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Original Articles

MiR-152 functions as a tumor suppressor in glioblastoma stem cells by targeting Krüppel-like factor 4

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ABSTRACT

Glioblastoma (GBM) is the most common central nervous system tumor and the molecular mechanism driving its development is still largely unknown, limiting the treatment of this disease. In the present study, we explored the potential role of miR-152 in glioblastoma stem cells (GSCs) as well as the possible molecular mechanisms. Our results proved that miR-152 was down-regulated in human GSCs. Restoring the expression of miR-152 dramatically reduced the cell proliferation, cell migration and invasion as well as inducing apoptosis. Mechanistic investigations defined Krüppel-like factor 4 (KLF4) as a direct and functional downstream target of miR-152, which was involved in the miR-152-mediated tumor-suppressive effects in GSCs. Meanwhile, this process was coincided with the down-regulated LGALS3 that could be bound and promoted by KLF4, leading to attenuate the activation of MEK1/2 and PI3K signal pathways. Moreover, the in vivo study showed that miR-152 over-expression and KLF4 knockdown produced the smallest tumor volume and the longest survival in nude mice. Taken together, these results elucidated the function of miR-152 in GSCs progression and suggested a promising application of it in glioma treatment.

Introduction

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults and a challenging disease to treat [1]. More than 50% of the patients who are diagnosed with primary malignant brain cancers are reported to be GBM in U.S. [2,3]. Numerous recent studies have demonstrated that GBM contains a subset of cells referred to as glioblastoma stem cells (GSCs) that are tumorigenic, resistant to therapy and have a self-renewing ability [4–7]. Studies have shown that GSCs play crucial roles in the development of GBM and may be a potential target for therapeutic manipulation [8]. However, the regulative mechanism of GSCs in the development of GBM is still unknown. Thus, we focused on the biological significance of GSCs isolated from surgically excised GBM specimens.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that play regulatory roles by targeting the sequences in the 3'-untranslated region (3'-UTR) of target mRNAs, which results in the reduction of mRNA expression or in the inhibition of mRNA translation [9,10]. Accumulating evidence shows that miRNAs can function as oncogenes or tumor suppressors depending on their specific target genes and the type of cancer [11,12]. They are thought to participate in various cancer processes, such as proliferation, cell cycle, and apoptosis [13]. Studies of miRNAs have shown that miR-34a was a negative regulator of the tumorigenic properties of lung cancer stem cells that over-expressed CD44 [14], and miR-98 could effectively inhibit the cell proliferation of human ovarian cancer stem cells [15]. In addition, miR-300 could promote self-renew and inhibit differentiation of glioma stem-like cells [16]. Consequently, the alteration in the expression and specific functions of miRNAs could affect the biological behaviors of cancer stem cells. However, the expression and functions of miRNAs in GSCs still need to be well documented.

MiR-152 belongs to the miR-148/152 family that is involved in a series of cellular activities such as cell proliferation, invasion and angiogenesis [17,18]. Recent studies showed that miR-148a expression was decreased in hematopoietic stem cells [19] and mesenchymal stem cells [20,21]. Previously, it has been found that miR-152 is expressed and acts as a tumor suppressor in several types of cancers such as breast cancer [22], endometrial cancer [23], ovarian cancer [24] and cholangiocarcinoma [25]. However, the function of miR-152 in stem cells, particularly in GSCs, has not yet been reported.

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The aim of the present study was to determine the role of miR-152 in GSCs isolated from surgically excised GBM specimens. The potential mechanisms of miR-152 in regulating the biological behaviors of GSCs were also investigated. Our findings will help to elucidate the function of miR-152 in the GSCs progression and will benefit the future glioma treatment.

Materials and methods

Human tissue samples

Glioma samples and normal brain tissues (NBTs) were collected from patients undergoing complete or partial surgical resection at the Department of Neurosurgery, Shengjing Hospital of China Medical University. The procedures were in accordance with Declaration of Helsinki. All participants provided their written informed consent, and the hospital ethical committee approved the experiments. The samples were obtained from those without necrosis and coagulation parts. The diagnosis was established histologically by two experienced clinical pathologists according to the WHO classification.

Cell culture

GSCs were obtained from GBM tissues (GBMs) which were washed, dissociated and subjected to enzymatic dissociation, as described previously [5,26]. The cultures were maintained in the serum-free medium of Dulbecco's Modified Eagle Medium (DMEM)/F12 (Life Technologies Corporation, Grand Island, NY, USA) supplemented with 20 ng/ml of basic fibroblast growth factor (bFGF, Life Technologies Corporation, Carlsbad, CA, USA), 20 ng/ml of epidermal growth factor (EGF, Life Technologies Corporation, Gaithersburg, MD, USA) and 2% B27 (50×, Life Technologies Corporation, Grand Island, NY, USA). HEK 293T were maintained in DMEM containing 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA). All cultures were maintained at 37 °C in an atmosphere of 5% CO_2.

Isolation and identification of GSCs

Cultures were formed primary spheres. The serum-free medium was refreshed every 2 days until the spheres were visible under microscopy. The spheres were harvested by mechanical centrifugation, trypsinized into single-cell suspensions and plated into a 96-well plate for the limiting dilution assay and colon sphere formation by limiting dilution as described previously [5]. For differentiation assay, the spheres were transferred onto glass coverslips coated with poly-L-ornithine (BD Biosciences, Franklin Lakes, NJ, USA) in cultural medium with 10% FBS. For immunostaining of undifferentiated spheres, cells were stained with anti-Nestin antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CD133 antibody (1:100, Santa Cruz Biotechnology). For differentiated spheres, cells were stained with anti-GFAP (1:100, Abcam, Cambridge, MA, USA) and anti-beta-tubulinIII (1:100, Santa Cruz Biotechnology). Cells were also counterstained with DAPI to identify all nuclei.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from cells using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA). RNA concentration and quality were determined for each sample by the 260/280 nm ratio using a Nanodrop Spectrophotometer (ND-100). Real-Time PCR analysis was performed to test the expression levels of miR-152 and KLF4 by means of a 7500 Fast Real-Time PCR System. For quantification of miR-152 expression, reverse transcription and real-time PCR amplification were carried out using Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-152 and U6 (Applied Biosystems, Foster City, CA, USA), respectively. For quantification of KLF4 mRNA, reverse transcription and real-time PCR amplification were carried out using the High Capacity cDNA Reverse Transcription Kits and TaqMan Universal Master Mix II with the gene expression assays of KLF4 and GAPDH (Applied Biosystems), respectively. Fold changes were calculated by relative quantification ($2^{-\Delta\Delta Ct}$) method.

Lentivirus vector construction and infection

Human full-length KLF4 gene with its 3'-UTR sequences and short hairpin RNA directed against human KLF4 gene were ligated into the LV5-CMV-GFP-EF1a-Puro vector and LV3-CMV-GFP-Puro vector (GenePharma, Shanghai, China), respectively. Lentiviral vectors or control lentivirus vectors (NC), and packaging vectors were cotransfected into HEK 293T cells using Lipofectamine 2000. Virus particles were harvested 48 h after transfection. Cells were then infected with Lentivirus or their NC. GFP-positive cells were picked to select KLF4 (+)-NC, KLF4 (+), KLF4 (−)-NC and KLF4 (−) cells and further propagated.

Cell transfection of miRNA

MiR-152 agomir, miR-152 antagomir and their respective negative control molecules (NC) were synthesized (GenePharma). Transfections were conducted using Lipofectamine 2000. The transfected efficacy of miR-152 agomir and miR-152 antagomir were evaluated by quantitative RT-PCR, and the high transfected efficacy of these could sustain 7 days. The time after transfected 72 h was considered as the harvested time in the subsequent experiments. GSCs transfected with miRNAs were divided into five groups: control group given no miRNAs, pre-miR-152-NC group transfected with miR-152 agomir NC, pre-miR-152 group transfected with miR-152 agomir, anti-miR-152-NC group transfected with miR-152 antagomir NC, anti-miR-152 group transfected with miR-152 antagomir. Those stable expressing cells co-transfected with miR-152 agomir (or miR-152 antagomir) were divided into nine groups: control group, pre-miR-152-NC + KLF4(+)-NC group (KLF4(+)-NC stable expressing cells co-transfected miR-152 agomir NC), pre-miR-152 + KLF4(+) group (KLF4(+) stable expressing cells co-transfected miR-152 agomir), pre-miR-152-NC + KLF4(-)-NC group (KLF4(-)-NC stable expressing cells co-transfected miR-152 agomir NC), pre-miR-152 + KLF4(-) group (KLF4(-) stable expressing cells co-transfected miR-152 agomir), anti-miR-152-NC + KLF4(+)-NC group (KLF4(+)-NC stable expressing cells co-transfected miR-152 antagomir NC), anti-miR-152 + KLF4(+) group (KLF4(+) stable expressing cells co-transfected miR-152 antagomir), anti-miR-152-NC + KLF4 (-)-NC group (KLF4(-)-NC stable expressing cells co-transfected miR-152 antagomir NC), anti-miR-152 + KLF4(−) group (KLF4(-) stable expressing cells co-transfected miR-152 antagomir).

Cell proliferation assay

Cell proliferation assays were performed using the Cell Counting Kit-8 (CCK8, Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Cells were seeded at a density of 2000 cells per well in 96-well cell culture plates with five replicate wells for each group. Twenty microliters of CCK8 was added into each well and incubated for another 4 h, and the absorbance was finally measured at the wavelength of 450 nm.

Quantization of apoptosis by flow cytometry

Cell apoptosis was quantified by Annexin V-PE/7-AAD staining (SouthernBiotech, Birmingham, AL, USA). The cells were harvested and stained with Annexin V-PE and 7-AAD according to the instruction of the manufacturer. Cells were acquired by flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA) and analyzed by CELL Quest 3.0 software.

Cell migration and invasion assay

To examine the effects of cell migration and invasion, the 24-well transwell chambers with 8 μm pore size polycarbonate membrane (Corning Incorporated, Corning, NY, USA) were used. In brief, cells were re-suspended in serum-free medium, and then seeded on the top side of membrane (without Matrigel solution for cell migration assay) or plated on the top side of membrane pre-coated with Matrigel solution (BD, Franklin Lakes, NJ, USA) (for cell invasion ability assay) and incubated at 37 °C for 48 h, followed by removal of cells inside the upper chamber with cottons swabs. Migrated and invaded cells on the lower membrane surface were fixed and then stained with 20% Giemsa solution. The cell number was counted in five randomly selected fields.

Western blot analysis

Cells were lysed using ice-cold RIPA buffer and centrifuged at 14,000 × g 4 °C for 5 min. Protein concentrations were determined using the BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts of total protein were resolved on SDS-PAGE and electro-transferred onto PVDF membranes. Membranes were incubated in 5% skim milk dissolved in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 3 h at room temperature and then incubated with primary antibodies as follows: KLF4 (1:500, Santa Cruz Biotechnology), LGALS3 (1:1000, Santa Cruz Biotechnology), MEK1/2, p-MEK1/2, PI3K, p-PI3K (1:500, Santa Cruz Biotechnology) and GAPDH (1:1000, Santa Cruz Biotechnology), followed by incubation with appropriate correlated HRP-conjugated secondary antibody. Immunoblots were visualized by enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology) and scanned using ChemImager 5500 V2.03 software. The relative integrated density values (IDVs) were calculated by the FluorChem 2.0 software and normalized with GAPDH.

Reporter vectors constructs and luciferase reporter assay

KLF4 3'-UTR sequences were amplified by PCR and cloned into a pmirGlo Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to form 3'-UTR-luciferase reporter vector (KLF4-3'UTR-Wt) (GenePharma). To mutate the putative binding site of miR-152 in the 3'-UTR-containing vector, the sequence of putative binding site was replaced as indicated (KLF4-3'UTR-Mut). HEK 293T cells were seeded in 24-well plates and co-transfected with KLF4-3'UTR-Wt (or KLF4-3'UTR-Mut) and