. LN-229 cells were assigned with untreated (control), mimic control and miR-454-3p mimic groups. For the cell invasion assay, Matrigel (BD Biosciences) was used to pre-coat the upper side of the membrane, which was subsequently incubated at 37°C for 1 h for gel formation, and hydrated in FBS for 2 h prior to use. In the lower chamber, 600 µl DMEM containing 10% FBS was added, and the three groups cells were added to the upper chamber at a density of 1x10⁵ cells/well respectively. Following incubation at 37°C for 24 h. The level of migration was observed under an optical microscope (Leica DM16000B; Leica Microsystems GmbH, Wetzlar, Germany), and counted for 5 random (magnification, x100) fields per well. Cell counts are expressed as the mean number of cells per field of view (26).

For the cell migration assay, the LN-229 cells in the logarithmic phase were cultured in DMEM in 24-well plates at a density of 10⁵ cells/well to obtain a monolayer culture. The monolayer was carefully scratched with a new 1-ml pipette tip across the center of the well, with the long axis of the tip remaining perpendicular to the bottom of the well, such that the width of the scratch was equal to the outer diameter of the tip. Following scratching, the cells were incubated at 37°C with 5% CO₂ for 24 h. Images of the migrated cells were captured at 0 and 24 h and were captured from 5 random (magnification, x5) fields in each sample.

Target prediction. According to TargetScan 7.1 (http://www.targetscan.org/vert_71/), it was predicted that CPEB1 may be targeted by miR-454-3p.

Luciferase reporter assays. For the luciferase reporter assay, LN-229 cells were seeded in 96-well plates at a density of 10⁴ cells/well and incubated at 37°C with 5% CO₂ for 24 h. Subsequently, pGL3-CPEB1-3′untranslated region (UTR)-wild type (WT) or pGL3-CPEB1-3′UTR-mutant plasmids (constructed by Beyotime Institute of Biotechnology, Haimen, China) were transfected into the control, miR-454-3p mimics and negative control miR-454-3p mimics groups using Lipofectamine 2000. The transfection efficiency was normalised with an internal control Renilla luciferase vector (pRL-CMV; Promega Corporation, Madison, WI, USA). Following 48 h incubation, a luciferase assay kit (cat. no. RG005; Beyotime Institute of Biotechnology) was used to measure the reporter activity according to the manufacturer's protocols.

Western blot analysis. After LN-229 cells were untransfected or transfected with mimic control or miR-454-3p mimic cells were washed with cold PBS and treated with a lysis buffer (cat. no. C3702; Beyotime Institute of Biotechnology). The quality was detected using BCA method and protein concentration was measured with a NanoDrop instrument (NanoDrop; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). A total of 30 µg protein samples were separated by 15% SDS-PAGE, and the proteins were transferred onto polyvinylidene difluoride membranes. Subsequently, the membranes were blocked with a buffer containing 10% non-fat milk in PBS with 0.05% Tween-20 for 2 h at room temperature, and then incubated with primary antibodies against metadherin, AEG-1, MMP-9, CPEB1 and GAPDH for 1 h at room temperature. Additionally, the membranes were incubated at room temperature with horseradish peroxidase-labeled goat anti-rabbit antibody (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology, Haimen, China) conjugated to horseradish peroxidase for 45 min. Protein bands were visualized by enhanced chemiluminescence (Abbott Laboratories, Arlington Heights, IL, USA). Evaluation of target protein expression was performed using ImageJ version 1.38 (National Institutes of Health, Bethesda, MD, USA).

The primary antibodies [anti-metadherin (1:500; cat. no. MA515564), MMP-9 (1:1,000; cat. no. PA516851), CPEB1 (1:1,000; cat. no. P8250561) and GAPDH (1:500; cat. no. P8519440)] were purchased from Wuhan Khayal Bio-Technology Co., Ltd. (Wuhan, China); anti-AEG-1 (1:1,000; cat. no. PL032231R) was purchased from Otwo Biotech (Shenzhen), Inc. (Shenzhen, China).

Statistical analysis. All results were confirmed in at least three independent experiments, and the qualitative data from single representative experiments are presented. All quantitative data are presented as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). A Student's t-test was used for comparisons between two groups. One-way analysis of variance followed by a least significant difference post hoc test was used for comparisons between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-454-3p expression is significantly downregulated in glioma tissues and GBM cell lines. To investigate the role of miR-454-3p in GBM development, the expression levels of miR-454-3p in tissue samples and GBM cell lines were analyzed by RT-qPCR. As presented in Fig. 1, it was observed that miR-454-3p levels were significantly lowered in glioma tissues and GBM cell lines (LN-229, A172 and GL15) compared with adjacent tissues and normal human astrocyte cells (HA1800) (P<0.01), respectively. From the results of Fig. 1, the miR-454-3p levels were significantly lower in LN-299 cell than in A172 and GL15 cells, so LN-229 cells were chosen for further analysis.

Overexpression of miR-454-3p inhibits the proliferation of LN-229 cells. To investigate whether miR-454-3p serves a role as a tumor suppressor in GBM, the effects of miR-454-3p overexpression on the proliferation of GBM cells were analyzed. LN-229 cells were transfected with control miR, miR-454-3p mimic or negative control miR-454-3p mimic, and the relative expression of miR-454-3p was verified by RT-qPCR (Fig. 2A). As was demonstrated in Fig. 2A, the miR-454-3p level was significantly increased following overexpression of miR-454-3p compared with the negative control miR-454-3p mimic (P<0.01). In addition, the effects of miR-454-3p on cell proliferation was investigated via a CCK-8 assay. Analysis of the results indicated that the overexpression of miR-454-3p significantly suppressed LN-229 cell proliferation compared with the negative control
miR-454-3p regulates glioblastoma cells by targeting CPEB1

3968

HUI et al: miR-454-3p regulates glioblastoma cells by targeting CPEB1

Figure 1. miR-454-3p expression is downregulated in glioma tissues and various human GBM cell lines. (A) Relative expression levels of miR-454-3p were determined by RT-qPCR in human glioma and adjacent tissues. (B) Relative expression levels of miR-454-3p were determined by RT-qPCR in human GBM cell lines (LN-229, A172 and GL15) and a normal human astrocyte cell line (HA1800). ***P<0.001 vs. HA1800 and ****P<0.001. GBM, glioblastoma; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Figure 2. Overexpression of miR-454-3p inhibits the proliferation of LN-229 cells. LN-229 cells were divided into three transfection groups: Control, mimic control and miR-454-3p mimic groups. (A) Relative expression levels of miR-454-3p were measured by reverse transcription-quantitative polymerase chain reaction. (B) Cell proliferation was assessed by a Cell Counting kit-8 assay; the OD value of each group in LN-229 cells was determined at 570 nm. *P<0.05, **P<0.01 vs. mimic control. miR, microRNA; OD, optical density.

group (Fig. 2B). Above date suggested that miR-454-3p overexpression could inhibit the proliferation of LN-229 cells.

Overexpression of miR-454-3p inhibits the invasion of LN-229 cells. As migration and invasion are two key stages associated with malignant progression and metastasis (27), the effects of miR-454-3p on the migration and invasion of LN-229 cells was investigated in the present study. The results of the Transwell and scratch assays indicated that the overexpression of miR-454-3p significantly inhibited the invasion and migration of LN-229 cells compared with the negative control group (Figs. 3 and 4). Collectively, these results suggested that miR-454-3p overexpression exerted tumor suppressive effects by suppressing the proliferation, migration and invasion of the LN-229 GBM cell line.

Metadherin has been reported to regulate mesenchymal marker protein expression in numerous types of tumor and to promote cancer metastasis (28-30). Additionally, AEG-1 has been reported to serve a pivotal oncogenic role in tumorigenesis (31,32), and MMPs, a family of zinc-binding proteins, including MMP-9, have been demonstrated to serve important roles in tumor cell invasion and metastasis due to their ability to degrade the extracellular matrix (33,34). In the present study, western blotting and RT-qPCR revealed that miR-454-3p significantly decreased the protein and mRNA expression levels of CPEB1, metadherin, AEG-1 and MMP-9 compared with in the control group (Fig. 5).

CPEB1 is a target gene of miR-454-3p. The TargetScan database was used to identify the potential target mRNA of miR-454-3p in the present study. The results demonstrated that CPEB1 was a putative target gene of miR-454-3p (Fig. 6A). Subsequently, a dual-luciferase activity assay revealed that miR-454-3p significantly suppressed the luciferase activity of the WT 3'-UTR of CPEB1 compared with that of the mutant
type (Fig. 6B). Furthermore, the overexpression of miR-454-3p was observed to suppress the protein expression of CPEB1 in the LN-229 cells by western blot analysis (Fig. 5B). These data indicated that CPEB1 is a direct target of miR-454-3p in GBM LN-229 cells.

**Discussion**

GBM is among the most lethal and aggressive forms of brain cancer, and accounts for 15% of brain malignancies (35). Despite technological improvements in the diagnosis and
treatment of GBM, the rates of treatment failure and recurrence remain an issue. The poor prognosis of patients with GBM is primarily due to late diagnoses and poor responses to chemotherapy (36). Therefore, further investigation is urgently required to identify and develop novel therapeutic approaches for the prevention and treatment of GBM.

Recent studies have revealed that miRNAs contribute to the progression and development of GBM (37,38). For example, decreased expression levels of miR-146a in GBM have been associated with the regulation of cell proliferation and apoptosis via the targeting of notch 1 (39). In addition, upregulated miR-622 has been observed to inhibit cell proliferation, motility and invasion by repressing KRAS proto-oncogene GTPase in GBM (40). Additionally, miR-10b in GBM may mediate TGF-β1-regulated cell proliferation, migration and EMT (41); however, to the best of our knowledge, the roles of miR-454-3p, which is located on chromosome 17q22, in the progression of GBM, particularly its effects on cell proliferation and migration and target gene regulation, have not yet been studied.

In the present study, downregulation of miR-454-3p in glioma tissues and GBM cell lines was observed. Functional experiments further revealed that the overexpression of miR-454-3p may inhibit the proliferation, migration and invasion of the LN-229 GBM cell line. Recent studies have reported that miR-454 may act as an oncogene or tumor suppressor in cancer (17,42). For instance, miR-454-3p was reported to be upregulated and to function as an oncogene in hepatocellular carcinoma, non-small cell lung cancer (11) and colorectal cancer (12); however, the present study revealed that miR-454-3p served a tumor suppressing role in GBM LN-229 cells. Thus, miR-454-3p may serve a variety of roles in different types of tumor. To the best of our knowledge, there have been no reports regarding the association between miR-454-3p and CPEB1. The results of the present study indicated that miR-454-3p negatively regulated CPEB1, and identified CPEB1 as a direct target of miR-454-3p. Therefore, the findings of the present study collectively indicated that miR-454-3p may be an important miRNA that is potentially associated with the proliferation, migration and invasion of GBM cells.

Metastasis is one of the principal factors that contributes to the mortality of patients with GBM (43). The present study reported that the overexpression of miR-454-3p inhibited the migration and invasion of GBM cells in vitro. In epithelial cancers, EMT is regarded as one of the major mechanisms that promote cell migration, invasion and metastasis. A recent study demonstrated that CPEB1 mediates EMT and metastasis in breast cancer (22). In the present study, miR-454-3p was suggested to inhibit the migration and invasion of GBM cells by negatively regulating CPEB1. In addition, MMPs are proteolytic enzymes that serve a pivotal role in the transformation and progression of tumors at all stages, particularly invasion and metastasis (44). The present study revealed that within GBM cells, miR-454-3p significantly inhibited the
expression of MMP-9. Collectively, these results indicated that the upregulation of miR-454-3p in GBM cells suppressed the migration and invasion of the tumor cells by inhibiting CPEB1 and MMP-9 expression.

In conclusion, the present study demonstrated that miR-454-3p was significantly downregulated in GBM cell lines, and that miR-454-3p overexpression suppressed cell proliferation, migration and invasion, potentially by targeting CPEB1 in the GBM cells. These findings indicated a novel tumor suppressive role of miR-454 in the development of GBM. Furthermore, miR-454-3p and CPEB1 may be potential therapeutic targets in the treatment of GBM.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XH and SZ searched the literature and designed the study. XH and YW performed the experiments and interpreted the data. YW contributed to the materials. XH wrote and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Hua'an First People's Hospital. All tissue samples were collected for use after obtaining informed consent from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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