Original Article

MicroRNA-132 inhibits human osteosarcoma cell proliferation and migration by targeting SOX4

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Abstract: Dysregulation of microRNA-132 (miR-132) has been observed in various types of human cancers. We investigated the biological functions and associated molecular mechanisms of miR-132 activity in human osteosarcoma tissues and cells. miR-132 expression was measured in human osteosarcoma tissues by quantitative real-time polymerase chain reaction. miR-132 mimics, inhibitors, and negative controls were transfected into osteosarcoma cells. Cell proliferation and migration were measured by CCK-8 and wound healing assays, respectively. Luciferase reporter assays and western blotting were performed to confirm the expression of miR-132 target genes. miR-132 expression in osteosarcoma tissues was dramatically decreased compared to that in the adjacent normal tissues. Additionally, miR-132 overexpression significantly suppressed the proliferation and migration ability of osteosarcoma cells in vitro, whereas the opposite effect was observed after transfection of a miR-132 inhibitor. Moreover, sex-determining region Y-related high mobility group box 4 (SOX4), identified as a target gene of miR-132, was inversely correlated with miR-132 expression in osteosarcoma tissues. Furthermore, downregulation of SOX4 expression by siRNA inhibited proliferation and invasion in MG63 cells. Our findings indicate that miR-132 overexpression inhibits the proliferation and migration of osteosarcoma cells by downregulating SOX4. These results suggest that miR-132 could be considered a potential therapeutic target in osteosarcoma.

Keywords: miR-132, SOX4, osteosarcoma, proliferation, migration

Introduction

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents, and is associated with high mortality [1]. Current therapies incorporate surgical resection and combinational chemotherapy (doxorubicin and cisplatin with or without methotrexate), which are curative in approximately 70% of patients [2]. However, the survival rate of patients with metastatic or relapsed osteosarcoma has remained virtually unchanged over the previous few decades, with an overall 5-year survival rate of approximately 50-70% [3, 4]. Additionally, studies have demonstrated that different genetic alterations might occur within the same clinical tumor stage, varying the response to chemotherapy and metastatic capabilities in some tumors [5]. Therefore, a better understanding of the pathogenesis and molecular mechanisms involved in osteosarcoma progression is essential to develop novel strategies for targeted therapy.

The sex-determining region Y-box 4 (SOX4) gene, part of the SOX family, has been shown to play an important role in the development and progression of cancer [6]. SOX4 is highly conserved in vertebrates [7, 8]. Recently, increasing evidence has suggested that Sox4 expression is significantly elevated in various cancers such as breast, prostate, and colon [9-11]. Sox4 overexpression is also associated with poor clinical prognosis and it is therefore an ideal marker to predict treatment outcomes [12].

MicroRNAs (miRNAs) are small, non-coding RNA molecules that bind to the 3’-untranslated region (UTR) of target mRNAs, resulting in translational repression or mRNA degradation. Thus, these molecules play important roles in a vari-
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A variety of biological events such as proliferation, development, differentiation, and apoptosis [13, 14]. Recently, several miRNA profiling studies have revealed that miRNA-132, a member of the miR-212/132 family, is abnormally expressed in various human cancers such as lung cancer, breast cancer, and colorectal carcinoma. Additionally, miR-132 might modulate tumorigenesis and the behavior of cancer cells by suppressing a number of oncogenes [15-17]. Gougelet et al [18] showed that miR-132 expression is decreased in osteosarcoma and has the statistically significant ability to predict ifosfamide response. Additionally, a precursor of miR-132 was shown to significantly prevent osteosarcoma cell proliferation in vitro and in vivo tumor growth via the downregulation of cyclin E1 [19]. However, the link between miR-132 dysregulation and the clinicopathological characteristics of osteosarcoma remains unknown.

In this study, we determined the expression and function of miR-132 in osteosarcoma. Furthermore, we investigated the molecular mechanism of miR-132 involvement in this disease by identifying a possible target gene, namely SOX4.

Materials and methods

Patients and samples

Human osteosarcoma tissues and adjacent normal tissues were collected at the time of surgical resection from 96 patients from June 2012 to May 2014 at the Department of Orthopaedics, the Second Affiliated Hospital of Nantong University. Human tissues were immediately aliquoted, labeled, and stored at -80°C until use. Signed informed consent was obtained from all patients and the study was approved by the Institute Research Ethics Committee of the Second Affiliated Hospital of Nantong University.

Cell lines and culture

Osteosarcoma cell lines (MG63, U-2OS, and Saos-2) were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI 1640 medium (GIBCO, Shanghai, China) supplemented with 10% fetal bovine serum (GIBCO). Cultures were incubated at 37°C with 5% CO₂ in a humidified incubator.

Cell transfection

The miR-132 mimics, as well as inhibitor, and non-specific miRNA negative control molecules were purchased from RiboBio Company (China). Small interfering RNA against SOX4 (si-SOX4) and the negative control (si-NC) were designed by Genepharma Company (China). For transfection, cells were cultured in a 12-well plate and transiently transfected at 40% to 50% confluence using riboFECT™ CP Reagent and Buffer according to the manufacturer protocol, at a final concentration of 50 nM. After transfection and culture for 48 h, cells were collected for western blot and quantitative real-time polymerase chain reaction (qRT-PCR) analyses.

RNA isolation and quantitative real-time PCR

Total RNA from tissue samples and cell lines was harvested using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Expression of mature miRNAs was assayed using a Taqman MicroRNA Assay (Applied Biosystems) specific for hsa-miR-132, whereas qRT-PCR was performed using an Applied Biosystems 7900 Realtime PCR System and a TaqMan Universal PCR Master Mix according to the manufacturer’s protocol. All primers were obtained from TaqMan miRNA Assays. Small nuclear U6 snRNA (Applied Biosystems) was used as an internal control. Differences in gene expression, expressed as fold-changes, were calculated using the 2-ΔΔCt method.

Cell proliferation assay

The Cell Counting Kit-8 (CCK-8, Beyotime Biotech, Jiangsu, China) assay was used for cell proliferation analysis in accordance with the manufacturer’s protocol. Cells were seeded in 96-well culture plates and incubated for 24, 36, and 48 h at 37°C in a humidified atmosphere with 5% CO₂. The absorbance at 450 nm was then measured using a microplate reader (BioTek, USA).

Cell migration assay

Migration ability was determined using a wound-healing assay. The cells were plated in 6-well plates without antibiotics; 24 h later, normal and transfected cells were wounded using a sterile plastic 100-μL micropipette tip. Floating debris was washed with phosphate-buffered saline (PBS) and cells were subse-
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...subsequently cultured in serum-free medium. The width of the wound was measured at 0, 12, and 24 h, and three or four different locations were visualized and photographed under a phase-contrast inverted microscope.

**Dual-luciferase reporter assay**

The 3'-UTR sequence of SOX4, which was predicted to interact with miR-132, or a mutant sequence with the predicted target sites, were synthesized and inserted into pMir-Report (Ambion, USA), yielding pMir-Report-SOX4. Mutations within the potential miR-132 binding sites were generated by nucleotide replacement of the wild-type sequence to inhibit miR-132 binding. Cells were cultured in 12-well plates and transfected with the pMir-Report vectors containing the 3'-UTR variants and miR-132 mimics for 48 h. The pRL-SV40 vector (Promega, USA) carrying the Renilla luciferase gene was used as an internal control to normalize for transfection efficiency. Luciferase values were determined using the Dual Luciferase Reporter Assay (Promega, USA).

**Protein isolation and western blot analysis**

Tissues and cells were washed with PBS, lysed on ice using lysis buffer, and cleared by centrifugation at 12,000 rpm at 4°C for 10 min. Proteins were quantified, separated on a 10% sodium dodecyl sulfate-agarose gel, and transferred to a nitrocellulose membrane (Amer- sham Bioscience, Buckinghamshire, U.K.). The membranes were blocked with 5% non-fat, dry milk in tris-buffered saline with 0.1% tween-20 at room temperature for 2 h, and incubated with anti- Sox4 (1:1,000, Santa Cruz) or GAPDH antibody (1:3,000, CST) at 4°C overnight. Blots were washed and incubated with appropriate horseradish peroxide-conjugated secondary antibody at room temperature for 2 h. Antibody complexes were visualized and quantified using an enhanced chemiluminescence-western blotting detection system (Tanon, Shanghai China).

**Statistics**

Data are expressed as the mean ± standard error of the mean (SEM) from at least four separate experiments. Differences between groups were analyzed using a Student's t-test or one-way analysis of variance analysis. A P value of < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 20.0 (SPSS, Chicago, IL).

**Results**

**miR-132 is downregulated in osteosarcoma tissues**

To determine the levels of miR-132 in osteosarcoma tissues, 96 human osteosarcoma tissues and adjacent non-tumor tissues (NC) were used. As shown in **Figure 1**, results from qRT-PCR showed that miR-132 was significantly downregulated in osteosarcoma tissues in comparison with adjacent normal tissues (0.41 ± 0.16 versus [vs.] 1 ± 0.39, P < 0.01).

**miR-132 plays a role in cell proliferation and migration in vitro**

To investigate the role of miR-132 in the pathogenesis of osteosarcoma, miR-132 mimics, an inhibitor, and the corresponding negative control were synthesized and transfected into human osteosarcoma cell lines, namely MG63, U-20S, and Saos-2. The results from qRT-PCR analysis showed that the mRNA level of miR-132 was significantly upregulated in the miR-132-mimic group and downregulated in the miR-132-inhibitor group compared to that in the negative control group (all P < 0.01, **Figure 2A-C**). To determine the effect of miR-132 on the proliferation of osteosarcoma cells, we per-
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Figure 2. Effect of miR-132 on osteosarcoma cell lines. A-C: miR-132 mimics increased the expression of miR-132, and a miR-132 inhibitor decreased the expression of miR-132, compared to that in the miR-132 negative control in osteosarcoma cell lines (MG63, U-2OS, and Saos-2). D-F: A CCK-8 proliferation assay showed that overexpression of miR-132 significantly inhibited the proliferation of osteosarcoma cells compared to the negative control. Conversely, a miR-132 inhibitor promoted cell proliferation. G-I: A wound-healing assay showed that overexpression of miR-132 significantly inhibited the migration of osteosarcoma cells compared to that of the negative control. Conversely, a miR-132 inhibitor promoted cell migration. *P < 0.05 and **P < 0.01 vs. negative control group.

formed a CCK-8 assay. The results demonstrated that compared to the negative control, the proliferation rate was markedly suppressed through overexpression of miR-132 (all P < 0.01, Figure 2D-F). To assess whether miR-132 affects the migration of osteosarcoma cells, a wound-healing assay was performed. Our results showed that miR-132 mimics significantly reduced the migration of osteosarcoma cells, when compared to the negative control, at 24 h (P < 0.05, Figure 2G-I). As expected, when cells were transfected with a miR-132 inhibitor, we found that downregulation of miR-132 promoted the growth and migration of osteosarcoma cells, compared to negative control cells (Figure 2D-I). In conclusion, our results indicate that upregulation of miR-132 could suppress cell proliferation and migration in osteosarcoma cells.

miR-132 directly targets SOX4 in osteosarcoma cells

To investigate how miR-132 inhibits cell proliferation and migration, we identified its targets using bioinformatics software (TargetScan). The results showed that the gene encoding
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Figure 3. Sox4 is a direct target of miR-132. A: Computer prediction showing that the 3'-UTR of SOX4 mRNA contained a target site for miR-132. B: A luciferase activity assay revealed that miR-132 suppressed Wt (wild type) SOX4 3'-UTR luciferase activity, whereas it had no effect on a Mut (mutant) SOX4 3'-UTR luciferase activity, when compared to that in control MG63 cells. C: The expression levels of SOX4 were inversely correlated with miR-132 in osteosarcoma tissues. D, E: The protein level of Sox4 was detected by western blotting after transfection with miR-132 mimics, an inhibitor, or a negative control in MG63 cells. GAPDH was chosen as a loading control. *P < 0.05 and **P < 0.01 vs. negative control group.

SOX4 harbored a potential miR-132-binding site (Figure 3A). Subsequently, the 3'-UTR of SOX4 was cloned and inserted into a luciferase reporter construct in MG63 cells. Overexpression of miR-132 led to an appropriate 70% reduction of luciferase activity in cells carrying the vector with the wild-type 3'-UTR when compared to activity in the negative control (P < 0.01, Figure 3B). However, mutation of the potential miR-132 binding site abolished the inhibitory effect of miR-132 (Figure 3B).

Sox4 is inversely associated with miR-132 expression in osteosarcoma tissues and cells

The expression of Sox4 in osteosarcoma tissues was significantly higher than that in adjacent non-tumor tissues. A negative association between the expression of Sox4 and miR-132 in osteosarcoma tissues was observed. We calculated the following linear regression equation: Sox4 = -2.58 × miR-132 + 3.13, with $R^2 = 0.35$ (P < 0.01, Figure 3C). MG63 cells were then transfected with a negative control, miR-132 mimics, and an inhibitor. As expected, up-regulation of miR-132 decreased the relative expression of Sox4 (0.38 ± 0.09 vs. 1.0 ± 0.22, P < 0.01, Figure 3D, 3E) and the downregulation miR-132 increased the relative expression of Sox4 (2.36 ± 0.25 vs. 1.0 ± 0.22, P < 0.01, Figure 3D, 3E). These results suggest that miR-132 inversely regulates Sox4 protein expression in osteosarcoma tissues and cells.

Knockdown of SOX4 showed similar effects as miR-132 overexpression

To further verify its role in osteosarcoma, we knocked down the expression of SOX4 using siRNA oligonucleotides (Figure 4A, 4B). As expected, SOX4 knockdown inhibited the proliferation and migration of MG63 cells (Figure 4C, 4D), with higher optical density values and migration rates as compared to those of negative control at the same time points (P < 0.05). This suggests that ablation of SOX4 mirrors the effects of miR-132 overexpression.

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Discussion

In this study, we found that the expression of miR-132 was decreased in osteosarcoma tissues and cell lines (MG63, U-20S, and Saos-2). Overexpression of miR-132 inhibited cell proliferation and migration in the three selected cell lines used in this study, whereas the opposite effect was observed with the transfection of a miR-132 inhibitor. Furthermore, bioinformatics, qRT-PCR, western blotting, and luciferase reporter assays revealed that miR-132 targets SOX4. We also observed that Sox4 expression is inversely proportional to miR-132 expression in osteosarcoma tissues and cells and that SOX4 knockdown resulted in similar effects as miR-132 overexpression.

Previous studies have demonstrated that the dysregulation of small yet important molecules, namely miRNAs, in different types of cancers including osteosarcoma, is frequently associated with cancer progression [20-22]. miR-132, arising from the miR-212/132 cluster, is located in the intron of a non-coding gene on chromosome 17 in humans [23]. It has been reported that downregulation of miR-132 inhibits proliferation, invasion, migration, and metastasis in breast cancer by targeting HN1 [24]. Recent studies have shown that miR-132 overexpression induces G1/S cell cycle arrest in osteosarcoma cells by interacting with the 3'-untranslated region of the gene encoding cyclin E1 (CCNE1), repressing its expression. Additionally, Yang et al [25] demonstrated that

Figure 4. Effect of Sox4 on cell proliferation and invasion. A, B: The protein level of Sox4 was detected by western blot after transfection with si-negative control and si-SOX4. C, D: Knockdown of Sox4 markedly decreased the proliferation and migration capacity of MG63 cells compared to the negative control. *P < 0.05 and **P < 0.01 vs. negative control group.
miR-132 expression is decreased in osteosarcoma specimens with advanced clinical stage, which correlates with positive distant metastasis and poor response to chemotherapy. Moreover, both univariate and multivariate analyses have shown that osteosarcoma patients with low miR-132 expression have poorer overall and disease-free survival, and that low miR-132 expression is an independent prognostic factor for both overall survival and disease-free survival. In our study, we obtained similar results, wherein overexpression of miR-132 inhibited cell proliferation and migration in three selected cell lines, whereas the opposite effect was observed with an inhibitor of this molecule.

At the molecular level, our results revealed that SOX4 is a direct target of miR-132 in osteosarcoma cells. Recent studies have demonstrated that SOX4 might be an important oncogene contributing to tumor progression, since SOX4 levels are significantly elevated in multiple human cancers, correlating with tumor progression and metastasis. In addition, SOX4 regulates several key signaling pathways [7, 8, 11, 26, 27], and is a transcriptional inducer of epithelial to mesenchymal transition (EMT) [26]. EMT transcriptional inducers have been identified as part of a key developmental program that is associated with cancer progression and metastasis [28]. Zhang et al [11] identified that the growth-promoting function of SOX4 during the progression of breast cancer occurs through the coordination of EMT, thereby showing that SOX4 can be considered a prognostic marker for this disease. Furthermore, it has been demonstrated that increased expression of SOX4 is required for the induction of a mesenchymal phenotype during EMT [27]. Thus, SOX4/EMT is an attractive target for therapeutic interventions, and thus it is necessary to understand its role in the progression of carcinoma to a de-differentiated and more malignant state [29].

Moreover, previous studies have shown that miRNAs may play an important role in the regulation of SOX4. Wang et al [30] reported that miR-211 inhibits gastric cancer cell proliferation and invasion, partially by downregulating SOX4. Additionally, Yeh et al [31] demonstrated the role and clinical relevance of miR-138 in ovarian cancer invasion and metastasis, suggesting a potential therapeutic strategy for the suppression of ovarian cancer metastasis by targeting the SOX4 and HIF-1α pathways. Our study showed that the expression of SOX4 is inversely correlated with miR-132 in osteosarcoma tissues. We demonstrated that SOX4 is a functional target of miR-132 in osteosarcoma and that miR-132 overexpression is correlated with the downregulation of SOX4, leading to the inhibition of osteosarcoma cell proliferation and migration.

In conclusion, the findings of our study indicate that miR-132 is downregulated, whereas SOX4 is upregulated in osteosarcoma. We demonstrated that SOX4 is a direct target of miR-132. Overexpression of miR-132 inhibits osteosarcoma cell proliferation and migration by targeting SOX4. These results suggest that miR-132 might be a potential therapeutic target for osteosarcoma treatment.

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Disclosure of conflict of interest

None.

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