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MicroRNA-222 promotes tumorigenesis via targeting DKK2 and activating the Wnt/β-catenin signaling pathway

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ABSTRACT

MiR-222 in glioma can regulate cell cycle progression and apoptosis. However, the relationship between miR-222 and Wnt/β-catenin signaling pathway in glioma remains unknown. Here, we found that the Dickkopf-2 gene (*DKK2*) was a direct target of miR-222 by target prediction analysis and dual luciferase reporter assay. RNA interference silencing of *DKK2* proved that miR-222 overexpression led to constitutive activation of β-catenin through inhibition of *DKK2* expression in glioma cells. Furthermore, miR-222 siRNA significantly inhibited tumorigenesis in vivo. Finally, Western blot analysis showed that miR-222 could regulate the expression of β-catenin and the downstream genes of Wnt/β-catenin signaling pathway. Taken together, our findings reveal a new regulatory mechanism of miR-222 and suggest that miR-222 might be a potential target in glioma therapy.

1. Introduction

Gliomas are the most common form of malignancy in the central nervous system [1]. Despite therapeutic advances, the median survival time for high-grade gliomas is only 15 months [2]. Previous studies have suggested that the formation of gliomas is related to the rates of cell proliferation and apoptosis. Therefore, understanding the main regulatory mechanism of this malignancy is the key to developing novel and effective therapeutic strategies for gliomas.

MicroRNAs (miRNAs) are non-coding RNA molecules that regulate the expression of a wide variety of genes through sequence-specific binding to their mRNA targets, resulting in translational inhibition [3]. They have pivotal roles in various physiological and pathological processes, including proliferation and apoptosis [4]. An increasing body of evidence has suggested that the aberrant expression of miRNAs may lead to the development and progression of malignancy [5,6]. In recent years, miR-222 has been reported to be overexpressed in human carcinomas, including thyroid papillary tumors and glioblastoma [7,8]. Functional studies have demonstrated that miR-222 may target multiple signaling pathway and genes, such as p27Kip1 and PUMA, to promote the development and progression of malignancy [9,10]. Thus, miR-222 can be classified as an oncomir, and the decreased expression of miR-222 might be a promising strategy for cancer therapeutics. It has been demonstrated that abnormally high activation of the Wnt/β-catenin pathway is required for the initiation and progression of glioma[11]; however, until now, functional analysis of miR-222 and the Wnt/β-catenin pathway has not been well-documented.

In the present study, we investigated the potential involvement of miR-222 in glioma. We examined the expression level of miR-222 in glioma cells, and tested its effects on cell growth, apoptosis, and colony formation in vitro and tumorigenesis in vivo. Furthermore, we explored the underlying role of miR-222 in glioma. Finally, we also analyzed the possible relationship between miR-222 and the genes downstream of the Wnt/β-catenin pathway in glioma development and progression. Our study will provide a better understanding of glioma pathogenesis.

2. Materials and methods

2.1. Reagents

Antibodies against β-actin, Bcl2, Bax, β-catenin, c-myc, DKK2, and c-Jun were obtained from Santa Cruz Biotechnology Inc., (CA, USA). A dual luciferase reporter assay system was obtained from Promega Corporation (WI, USA). PGL3-promoter, PGL3-basic, and PRL-TK vectors were also purchased from Promega. All other chemicals were purchased from Sigma–Aldrich unless otherwise stated.

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2.2. Cell lines and cultures

Glioma cell lines, including U251, U87, SHG44, A172, and a human astrocyte cell line (HA) (Cell Bank of Chinese Academy of Science Shanghai China), were cultured in RPMI 1640 medium (Gibco Industries, Inc., Carlsbad, CA) with 10% fetal bovine serum (Gibco Industries, Inc). U87 and U87-miR-222-disrupted fluorescent (EGFP)-labeled cells were developed (Neuron Biotech co., Ltd, Shanghai, China) and are referred to as Control and GR232 for conciseness.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The rate of proliferation was assessed using the colorimetric MTT assay as described previously [12]. All cells were seeded into 96-well culture plates (2×10^3 cells/well). The cells were transfected with a miR-222 mimics, control oligonucleotide, or inhibitor (antisense oligonucleotide-miR-222) and grown for 4 days. One plate was developed immediately after the medium was changed, and other plates were developed every 24 h for 4 d. Assays were initiated by adding 20 μl of MTT substrate to each well, followed by an additional 3 h incubation. Finally, the media was removed, and 200 μl DMSO was added to each well. Plates were read at a wavelength of 570 nm in an Automated Microplate Reader (Multiskan Ex, Lab systems, FIN). All experiments were biologically repeated in triplicate to confirm consistency of the results obtained.

2.4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The relative expression levels of miRNAs were determined. Total RNA was isolated using TRIZOL™ reagent (Promega Corporation, WI, USA) according to the supplier's instructions. qRT-PCR was performed using One Step PrimeScript® miRNA cDNA Synthesis Kit (TaKaRa Biotechnology Ltd, Shandong, China). Real-time PCR was performed using SYBR Green Supermix with an iCycler®thermal cycler (Bio-Rad Laboratories Inc, CA, USA). Primers of all genes are described in the Supplementary data. Data were collected and analyzed by the comparative Ct (threshold cycle) method using *GADPH* as the reference gene.

2.5. Flow cytometric analysis of apoptosis

The extent of apoptosis was measured through the Annexin V-FITC apoptosis detection kit (Invitrogen Corporation, CA, USA) according to the manufacturer's instructions. The cells (6×10^4/well) were seeded in six-well plates in antibiotic-free media, followed by transfection with control oligonucleotide (100 nM) or mimics (100 nM) using Lipofectamine 2000 (Invitrogen Corporation, CA, USA). After 48 h, the cells were collected, washed with cold PBS twice, and gently resuspended in 400 μl 1*binding buffer. After adding 5 μl of Annexin V-FITC, the cells were gently vortexed and incubated for 10 min at 4 °C in the dark. Then, the tube was incubated with 10 μl of PI for another 5 min at 4 °C in the dark. Finally, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, NJ, USA). In Fig. 2C, cells in the lower right quadrant represent early apoptosis, and those in the upper right quadrant are representative of late apoptotic cells.

2.6. Luciferase activity assay

The 3′ UTR of human *DKK2* (GeneBank accession number NM_000633) cDNA, containing the putative target sites for miR-222, was amplified by PCR using the following primers: 5′-GATCGCCGTGTAATTCTAGAGTTTCATTGCCCTCT-3′ (sense) and 5′-CGTCGATAACTGAAACCAAAGTCAATAGCTGC-3′ (anti-sense). Then, it was inserted into the *Xba*I restriction site, immediately downstream of the luciferase gene in the pGL3-promoter-vector (Promega, Madison, WI). A mutant version with a deletion of 7 bp from a site of perfect complementarity was also generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA). Wild-type and mutant inserts were confirmed by sequencing. Twenty-four hours after transfection, the cells were seeded into 96-well plates (8×10^3 viable cells/well) and allowed to adhere overnight. Then, 200 ng of pGL3-DKK2-3′–UTR or pGL3-mut-DKK2-3′–UTR plus 80 ng pRL-SV40 (Promega Corporation, WI, USA) was transfected alone or in combination with control oligonucleotide (final concentration of 100 nM) inhibitor (100 nM) or mimics (100 nM) using Lipofectamine 2000 (Invitrogen Corporation, CA, USA) according to the manufacturer's protocol. Luciferase activity was measured 48 h post-transfection using the Dual Luciferase Reporter Assay System (Promega Corporation, WI, USA). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. Three independent experiments were performed in triplicate.

2.7. Western blot analysis

The U251 and U87 cells were seeded in six-well plates (4×10 cells/well) in antibiotic-free media, followed by transfection withcontrol oligonucleotide (100 nM), inhibitor (100 nM), or mimics (100 nM) using Lipofectamine 2000 (Invitrogen Corporation, CA, USA). After 48 h of culture, the cells were harvested and homogenized with lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Non-idet P-40, 0.5% sodium deoxycholate, 0.02% sodium azide, 100 g/ml PMSF, 1 g/ml aprotinin). Total protein extract (40 μg) was separated on 12% SDS–PAGE gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk in TBS, the membrane was probed with primary monoclonal antibody specific to DKK2 (1:100; Cell Signaling Technology) which was used as an internal control for protein loading. The membrane was further probed with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:2000; Santa Cruz), and the protein bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Corp., Piscataway, NJ). β-Actin was used as a loading control.

2.8. Subcutaneous and orthotopic xenografts

For subcutaneous implantation, 5×10^6 Control cells (U87 cells) and GR232 cells (miR-222-disrupted U87 cells) suspended in 100 μl PBS were injected subcutaneously into the flank of BALB/C nude mice (Cancer Institute of the Chinese Academy of Medical Science). The mice were randomly divided into two groups (five mice per group). Tumor growth in mice was detected by the Kodak In-Vivo FX Pro system (Kodak, New York, USA). Tumor volume of xenografts was detected every 5 days over the course of the study with fluorescence signaling. Mice were sacrificed and examined 5 weeks after the subcutaneous implantation.

All experiments were carried out under the approval of the Administrative Panel on Laboratory Animal Care of the Xinhua Hospital.

2.9. In vivo optical imaging

Prior to in vivo imaging, the mice were anesthetized with Phenobarbital sodium. Fluorescence imaging was obtained with an excitation wavelength of 490 nm and emission wavelength of 535 nm. Exposure times ranged from 1 to 2 min.