Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MicroRNA-124-3p represses cell growth and cell motility by targeting EphA2 in glioma

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A R T I C L E I N F O

Article history:
Received 20 June 2018
Accepted 30 June 2018
Available online xxx

Keywords:
MicroRNA-124-3p
EphA2
Glioma
Motility

A B S T R A C T

MiR-124-3p and EphA2 are aberrantly expressed in glioma tissue specimens. In the present study, we firstly investigated that miR-124-3p inhibits EphA2 expression mediated by binding its 3′-UTR to regulate the progression of human glioma. The U87MG and LN229 cells were transfected with miR-124-3p mimics and/or siRNA-EphA2, and then the role of miR-124-3p and EphA2 in the colony-formation, cell-cycle, migration and invasion of glioma cells in vitro were examined. Proteins involved in the epithelial-mesenchymal transition were examined using western blot. The results showed that miR-124-3p was significantly downregulated in glioma tissues, whereas a marked upregulation of EphA2 expression was found. Colony-formation and flow cytometry assays demonstrated that EphA2 downregulation or miR-124-3p mimics caused growth and cell-cycle inhibition in glioma. Transwell migration and invasion assays demonstrated that EphA2 downregulation or miR-124-3p mimics suppressed the migration and invasion of glioma cells. EphA2 downregulation or miR-124-3p mimics reduced the level of vimentin in U87MG and LN229 cells. In conclusion, miR-124-3p was found to suppress the growth, migration and invasion of glioma cells in vitro via EphA2. Furthermore, we validated miR-124-3p enforced its biological modulation via targeting EphA2 through the rescue experiment. Conclusively, our study proclaimed that miR-124-3p can counteract the malignant phenotypes of glioma cells by the inhibitory effect of the EphA2.

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1. Introduction

Glioma appears to be the most common and most aggressive malignant primary brain tumor in adults. Glioma causes large mortality around the world each year [1]. The malignancy of glioma is characterized by rapid cell growth, high invasion, angiogenesis and poor survival [2]. Although the recent availability of advanced techniques offers several benefits in the diagnosis and treatment of glioma, the poor prognosis and prolonging survival still remains, to date, challenging [3,4]. Thus, it is important to further explore the underlying mechanism and develop novel therapy strategies based on new molecular interaction networks must be achieved for glioma.

EphA2, belongs to the membrane-bound tyrosine kinase family, serves as an oncogene involved in promoting the formation and progression in different types of malignancies. Emerging evidences have implicated elevated EphA2 expression in several types of cancers, such as melanoma, breast carcinoma, oral squamous cell carcinoma, and prostate carcinoma [5–7]. Abnormal expression of EphA2 in cancers participates in modulating malignant tumor transformation, metastasis and tumor outcome. Additional EphA2 has been implicated in promoting cell proliferation, metastasis and epithelial-to-mesenchymal transition (EMT) [8], while opposite activities exist upon EphA2 deletion. Increasing evidence suggests that EphA2 expression may be causally related to glioma.

MicroRNAs (miRNAs), a class of small endogenous non-coding oligonucleotides of ~22 nt, participate in regulating the target gene expression by transcript degradation or translational repression. Ongoing research has identified miRNAs serve as tumor-suppressors or oncogenes by binding the 3′-untranslated region

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https://doi.org/10.1016/j.bbrc.2018.06.173
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(UTR) of target gene. Aberrant expression and dysregulation of miRNAs are correlated with a variety of the pivotal tumorigenic processes, such as proliferation, migration, and invasion in diverse kinds of malignancies, including glioma [9–11]. Among them, miR-124 drew great attention as one of the most frequent miRNA isolated in the nervous system and plays important roles in many aspects of neuronal physiology, such as neuronal development and neural plasticity [12]. MiR-124 has been found to decrease in glioma and restoration of miR-124 contributes to explicitly inhibitory impact on malignancies [13]. Decreased expression of miR-124 was correlated with poor prognosis in patients with glioblastoma.

In our present study, we found miR-124-3p was dysregulated and conduced to the abrogation of the malignant phenotypes of glioma cells by directly pairing with the 3′-UTR of EphA2. Additionally, we proved that high expression of EphA2 might be involved in modulating the malignancy of glioma in vitro. Furthermore, miR-124-3p suppressed EphA2-mediated signaling pathway in the progression of glioma. Together, our finding provides a potential supplementary therapeutic strategy concerning the molecular mechanism of glioma in the future.

2. Materials and methods

2.1. Clinical human glioma specimens and immunocytochemical staining

Twenty paired human glioma tissues and normal brain tissues were collected from Tianjin Huanhu Hospital during surgery. Informed consent was obtained from every patient and ethics approval was granted by the hospital institutional review board. The large RNA and small RNA of tissue samples were extracted and analysed for the expression levels of miR-124-3p and EphA2 by reverse transcription and real-time PCR. Immunocytochemical analysis was used to visualize the expression of EphA2 in glioma tissues. U6 and β-actin were served as an endogenous control.

2.2. miRNA target prediction and luciferase reporter assay

The miR-124 mimics, negative control (miR-NC)or miR-124 mutant were commercially synthesized by GenePharma (Shanghai, China). The miRNA sequences were listed below: miR-NC 5′-UUCUCCGAACGUCACGUTT-3'; miR-124 mimics 5′-UAAGGCACGCGGUGAAUGCC-3'. miR-124 mutant 5′-CCCUUACAGCGGUGAAUGCC-3' (CCUUACA instead of AAGGCAC).

The analysis of miR-124-3p predicted targets was performed using the TargetScan, PicTar and miRanda algorithms. The related functions of the targets were also considered.

The 3′-UTR of EphA2 reporter was created containing one putative miR-124-3p targeting site and one randomly scrambled sequence inserted into the pGL3 vector (Promega, Madison, WI, USA). For the luciferase activity assay, U87MG cells plated into 96-well plates were cotransfected with the wild or mutant plasmid and miR-124 or miR-NC. Following 48 h cultivation, Luciferase activity assay was performed with the Dual-Luciferase Reporter System (Promega, Madison, WI, USA).

2.3. Cell culture and transfection

Two human glioblastoma cell lines U87MG and LN229 were obtained from the Peking Union Medical College cell library (Beijing, China). Both the two cells were normally cultured in completely medium containing Dulbecco's modified Eagle medium (Gibco, Los Angeles, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 units/ml penicillin/streptomycin, maintained in a humidified atmosphere at 37 °C containing 5% CO^2. Cell transfetion was executed using Lipofectamine 2000 (Invitrogen, California, USA) as the mamufacturer's manuals.

Has-miR-124-3p mimics, miR-124-3p inhibitor, EphA2 siRNA, were all chemically artificialized by GenePharma (Shanghai, China) as well as their relative negative control. The cDNA encoding EphA2 was purchased from Genechem (Shanghai, China). Transient transfection was performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad,CA) in antibiotic-free Opti-MEM medium (Invitrogen, Carlsbad,CA) following the Manufacture's protocol.

2.4. Colony-formation assay and cell cycle assay

The glioma cell lines U87MG and LN229 cells were transfected with plasmids miR-124 mimics or si-EphA2 or empty control vector. For colony-forming activity, the post-transfected U87MG and LN229 cells were seeded into 6-well plates at a density of 500 cells per well and were allowed to grow for 10 days to form visible colonies. Then the cells were stained with 0.1% crystal violet and photographed under a microscope. Each treatment was performed in triplicate.

For cell cycle analysis, cells were also trypsinized and harvested. After being washed using PBS, cells were stained with propidium iodide (PI) using Cycletest Plus DNA Reagent Kit (BD Biosciences) following the manufacturer's protocol, and the cell cycle distribution was analyzed by FACSVerse flowcytometer (BD Biosciences). The percentages of cells in G0/G1, S, and G2/M phases were counted and compared. The experiments were carried out in triplicate.

2.5. Xenograft model with nude mice

SPF grade male BALB/C-A nude mice (4 weeks old), which were obtained from Laboratory Animal Center of the PLA Military Academy of Medical Sciences, were used in the evaluation of miR-124-3p on the glioma tumorigenicity in vivo. All the procedures were performed as the protocols approved by the Animal Care and Use Committee of Tianjin Medical University. The U87MG subcutaneous inoculation was performed in the dorsal area of the mice. Ten days later the mice were divided randomly into two treatment groups, which were injected with 200 pmol miR-124 or miR-NC mimics in Lipofectamine (2000) into xenograft model every 3 days as well as the measurement of tumor volume (volume $=$ length $\times$ width^2)/2 and the calculation of the xenograft tumor growth curves. 18 days after the inoculation, the mice were euthanized, and the tumor was removed for next Western blot and immunohistochemical assay for the expression of EphA2.

2.6. Migration and invasion assays

The migration and invasion assays was performed with the 24-well chambers (8 μm pore size, Corning, Cambridge, USA) with or without Matrigel (BD Biosciences, USA) respectively. Post-transfected cells (5×10^4/insert) were collected and resuspended with 200 μL DMEM and injected into the upper chambers. Meanwhile 600 μL DMEM containing 10% FBS were added to the bottom of each chamber served as the stimulated agent. After 24 h incubated, the cells on the upper surface of the membrance were removed and the cells adhering to the other side surface were fixed and stained with 1% crystal violet (Sigma-Aldrich, St, Louis, MO, USA). The labeled cells were counted under an inverted fluorescence microscope.

2.7. Western blot

Cells prepared at 72 h post-transfection were lysed using Mammalian Protein Extraction Reagent (CW Biotech, Beijing,

Please cite this article in press as: Q. Wu, et al., MicroRNA-124-3p represses cell growth and cell motility by targeting EphA2 in glioma, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.06.173

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