Original Article
MicroRNA-370 directly targets FOXM1 to inhibit cell growth and metastasis in osteosarcoma cells

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Abstract: MicroRNAs (miRNAs) are endogenous, non-coding, small RNAs, which play a critical role in regulating varieties of the biological and pathologic processes. Several reports have indicated that miR-370 acts as a tumor suppressor in varieties of tumors. However, the roles of miR-370 in osteosarcoma have not been reported. In this study, our objective was to explore the biological functions and its molecular mechanism of miR-370 in osteosarcoma cell lines, finding a therapeutic target of osteosarcoma. Our data demonstrated that miR-370 was evidently reduced in osteosarcoma cell lines, whereas FOXM1 expression was markedly increased. Up-regulation of miR-370 suppressed proliferation, arrested cell cycle and induced apoptosis in osteosarcoma cells. Besides, invasion and EMT of osteosarcoma cells was also inhibited by introduction of miR-370. Next, we found that FOXM1 expression was significantly reduced by up-regulation of miR-370. Bioinformatics analysis predicted that the FOXM1 was a potential target gene of miR-370. Luciferase reporter assay further confirmed that miR-370 could directly target the 3'UTR of FOXM1. Overexpression of FOXM1 in osteosarcoma cells transfected with miR-370 mimic partially reversed the effects of miR-370. In conclusion, miR-370 inhibited cell growth and metastasis in osteosarcoma cells by down-regulation of FOXM1.

Keywords: Osteosarcoma, miR-370, FOXM1, proliferation, invasion, EMT

Introduction

Osteosarcoma (OS) is the most frequent type of aggressive primary bone tumor and a major cause of tumor death in the pediatric age group because of its fast proliferation and early metastasis [1, 2]. Recently, the cure rate of patients with osteosarcoma is still very low in despite of the flying start in multimodal treatments including surgery, adjuvant chemo- and radiotherapy [3]. In addition, after surgery resection of the primary tumor and intensive chemotherapy, prognosis of patients with OS is also poor due to high risk of local relapse or distant metastasis [4]. However, the exact molecular mechanisms of OS are not clarified yet. Therefore, identification of novel targets for regulation of tumor growth and metastasis is important for the diagnosis, treatment, and prognosis of OS.

MicroRNAs (miRNAs) are a class of endogenous, small (approximately 22 nucleotides), non-coding RNAs [5], which regulate translation of their target genes by binding to complementary sequences in the 3'UTRs of targeted mRNAs. It has been reported that their target genes regulate a series of cell functions including cell proliferation, apoptosis, invasion and differentiation [6, 7]. Increasing reports showed that miRNAs are frequently involved in numerous cancers, including colon, lung, breast cancers and OS [8-11]. MiRNAs act as tumor suppressors or oncogenes in OS, which is dependent on the role of their target genes, such as miR-300 [12], miR-196a [13], miR-202 [14], miR-153 [15], miR-195 [16], miR-454 [17] and miR-144 [18]. These outcomes indicated that miRNAs are closely related to tumorigenesis, tumor progression and metastasis.

In recent years, it has been reported that miR-370 is decreased and acts as a tumor suppressor in non-small cell lung cancer (NSCLC) and laryngeal squamous cell carcinoma (LSCC) [19, 20], and is increased and acts as an oncogene
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in prostate cancer and gastric carcinoma [21, 22]. It has been shown that overexpression of miR-370 significantly inhibited cell proliferation and induced cell apoptosis of NSCLC cells by targeting tumor necrosis factor receptor-associated factor 4 (TRAF4) [19]. MiR-370 inhibited cell proliferation in Hep2 cells through down-regulation of forkhead box protein M1 (FOXM1) [20], whereas introduction of miR-370 promoted cell proliferation in prostate and gastric cells via suppression of forkhead box protein 01 (FOXO1) [21, 22]. However, the expression and roles of miR-370 in OS remain unclear.

In this study, we confirmed frequent down-regulation of miR-370 in human OS cell lines. Introduction of miR-370 inhibited cell proliferation arrested cell cycle and induced cell apoptosis of OS cells. Besides, invasion and epithelial-to-mesenchymal transition (EMT) of OS cells were suppressed by overexpression of miR-370. Next, we found that FOXM1, a tumor suppressor gene, was the direct target of miR-370 in OS. Restoration of FOXM1 reversed the inhibitory effects of miR-370. Therefore, our outcomes showed that miR-370 and FOXM1 might be promising therapeutic targets in OS.

**Material and methods**

**Cell culture**

Osteosarcoma cell lines including HOS, U2OS, SOSP-9607, MG63, 143B, SaOS-2 and one human normal bone cell line hFOB cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco Co., New York, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Co., New York, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO₂ on 0.1% gelatin-coated culture flasks.

**Transient transfection**

MG63 and U2OS cells were seeded in 6-well plates and incubated for 24 h, then transiently transfected with miR-370 mimic or miR-negative control of mimics (miR-NC) at a final concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s protocols. miR-370 mimic and miR-NC were purchased from RiboBio (Guangzhou, China). pcDNA3.1-FOXM1 and pcDNA3.1 vector were purchased from GeneChem (Shanghai, China).

**RNA isolation and quantitative real-time polymerase chain reaction (PCR)**

Total miRNA of MG63 and U2OS cells was isolated by Trizol reagent (Invitrogen) following the manufacturer’s instruments. Ten microgram RNA was used for gene-specific reverse transcription PCR using one-step RT-PCR kit (Qiagen, Venlo, The Netherlands) following the manufacturer’s protocols. Denaturation was performed at 95°C for 5 min, followed at 94°C for 15 s for 30 cycles, and 75°C for 2 min. Small unclear RNA U6 was used to normalize. All real-time experiments were conducted in triplicate.

**Cell counting kit-8 assay**

Cell proliferation was detected by the Cell Counting Kit-8 assay (CCK-8, Dojindo, Shanghai, China). Cells (1×10³ cells/well) were seeded in 96-well plates overnight. Then, cells were transfected with miR-370 mimic or miR-NC for 24 h. After that, cells were incubated in normal medium containing WST-8 substrate at 37°C for 2 h. Absorbance (450 nm) of the medium was detected using a spectrophotometer by assessing the cell proliferation.

**Cell cycle analysis**

Cells were transfected with miR-370 mimic or miR-NC for 24 h. After transfection, Cells were collected by trypsinization, washed with ice-cold phosphate buffer saline (PBS), and fixed in ice-cold 70% methanol overnight. Then, cells were centrifuged, resuspended in ice-cold PBS, and incubated with RNase (Sigma Chemical Co., USA) for 30 min at 37°C, and then were incubated with propidium iodide (PI; Sigma Chemical Co., USA) at room temperature for 30 min. The analyses of cell cycle distribution were performed by FACScan flow cytometer (BD Biosciences, San Jose, CA, USA).

**Annexin V-FITC/PI analysis**

Cells were transfected with miR-370 mimic or miR-NC for 24 h. After transfection, Cells were collected by trypsinization, washed with ice-cold phosphate buffer saline (PBS), and fixed in ice-cold 70% methanol overnight. Then, cells were centrifuged, resuspended in ice-cold PBS, and incubated with RNase (Sigma Chemical Co., USA) for 30 min at 37°C, and then were incubated with propidium iodide (PI; Sigma Chemical Co., USA) at room temperature for 30 min. The analyses of cell cycle distribution were performed by FACScan flow cytometer (BD Biosciences, San Jose, CA, USA).
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Kit (BD Biosciences) following the manufacturer's protocols. Then, each sample was quantitatively analyzed at 488 nm emission and 570 nm excitation by FACSCalibur flow cytometer (BD Biosciences).

Transwell invasion assay

To determine the effect of miR-370 on the cell invasion, we used Transwell matrigel invasion assay. Transwell chambers (8-mm pore size; Minipore) precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ) that contained extracellular matrix proteins was used following the manufacturer's protocol. Briefly, cells were transfected with miR-370 mimic or miR-NC. After transfection, cells were suspended in 100 μl serum-free DMEM and seeded on the upper chamber. 600 μl DMEM containing 10% FBS was added to the lower chamber. After 24 h incubation at 37°C in a 5% CO₂ atmosphere, cells on the surface of the upper chamber were removed by cotton swabs and invading cells were fixed in 70% methanol, and then stained with 0.1% crystal violet. Cell invasion was quantified by counting cells on the lower surface using phase contrast microscope (Olympus, Tokyo, Japan).

Western blot analysis

Cells were harvested and then lysed in RIPA (Beyotime Institute of Biotechnology Jiangsu, China). The protein concentration of cell lysates was quantified by BCA Kit (Beyotime Institute of Biotechnology Jiangsu, China), and equal amounts of proteins were separated by 8% SDS-PAGE, and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked in 5% non-fat milk at room temperature for 1 h and incubated overnight at 4°C with specific primary antibody respectively: anti-FOXM1 (1:1000; Abcam, USA); anti-E-cadherin and anti-N-cadherin (1:1000; Cell Signaling Technology Inc, MA, USA). The membranes were then incubated with a goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase secondary antibody (1:1000; Cell Signaling Technology Inc, MA, USA) for 3 h. The proteins were visualized using ECL reagents (Amersham Biosciences, Sweden). The density of the bands was assessed by the Image J software (USA), and values were normalized to the densitometric values of α-tubulin in each sample.

Luciferase reporter assay

Cells (1×10⁵/well) were seeded in 24-well plates and incubated for one day before transfection. pMiR-FOXM1-3'UTR wild-type or mutant reporter plasmid and pRL-SV40 renilla plasmid (Promega, USA) were co-transfected with miR-370 mimic or miR-NC into cells using Lipofectamine 2000. After 24 h, luciferase activity was quantified using the dual-luciferase assay reporter system (Promega, Fitchburg, WI, USA). The relative ratios of firefly to Renilla activity were reported. All experiments were performed in triplicate.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA). Data from each group were expressed as mean ± standard error of the mean (S.E.M.) and statistically analyzed by Student’s t test. Differences were considered statistically significant at a P value of <0.05.

Results

MiR-370 expression was reduced in osteosarcoma cell lines

To detect the expression of miR-370 in OS cells, six osteosarcoma cell lines (HOS, U2OS, SOSP-9607, MG63, 143B and SaOS-2) and hFOB,
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Figure 2. Effects of miR-370 overexpression on cell proliferation, cell cycle and apoptosis in MG63 and U2OS cells. MG63 and U2OS cells were transfected with miR-370 mimic or miR-NC for 24 h. A: The mRNA levels of miR-370 in MG63 and U2OS cells were determined by RT-PCR. B: Cell proliferation was assessed by CCK-8 assay. C: Cell cycle was detected by flow cytometry. D: Cell apoptosis was measured by flow cytometric analysis of cells labeled with Annexin-V/PI double staining. All data are presented as mean ± SEM, n=6. ##P<0.01 vs. miR-NC.
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a human normal bone cell line, were used to determine the expression of miR-370 by RT-PCR. Our findings showed that the expression of miR-370 was markedly down-regulated in these six OS cell lines compared to that in hFOB cells, as shown in Figure 1.

MiR-370 inhibited cell proliferation, induced G1-phase arrest and cell apoptosis in osteosarcoma cells

According to the down-regulation of miR-370 in osteosarcoma cells, we considered that miR-370 could function as a tumor suppressor. Among these OS cell lines, MG63 and U2OS cells were used to study further. We transfected miR-370 mimic into MG63 and U2OS cells. After transfection with miR-370 mimic, the RT-PCR analysis showed that mRNA level of miR-370 was significantly up-regulated in miR-370 mimic group compared to miR-NC group (Figure 2A). These data demonstrated that we efficiently enhanced miR-370 expression in MG63 and U2OS cells. The CCK-8 assays confirmed that introduction of miR-370 dramatically inhibited the proliferation of MG63 and U2OS cells (Figure 2B). Since miR-370 evidently suppressed proliferation of MG63 and U2OS cells, we guessed that miR-370 could block G1-to-S transition in osteosarcoma cells. Next, we used low cytometry to prove this hypothesis. We found that overexpression of miR-370 caused an obvious G1-phase arrest in both MG63 and U2OS cells compared with cells transfected with miR-NC (Figure 2C). Therefore, miR-370 might inhibit the proliferation of osteosarcoma cells by blocking the G1/S cell cycle transition. Furthermore, we also detected the pro-apoptotic effect of miR-370 on MG63 and U2OS cells. Then, the total apoptosis rates of MG63 and U2OS cells were detected by flow cytometry analysis. As shown in Figure 2D, the data showed that the number of apoptotic MG63 and U2OS cells was higher in miR-370 mimic group than that in miR-NC group.

Up-regulation of miR-370 suppressed invasion and EMT of osteosarcoma cells

To explore the effects of miR-370 on invasion and EMT in osteosarcoma cells, we used Transwell invasion assays to estimate the invasion potential of MG63 and U2OS cells. Our data showed that the invasion potential of osteosarcoma cells was significantly inhibited in miR-370 mimic group compared to miR-NC group (Figure 3A). Besides, we used Western blotting to confirm the effects of miR-370 mimic on the expressions of EMT markers in MG63 and U2OS cells. Introduction of miR-370 could enhance the expression of epithelial marker E-cadherin, and reduce the expression of mesenchymal marker N-cadherin in MG63 and U2OS cells (Figure 3B). Altogether, our data demonstrated that miR-370 could suppress the invasion and EMT in osteosarcoma cells.

miR-370 negatively regulated FOXM1 gene expression by directly targeting its 3’-UTR

We used the TargetScan 6.2, a miRNA target analysis tool, to investigate the potential target of miR-370. As a result, FOXM1 was a binding target of miR-370 (Figure 4A). Besides, we performed Western blotting to observe the expression of FOXM1 on protein level in MG63 and U2OS cells transfected with miR-370 mimic. Overexpression of miR-370 in MG63 and U2OS cells remarkably decreased the protein level of FOXM1 (Figure 4B). Next, we further demonstrated whether FOXM1 was a direct target of miR-370 by using luciferase reporter assay. Then, the wild-type (WT) FOXM1 3’-UTR was cloned into a luciferase reporter vector and the putative miR-370 binding site in the FOXM1 3’-UTR was mutated. The results showed that overexpression of miR-370 significantly decreased the luciferase activity of pMIR-FOXM1 3’-UTR WT, whereas mutation of the miR-370-binding site in the FOXM1 3’-UTR abolished the effect of miR-370 (Figure 4C). These results suggested that FOXM1 was directly and negatively regulated by miR-370.

Reintroduction of FOXM1 reversed the effects of miR-370 in osteosarcoma cells

To confirm whether FOXM1 indeed acted as a direct target of miR-370, we reintroduced FOXM1 into MG63 and U2OS cells transfected with miR-370 mimic to investigate. Reintroduction of FOXM1 could significantly increase the expression of FOXM1 compared with MG63 and U2OS cells transfected with miR-370 mimic and pcDNA vector (Figure 5A). Analysis by CCK-8 assay showed that up-regulation of FOXM1 in cells transfected with the miR-370 mimic enhanced the proliferation of osteosarcoma cells (Figure 5B). The Transwell assay showed that reintroduction of FOXM1 significantly reversed the inhibitory effect of the miR-370 mimic on invasion of osteosarcoma cells.
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Furthermore, increased FOXM1 expression decreased E-cadherin expression, and increased N-cadherin expression in MG63 and U2OS cells transfected with miR-370 mimic (Figure 5D). Therefore, the inhibitory effects of miR-370 were reversed by FOXM1 over-expression.

**Discussion**

Several reports have indicated that miR-370 functions as a tumor suppressive gene or oncogene and plays a critical role in human cancers. MiR-370 was down-regulated in NSCLC [19] and LSCC [20]. However, Wu et al. reported...
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miR-370 was up-regulated in prostate cancer cell lines [21]. MiR-370 was increased in patients with gastric carcinoma [22]. The precise roles of miR-370 in OS remained unclear because of its tumor-suppressing or tumor-promoting function. Therefore, in this study, we were aimed to elucidate the expression and biological functions of miR-370 in OS. Our findings showed that miR-370 was evidently decreased in OS cell lines compared to human normal bone cell line. Based on these results, we guessed that miR-370 might be a potential anti-oncogene in OS. As far as we knew, introduction of miR-370 significantly suppressed cell proliferation, invasion, EMT and induced apoptosis of MG63 and U2OS cells. Our current findings indicated that miR-370 played critical roles in regulation of proliferation, cell cycle,
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Figure 5. Overexpression of FOXM1 partially rescued miR-370-inhibited cell proliferation, invasion and EMT in osteosarcoma cells. MG63 and U2OS cells were transfected with either miR-370 mimic with or without pcDNA-FOXM1 vector. A: The protein expression of FOXM1 was determined by Western blot. α-tubulin was detected as a loading control. B: Cell proliferation was assessed by CCK-8 assay. C: The invasion of MG63 and U2OS cells was assessed by Transwell assay. D: The expressions of E-cadherin and N-cadherin were determined by Western blotting in MG63 and U2OS cells, respectively. α-tubulin was detected as a loading control. All data are presented as mean ± SEM, n=6. **P<0.01, ***P<0.001 vs. miR-370 mimic + pcDNA.
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apoptosis, invasion and EMT in OS and might be potential therapeutic target.

Next, we explored the exact molecular mechanism of miR-370 in suppressing proliferation, invasion, EMT and inducing apoptosis in OS cells. The real-time PCR, Western blot and luciferase reporter assay demonstrated that FOXM1 was a direct target of miR-370. Importantly, we also showed that up-regulating FOXM1 expression partly reversed the inhibitory effects of miR-370 overexpression on proliferation, invasion and EMT of OS cells. Consequently, we demonstrated that miR-370 played critical roles in the inhibition of proliferation, invasion and EMT in OS cells partially by down-regulating FOXM1 expression.

In this study, CCK-8 assays showed that up-regulation of miR-370 could evidently suppress the proliferation of MG63 and U2OS cells. Cell cycle analyses also showed that the percentage of cells in the G1-phase was increased and the percentage of cells in the S-phase was decreased in cells transfected with miR-370 mimic compared to cells transfected with miR-NC. Moreover, flow cytometry analysis demonstrated that miR-370 mimic could evidently induce apoptosis of MG63 and U2OS cells compared with miR-NC group. In addition, Transwell assay showed that miR-370 mimic dramatically inhibited the invasion of MG63 and U2OS cells compared with miR-NC group. Furthermore, we determined the change of EMT markers in MG63 and U2OS cells transfected with miR-370 mimic. EMT, characterized by different regulations of epithelial and mesenchymal genes, played important role in the process of tumor metastasis [23], which not only changed cell morphology but also induced cells to acquire essential new functions like migration and invasion. The up-regulation of mesenchymal marker N-cadherin and the down-regulation of epithelial marker E-cadherin were closely related to EMT [24, 25]. Our results showed that up-regulation of miR-370 could markedly suppress invasive ability of OS cells by dramatically up-regulating E-cadherin expression and down-regulating N-cadherin expression, which supported that miR-370 might suppress EMT process to restrain cell invasion and metastasis.

FoxM1 is a member of an evolutionarily conserved family of transcription factors characterized by a DNA-binding domain called the Forkhead Box [26-28], and it has been shown to be up-regulated in multiple human cancers, such as cervical cancer [29], bladder cancer [30], breast cancer [31] and acute myeloid leukemia [32]. Overexpression of FOXM1 was found to be associated with aggressive phenotype and poor prognosis in patients with breast cancer [31]. Furthermore, Yang et al. reported that FOXM1 also promotes the EMT of breast cancer cells through stimulating the transcription of Slug [33]. These data strongly indicate that FOXM1 plays an important role in the growth and metastasis of human breast cancer. In this study, our results demonstrated that FOXM1 was a target of miR-370. Besides, restoration of FOXM1 reversed the inhibitory effects of miR-370, suggesting that FOXM1 might play a critical role in progression and metastasis of OS.

In conclusion, our data have showed that the level of miR-370 was significantly decreased in OS cells. Introduction of miR-370 inhibited proliferation, invasion, EMT and induced apoptosis of OS cells via directly targeting FOXM1. This novel miR-370/FOXM1 axis might provide new insights into the molecular mechanisms underlying progression and metastasis of tumor, and overexpression of miR-370 might be a potential therapeutic target for OS treatment in the future.

Disclosure of conflict of interest

None.

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