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miR-451 inhibits cell growth, migration and angiogenesis in human osteosarcoma via down-regulating IL 6R

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ABSTRACT

Osteosarcoma (OS) has become one of the most common primary malignant tumors in the children and adolescents with a poor prognosis owing to its high malignant and metastatic potential. Although increasing evidence indicates that miR-451 could inhibit the growth and metastasis of OS, its effect on angiogenesis in OS is still very poor. What is more, the mechanism by which miR-451 affects the OS has not been fully elucidated. In the present study, miR-451 was reduced in human osteosarcoma tissues compared with the adjacent bone tissues, and the introduction of miR-451 dramatically inhibited the growth, migration and angiogenesis in OS. Additionally, it was suggested that IL 6R is a direct target gene of miR-451. Silencing of IL 6R suppressed the growth, migration and angiogenesis of OS, which was consistent with the effect of overexpression of miR-451. In conclusion, our data demonstrate that miR-451 may function as a potential suppressor of tumor growth, migration and angiogenesis in OS via down-regulating IL 6R, suggesting a promising therapeutic avenue for managing OS.

1. Introduction

Osteosarcoma (OS), one of the most common primary malignant tumors in the children and adolescents, is characterized by rapid progression, high metastatic potential and poor clinical prognosis [1,2]. Despite recent advances in both diagnostic methods and therapeutic strategies including maximal safe surgical resection combined with standard chemotherapy and radiation, the 5 years survival rate for patients with OS was still very low [3,4]. This disappointing outcome strongly suggests us to better understand the molecular mechanisms involving OS formation and development.

Recent studies have demonstrated that dysfunction of small non-coding RNAs called microRNAs (miRNAs) is related to the initiation and progression of many cancers, including OS [5,6]. What is more, several target key gene products of ectopic miRNAs have been testified to be involved in the cell proliferation and metabasis, indicating that miRNAs can act as excellent biomarker candidates and potential therapeutic tools [7,8].

It has been demonstrated that miR-451, which acts as a tumor suppressor, is associated with several types of human cancers, including lung cancer [9] hepatocellular carcinoma [10], and gliomas [11]. More recently, miR-451 was suggested to inhibite the proliferation, migration and invasion of OS cells, indicating miR-451 acts a tumor suppressor for OS [12,13]. Angiogenesis is a prerequisite for the development and metastasis of cancer cells through supplying oxygen and nutrients [14,15]. Previous studies have shown that angiogenesis is associated with tumor metastasis in OS [16,17]. However, the relationship between miR-451 and angiogenesis in OS has not been fully understood.

IL 6, a multifunctional cytokine, regulates the immune response, haemopoiesis and inflammation, and therefore plays a central role in diverse biological processes, including cellular proliferation, differentiation and apoptosis [18,19]. It exerts its biological activities through activating its receptor IL 6R. High expression of IL 6R and IL 6 have been widely documented in many tumors [20,21]. Moreover, as a key tumor suppressor, IL 6R is becoming a critical target in inhibiting tumorigenesis [20]. Thus, elucidating the molecular mechanism of IL 6R regulation in OS is important for future OS treatment.

Therefore, we confirmed the expression of miR-451 in OS tissue specimens and OS cell lines by quantitative reverse transcription-polymeras chain reaction (qRT-PCR). Moreover, we investigated the effects and mechanisms of miR-451 on the growth, migration

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and angiogenesis in OS cells.

2. Materials and methods

2.1. Cell culture and tumor specimens

The Human OS cell lines (U2OS, HOS and MG-63) and the normal human osteoplastic cell line (NHOst) were purchased from the Type Culture Collection of the Chinese Academy of Sciences(-Shanghai, China). They were cultured in Dulbecco modified Eagle medium (DMEM) contained 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 ng/mL streptomycin. OS samples and the matched adjacent noncancerous tissues were collected from 10 patients undergoing OS resection. All procedures were approved with the Local Research Ethics committee.

2.2. Oligonucleotides and cell transfection and generation of stablytransfected cell lines

MG 63 cells were seeded into 6-well, 12-well, 24-well, or 96-well plates and incubated at 37 °C and 5% CO_2 overnight. MiR-451 mimics and miR control were chemically synthesized by GenePharma (Shanghai, China). Cells were transfected with miR-451 mimics or miR control using lipofectamine reagent (Invitrogen, California) and transfected with si IL 6R or siRNA controls using Lipofactamine 2000 reagent after cells reached 60%–70% confluence. Cells were harvested for further experiments 24 h after transfection. Tumor conditioned medium(TCM) was centrifuged sequentially at 500 g to discard the detached cells, followed by 10,000*g* centrifugation to remove cell debris at 4 °C for 10 min. The supernatant was then gathered and stored at −80 °C for the subsequent experiments.

2.3. RNA extraction and real-time reverse transcription polymerase chain reaction

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen) and stored at −80 °C until use. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis for mature miR-451 was carried out in triplicate using the Prime Script RT Reagent kit (Takara, Dalian, China) according to the manufacturer's instructions. Total RNA (500 ng) was converted to complementary DNA for qRT-PCR and was performed using SYBR Premix DimerEraser (Takara) on a 7900HT system. The expression of miR-451 in each group was calculated relative to that of U6, and fold changes were calculated by relative quantification ($2^{-\triangle\triangle Ct}$).

2.4. Cell proliferation assay

Cell viability was monitored by Cell Counting kit (CCK)-8 (Dojindo, Kumamoto, Japan). For MG 63 cells proliferation assay, MG 63 Cells(5×10^3) were seeded onto 96-well plates and incubated for 1, 2, 3 and 4 days. For HUVECs proliferation assay, HUVECs were seeded onto 96-well plates at the density of 4×10^3 cells/well. Then, cells were incubated with the different TCM for 1, 2 and 3 days. Subsequently, 10 μl of CCK8 reagents were added to the 96-well plates, after 2 h incubation at 37 °C, the absorbance at 450 nm was measured to evaluate the number of viable cells by a microplate reader.

2.5. Colony formation assay

MG 63 cells were plated in 6-well culture plates at a density of 100 cells/well. After incubation for 10 days at 37 °C, cells were washed three times with phosphate buffered saline(PBS) and stained with 0.1% crystal violet for 20 min. The number of colonies containing >50 cells was counted under a microscope.

2.6. Migration assay

The migration assay of MG 63 cells was conducted in 24-well BD Matrigel invasion chambers (BD Biosciences, Cowley, United Kingdom) according to the manufacturer's instructions. Briefly, 5×10^4 MG 63 cells were seeded in the upper well of the migration chamber in DMEM without serum, and 500 μl DMEM supplemented with 10% FBS were added to the lower chamber well. After 24 h incubation, the cells on the top of the well were removed with a cotton swab, and the bottom cells were fixed with 4% paraformaldehyde, subsequently stained with 0.1% crystal violet for 30 min. Images were captured in 5 independent fields.

2.7. Wound healing assay

For the wound healing assay, 1.5×10^5 cells/well were seeded on 6-well plates supplemented with culture medium. Cells were cultured in serum-free medium for 10 h prior to the assay, and wounds were subsequently scratched into the confluent cell monolayer using a P200 pipette tip. The migration of the cells and the closing of the scratch wound were observed and the images were captured at 0 h and 24 h. The experiments were performed in triplicate and the whole assay was repeated three times.

2.8. Western blot

Cells were treated as described previously. Then, cells were harvested and lysed on ice for 30 min in radioimmunoprecipitation assay buffer supplemented with protease inhibitors. After a centrifugation, protein concentrations were determined by the bicinchoninic acid method (Beyotime, China) and separated by 10% SDS-polyacrylamide gel electrophoresis. Subsequently, proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, USA). The membranes were blocked with 5% non-fat milk and incubated with IL 6R anti-body (Abcam, Southampton, UK), VEGF anti-body and GAPDH (Bioworld Technology [Nanjing, China]). The protein complex was detected with enhanced chemiluminescence reagents (Pierce IL, USA).

2.9. Luciferase reporter assay

The wild-type (wt) and mutant (mut) 3'UTR of IL-6R predicted to interact with miR-451 by TargetScan software (http://www.targetscan.org) were constructed and cloned to the firefly luciferase-expressing vector psiCHECK™ (Promega, Madison, WI, USA). Constructs were transfected into MG 63 cells in 24-well plates and cotransfected with miR-451 or miR-con. After 24 h incubation, luciferas assays were performed using the Dual-Luciferase Reporte Assay System (Promega, Wisconsin).

2.10. Tube formation analysis in vitro

The angiogenic ability was analyzed by tube formation assay. Briefly, a 24-well plate was precoated with Matrigel (BD Pharmingen, San Jose, CA, USA) overnight. Then, HUVECs were seeded into the plate at the density of 3×10^4 cells/well and cultured in the absence or presence of TCM for 12 h. The formation of capillary-like structures were photographed under an inverted microscope and by counting branch points in six random fields per well.

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