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MiR-615 inhibits cell proliferation, migration and invasion by targeting EGFR in human glioblastoma

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ABSTRACT

MiR-615 and epidermal growth factor receptor (EGFR) are associated with a number of disease processes and pathogenesis. However, little is known about the mechanisms of miR-615 and EGFR in human glioblastoma multiforme (GBM). Here, we found that down-regulation of miR-615 expression occurred in GBM tissues and cells, and was inversely correlated with overall survival, relapse-free survival, WHO grade as well as EGFR expression. We further determined that miR-615 functions as a tumor suppressor by inhibiting GBM cell proliferation, cell cycle, migration and invasion, and promoting cell apoptosis. In-vivo assay validated the inhibition effect of miR-615 on tumor growth and EGFR expression. Luciferase reporter assays demonstrated that miR-615 targeted the 3′-untranslated region (3′-UTR) of EGFR. Besides, over-expression of EGFR reversed the inhibition effects of miR-615, while silencing of EGFR aggravated these inhibition effects. In conclusions, we identified that miR-615 plays a tumor suppressor role in GBM cell proliferation, migration and invasion by targeting EGFR expression, and miR-615 may act as a novel biomarker for early diagnosis or therapeutic targets of GBM.

1. Introduction

Glioma is the most common malignant tumor in human central nervous systems, which accounts for approximately 50% in all cerebral tumors [1]. Despite more advances in the diagnosis and treatment such as chemo-/radiotherapy and resection, the prognosis of glioma patients was not satisfactory [2,3]. It has been reported that the clinical prognosis remains poor with a median overall survival of 15–17 months. Notably, the chemo-/radiotherapy resistance markedly affects the therapy outcome of glioma patients [4,5]. Therefore, it is crucial to figure out the molecular mechanism underlying the progression of glioma cells.

MicroRNA (miRNA) is a kind of non-coding RNA with the length of 19–23 nucleotides, which binds to the 3′ untranslated region of mRNA of the target genes to regulate the expression of the target genes [6–9]. miR-615 has been demonstrated to be aberrantly expressed in different human tumors with varied roles in the development of cancers [10]. miR-615 represents a potential anti-onco-miR and participates in breast cancer carcinogenesis by suppressing AKT2 expression [11]. However, the specific roles and regulatory mechanisms of miR-615 in the progression of GBM are rarely explored. Additionally, EGFR expression was identified to be highly expressed in a variety of human cancers, involving cervical cancer, gastric adenocarcinomas and colon cancer [12–14]. However, it is not clear whether miR-615 can regulate the expression of EGFR in GBM.

In this study, we firstly demonstrated miR-615 expression decreased in GBM tissues and cells. Then, we found that EGFR was a direct target of miR-615 in inducing proliferation, migration and invasion of GBM. Finally, our findings revealed the miR-615/EGFR signaling was a novel molecular circuit in the development of GBM.

2. Materials and methods

2.1. Patients and tissue samples

Tissues specimens were obtained from glioma patients who were diagnosed at the Shandong Provincial Third Hospital and Shandong Provincial Hospital between 2008 and 2011. Adjacent non-tumor tissues located at least 5 cm from tumors were selected as controls. Surgically resected tissues were diagnosed by two

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pathologists according to World Health Organization (WHO) histological criteria. Written informed consent was obtained from each patient and the present study was approved by the Ethics Committee of Shandong Provincial Third Hospital. The survival time was defined as the time between the day of the initial diagnosis and the day of patient mortality due to the glioma. The specimens were obtained after surgical resection, immediately frozen, and stored in liquid nitrogen. None of patients had received chemotherapy, immunotherapy or radiotherapy prior to the surgery.

2.2. Cell culture

Neuroglioma cell lines U87, U251 and SHG-44 and the normal astrocyte cell line NHA were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS), 0.25% trypsin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Thermo Fisher Scientific, Inc. Waltham, MA, USA). Other consumables for cell culture were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Human embryonic kidney-293T (HEK293T) cells were obtained from The Second Affiliated Hospital of Dalian Medical University, and were grown in DMEM supplemented with10% FBS in a 5% CO_2 incubator that was maintained at 37 °C.

2.3. Cell transfection

For cell transfection, negative control miRNAs (miR-NC), miR-615 mimics for miR-615 upregulation, and pc-DNA3.1-EGFR plasmids for EGFR over-expression were diluted in OPTI-MEM (Thermo Fisher Scientific, Inc.), which was then added with diluted Lipofectamine 2000. Following incubation for 20 min at room temperature, the mixture was added into the cell suspension. For silencing of EGFR, cells were grown at a density of 100.000 cells/well in a 6-well plate, and transfected by using Attractene Transfection Reagent (cat. number.1051531, Quiagen) for 6 h with 0.2 mg/ml EGFR siRNA (Origene Technologies) or scramble negative control (Origene Technologies) at the same dose following manufacturer's indications.

2.4. RNA extraction and real-time quantitative RT-PCR

Total RNA or miRNA was extracted using RNeasy kit (Qiagen, USA) or miRNA Easy kit (Qiagen) according to manufacturer's instructions. RNA purity and concentration was determined with a spectrophotometer (ND-1000; Nano-Drop Technologies). cDNAs were synthesized using miScript II RT kit (QIAGEN). Quantitative PCR were performed using miScript SYBR Green PCR kit (QIAGEN) in an ABI-7900HT machine following manufacturer's instructions. Relative miRNA expression normalized to U6 was carried out using the 2-ΔΔCt method. Relative EGFR mRNA expression normalized to GAPDH was performed using 2-ΔΔCt method.

2.5. Western blot

Tissues and cells were lysed in lysis buffer (Roche, Basel, Switzerland) for protein extraction. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Maibio, Shanghai, China). After blocking in 5% skim milk for 2 h at room temperature, the membranes were incubated with the specific primary antibodies at 4 °C overnight. GAPDH was used as an internal control. The membranes were washed in PBS 3 times and incubated in HRP-conjugated secondary antibodies for 1 h at room temperature. Positive signals were developed by ECL Plus Western Blotting Substrate (Thermo Scientific) and analyzed by Image J (National Institutes of Health, Bethesda, MD).

2.6. Cell proliferation assay

In brief, cells (2×10^4 cells/well) in different groups were seeded into 96-well culture plates and cultured in RPM1640 medium containing 10% FBS. After transfection for 24, 48 or 72 h, MTT reagent (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well, followed by incubation at 37 °C for an additional 4 h. Subsequently, 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to dissolve the crystals for 10 min at 37 °C. The spectrometric absorbance at 490 nm was measured by an EnSpire Multimode Plate Reader (PerkinElmer, Inc., Waltham, MA, USA).

2.7. Cell cycle analysis

Cell cycle analysis was carried out using the Muse Cell Cycle Kit and Muse Cell Analyzer (Merck Millipore, Darmstadt, Germany). Cells were harvested by trypsinization and fixed by 70% ice-cold ethanol. After fixation, cell pellets were resuspended in Muse Cell Cycle Kit reagent, incubated for 30 min, and protected from light until further analysis. Cell cycle analysis carried out by the method supplied by the manufacturer.

2.8. Apoptosis analysis

The annexin V-FITC/propidium iodide (PI) staining assay was used to detect the apoptosis of cells. The results were analyzed according to the manufacturer's instructions. The experiments were performed at least three times independently, and a representative is shown.

2.9. Cell migration and invasion assays

Cells (5×10^4 for migration assays and 2×10^5 for invasion assays) were seeded into the upper chamber in serum-free medium. Culture medium containing 10% FBS was added to the lower chamber as the chemoattractant. After the cells were incubated in a humidified incubator at 37 °C for 24 h (migration assay) or 36 h (invasion assay), cells on the upper surface of the membrane were removed by scraping with a cotton swab, whereas cells attached to the lower surface were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 10 min. Five randomly selected fields of the fixed cells were imaged and counted using a X71 light invert microscope (magnification, × 200; Olympus, Tokyo, Japan).

2.10. Xenograft model

Animal experiments were conducted in accordance with the guideline for the Regulations for Animal Experiments and Related Activities at Shandong Provincial Third Hospital. The backs of 6-week- old female Balb/c nude mice were inoculated with 5.0×10^6 cells. Tumor size was monitored at 2-day intervals by measuring the length and width with calipers, and its volumes were calculated with the formula: $(L \times W2) \times 0.5$, where L is length and W is width of each tumor. Mice were sacrificed by cervical spine fracture dislocation and organs were collected for pathological examination.

2.11. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of at

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