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MicroRNA-585 suppresses tumor proliferation and migration in gastric cancer by directly targeting MAPK1

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ABSTRACT

Increasing evidence reveals that microRNA contributes to the development and progression of gastric cancer (GC), but the roles of miR-585 in GC remain unclear. In this study, we confirmed that miR-585 was down-regulated in GC tissues and cell lines and that miR-585 expression was related to extent of invasion, TNM stage, lymph node invasion and poor prognosis. Our results showed that miR-585 inhibits the proliferation and migration of GC, and MAPK1 is a direct and functional target of miR-585. Our study sheds light on the role of miR-585 as a suppressor for tumor growth and metastasis and raises the intriguing possibility that miR-585 may serve as a new potential marker for monitoring and treating GC.

1. Introduction

Gastric cancer (GC) is one of the most common malignant tumors that seriously endanger human health, ranking second in tumor-related mortality [1,2]. The incidence of GC is particularly high in China, which ranked the second in the nation and the mortality rate ranked third in all types of cancers [3]. The occurrence and development of GC involves a variety of oncogenes, tumor suppressor genes, resulting a complex process of disease [4]. With the rapid development of technology and a deeper understanding of molecular biology, many scholars have carried out comprehensive studies of GC. But the exact mechanism of the development of GC is still unclear, which also greatly limits the clinical treatment of GC. Therefore, it is of great clinical value and social benefit to study the pathogenesis of GC from molecular level.

MicroRNAs (miRNAs) are a group of recently discovered non-coding RNAs with a length of about 22 nucleotides, which can directly bind to the target region of the non-coding region (3′-UTR) to play an important regulatory role on post-transcriptional level [5,6]. miRNAs have a very wide range of biological functions, such as biological processes involved in cell development, proliferation, metastasis, metabolism, differentiation, cell cycle and apoptosis [7]. Abnormal miR-585 expression was reported in non-small-cell lung cancer, suggesting that miR-585 may be important in cancer pathogenesis and progression [8]. However, miR-585 expression and function in the GC remains unclear. In this study, we investigated the expression of miR-585 in GC tissues and studied their effect on the development of GC.

2. Materials and methods

2.1. Tissue samples

This study was approved by the Ethic Committee of The First Affiliated Hospital of University of Science and Technology of China, and all patients signed informed consents. GC and paired adjacent non-cancer tissues were obtained from 65 GC patients who were accepted with surgical operation between November 2011 and March 2010 at the Department of General Surgery, The First Affiliated Hospital of University of Science and Technology of China. All patients had no prior radiotherapy or chemotherapy before surgery. Tissues were immediately were frozen in liquid nitrogen after obtaining fresh stomach tissue samples to ensure total RNA

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extraction for subsequent studies.

2.2. Cell culture, lentivirus, and infection

MKN-28, MKN-45, BGC-803, SGC-7901, BGC-823, GES-1, and HEK- 293T were bought from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. All cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and streptomycin (100 mg/ml)/penicillin (100 U/ml) and incubated at 37 °C in a humidified incubator culture containing 5% CO_2. Lentivirus vector of miR-585 and negative control were purchased from GenePharma (Shanghai, China). Lentiviral transfection was performed according to the manufacturer's instructions to create BGC-823 cells stably expressing miR-585. Post-transfection BGC-823 cells were further selected in medium with blasticid for 2 weeks to establish stably transduced cells.

2.3. Transient transfection

miR-585 inhibitors vector (miR-585-in) and its control vector (NC-in) were purchased from GenePharma (Shanghai, China). Cells were transfected with vectors using lipofectamine 2000 according to manufacturer's instructions. qRT-PCR was performed to calculated transfection efficiency and cells were used for experiment 48 h after transfection.

2.4. Total RNA extraction and quantitative real time RT-PCR (qRT-PCR)

According to the manufacturer's protocol, we extracted Total RNA from the tissues and cells using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription of Total RNA to cDNA was obtained using PrimeScript RT Master Mix (TaKaRa, Shiga, Japan). qRT-PCR was performed as described in the instructions provided with SYBR Green (Applied Biosystems, Foster City, CA, USA). The primers used for amplification were as follows: miR-585 forward 5′-ACGCGTTCTCCTTACCATCCCTGA -3′, reverse 5′- CGATCTGGAAG-TAACCCAAGCC -3′; MAPK1 forward 5′- AATTGTATAA-TAAATTTAAAAGCTAG -3′, reverse 5′- GTAATACTGCTCCAGATATGG -3′. GAPDH or U6 was used as internal control.

2.5. Western blot

Total protein of cells were extracted in RIPA buffer (Beyotime) containing 1% PMSF and 1% protease inhibitor cocktail. Then, western blot assay was performed as previously described [9]. Primary antibodies were anti-MAPK1 (1:1000; Abcam, Cambridge, MA, USA) and anti- GAPDH (1:5000; Abcam, Cambridge, MA, USA).

2.6. Cell proliferation assay and colony formation assay

CCK-8 (Dojindo, Kumamoto, Japan) was used to assay cell proliferation. The cell types at a concentration of 1.5×10^3 cells/200 μl were seeded and cultured in 96 well plates. Absorbance was measured at 450 nm with a Microplate Autoreader (Bio-Rad, Hercules, CA, USA). For the colony formation assay, 1.0×10^3 cells were seeded into a 6-well plate and cultured in 4 mL culture media containing 10% fetal calf which was replaced every 3–4 days and incubated for 2 weeks. All cells were fixed with methanol and stained with 0.5% crystal violet in PBS for 20 min. Visible colonies was counted using a phase-contrast microscope at a magnification of 4 × . All experiments were performed in triplicate.

2.7. Cell apoptosis and cycle analysis

For apoptosis analysis, an Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, USA) was used to detect cell apoptosis according to the manufacturer's instructions. Briefly, the cells were washed with PBS and the cells were resuspended in 1 × binding buffer, then, 5 μl propidium iodide (PI) and 5 μl FITC Annexin V were added to 100 μl of the cell suspension and incubated for 15 min in the dark. 400 μl 1 × Binding Buffer was added into cells after incubation. The apoptosis was analysed by FACScan using the Cell-Quest software. PI-negative and Annexin V-FITC-positive cells were undergoing apoptosis.

For cell cycle analysis, GC cells were fixed in 70% ethanol and then stored at 4 °C overnight. Then, fixed cells were washed three times with PBS and incubated with 100 μg/ml RNase A for 30 min at 37 °C. After staining with PI (50 μg/ml), the cells were subjected to fluorescence activated cell sorting (FACS) on a FACScan (Beckman Instruments, USA). The cell populations were quantified using the Modfit software at the G0/G1, S and G2/M phases.

2.8. Wound healing assay and transwell migration assay

In order to obtain the desired effect, cells were cultured to reach confluence and wounded with 20-μL pipette tips. Rinse the cells with PBS and culture the cells in serum-free media. The wound closing procedure was observed and photographed at 0 and 48 h. For migration assay, 200 μL serum-free medium containing 1×10^5 GC cells was added into the upper transwell chamber and 700 μL medium with 10% serum was added into the lower transwell chamber. After cultured for 24 h in 37 °C, 5% CO_2 condition, cells that did not move through the membranes were removed and then cells that migrated to the bottom of the membrane were stained with 0.5% crystal violet for 30 min. The migration cells were counted and photographed in five different areas using an inverted microscope.

2.9. In vivo tumorigenesis assay

All animal experiments were conducted according to the guidelines for animal experimentation and were approved by the Institutional Animal Care Committee of The First Affiliated Hospital of University of Science and Technology of China. Stably infected GC cells with the miR-585 or NC (1×10^6 in 100 μL) were injected subcutaneously of 4-week-old male nude mice (Institute of Zoology, Chinese Academy of Sciences, Shanghai, China). Tumor width (W) and length (L) were measured every week. Tumor volume was evaluated using the following formula: tumor volume = $LW^2 \times 0.52$. Mice were sacrificed after 30 days. All tumors were excised, weighed, harvested and embedded in paraffin. Proliferation index and apoptosis index were detected by IHC (expression of Ki-67) and Tunnel apoptotic fluorescence method according to manufacturer's instructions.

2.10. Luciferase reporter assay

Plasmids containing wild-type 3′-UTR of MAPK1 gene or the mutant for potential miR-585 target sites were amplified and subcloned into pMIR-Report luciferase reporter vector. HEK-293 cells were seeded in a 24-well plate with a density of 1×10^5 cells/per well, and co-transfected with wild-type 3′-UTR or mutant 3′-UTR and miR-585 lentivirus or miR-585 inhibitors. At 48 h after transfection, luciferase activity was determined using a dual-luciferase reporter assay (Promega, USA) according to the manufacturer's protocol. Renilla luciferase activity was normalized to that of firefly luciferase.

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