MicroRNA-150 functions as an antioncogenic regulator in osteosarcoma

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Abstract. Numerous studies have demonstrated that microRNAs (miRs) are involved in several physiological and pathological processes, and participate in cancer initiation and progression. The abnormal expression of miR-150 has been reported in numerous types of human cancer. However, at present there are no studies of miR-150 in osteosarcoma (OS). Reverse transcription-quantitative polymerase chain reaction was performed to measure miR-150 expression levels in OS tissues and cell lines. Subsequent to transfection with miR-150 mimics or zinc finger E-box binding homeobox 1 (ZEB1) small interfering RNA, an MTT assay, Transwell migration and invasion assays, western blotting and a Dual-Luciferase reporter assay were performed in human OS cell lines. The present study revealed that miR-150 was downregulated in OS tissues and cell lines. In addition, the expression levels of miR-150 were correlated with the clinical stage and degree of distant metastasis of patients with OS. In addition, ZEB1 was identified as a direct target of miR-150 in vitro. In conclusion, miR-150 targeted ZEB1 to function as an antioncogenic regulator in OS. These findings elucidated a novel underlying mechanism for the pathogenic process in OS carcinogenesis and progression, and may provide novel targeted therapeutic regimens for patients with OS.

Introduction

Osteosarcoma (OS) is the most common type of primary malignancy of the bones and joints, and accounts for $\sim 2.4\%$ of all malignancies in child and adolescent patients, and $\sim 20\%$ of all types of primary bone cancer (1,2). The estimated

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incidence of OS is four to five cases per million worldwide, with a peak incidence at 15-19 years old (3). Currently, the main standard therapeutic methods for OS include local control of the primary lesion by surgery and the use of combinational chemotherapy (4). OS cells are characteristically aggressive, with capabilities of rapid growth and early metastasis. Lymph node and/or distant metastasis is developed in >30% of patients with locally advanced OS (5,6). Although progress in therapeutic treatments has occurred, prognosis remains poor. The 5-year overall survival rate for locally advanced patients is 60-70%, whereas for patients who present with metastatic disease it is <30% (7,8). Understanding the molecular mechanisms underlying the rapid growth and early metastasis of OS and investigating novel therapeutic regimens to prevent metastasis during the early stages is, therefore, important.

microRNAs (miRNAs/miRs) are a group of endogenous, non-protein-coding and short RNAs (18-25 nucleotides) with highly conserved sequences in plants, animals and DNA viruses (9). Several studies have demonstrated that miRNAs regulate mRNA expression in tumor and normal cells, by binding to sites in the 3' untranslated regions (3'UTR) of mRNAs in a base-pairing manner, resulting in the degradation of mRNAs or translational inhibition at the post-transcription level (10-12). It has been estimated that miRNAs regulate more than two-thirds of human genes (13). Abnormal expression of miRNAs has been reported in various diseases, particularly in cancer (14). Numerous studies have suggested that the abnormal expression of miRNAs in cancer serves a crucial function in several physiological and pathological processes, including cell growth, differentiation, the cell cycle, apoptosis, survival, migration and invasion (15,16). miRNAs may act as tumor suppressors or oncogenes in the initiation and development of various types of human malignancies, depending on the roles of the target mRNAs (17). Therefore, an investigation into miRNAs may reveal the prognostic value and therapeutic potential of miRNAs in OS.

The present study aimed to investigate the expression, functions and molecular mechanisms of miR-150 in OS carcinogenesis and progression. In the present study, the miR-150 expression levels in OS tissues and cell lines were analyzed, followed by functional studies of miR-150 in human OS cell lines. The results of the present study revealed that miR-150 was significantly downregulated in OS tissues and

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cell lines. Low expression levels of miR-150 were associated with clinical stage and distant metastasis in patients with OS. In addition, miR-150 inhibited OS cell growth, migration and invasion. Additionally, zinc finger E-box binding homeobox 1 (ZEB1) was identified as a direct target of miR-150. Therefore, miR-150 was determined to be an antioncogenic regulator in OS via the direct targeting of ZEB1. These findings indicated a novel molecular mechanism underlying the pathogenic process in OS carcinogenesis and progression, and may facilitate the development of novel targeted therapeutic regimens for patients with OS.

Materials and methods

Clinical specimens. The current study was approved by the Ethical Review Committee of Tianjin Hospital (Tianjin, China). In addition, written informed consent and clinicopathological information was obtained from each patient with OS involved in the present study. A total of 67 pairs of OS tissues and matched normal adjacent tissues (NATs) were obtained from patients (39 male and 28 female; age range, 16-65 years) who underwent surgical resection at Tianjin Hospital between June 2013 and January 2015. All the patients with OS had not received any therapeutic treatments prior to surgery. Specimens had been histologically and clinically diagnosed following surgery. Tissues were snap-frozen in liquid nitrogen and stored at -80°C until use.

Cell culture. The HOS, U2OS, MG-63 and SAOS-2 human OS cell lines and the human normal osteoblastic hFOB 1.19 cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). The HEK293T cell line was obtained from the Chinese Center for Type Culture Collection (Wuhan, China). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher, Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Cell transfection. miR-150 mimics and negative controls (NC) were obtained from GenePharma Co., Ltd. (Shanghai, China). ZEB1 small interfering RNA (siRNA) and negative control (NC) siRNA were purchased from Guangzhou RiboBio (Guangzhou, China). When the growth of the cells reached the exponential phase they were plated into 6-well plates at a density of 7.5x10⁵ per well and maintained in DMEM containing 10% FBS without antibiotics. The cells were transfected with miR-150 mimics, NC, ZEB1 siRNA or NC siRNA using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues and cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized using a PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan). RT-qPCR was carried out to evaluate miR-150 expression with a SYBR Premix Ex TaqTM kit (Takara Biotechnology Co., Ltd., Dalian, China), and U6 small nuclear RNA was used as an internal control. The thermocycling conditions for qPCR of miR-150 and U6 were as follows: 95°C for 30 sec; 40 cycles of 95°C for 5 sec; 60°C for 30 sec. ZEB1 mRNA expression was analyzed using SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), and GADPH was used as the internal reference gene. The thermocycling conditions for qPCR of ZEB1 and GADPH were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 sec; 60°C for 1 min. RT-qPCR was performed on an Applied Biosystems 7500 Real-time PCR detection system (ABI; Thermo Fisher Scientific, Inc.). Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (18).

MTT assay. An MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was performed to assess the OS cell viability. After 24 h of transfection at 37°C, the cells were collected and seeded into 96-well plates at a density of 3,000 cells/well. Cells were cultured in a cell culture box at 37°C with 5% CO₂ for 1, 2, 3 and 4 days. A total of 20 μ l MTT solution (5 mg/ml) was added into each well and incubated for a further 4 h at 37°C. Then, the cells were lysed in 150 μ l dimethyl sulfoxide for 10 min at 37°C. The absorbance was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a 490 nm wavelength. Each sample was evaluated in triplicate.

Transwell migration and invasion assays. Cell migration and invasion assays were used to evaluate OS cell motility ability using Transwell chambers (8 μ m pore size; Costar, Cambridge, MA, USA). For the cell invasion assay, the Transwell chamber was coated with 50 μ g Matrigel (BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocol. After 48 h of transfection, cells were collected, 1x10⁵ cells were resuspended in 200 μ l DMEM without FBS and were then added into the upper chamber, while the lower chamber was filled with 500 µl DMEM supplemented with 20% FBS. After 24 h of incubation, the cells were fixed with 100% methanol for 10 min and stained with 0.5% crystal violet for 20 min. Subsequently, cells that had not migrated or invaded to the lower membrane were carefully removed with cotton swabs. The cells in >5 randomly selected fields (magnification, x100) were counted under an inverted microscope (CKX41; Olympus Corporation, Tokyo, Japan). All experiments were repeated at least three times.

miR-150 targets prediction. The target genes of miR-150 were predicted using the following TargetScan (http://www.targetscan.org/index. html), PicTar (http://pictar.mdc-berlin.de/) and miRanda (http://www.microrna.org).

Western blot. After a 72-h transfection, proteins were harvested from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The protein concentration was quantified using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amount of proteins (20 μ g) were subjected to 10% SDS-PAGE and electrotransferred to polyvinylidene



Figure 1. miR-150 expression in OS tissues and cell lines. (A and B) miR-150 was significantly downregulated in OS tissues compared with in NATs. (C) Expression levels of miR-150 decreased in HOS, U2OS, MG-63 and SAOS-2 cell lines compared with in hFOB 1.19 cells. *P<0.05, compared with the respective controls. miR, microRNA; OS, osteosarcoma; NATs, normal adjacent tissues.

fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) at room temperature for 2 h. Then, the membranes were incubated with primary antibodies, including a mouse anti-human monoclonal ZEB1 antibody (1:1,000 dilution; cat. no. sc-81428; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and an anti-human monoclonal GADPH antibody (1:1,000 dilution; cat. no. sc-59540; Santa Cruz Biotechnology, Inc.), overnight at 4°C. Subsequent to washing with TBS/Tween-20 three times, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilution; cat. no. A0192; Beyotime Institute of Biotechnology) at room temperature for 1 h. The protein blots were visualized with enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). GADPH was used as a loading control.

Dual-Luciferase reporter assay. PGL3-ZEB1-3'UTR wild type (Wt) and PGL3-ZEB1-3'UTR mutant (Mut) was obtained from GenePharma Co., Ltd. HEK293T cells were seeded into 12-well plates and transfected with miR-150 mimics or NC, and PGL3-ZEB1-3'UTR Wt or PGL3-ZEB1-3'UTR Mut using Lipofectamine[®] 2000. After 48 h of transfection, firefly and *Renilla* luciferase activities were measured using a Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Firefly luciferase activities were normalized to *Renilla* luciferase activities for each well. Statistical analysis. The data are presented as the mean \pm standard deviation, and were compared with Student's t-tests or one-way analysis of variance and multiple comparisons using the SPSS version 16.0 statistical software package (SPSS, Inc., Chicago, IL, USA). SNK was utilized to compare the two groups in multiple groups studies. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-150 expression is decreased in OS tissues and cell lines. RT-qPCR was performed in order to evaluate miR-150 expression in OS tissues, NATs, OS cell lines and the human hFOB 1.19 normal osteoblastic cell line. As presented in Fig. 1A and B, miR-150 expression levels in OS tissues were significantly lower compared with in NATs (P<0.05). miR-150 was also downregulated in HOS, U2OS, MG-63 and SAOS-2 cell lines, as compared with in hFOB 1.19 cells (P<0.05; Fig. 1C). These results suggested that miR-150 may serve an important role in OS.

Correlation between miR-150 expression and clinicopathological features in patients with OS. In the present study, an investigation was performed into whether the expression levels of miR-150 were associated with clinicopathological features in patients with OS. As presented in Table I, statistical analysis revealed that miR-150 expression was significantly associated with the clinical stage (P=0.016) and distant metastasis (P=0.027) in patients with OS. However,

Table I. Correlation between exp	ression of miR-150 and clini-
copathological features in patient	ts with osteosarcoma.

Clinical features	Patient no.	miR-150 expression		
		Low (n=38)	High (n=29)	P-value
Sex				0.803
Male	39	23	16	
Female	28	15	13	
Age				0.204
<50 years	40	26	14	
≥50 years	27	12	15	
Anatomical location				1.000
Tibia/femur	39	22	17	
Elsewhere	28	16	12	
Tumor size (cm)				0.624
<8 cm	33	20	13	
≥8 cm	34	18	16	
Clinical stage				0.016 ^a
I-II	35	15	20	
III	32	23	9	
Distant metastasis				0.027ª
Present	34	24	10	
Absent	33	14	19	

^aP<0.05, vs. control; miR, microRNA.

no correlation was observed between miR-150 expression and other clinicopathological factors, including sex, age, anatomical location and tumor size.

miR-150 inhibits the proliferation of OS cells. To investigate the functional roles of miR-150 in OS, the present study transfected miR-150 mimics into human OS cells. miR-150 expression levels in HOS and U2OS cells were low, as compared with in the other cell lines investigated. Thus, HOS and U2OS cells were selected for transfection with miR-150 mimics or the NC. Subsequent to a transfection of 48 h, miR-150 expression was quantified by RT-qPCR. As presented in Fig. 2A, miR-150 expression level was markedly elevated by miR-150 mimics in HOS and U2OS cells (P<0.05).

MTT assays were used to measure OS cell proliferation subsequent to transfection with miR-150 mimics or NC. As depicted in Fig. 2B, miR-150 inhibited the growth of HOS and U2OS cells. After 96 h of transfection, the rate at which miR-150 suppresses cell proliferation reached $26.05 \pm 4.24\%$ in HOS cells and $30.87 \pm 5.57\%$ in U2OS cells. These results indicated that miR-150 may function as a novel tumor suppressor in OS.

miR-150 inhibits the migration and invasion abilities of OS cells. To evaluate the functions of miR-150 in OS metastasis, migration and invasion assays were performed using Transwell

chambers. As presented in Fig. 3, miR-150 inhibited HOS and U2OS cell migratory and invasive abilities (P<0.05). These findings suggest that miR-150 may serve a critical role in OS metastasis.

ZEB1 is a direct target of miR-150 in vitro. TargetScan, PicTar and miRanda were used to explore the molecular mechanism of miR-150 in OS. ZEB1 was identified as a target of miR-150 in all three prediction programs (Fig. 4A). RT-qPCR and western blotting were then performed to measure ZEB1 expression at the mRNA and protein levels subsequent to transfection with miR-150 mimics. As indicated in Fig. 4B, ZEB1 was significantly downregulated at the mRNA level in HOS and U2OS cells subsequent to transfection with miR-150 mimics (P<0.05). Similarly, western blotting revealed that ZEB1 protein expression was downregulated in miR-150 mimic-transfected HOS and U2OS cells (P<0.05; Fig. 4C).

Finally, Dual-Luciferase reporter assays were performed to explore whether miR-150 directly targets the 3'UTR of ZEB1. As presented in Fig. 4D, miR-150 significantly inhibited PGL3-ZEB1-3'UTR Wt luciferase activity, but not the PGL3-ZEB1-3'UTR Mut luciferase activity, in HEK293T cells (P<0.05). These results demonstrate that ZEB1 is a direct target gene of miR-150 *in vitro*.

ZEB1 is involved in miR-150-mediated tumor suppression functions in OS cells. To determine whether ZEB1 serves as a critical mediator of the suppressive functions of miR-150 on OS cell proliferation, migration and invasion, the present study transfected ZEB1 siRNA or NC siRNA into HOS and U2OS cells. After 72 h of transfection, western blot analysis was performed to determine ZEB1 protein expression. As indicated in Fig. 5A, ZEB1 was significantly downregulated in miR-150 mimic-transfected HOS and U2OS cells (P<0.05).

In the MTT assay, the knockdown of ZEB1 decreased HOS and U2OS cell proliferation (P<0.05; Fig. 5B). In addition, in migration and invasion assays, silencing of ZEB1 inhibited HOS and U2OS cell migratory and invasive abilities (P<0.05; Fig. 5C). These results demonstrated that the functions of ZEB1 siRNA were similar to those induced by miR-150 in HOS and U2OS cells, suggesting ZEB1 may be a functional target of miR-150 in OS.

Discussion

Since their discovery, miRNAs have received considerable attention (19). Several studies have indicated that miRNAs contribute to various physiological and pathological processes, and participate in the initiation and progression of cancer (20,21). Numerous studies have demonstrated that miR-150 is downregulated in certain types of human cancer, including pancreatic cancer (22), esophageal squamous cell carcinoma (23), colorectal cancer (24), hepatocellular carcinoma (25), ovarian cancer (26) and malignant lymphoma (27). However, miR-150 was also reported to be upregulated in prostate (28), non-small cell lung (29), breast (30) and gastric cancer (31). These conflicting studies suggest that miR-150 expression levels in cancer exhibit tissue specificity.



Figure 2. Upregulation of miR-150 inhibited OS cell proliferation. (A) miR-150 was significantly upregulated in miR-150 mimic-transfected HOS and U2OS cells, compared with in cells transfected with the NC. (B) MTT assays revealed that miR-150 suppressed growth in HOS and U2OS cells. *P<0.05, compared with the respective controls. miR, microRNA; OS, osteosarcoma; NC, negative control.



Figure 3. Cell migration and invasion was evaluated using Transwell chambers. miR-150 was revealed to suppress HOS and U2OS cell migration and invasion abilities. *P<0.05, compared with the respective controls. miR, microRNA; NC, negative control.

In the present study, miR-195 was revealed to be significantly downregulated in OS tissues and cell lines. In addition, a low expression level of miR-150 was significantly associated with clinical stage and distant metastasis. These results suggest that miR-150 may exhibit tumor-suppressive roles in OS carcinogenesis and development.

The collective results from numerous previous functional studies demonstrated that miR-150 may be a tumor



Figure 4. ZEB1 was a direct target gene of miR-150. (A) miR-150 binding site in the 3'-UTR of ZEB1 and the ZEB1 3'-UTR mutant sequence. (B) Reverse transcription-quantitative polymerase chain reaction revealed that ZEB1 was downregulated at the mRNA level in HOS and U2OS cells subsequent to transfection with miR-150 mimics. (C) Western blotting demonstrated that ZEB1 protein expression was decreased in miR-150 mimic-transfected HOS and U2OS cells. (D) miR-150 inhibited the PGL3-ZEB1-3'UTR wild type luciferase activity, but not the PGL3- ZEB1-3'UTR mutant luciferase activity in HEK293T cells. *P<0.05, compared with the respective controls. ZEB1, zinc finger E-box binding homeobox 1; miR, microRNA; 3'-UTR, 3' untranslated region; NC, negative control; Mut, mutated; Wt, wild-type.

suppressor. For example, in pancreatic cancer, patients whose tumors were associated with low miR-150 expression exhibited higher mortality rates, compared with patients whose tumors exhibited high miR-150 expression. In addition, the upregulation of miR-150 decreased pancreatic cancer cell proliferation, migration, invasion, clonogenicity and cell cycle progression, and promoted apoptosis via the blockade of c-Myb and mucin 4, cell surface associated (32). In colorectal cancer, a low miR-150 expression group exhibited shorter survival rate and worse response to adjuvant chemotherapy compared with a high miR-150 expression group (33). miR-150 inhibited colorectal cancer cell growth and induced cell apoptosis by directly targeting c-Myb (34). Yokobori et al (23) revealed that low expression levels of miR-150 in esophageal squamous cell carcinoma were significantly associated with tumor depth, lymph node metastasis, lymphatic invasion, venous invasion, clinical staging and poor prognosis. In the aforementioned study, the upregulation of miR-150 inhibited esophageal squamous cell carcinoma cell proliferation and tumorigenicity in vivo. Therefore, upregulating miR-150 or providing analogous pharmaceutical compounds exogenously, may be an effective therapy for tumors resulting from the activation or overexpression of these oncogenes.

The functions of miRNAs are tissue-type dependent. miR-150 has been verified as an oncogene in a number of different types of cancer (28,29,35). For example, in prostate cancer, miR-150 was markedly upregulated, and the high expression of miR-150 was positively associated with tumor recurrence and metastasis in prostate cancer (28). In addition, patients with prostate cancer and high miR-150 expression exhibited significantly poorer overall survival and disease-free survival compared with those patients with low miR-150 expression (28). The 5-year overall survival rate was 55.93% in patients with prostate cancer with low miR-150 expression, whereas it was 35.19% in patients with high miR-150 expression (28). In non-small cell lung cancer, a high expression level of miR-150 was correlated with lymph node metastasis, distant metastasis and clinical tumor node metastasis stage. The 5-year overall survival rate was 69.2% in the low miR-150 expression group; however, in the high miR-150 expression group, it was 40.8% (36). In addition, the downregulation of miR-150 enhanced non-small cell lung cancer proliferation and migration, and inhibited cell apoptosis through targeting B-cell lymphoma 2 antagonist/killer 1, SRC kinase signaling inhibitor 1 and tumor protein 53 (29,36,37). Huang et al (30) revealed that the ectopic expression of miR-150 induced breast cancer cell proliferation and clonogenicity, and suppressed cell apoptosis by directly targeting PX27. These findings also suggested that miR-150 may have important functions in these types of cancer, and may be investigated as a potential therapeutic gene for the treatment of these cancer types.

In the present study, miR-150 was revealed to inhibit OS cell proliferation, migration and invasion *in vitro*. Identification of miR-150 target mRNAs is important for understanding the functions of miR-150 in OS carcinogenesis and progression, and to investigate novel targeted therapies for OS. The present study identified ZEB1 as a direct target gene of miR-150 *in vitro*. ZEB1 is a member of the zinc finger family, which is located on the short arm of human chromosome 10 (38). Wang *et al* (39)



Figure 5. Effects of ZEB1 on OS cell growth, migration and invasion. (A) ZEB1 was downregulated in HOS and U2OS cells subsequent to transfection with ZEB1 siRNA. (B) ZEB1 siRNA significantly inhibited HOS and U2OS cell proliferation. (C) ZEB1 siRNA suppressed HOS and U2OS cell migration and invasion abilities. *P<0.05, compared with the respective controls. ZEB1, zinc finger E-box binding homeobox 1; OS, osteosarcoma; siRNA, small interfering RNA; NC, negative control.

verified that ZEB1 is involved in cancer progression, and that it is considered an important transcriptional regulator of E-cadherin. In OS, ZEB1 was revealed to be upregulated in patients with lung metastases compared with patients without lung metastases. In addition, the expression of ZEB1 in OS tissues was increased with increasing Enneking stage (38). These results indicated that ZEB1 may contribute to OS metastasis. Therefore, additional studies are required with respect to ZEB1 as a potential target for the inhibition of OS metastasis. In conclusion, the present study demonstrated that miR-150 was significantly downregulated in OS tissues and cell lines. Low expression of miR-150 was associated with clinical stage and distant metastasis. In addition, miR-150 inhibited OS cell growth, migration and invasion, and ZEB1 was identified as a direct target of miR-150 *in vitro*. These findings suggest that miR-150 targets ZEB1 to inhibit OS growth and metastasis, a mechanism that may be investigated as a therapeutic regimen to prevent rapid growth and early metastasis in OS.

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