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Hsa_circ_0007534/miR-761/ZIC5 regulatory loop modulates the proliferation and migration of glioma cells

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ABSTRACT

Increasing evidences demonstrate the essential roles of circular RNAs (circRNAs) in human cancers. However, the study about the functions of circRNAs in glioma remains very limited. In the present study, we found that circRNA hsa_circ_0007534 was highly expressed in glioma tissues compared to normal brain tissues. Furthermore, we found that knockdown of hsa_circ_0007534 significantly inhibited the proliferation and migration of glioma cells. In mechanism, we showed that hsa_circ_0007534 could sponge miR-761 to repress its availability in glioma cells. We found that inhibition of miR-761 could rescue the suppressed proliferation and migration of glioma cells by hsa_circ_0007534 knockdown. Moreover, we explored the downstream mechanism and found that ZIC5 was a target of miR-761. We showed that hsa_circ_0007534 promoted the expression of ZIC5 by inhibiting miR-761 in glioma cells. And restoration of ZIC5 expression significantly reversed the effects of hsa_circ_0007534 knockdown on glioma cell proliferation and migration. In summary, our results demonstrated that hsa_circ_0007534 serves as an oncogene in glioma via promoting ZIC5 expression by repressing miR-761 availability. Our results suggested that hsa_circ_0007534/miR-761/ZIC5 regulatory loop might be a promising therapeutic target for glioma treatment.

1. Introduction

Glioma is most common and malignant intracranial tumor, which leads to a large number of cancer-related deaths worldwide [1]. According to the malignant degree determined by histopathologic characteristics, glioma could be grouped into low-grade (WHO grade I and II) and high-grade (WHO grade III and IV) glioma [2]. Nowadays, surgical resection combined with postoperative radiochemotherapy is the main therapeutic approach for glioma patients [3]. However, Due to the high recurrence rate and mortality rate, the outcomes of glioma patients remains rather poor [4]. Therefore, it is necessary to determine the mechanism of glioma progression and identify novel therapeutic targets for glioma treatment.

Among all human transcripts, about 90% are noncoding RNAs (ncRNAs), including microRNAs (miRNAs), long noncoding RNAs (lncRNA), circular RNAs (circRNAs) [5,6]. Recently, as the development of sequencing technology, the functions of circRNAs are gradually uncovered. More and more reports indicate that circRNAs are involved in regulation of human cancer progression and serve as essential regulators [7]. CircRNAs are demonstrated to regulate the proliferation, migration, invasion and apoptosis of various cancer cells [8]. For instance, Yang et al. reported that circular RNA circ-ITCH inhibits bladder cancer cell proliferation via sponging miR-17/miR-224 and regulating p21, PTEN expression [9]. Xu et al. showed that downregulated circular RNA hsa_circ_0001649 regulates proliferation, migration and invasion in cholangiocarcinoma cells [10]. However, the study about the function of circRNAs is just emerging. Moreover, the knowledge of circRNAs in glioma remains very limited.

A previous report showed that hsa_circ_0007534 promotes the proliferation of colorectal cancer cells [11]. However, the role of hsa_circ_0007534 in glioma and its functional mechanism are largely unknown. In this study, we found that hsa_circ_0007534 was significantly upregulated in glioma tissues compared to normal brain tissues. And hsa_circ_0007534 knockdown markedly inhibited the proliferation and migration of glioma cells. In mechanism, we revealed that hsa_circ_0007534 promoted the expression of

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ZIC5 by inhibiting miR-761 availability. Consequently, enhanced expression of ZIC5 contributed to the proliferation and migration of glioma cells. Taken together, our study for the first time demonstrated the key role of hsa_circ_0007534/miR-761/ZIC5 regulatory loop in glioma progression.

2. Materials and methods

2.1. Patient samples and cell lines

A total of 35 glioma samples were obtained from patients who experienced surgical resection at Linyi Central Hospital. Normal brain tissues were obtained from 35 individuals who died in traffic accidents at Linyi Central Hospital. None of the patients had received radiotherapy or chemotherapy before surgery. This project was approved by the Ethic Committee of Linyi Central Hospital. All tissues were immediately stored in liquid nitrogen until the total RNA was extracted.

Human glioma cell lines, U251, U118, LN229, and U87MG, as well as normal human astrocytes (NHA) were purchased from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 mg/ml at 37 °C under a humidified atmosphere of 5% CO_2.

2.2. Cell transfection

For cell transfection, glioma cell lines were cultured in 6-well plates with serum-free medium overnight and then transfected with a short interfering RNA targeting the junction region of hsa_circ_0007534 (siRNA sequence: 5′-GATCATTCA-GAGCTATTTTGA-3′) plasmid (100 nM), miR-761 mimics and their corresponding controls using the Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions for at least 5 h before the medium was replaced with fresh medium. After culture at 37 °C for 72 h, the treated cells were harvested for the subsequent experiments.

2.3. Cell proliferation assay

Cell proliferation was detected by using the Cell Counting Kit-8 (7 sea biotech, Shanghai, China). Cells were grown in 96-well plates with 1×10^4 cells per well and incubated at 37 °C in 5% CO_2 until cell confluence reached 70%. After 48 h of plasmid transfection, cells were incubated for an additional 24, 48, 72 and 96 h. A volume of 10 μL of CCK8 solution was added into each well. The absorbance at 450 nm was measured with the SUNRISE Microplate Reader (Tecan, Switzerland).

2.4. In vitro cell migration assay

Twenty-four-well plates containing transwell chambers with 8-μm pore size polycarbonate membrane inserts (Corning Incorporated, Corning, NY, USA) were used for cell migration assay. For cell migration assays, 5×10^4 cells in serum-free RPMI 1640 medium were seeded in each well on Matrigel-free chamber. After 24 h of incubation, cotton swabs were used to remove the cells inside the upper chamber, while the cells on the other side of the membrane were fixed and stained with 0.5% crystal violet solution. Five random fields were counted for each well.

2.5. RNA isolation and qRT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen), followed by removal of DNA with the TurboDNase Kit (Ambion). Quantification of extracted RNA was performed using NanoDrop. cDNA synthesis was performed using PrimeScriptRT reagent KIT (Takara) using 1000 ng of total RNA. QRT-PCR was performed using the SYBR Select Master Mix (Applied Biosystems) on an ABI 7900 system (Applied Biosystem). The level of GAPDH was used as a control. The Ct value was calculated based on the ΔΔCt-method. Fold change of gene expression was expressed as $2^{-\Delta\Delta Ct}$ method.

2.6. Western blot

Total protein was extracted from cells and tissues using RIPA lysis buffer according to manufacturer's protocol. Then 40 mg of protein per lane was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. After a blocking incubation with 5% milk-TBST, membranes were incubated with primary antibodies against ZIC5 and GAPDH at 4 °C overnight. After washing twice with TBST, the membranes were incubated 1 h in a secondary antibody that was conjugated to horseradish peroxidase (1:10,000 dilution). Afterwards, protein bands were visualized with an ECL Detection System (Thermo, Scientific).

2.7. Luciferase reporter assay

Glioma cells were seeded into a 24-well plate. Cells were co-transfected with wild-type or mutated hsa_circ_0007534 or ZIC5 3′-UTR reporter plasmids, and with miR-761 mimics or negative controls. Luciferase assays were conducted 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega, WI, USA).

2.8. Statistical analysis

All experiments were conducted at least three times. Data were displayed as mean ± SD. Analyses were performed with SPSS 20.0 software (SPSS, Chicago, IL, USA). Student's t-test was used for differences between two groups and one-way ANOVA for multiple comparisons. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Hsa_circ_0007534 knockdown inhibited glioma cell proliferation and migration

To investigate the role of hsa_circ_0007534 in glioma, we first analyzed the expression levels of hsa_circ_0007534 in glioma tissues and normal brain tissues by qRT-PCR. We found that hsa_circ_0007534 expression was significantly upregulated in glioma tissues (Fig. 1A). Furthermore, we showed that high hsa_circ_0007534 expression levels were positively correlated with advanced tumor grade (III/IV) in glioma (Fig. 1B). We then measured the expression of hsa_circ_0007534 in glioma cell lines, including U118, LN229, U87MG and U251 cells, and normal human astrocytes (NHA) cells. The results indicated that hsa_circ_0007534 expression was higher in glioma cell lines compared to NHA cells (Fig. 1C). These data suggested that hsa_circ_0007534 overexpression might be involved in glioma progression.

Then we knocked down hsa_circ_0007534 in LN229 and U251 cells by specific siRNA against hsa_circ_0007534. qRT-PCR analysis showed that hsa_circ_0007534 expression levels were significantly downregulated in LN229 and U251 cells transfected with siCirc (Fig. 1D). Then we performed CCK8 and transwell assays to evaluate the effects of hsa_circ_0007534 on glioma cell proliferation and migration. The results indicated that hsa_circ_0007534

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