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Hsa_circ_0009910 promotes carcinogenesis by promoting the expression of miR-449a target IL6R in osteosarcoma

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ABSTRACT

Circular RNAs (circRNAs) have recently shown capabilities as gene regulators in mammals. Some of them interact with microRNAs (miRNAs) and function as sponges to affect related miRNAs' activities. In this study, the molecular function of circRNA_0009910 and its potential downstream miRNA targets were explored. The expression levels of hsa_circ_0009910 were found to be overexpressed in osteosarcoma (OS) cells. Knockdown of circ_0009910 induced cell proliferation inhibition, cell cycle arrest, and apoptosis in OS cells. The target miRNA was predicted to be miR-449a, whose expression was down-regulated in OS cells. Inhibition of miR-449a abolished the effect of circ_0009910 knockdown on cell growth and apoptosis. The expression of miR-449a were found to be negatively correlated with that of circ_0009910 in OS tissues. Direct interaction of circ_0009910 and miR-449a was confirmed through dual-luciferase assays. Moreover, IL6R was predicted as a potential target of miR-449a. Overexpression of miR-449a decreased the mRNA and protein levels of IL6R. Restoration of IL6R impaired the miR-449a induced inhibition of cell proliferation, cell cycle arrest, and apoptosis. The mRNA expression of IL6R was inversely correlated with miR-449a in OS tissues. In addition, JAK1/STAT3 signaling pathway was regulated by circ_0009910/miR-449a/IL6R axis. Taken together, we suggested that circ_0009910 acted as a sponge of miR-449a and upregulated miR-449a functional target IL6R, thereby contributed to carcinogenesis of OS.

1. Introduction

Circular RNAs (circRNAs) are closed-loop RNAs produced through end-to-end joining of RNA transcription fragments during transcription [1]. Several studies have demonstrated the various molecular functions of circRNAs, including mediating alternative splicing [2], interacting with proteins [3], and acting as microRNAs (miRNAs) sponges [4,5]. Recently, many studies reveal that circRNAs may promote the progression of cancer. For example, upregulation of hsa_circ_001569 contributed to cell proliferation and invasion in colorectal cancer by sponging miR-145 [6]. Knockdown of cZNF292 suppressed cell proliferation and cell cycle progression in glioma via the Wnt/β-catenin signaling pathway [7]. Hsa_circ_0067934 silencing inhibited the proliferation and migration of esophageal squamous cell carcinoma (ESCC) cells [8]. CircPVT1, which was found to be independently associated with patients's survival, promoted cell proliferation by acting as a sponge of miR-125 family [9]. Hsa_circ_0009910 is located at chr1:12049221-12052747 with 315 bp of length in gene symbol MFN2. Sequencing analysis of the transcriptomes has revealed exon−exon junction reads connecting the 5′ end of exon 2 to the 3′ end of exon 3 [10–12], suggesting that exons 2 and 3 of MFN2 may be spliced together to form a covalently linked circularRNA (namely circ_0009910). However, its function and mechanism in the development of OS was unclear.

MiR-449a, which belongs to miR-34-5p/miR-449-5p family, was commonly reported as tumor suppressor in various human malignancies [13]. Overexpression of miR-449a induced cell-cycle arrest, apoptosis in prostate cancer cells [14]. Restoration of miR-449a suppressed cell growth and induced G1 phase arrest in bladder

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cancer [15]. Furthermore, by targeting c-Met, miR-449a was found to be a negative regulator of cell migration and invasion in non-small cell lung cancer [16]. Ectopic expression of miR-449a noticebly inhibited cell motility *in vitro* and pulmonary metastasis *in vivo* [17]. E2F1 and DNA methylation were involved in regulating miR-449a expression [18,19]. The expression of tumor-related miRNAs could be regulated by circRNAs [5]. It should be explored whether other mechanisms responsible for the dysregulation of miR-449a.

In the present study, the role circ_0009910 in the regulation of osteosarcoma (OS) cell growth, cell cycle, and apoptosis and the potential mechanism were investigated.

2. Materials and methods

2.1. Cell lines and tissue samples

Human OS cell lines (MG63, Saos-2 and U2OS) and fetal osteoblastic cell line hFOB were maintained in our laboratory. Cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco) and 100 U/ml penicillin/streptomycin solution, except for the U2OS cells that were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM). All the cell lines were cultured at 37 °C in a humidified incubator containing 5% CO_2.

A total of 30 OS tissues and 30 adjacent non-tumor tissues (located 3 cm away from the tumor) were obatined from patients who underwent surgical resection in our departemnt between Feb 2013 and Dec 2014. None of the patients had tumor history or received radiochemotherapy before surgery. This study was approved by the Institutional Research Ethics Committee of our hospital and informed consents were signed by enrolled patients before surgery. Tissues specimens were immediately frozen in liquid nitrogen after resection and stored at −80 °C until use.

2.2. Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was isolated from cultured cells and fresh tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, 500 ng of total RNA was reverse transcribed in a final volume of 10 μl with the Prime Script RT Master Mix (Takara, Japan). QRT-PCR was performed using the SYBR Master Mix (ABI, Foster City, CA, USA) on the ABI 7500 system (ABI). All primers were as follows: circ_0009910 (divergent primers), forward, 5′-TGAGAGGCATCAGTGAGGTG-3′ and reverse, 5′-AAGTGCTTAAGTGGGGATGC-3’; miR-499a, forward, 5′-TGCGGTGGCAGTGTATTGTTAGC-3′ and reverse, 5′-CCAGTGCAGGGTCCGAGGT-3’; U6, forward, 5′-AGCCCGCACTCAGAACATC-3′ and reverse, 5′-GCCACCAAGACAATCATCC-3’; IL6R, forward, 5′-CCCCTCAGCAATGTTGTTTGT-3′ and reverse, 5′-CTCCGGGACTGCTAACTGG-3’; β-actin forward, 5′-GGCACTCTTCCAGCCTTCC-3′ and reverse, 5′-GAGCCGCCGATCCACAC-3’. β-actin and U6 were uased as an internal control. RNA expression was quantified using $2^{-\Delta\Delta Ct}$ method.

2.3. Plasmid construction, oligonucleotide synthesis and transfection

All oligonucleotide sequences were synthesized by GenePharma (Shanghai, China). A specific siRNA for circ_0009910 was designed to target covalent closed junction. Construction of pcDNA3.1 plasmids containing circ_0009910 siRNAs (si-circ; sequence: 3′-TGGCCGCGCAATGTCCCTGCT-5′) was used to knockdown circ_0009910 and a control scrambled siRNA (si-NC; sequence: 3′-UCACCCAGAUGCCGCUAU-5′) was used as control. miR-449a mimics sequence was 5′-UGGCAGUGUAUUGUUAGCUGGU-3’; the mimic control (mim-ctrl), 5′-UUAUCUCCUGUGCGATT-3’; miR-449a inhibitor, 5′-CCACGAUGCUACGUUU-3’; inhibitor control (inh-ctrl), 5′-CAGUACAUUGGUUCUGCAA-3’. Cells were transfected with 50 pmol/ml miR-449a mimic, mim-ctrl, miR-449a inhibitor, inh-ctrl using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The medium was replaced with culture medium at 6 h after transfection. IL6R cDNA without its 3′-UTR were inserted into pcDNA3.1(+) (Invitrogen) to generate the recombinant vector pcIL6R and the empty vector (pcDNA3.1) was used as control.

2.4. Cell proliferation assay

MTT assay was used to evaluate cell proliferation. Transfected cells were seeded into 96-well plates (2000 cells per well) and cultured for 1, 2, 3, and 4 days. MTT (5 mg/ml) was added to each well for 4 h at 37 °C. Then, the medium was removed, and the reaction was stopped by 200 μl DMSO. Absorbance readings at 490 nm were obtained in triplicate using a spectrophotometric plate reader (Thermo Scientific, Waltham, MA, USA).

2.5. Cell cycle and apoptosis analysis

For the cell cycle analysis, at 48 h post-transfection, cells (2×10^5 cells) were digested by trypsin, washed twice with PBS, and fixed in 70% ethanol overnight at 4 °C. Then, cells were subject to be washed with PBS, centrifuged at 1500 rpm for 5 min and followed by treated with RNase A (0.1 mg/ml) propidium iodide (PI, 0.05 mg/ml, Sigma, St Louis, MO, USA) for 20 min at room temperature. Cell cycle analysis was performed with FACS flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

For measurement of cell apoptosis, cells were plated in 6-well plates (6×10^5 cells/well) and cultured for 24 h. Then, the cells were transfected and further incubated for 48 h. The cells were washed twice with cold PBS and stained using the Annexin V/PI detection kit (BD Biosciences). The apoptotic cells were detected using flow cytometry (BD Biosciences) according to the manufacturer's instructions. All the experiments were performed in triplicate.

2.6. Luciferase activity assay

A circRNA_0009910 segment (105bp) was synthesized with either mutant or wild-type seed region and cloned into the psiCHECK-2 vector (ABI). Five nucleotides in the seed region were mutated to obtain the mutant circ_0009910 sequences (wide type, seed sequences: 5′-CACTGCCA-3’; mutant, seed sequences: 5′-GUGACCCA-3′). MG63 cells (1×10^5 cells/well) were co-transfected with wild-type circ_0009910 or mutated type and miR-449a mimic or mimic control (mim-ctrl) using Lipofectamine 2000 (Invitrogen). After induction for 48 h, luciferase activity was assessed using the dual-luciferase reporter kit (Promega, Madison, WI, USA).

A wild-type 3′-UTR fragment of IL6R mRNA containing the putative miR-449a binding site was amplified by PCR and cloned into downstream of the firefly luciferase gene in the pMIR-REPORT vector (Thermo Scientific) to produce the pMIR-IL6R-3′UTR luciferase vector (IL6R-WT). The QuikChange Mutagenesis Kit (Stratagene, Palo Alto, CA, USA) was used to generate the mutant seed sequences and the mutated IL6R-3′UTR fragment was cloned into pMIR-REPORT vector to produce the pMIR-IL6R-3′UTR-mut vector (IL6R-MUT). For the luciferase assay in MG63 cells, cells were cotransfected in 48-well plates with IL6R-WT or IL6R-MUT, miR-449a mimics or mim-ctrl using Lipofectamine 2000 reagent (Invitrogen).

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