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Original Article

miR-143 inhibits osteosarcoma cell proliferation by down-regulating K-ras expression

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Abstract: Osteosarcoma is a primary bone malignancy, and it usually occurs in children and adolescents under 20 years old. Osteosarcoma is highly malignant, and the tumor cells will migrate to brain, lung, kidney, and other tissues. The mechanisms of osteosarcoma tumor genesis have not yet been fully studied. Some groups reported activation of Kras and abnormal expression of several miRNAs during osteosarcoma tumor progression. In this study, we focused on the regulatory relationship between miR-143 and Kras and its effects on osteosarcoma cell proliferation. Dual-luciferase reporter assay was performed to determine the regulatory relationship between miR-143 and Kras using wild type or mutated Kras 3'UTR inserted reporter vectors; Kras expression was detected by RT-qPCR and western blot after miR-143 mimic or miR-143 inhibitor transfection; CCK8 assay was used to check the role of miR-143 on osteosarcoma cell proliferation under different miR-143 expression levels. Dual-luciferase reporter assay showed that miR-143 targets the 3'UTR of Kras. High expression of miR-143 decreases Kras protein to 63%; while inhibited miR-143 expression elevates Kras protein to 1.67 fold. CCK8 assay revealed that increased miR-143 expression inhibits osteosarcoma cell proliferation. Kras is a target of miR-143; miR-143 inhibits osteosarcoma cell proliferation by repression of Kras.

Keywords: Osteosarcoma, miR-143, Kras

Introduction

Osteosarcoma is a common primay bone malignancy and accounts for about 22% of primary malignant bone tumors [1]. Osteosarcoma patients are mainly children and adolescents under 20 years old; the incidence rate of this disease is about 3/100,000 [2]. Osteosarcoma patients are usually with poor prognosis, because osteosarcoma is highly malignant, prone to invade and metastasize (to the lung, brain, kidney and other tissues and organs) [3, 4]. Cell proliferation and metastasis are critical in osteosarcoma invasion, so discovering the molecular mechanisms underlying osteosarcoma cell proliferation is important in control and treatment of osteosarcoma. Recent studies have found that abnormal expression of oncogenes and tumor suppressors, like P53 and Kras, are always correlated with osteosarcoma tissue [5]. Kras belongs to the ras family, and is located on chromosome 12 [6]. Kras has been found involved in regulating cell proliferation through cellular signaling pathways [7]. Misregulation of Kras expression will cause disorders of intracellular signal transduction, which will affect cell proliferation and finally lead to cancer [8]. However, the regulators of Kras expression are still elusive. The recently found small RNAs (microRNA and miRNA) provide a clue to answer this question. MicroRNAs (miRNAs) are small non-coding RNAs of 18-25 nucleotides. MiRNAs repress gene translation by binding to the 3'UTRs of their target mRNAs [9]. MiRNAs, which only account for 1% of genes, are predicted to regulate the expression about 30% of all genes [10]. Interestingly, the miR-143 has been shown to be down regulated in osteosarcoma tissues [11], however, the functional importance of miR-143 in osteosarcoma is not studied. Here we examined the proliferation of osteosarcoma cells and Kras expression under different miR-143 expression levels, and discussed how miR-143 regulates osteosarcoma cell proliferation.

Table 1. RT-qPCR target genes and primer sets

Target	Forward	Reverse
β-actin	GAGGGAAATCGTGCGTGAC	CTGGAAGGTGGACAGTGAG
FAS	CCTCTGGCACATGGCGAGAAGCGCTGG	CCGGATTGGGATTCCATCAGGCTTGACT
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
miR-143	AGUCAGUGAGAUGAAGCACUG	GTGCAGGGUCCGAGGU

Materials and methods

Equipments and reagents

Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA); Minimum Essential Medium Eagle (MEM) (Gibco, USA); Fetal Bovine Serum (FBS) (Gibco, USA); Trizol (Invitrogen, USA); RT-qPCR reagents (TOYOBO, Japan); SYBR Green Master Mix (Toyobo Life Science, Japan); Lipofectamine 2000 (Invitrogen, USA); Cell Counting Kit-8 (CCK8) (Dojindo, Japan); PBST (Sigma, USA); β-actin antibody (Abcam, USA); Kras antibody (Abcam, USA); Dual-Luciferase Reporter Assay System (Promega, USA); CO_2 incubator (Thermo Scientific, USA); RT-qPCR instruments (ABI, USA).

Cell culture

MG-63 human osteosarcoma cell line is from Shanghai Laboratory Animal Center, Chinese Academy Sciences, and cultured at 37°C with 5% CO_2.

Transfection

MG-63 cells are seeded in 12-well plates at 30%-50% confluence, with 1 ml 10% FBS/DMEM in each well. 24 h after seeding, old medium weas replaced with 800 μl fresh medium. Transfection was performed using Lipofectamine 2000 according to the manufacture's instructions. In brief, 2 μl siRNA diluted in 100 μl MEN and 3 μl lipofectamine in 100 μl MEN were mixed well and incubated for 5 mins at room temperature, then added to one well of the 12-well plate. 4-6 h after transfection, old medium was replaced with fresh medium. Gene expression levels were analyzed 48 h after transfection.

Dual-luciferase reporter assay

The dual-luciferase reporter assay system is from Promega. To do this assay, the 3'UTRs of Kras (wild type and mutant) were amplified by PCR, and then inserted into the pmirGLO dual luciferase vector through restriction enzyme digestion and ligation. pmirGLO dual luciferase vector inserted with wild type or mutated Kras 3'UTRs, pRL-TK luciferase control reporter vector, and miR-143 minic were transfected into MG-63 cells using Lipofectamine 2000. Dual-luciferase reporter assays were performed 48 h after transfection.

RNA isolation, cDNA systhesis, and RT-qPCR

Total RNA was isolated using Trizol reagent according to the manufacture's protocol. To synthesize cDNA, 1 μg RNA was incubated at 65°C for 5 mins, put in ice for 2 mins, and then mixed with iScript cDNA synthesis reagents, and incubated at 37°C for 15 mins, and 98°C for 5 mins. RT-qPCR was performed using the newly synthesized cDNA, specific primers (as listed in **Table 1**), and SYBR green mix in a 20 μl system with VIIA™ 7 Real-Time PCR System (Thermo Scientific, US). The RT-qPCR reaction started with a 5 mins denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 45 s, and extension at 72°C for 15 s.

Western blot to detect Kras expression

MG-63 cells were collected in EP tubes, lysed in RIPA buffer with protease inhibitors on ice for 30 mins, and then centrifuged at 12,000 g for 10 mins. Supernatants were collected and protein concentrations were measured using Bradford assay. To prepare the protein samples for SDS-page, desired amounts of RIPA buffer and 5xSDS sample buffer were added to the cell lysis to make the final protein concentration 2 μg/μl, and then boiled at 100°C for 5 mins. SDS-page was performed using 30 μl protien samples with a 7%-16% gradient separating gel and a 4% stacking gel at 12 mA. For Western blot, the polyacrylamide gel was transfered to a nitrocellulose membrane at 300 mA. The membrane was blocked with 5% nonfat dry milk in PBST for 2 h at room temperature. After 3 washes in PBST, membrane was incubated at 1:1000 diluted primary antibody at 4°C overnight. After washing for 3 times with PBST,

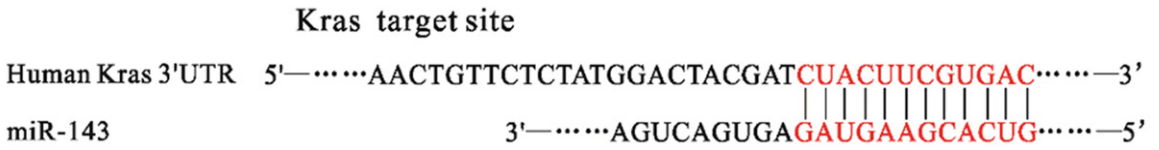


Figure 1. miR-143 targets the 3'UTR of Kras mRNA.

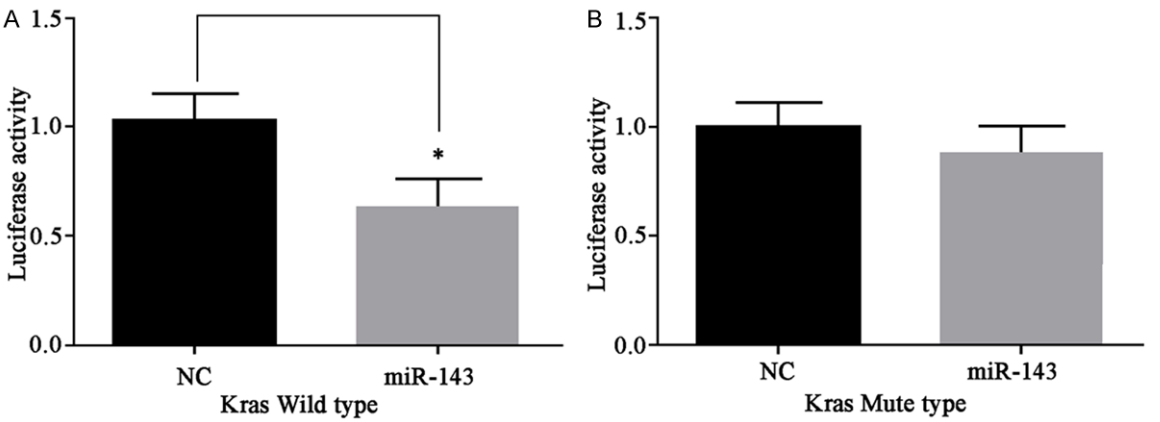


Figure 2. Dual-luciferase reporter assay using wild type (A) or mutant (B) luciferase reporter vectors (*P<0.05).

membrane was then incubated at 1:10000 diluted secondary antibody for 1 h at room temperature. Then membrane was treated with ECL and developed into a film. Band intensities were measured and quantified using the GIS-2020D Full Automatic Digital Gel Imaging Analysis System.

CCK8 cell viability assay

MG-63 cells were seeded in 96-well plates with 0.5x104 cells per well. Cells were divided into 6 groups: NC, miR-143 mimic, NC inhibitor, miR-143 inhibitor, siNC, and Kras RNAi, with 6 replicate wells per group. Culture medium was removed and 100 μl of 10% CCK8 solution was added to each well and incubated at 37°C for 4 h, 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, and 96 h after treatments. Absorption was measured using a microplate reader, and cell proliferation curve was generated by plotting OD values versus time.

Statistical analysis

All data were analyzed using SPSS 16.0 software. Numbers were showed in average ± s.d., and one-way analysis of variance (ANOVA) was used when comparing between groups. A *p*-value of <0.05 was considered significant.

Results

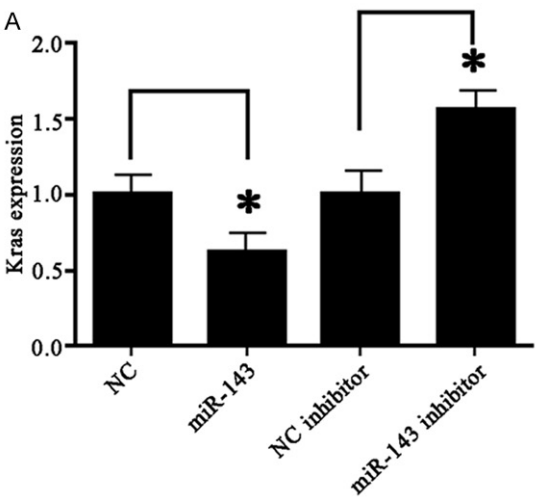
Identification of Kras as a target of miR-143

Using Targetscan and miRanda algorithms, we predicted that Kras might be targeted by miR-143, as shown in **Figure 1**.

A wild type and a mutant luciferase reporter vector were generated by cloning the wild type or mutated 3'UTRs of Kras into pimrGLO vector. Wildtype or mutant luciferase reporter vector was transfected into MG63 cells, together with miR-143 mimics. Dual-luciferase reporter assay showed that luciferase activity was significantly inhibited by miR-143 when conjugated with the wild type 3'UTR of Kras; while there were no obvious changes in luciferase activity by miR-143 when conjugated with mutated 3'UTR of Kras (**Figure 2**). These results suggest that miR-143 can target the 3'UTR of Kras mRNA, and inhibit its mRNA translation.

miR-143 regulates Kras expression

The dula-luciferase reporter assay proved that Kras is a target of miR-143. To study how miR-143 regulates Kras expresson in detail, MG-63 cells were transfected with NC (negative control), miR-143 mimic, NC inhibior, and miR-143



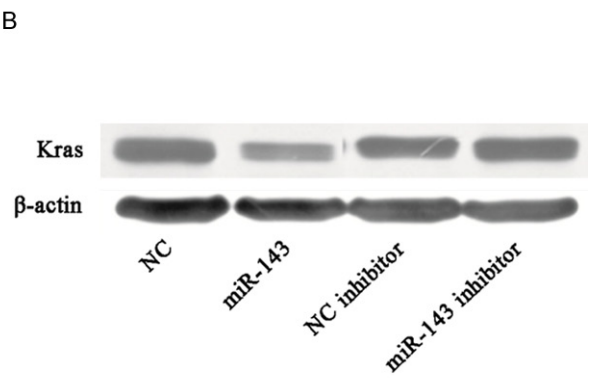


Figure 3. miR-143 regulates Kras expression at both mRNA (A) and protein (B) levels (*P<0.05).

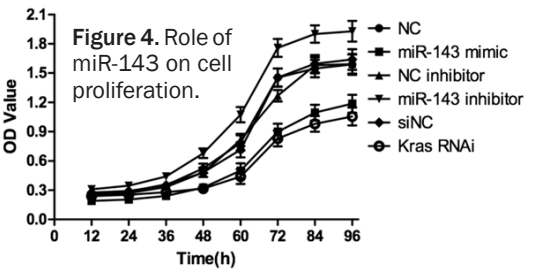


Figure 4. Role of miR-143 on cell proliferation.

inhibitor 24 h after seeding. 48 h after transfection, the mRNA level and protein expression of Kras were detected (**Figure 3**). Kras mRNA level deceased to 0.62 fold in miR-143 mimic transfected cells (high miR-143 level) compared with NC (P<0.05); Kras mRNA increased to 1.58 folds in miR-143 inhibitor transfected cells (low miR-143 level) compared with NC inhibitor transfected cells (P<0.05) (**Figure 3A**). Western blot data showed that Kras protein expression decreased in miR-143 mimic transfected cells compared to NC; while Kras protein increased in miR-143 inhibitor transfected cells compared with NC inhibitor (P<0.05) (**Figure 3B**). These data demonstrate that miR-143 regulates Kras expression at both mRNA and protein levels.

miR-143 regulates the proliferation of MG-63 human osteosarcoma cells

CCK8 assay was used to determine the effect of miR-143 on MG-63 cell proliferation. MG-63 cells were transfected with NC, miR-143 minic, NC inhibitor, miR-143 inhibitor, siNC, and Kras siRNA respectively. CCK8 assay was performed 12 h, 24 h, 36 h, 48 h, 69 h, 72 h, 84 h, and 96 h after transfection for each group. As shown in **Figure 4**, MG-63 cells transfected with miR-143 had a significant decrease in cell number compared with the NC transfected cells; likewise, cells transfected with the miR-143 inhibitors showed stimulated cell proliferation compared with NC inhibitor transfected cells. These data prove that miR-143 inhibits MG-63 cell proliferation by down-regulating Kras expression.

Discussion

Osteosarcoma is a common primary bone malignancy, and is likely to occur in children and adolescents younger than 20 years old. Osteosarcoma is highly malignanct, prone to invade and metastasize; tumor cells can migrate to other tissues and organs, like liver, kidney, and brain [12]. Researches have proved that the dysregulated proliferation of osteosarcoma cells is a cause for its metastasis, and that Kras is abnormally expressed during the development of osteosarcoma [13]. Kras belongs to the ras family, and its abnormal expression is associated with varieties of tumor growth, such as colorectal cancer, stomach cancer, osteosarcoma, and breast cancer [14]. Recently developed gene therapy in patients with osteosarcoma has brought new hope to improve the diagnosis and treatment of osteosarcoma. Gene therapy is a treatment based on specific genes, namely by altering the expression or function of specific genes in vivo to regulate proliferation and differentiation of tumor cells, to achieve the purpose of treatment [15]. So, studying the molecular mechanisms of

osteosarcoma cell growth and proliferation, and finding specific regulating genes, are of great significance for the diagnosis and treatment of osteosarcoma.

miRNAs are non-coding RNA sequences in eukaryotic, with highly conserved sequences and functions. Mature miRNA works by targeting 3'UTR of mRNA through uncompletely complementary binding, and hence inhibits the expression of the gene. It is known that miRNAs only account for about 1% of all genes, but can regulate the expression of about 30% genes. Due to its strong regulatory function, miRNAs play significant roles in cell proliferation, differentiation, and migration [16]. Researchers have found that miRNA expression was abnormal in a variety of tumors, for example miR-125b expresses at a low level in breast cancer [17]; miR-203 is down regulated in lung cancer [18]. It has been proved that miR-143 has a low expression level in osteosarcoma, gastric cancer, cervical cancer tissues, and that miR-143 can influence tumor formation by regulating TGF-β [19], ERK-5 [20], and Bcl-2 [21] pathways. Low expression of miR-143 in osteosarcoma cells affects the proliferation of osteosarcoma cells, however, the underlying molecular mechanisms of this phenomenon require further studies.

According to previous works, miR-143 and Kras are abnormally expressed in osteosarcoma tissues. Based on Targetscan and miRanda targeting gene prediction, Kras might be a target of miR-143. To confirm that miR-143 is a regulator of Kras in osteosarcoma cells, we constructed luciferase reporter plasmids with wild type and mutated 3'UTRs of Kras mRNA. By transfecting miR-143 mimic and Kras wild type (or mutant) reporter plasmid into MG-63 osteosarcoma cells, our dual luciferase reporter assay showed that intracellular luciferase fluorescence signal significantly decreased when miR-143 was transfected together with Kras wild type reporter plasmid; whereas there were no significant changes in luciferase fluorescence signal in miR-143 and Kras mutant reporter plasmid transfected cells. These results suggest that miR-143 can targer the 3'UTR of Kras mRNA, and hence inhibits Kras gene expression. To further prove that miR-143 regulates Kras gene expression, we changed the intracullular miR-143 expression level, and then detected the changes of Kras expression. Our results showed that in cells with high expression of miR-143, both the mRNA and protein of Kras were down regulated; while when miR-143 was inhibited, Kras expression increased at both mRNA and protein levels. Kras gene expression negatively correlates with miR-143 level in osteosarcoma cells, suggesting that miR-143 can regulate the Kras gene expression. Further more, we found that cells transfected with miR-143 mimic showed a diminished proliferation capacity compared with the NC transfected cells; while cells transfected with miR-143 inhibitor showed enhance cell proliferation (compared with NC transfected cells); at the same time, cells transfected with Kras siRNA proliferated more slowly compared with siNC transfected cells. These results demonstrate that high expression of miR-143 decreases osteosarcoma cell proliferation, and that low expression of Kras gene can inhibit the devision of osteosarcoma cells.

To sum up, this paper found that Kras gene is a target of miR-143, and miR-143 can affect the proliferation of osteosarcoma cells through regulating Kras gene expression. Osteosarcoma is caused by multiple interacting factors, and its occurrence and development is also regulated by different factors. This study found that miR-143 mediated down-regulation of Kras affects osteosarcoma cells proliferation, which is one of the key processes of steosarcoma formation. The mechanism of osteosarcoma needs further studies.

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Disclosure of conflict of interest

None.

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