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Original article

MicroRNA-155 targets MAP3K10 and regulates osteosarcoma cell growth

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ABSTRACT

Osteosarcoma is the most common type of bone cancer prevalent in young adults. Recent studies suggested that aberrant microRNA expression plays an essential role in osteosarcoma pathogenesis. In this study, we found miR-155 up-regulation in different osteosarcoma cell lines U2OS, Saos-2 and MG-63. Through bioinformatic prediction and analysis, we identified its target MAP3K10 that involved in cell proliferation and apoptosis. This work demonstrates novel interaction between microRNA, intercellular MAPK signaling and apoptosis in osteosarcoma, which will provide targets for therapeutic development.

1. Introduction

Osteosarcoma is the most common form of primary malignant bone cancer, especially prevalent in children and adolescents. It is reported that approximately 4–5 cases per million [1]. This type of aggressive sarcoma is characterized by a high level of chromosomal instability and diverse karyotypes [2]. While a number of amplifications of chromosomal region 6p12 and 8q24 have been observed in osteosarcoma, oncogenes such as RB and cMYC mutations also have been associated in osteosarcomagenesis [3,4], the detailed molecular pathways involved in osteosarcoma remain unclear. Interestingly, recent studies have shown that microRNAs (miRNAs) deregulation occurs in osteosarcoma [5,6].

miRNAs is a family of small non protein coding RNAs (20–25 nucleotides) that play a critical role in the posttranscriptional gene regulation by targeting mRNAs for translational repression and/or degradation.

MiR155 host gene BIC is abundantly expressed in most tissues and cell types [7], therefore, may play a critical role in a wide range of biological processes, such as hematopoiesis, inflammation. Moreover, miR155 levels are elevated in a broad array of cancers including lymphomas, leukemia and solid tumors. Deregulation of this miRNA may also significantly participate in the development of carcinogenesis.

In this study, we investigated the role of miR155 in human osteosarcoma. We found miR155 is upregulated in a number of osteosarcoma cell lines. Inhibition of this miRNA leads to cancer cell death. We identified the target of miR155 as MAP3K10. More importantly, inhibition of this miRNA in cancer cell reduced the tumor growth in vivo. Our results revealed a novel role of miR155 in cancer cell growth and provided a new possibility of therapeutic intervention in treatment of osteosarcoma.

2. Materials and methods

2.1. Cell lines

U2OS, Saos-2 and MG-63 cells were provided by Institute of Biochemistry and Cell Biology of Chinese Academy of Science and originated from American Type Culture Collection (ATCC).

2.2. RNA extraction

Total RNA of cultured cells was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions.

2.3. RT-PCR

One μg of total RNA per sample was synthesized using the RT reagent Kit (Formentas, USA) following the manufacturer's protocol. And then, real-time PCR was performed using the Fast-Start Universal SYBR Green Master kit (Roche, Switzerland) on the

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ABI 7500 thermocycler (Applied Biosystems, USA). Primers were designed and provided by Ruibo Biotech (Guangzhou, China).

2.4. MiRNA target prediction

MicroRNA target genes were predicted using the website program (www.microRNA.org).

2.5. Luciferase assay

293T cells were transfected with MAP3K10 3′UTR plasmid, Renilla luciferase pRL-TK vector (Promega USA), and miR-155 mimics(miR10004658-1-5, Ribobio) or miR-NC mimics(miR01201-1-5,Ribobio) using lipofectamine 2000 reagent (Invitrogen, USA). After 48 h, luminescence was examine using the Dual-Luciferase Reporter Assay System (Promega, USA). Luminescence results were normalized to the Renilla luminescence.

2.6. Western blot analysis

Cell protein extracts were immunoblotted with a primary antibody and then a secondary antibody. Primary antibodies, MAP3K10 antibody(867–880, SIGMA),were used at a diluted concentration of 1:2000 while the secondary antibody(A0545, SIGMA) was used at 1:10,000.

2.7. SiRNA transfection

The human MAP3K10 siRNA(anti-MAP3K10) and negative control oligonucleotide (anti-NC) were designed and provided by Ribobio (Guangzhou, Guangdong, China). MG-63 cells were transfected with 40 nM of anti-MAP3K10 or anti-NC by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.8. Apoptosis detection and analysis

Cell apoptosis was detected using the Annexin V/PI apoptosis detection kit(BMS500FI, eBiosicence) using the manufacturer's protocol. Briefly, cells were washed by ice-cold PBS and binding buffer and were then incubated for 15 min in 100 μl of binding buffer containing 5 μl of fluorochrome-conjugated Annexin V. Cells were washed using binding buffer and 5 μl of PI. Signals were detected using flow cytometry.

2.9. Animal experiments

MG-63 cells were transfected with anti-miR-155(miR20004658-1-5,Ribobio) or anti-miR-NC(miR02201-1-5,Ribobio). After 6 h, cells were injected subcutaneously into nude mouse. Tumor sizes are measured once per week.

We got the approval of using these mice from the Bioethics committee of the Zhengzhou University. The raising of the mice accorded with the "Guide for the Care and Use of Laboratory Animals".

2.10. Statistical analysis

Data were expressed as Mean ± SEM of three independent experiments. For all statistical tests, PRISM 5.0 (GraphPad Software Inc., USA) was used. P values less than 0.05 were identified as statistically significant.

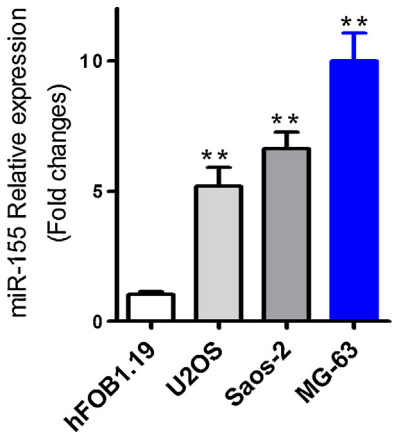

Fig. 1. Expressions of miR-155 are upregulated in human osteosarcoma cell lines. Mature miR-155 expressions were analyzed by real-time PCR and standardized to the endogenous control U6. Human osteosarcoma cell lines U2OS, Saos-2 and MG-63 were examined as well as the control cell hFOB1.19. (**$p < 0.01$).

3. Results

3.1. Human osteosarcoma cell lines overexpress miR-155

Osteosarcoma cells share some osteoblastic features, but they differ in many aspects, in particular the proliferation kinetics and osteoid production [8]. Thus, we compared three osteosarcoma cell lines of different origin, U2OS, Saos-2 and MG-63 [9], with normal human osteoblasts, hFOB1.19. We found that the expression of miR-155 is upregulated in all three osteosarcoma cell lines compared to normal osteoblasts (Fig. 1). This miRNA showed five-fold increase in U2OS, and about 10-fold increase in MG-63 cells. We focused on MG-63 cells for our following study.

3.2. MiR-155 deficiency leads to activation of apoptosis in MG-63 cells

To elucidate the function of miR155 in osteosarcoma cell line, we used anti-miRNA oligonucleotides, anti-miR-155. Unlike the transitional antisense techniques that suppress expression of target RNAs, anti-miRNA oligonucleotides appear to work primarily through a steric blocking mechanism of action. These compounds are synthetic reverse complements that tightly bind and inactivate the miRNA instead of decreasing its expression. An anti-scramble was used as negative control (anti-miR-NC). Interestingly, when MG-63 cells were transfected with anti-miR-155, their proliferation was significantly reduced (Fig. 2A) compared to U2OS and Saos-2 cells (Supplemental Fig. 1).

We next examined whether the reduced proliferation is due to apoptosis using Annexin V/PI staining. As shown in Fig. 2B, treatment of anti-miR-155 increased both doubled Annexin V positive population, and increased Annexin V and PI double positive cells about five fold. This suggests that miR155 deficiency leads to enhanced apoptosis and cell death. To confirm the activation of apoptosis upon miR155 inhibition, we tested Caspase-3/7 activity, which are the effector caspases that execute apoptosis by cleaving cellular proteins on specific Asp residues. Consistent with the Annexin V staining results, the Caspase-3/7 activity was increased when cells were treated with anti-miR-155, compared to cells treated with control anti-miR-NC (Fig. 2C). These findings demonstrate that miR-155 is important to inhibit the activation of apoptosis in osteosarcoma.