Long non-coding RNA MIAT competitively binds miR-150-5p to regulate ZEB1 expression in osteosarcoma

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Abstract. Long non-coding RNAs (LncRNAs), are significant in a number of biological stages and illnesses. The myocardial infarction associated transcript (MIAT) serves a function in numerous types of illness and physiological and pathological processes, including paranoid schizophrenia, diabetic retinopathy, myocardial infarction and neuroendocrine prostate cancer. However, the function of the lncRNA MIAT in the development of osteosarcoma is unknown. It has been identified that during the development of osteosarcoma, MIAT is upregulated in tumor tissues compared to adjacent non-tumor tissues. The spreading and proliferation of osteosarcoma cells was reduced by MIAT knockdown. These findings indicate that MIAT functions by competing with critical RNAs to target miR-150-5p and activate zinc finger E-box binding homeobox 1 to modulate the function of osteosarcoma cells. Together, the present findings may contribute to the understanding of the pathogenesis of osteosarcoma.

Introduction

Osteosarcoma primarily impacts children, juveniles and adults from an early age (1). Although the worldwide incidence rate of this rare disease is only 3-4 cases per million, osteosarcoma is the most common form of bone cancer (2). Various chemotherapeutic and radiation treatments have been developed over the past two decades; however, the survival rate is still low. Almost 50% of patients succumb to pulmonary metastasis in the terminal stages of the disease (3). Elucidating the underlying molecular mechanisms of osteosarcoma metastasis is therefore a desirable research outcome.

The epithelial-mesenchymal transition (EMT) is involved in the process of invasion and metastasis in many cancers, including osteosarcoma (4,5). Zinc finger E-box binding homeobox 1 (ZEB1), is a key protein that functions to regulate the phenotype of EMT during cancer progression (6). ZEB1 may promote prostatic cancer, and overexpression of ZEB1 leads to the promotion of lung cancer cell metastasis (7,8). Additionally, the overexpression of ZEB1 has been demonstrated to be associated with the development, carcinogenesis, invasion and metastasis of osteosarcoma (9).

Long non-coding RNAs (lncRNAs) are RNA molecules that have over 200 nucleotides and possess the potential for protein-coding (10). LncRNAs are involved in regulating cellular functions and the progression of various types of cancer (11). Several studies have demonstrated that lncRNAs serve a critical function in numerous cellular processes by competing with RNAs to regulate microRNAs (12-14). The myocardial infarction associated transcript (MIAT) may be expressed in postmitotic retinal precursor cells and mitotic progenitors (15). With regards to human malignancies, MIAT upregulation has been observed in conditions including neuroendocrine prostatic and gastric cancer (16,17). However, the role of MIAT in the regulation of osteosarcoma remains unresolved.

Materials and methods

Tissue samples. Patients with osteosarcoma provided six tissue samples and were operated on at The First Affiliated Hospital of Harbin Medical University, China. Samples were snap frozen at -80˚C until RNA extraction. Written informed consent was obtained from all patients. The study was approved by the Research Ethics Committee at Harbin Medical University (Harbin, China).

Cell culture and transfection. The Chinese Cell Bank of the Chinese Academy of Sciences (Shanghai, China) provided the osteoblast cell lines hFOB (OB3) and osteosarcoma cell lines Saos-2 and MG-63. Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare, Chicago, IL, USA) was used as cell medium at 37˚C, with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel) and with 95% air and 5% CO_2. siRNAs targeting MIAT (forward, 5'-GGACGTTCACAACCACAC TG-3' and reverse, 5'-TCCCACTTTGGCATTCTAGG-3') were designed by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Knockdown and overexpression of miR-150-5p

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were obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The sequences were as follows: Human miR-150-5p, 5'-UCUCCCAACCCUUGUACCAGUG-3' and 29-O-methyl modified miR-150 inhibitor, 5'-CACUGGUACAAGGGUUGGGAGA-3'. Cell transfections were performed using X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. siNC (50 μl DMEM was mixed with 20 pmol siNC; GCACCTTGAGTGAATGTCAGGGACTCCCTGATGATGTGA; Guangzhou RiboBio Co., Ltd.) was defined as the negative control.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol® reagent (Life Technologies; Thermo Fisher Scientific, Inc.) was used to extract RNA according to the manufacturers protocol. Invitrogen (Thermo Fisher Scientific, Inc.) provided the PCR primers. A NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to measure the concentration of extracted RNA. A TaqMan® miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) synthesized cDNA via RT in 5 ng of total RNA to find miR-150-5p levels. The $2^{-\Delta\Delta Cq}$ method was used to determine the expression levels of miR-150-5p (18). MIAT expression was divided into high and low groups using RT-qPCR, using the median value as the cut-off to differentiate between the high and low groups. Bioinformatics analysis was used (MicroRNA, Starbase version 2.0) to determine the potential complementarity between MIAT and miRNAs.

In the synthesis kit of cDNA, RNA synthesized the cDNAs via specific gene primers. (Invitrogen; Thermo Fisher Scientific, Inc.) to determine ZEB1 mRNA expression. qPCR was performed using a SYBR Green Real-Time PCR Master Mix kit (Toyobo Life Science, Osaka, Japan) according to the manufacturer's protocol, and the ABI 7500 Sequence Detection System (Life Technologies; Thermo Fisher Scientific, Inc.). In a total reaction volume of 20 μl, amplification was performed with 1 μl reverse primer, 1 μl forward primer, 10 μl SYBR Master Mix, 6 μl diethyl pyrocarbonate and 2 μl cDNA. The conditions for the reaction were as follows: 72˚C for 45 sec, 60˚C for 15 sec and 40 cycles of 95˚C for 15 sec. The internal control was GAPDH. Expression levels of ZEB1 were determined in relation to GAPDH by the $2^{-\Delta\Delta Cq}$ method (18). Table I presents the primer sequences.

Western blot analysis. Cells were washed using PBS three times at room temperature and lysed using radioimmunoprecipitation assay buffer with 1% protease inhibitor (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The protein concentration was measured by Nanodrop. Proteins (50 μg) were separated by SDS-PAGE (10% gel). Protein transfer was then conducted onto nitrocellulose membranes. Blocking was performed using 0.1% Tween 20 and 5% nonfat milk (BD Biosciences, Franklin Lakes, NJ, USA) in TBS for 2 h at room temperature. Samples were incubated with the proper primary antibodies (cat. no. ab203829; 1:1,000; rabbit; Abcam, Cambridge, MA, USA) at 4˚C overnight with gentle agitation followed by staining with the fluorochrome-labeled secondary antibody (cat. no. A10235; 1:8,000; rabbit anti-mouse; Alexa Fluor 800; LI-COR Biosciences, Lincoln, NE, USA) at room temperature for 1 h. The Odyssey fluorescent scanning system (LI-COR Biosciences) detected immunoreactivity and Image Studio software 4.0 (LI-COR Biosciences) was used to examine captured images. The loading control was β-actin.

Cell proliferation assay. The cell counting kit-8 kits (CCK-8) were used according to the manufacturer's instructions. MG63 and Saos-2 cells were seeded in 96-well plates at $1x10^4$ cells/well for 24 h. CCK-8 solution (10 μl) was added to each well, and the cells were incubated at 37˚C for 2 h. Absorbance at 450 nm was measured using a microplate reader. The assay was conducted in triplicate.

Wound healing assays. Osteosarcoma cells (MG63 and Saos-2 cells) were seeded in six-well plates and allowed to reach 80-90% confluence. A wound line was drawn across the surface of the plates using a 200-μl sterile plastic tip. PBS was used to wash the plates. Images were captured 24 h post wound infliction. Each test was conducted in triplicate.

Transwell assays. Transwell filters (8 μm pore size; BD Biosciences) were placed on a 24-well plate containing DMEM/F12 (Hyclone: GE Healthcare). The medium in the upper membrane was serum free, and in the lower chamber contained 10% FBS (Biological Industries, Beit-Haemek, Israel).

MG63 and Saos-2 cells were suspended in DMEM/F12 at a cell density of $2.5x10^5$ cells/ml for 24 h. After 24 h, cells present on the top of the membrane were cleared using a cotton swab. Cells present on the bottom portion of the membrane were fixed using 4% paraformaldehyde in PBS for 10 min and stained using crystal violet at room temperature for 15 min. Cell invasion was quantified as the average number of cells from 3 inserts present on the bottom portion of the membrane.

Statistical analysis. All data are expressed as the mean ± standard error of the mean. SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA) was used to analyze all data. Statistical comparison of two groups was performed using a Student's t-test. One-way analysis of variance was also used to compare ≥2 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MIAT levels are elevated in osteosarcoma. The expression levels of the lncRNA MIAT 6 were investigated in groups of osteosarcoma tissues using RT-qPCR. MIAT levels were decreased in adjacent non-tumor tissues compared with osteosarcoma tissues (Fig. 1A). The lncRNA MIAT expression levels were also investigated in MG63, Saos-2 and OB3 cells and it was identified that MIAT expression was lower in the OB3 cell line than in Saos-2 and MG63 cells (Fig. 1B). These results demonstrate that MIAT levels are potentially associated with osteosarcoma.

MIAT knockdown inhibits the proliferation and invasion of osteosarcoma cells. The function of MIAT in the proliferation and invasion of osteosarcoma cells was examined. Transfection of MG63 and Saos-2 cells with MIAT siRNAs

Table I. Primers used for RT-PCR.

Name	Sequence (5'-3')	Length (bp)
GAPDH forward	AAGAAGGTGGTGAAGCAGGC	20
GAPDH reverse	TCCACCACCCTGTTGCTGTA	20
miR-150-5p forward	GTCTCCCAACCCTTGTAC	18
miR-150-5p reverse	TATCCAGTGCGTGTCGTG	18
ZEB1 forward	FGCCAATAAGCAAACGATTCTG	22
ZEB1 reverse	TTTGGCTGGATCACTTTCAAG	21
U6 forward	CTCGCTTCGGCAGCACATATACT	23
U6 reverse	ACGCTTCACGAATTTGCGTGTC	22

ZEB1, zinc finger E-box binding homeobox 1; RT-PCR, reverse transcription-polymerase chain reaction.

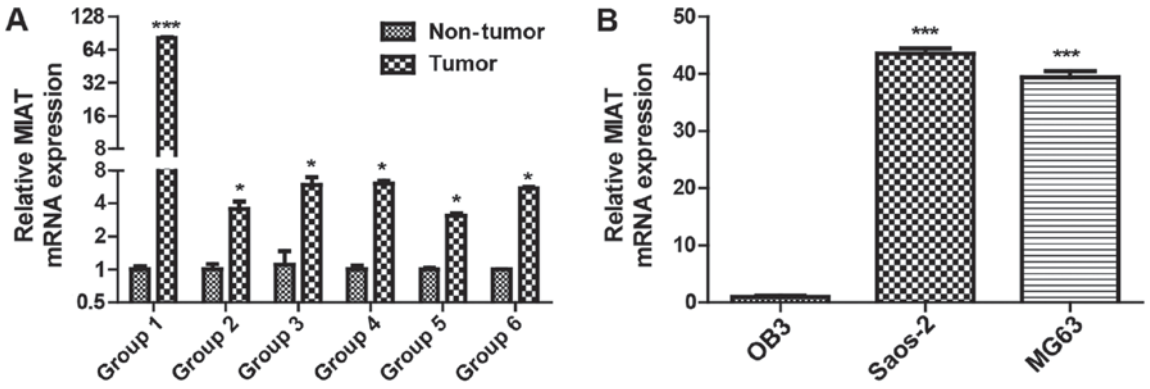

Figure 1. mRNA expression levels of MIAT are elevated in osteosarcoma. (A) MIAT mRNA expression levels in six groups of osteosarcoma tissues are significantly higher than those in non-tumor tissues as determined by reverse transcription-quantitative polymerase chain reaction. (B) MIAT mRNA expression levels in OB3, MG63 and Saos-2 cells. Data are expressed as the mean ± the standard error of the mean. n=3. *P<0.05 and ***P<0.001 compared with the control group. MIAT, myocardial infarction associated transcript.

was demonstrated to lower MIAT expression in comparison to control cells (Fig. 2A). CCK8 results indicated that knockdown of MIAT reduced MG63 and Saos-2 cell proliferation compared to cells transfected with siRNAs (Fig. 2B). MIAT knockdown also limited the proliferation and invasion of MG63 and Saos-2 cells (Fig. 2C and D) compared to cells transfected with siRNAs. Taken together, these data reveal that MIAT may promote proliferation and invasion of osteosarcoma cells *in vitro*.

MIAT promotes ZEB1 expression in osteosarcoma. ZEB1 is important for the proliferation and invasion of osteosarcoma cells (19). Thus, it was investigated whether MIAT affects ZEB1 expression in osteosarcoma cells. First, the association between MIAT and the ZEB1 expression levels was evaluated in 6 samples of tumor-adjacent tissue and osteosarcoma samples by western blotting and RT-qPCR.

It was identified that ZEB1 expression was notably increased in the high MIAT osteosarcoma tissue group compared with that in the low MIAT group (Fig. 3A and B). It was additionally noted that ZEB1 expression levels were decreased in OB3 cells compared with MG63 and Saos-2 cells (Fig. 3C and D). Furthermore, ZEB1 expression levels were evaluated in Saos-2 and MG63 cells transfected with MIAT siRNAs or siNC (negative control) and it was identified that MIAT knockdown by siRNAs resulted in lower ZEB1 expression compared to cells transfected with control siRNAs (Fig. 3E and F).

miR-150-5p is a downstream target of MIAT. To identify the potential downstream miRNA targets of MIAT and its interactions in osteosarcoma, bioinformatics analysis was used (MicroRNA, Starbase version 2.0) to determine the potential complementarity between MIAT and miRNAs. Bioinformatics predictions revealed that the MIAT sequence has four putative miRNA binding sites, including sites for miR-29a-3p, miR-29b-3p, miR-29c-3p, and miR-150-5p-5p. Yan *et al* (20) previously reported that miR-150-5p-5p focuses on MIAT in endothelial cells, and another study revealed that miR-150-5p suppresses ZEB1 in epithelial ovarian cancer (21). It may be observed that miR-150-5p levels were higher in adjacent non-tumor tissues than in osteosarcoma tissues (Fig. 4A) and lower in MG63 and Saos-2 cells than OB3 cells (Fig. 4B).

To determine whether miR-150-5p targets MIAT, miR-150-5p expression in Saos-2 and MG63 cells transfected with MIAT-siRNA or siNC was examined. The results revealed that miR-150-5p expression was visibly elevated in MG63 and Saos-2 cells transfected with MIAT-siRNA compared to control siRNA (Fig. 5A and B). Rescue experiments were subsequently performed by transfecting miR-150-5p in Saos-2

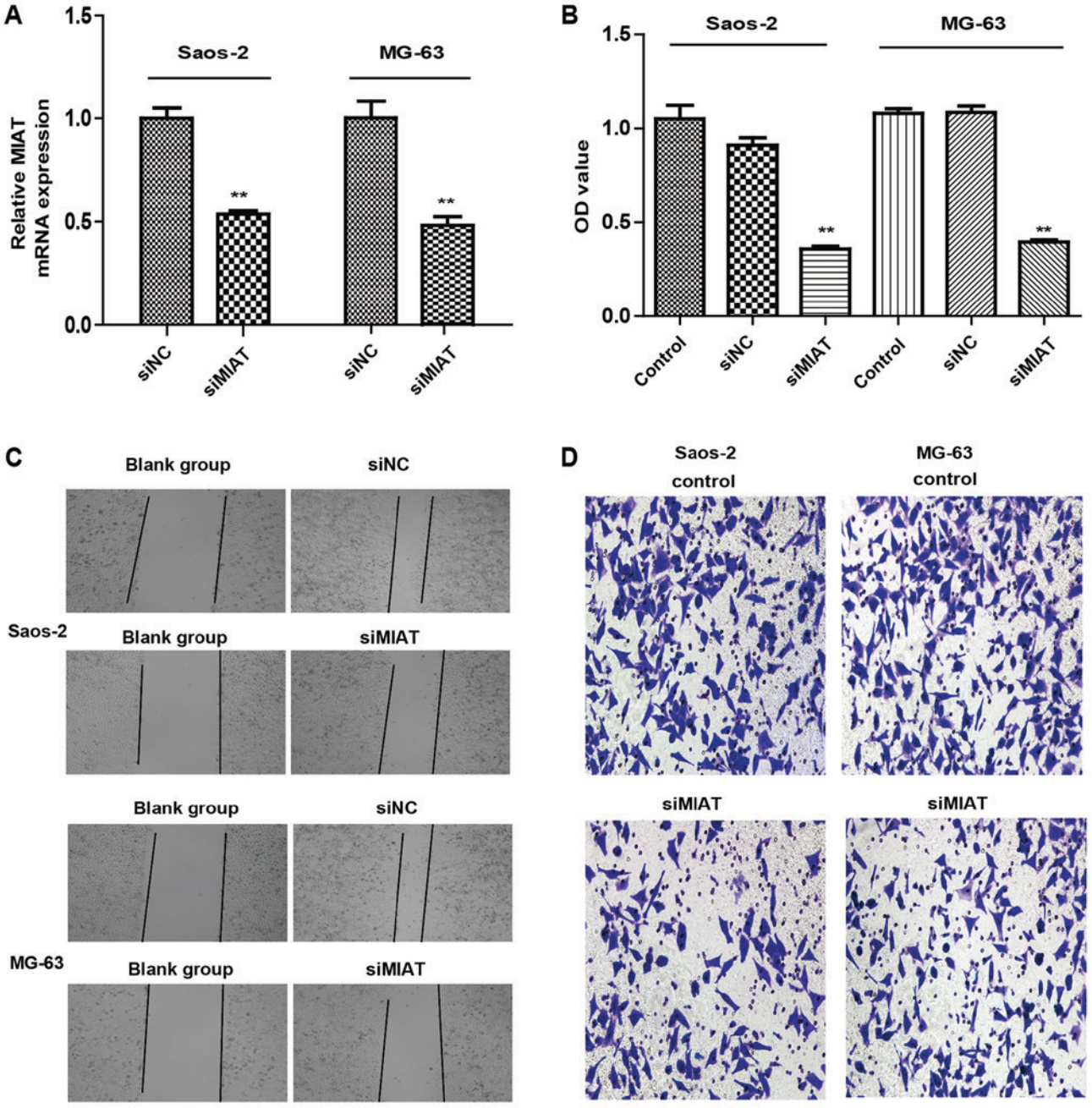

Figure 2. MIAT knockdown inhibits the proliferation and invasion of osteosarcoma cells. (A) Expression levels of MIAT in Saos-2 and MG-63 osteosarcoma cells following transfection with MIAT siRNAs. (B) Cell proliferation determined by cell counting kit 8. Knockdown of MIAT also reduced cell proliferation detected by (C) wound healing assays and (D) invasion assays. Data are expressed as the mean ± the standard error of the mean. n=3. **P<0.01 and ***P<0.001 compared with the control group. MIAT, myocardial infarction associated transcript.

and MG63 cells. Overexpressing miR-150-5p led to an increase in ZEB1 (Fig. 4C and D). Additionally, inhibiting MIAT limited ZEB1 levels by inhibiting miR-150-5p (Fig. 5A and B). Together, the results demonstrate the importance of MIAT in regulating ZEB1, by controlling miR-150-5p.

miR-150-5p reverses the effects of MIAT in osteosarcoma cells. These results demonstrated that miR-150-5p is a downstream target of MIAT. However, the function of miR-150-5p in the MIAT-mediated influence on osteosarcoma cells remains unclear. To determine whether MIAT may promote proliferation and invasion of osteosarcoma cells via the MIAT-miR-150-5p-ZEB1 axis, further experiments were carried out. RT-qPCR and western blotting revealed that reduced ZEB1 expression by inhibition of MIAT could be largely reversed by AMO-miR-150-5p (Fig. 5). Together, these results suggest that miR-150-5p was able to change the function of MIAT in osteosarcoma cells and that MIAT was able to promote the proliferation and invasion of osteosarcoma cells via the MIAT-miR-150-5p-ZEB1 axis.

Discussion

Osteosarcoma has been considered a common type of basic malignancy of bone and is derived from the progenitor mesenchymal cells of bone-forming cells (22). The morbidity associated with osteosarcoma is high since early diagnosis is difficult, and

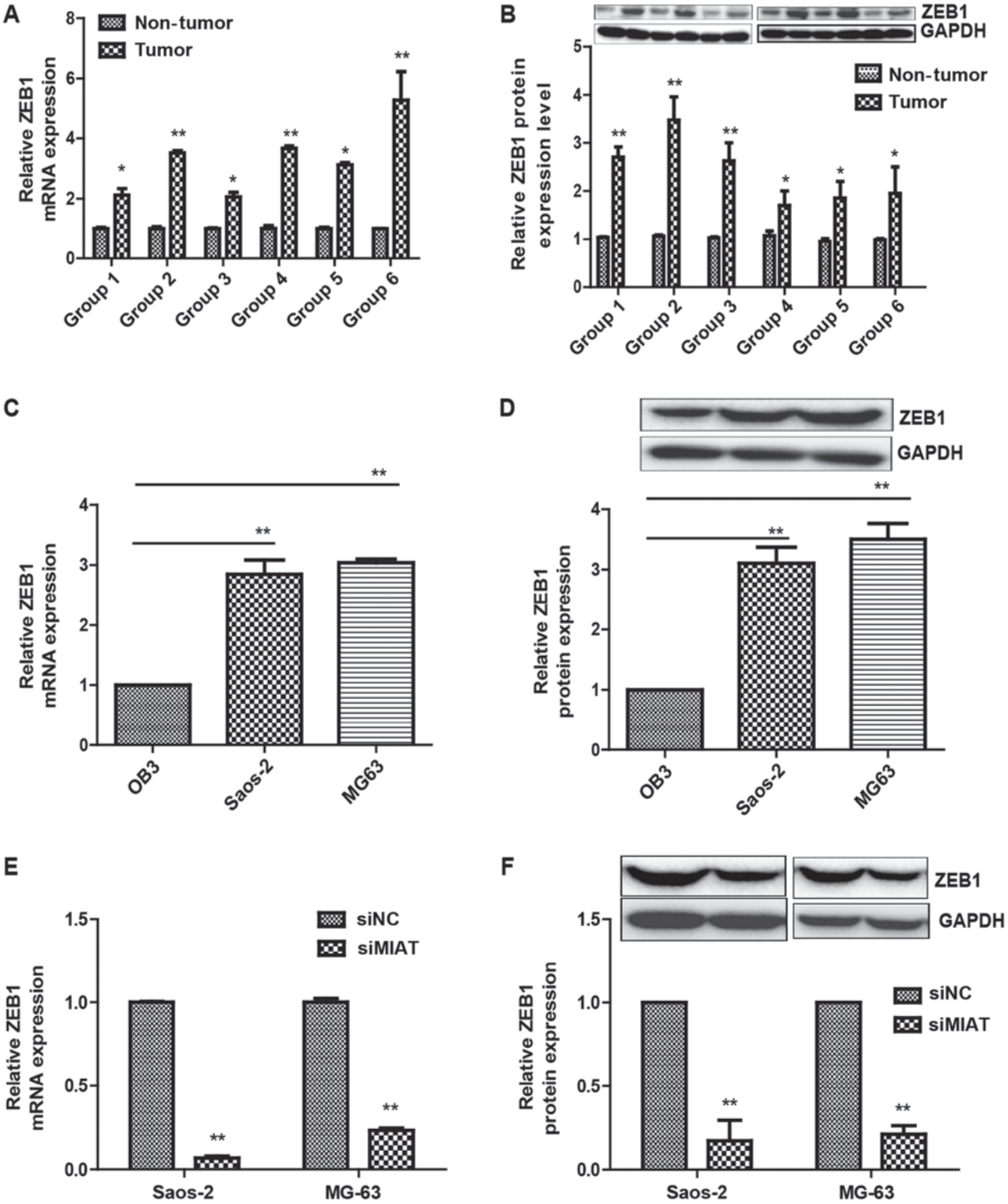

Figure 3. MIAT promotes ZEB1 expression in osteosarcoma. ZEB1 (A) mRNA and (B) protein expression levels are notably increased in the high MIAT osteosarcoma tissues groups compared to that in the low groups. The (C) mRNA and (D) protein expression levels of ZEB1are elevated in Saos-2 and MG-63 osteosarcoma cells compared with OB3 osteosarcoma cells. The (E) mRNA and (F) protein expression levels of ZEB1, MG63 and Saos-2 cells transfected with MIAT siRNAs had lower ZEB1 expression. Data are expressed as the mean ± the standard error of the mean. n=3. *P<0.05 and **P<0.01 compared with the control group. MIAT, myocardial infarction associated transcript; ZEB1, zinc finger E-box binding homeobox 1.

therapeutic solutions to osteosarcoma are lacking. Therefore, it is important to identify new molecules associated with developing osteosarcomas and develop novel targeted therapy strategies.

Previous studies have demonstrated the role of lncRNAs (23) and the molecular mechanisms through which lncRNAs affect human tumors (24-26). However, the mechanism involving the lncRNA MIAT in osteosarcoma has remained unknown. This research shows that highly overexpressed MIAT in cell lines and osteosarcoma tissues may have a monogenic role.

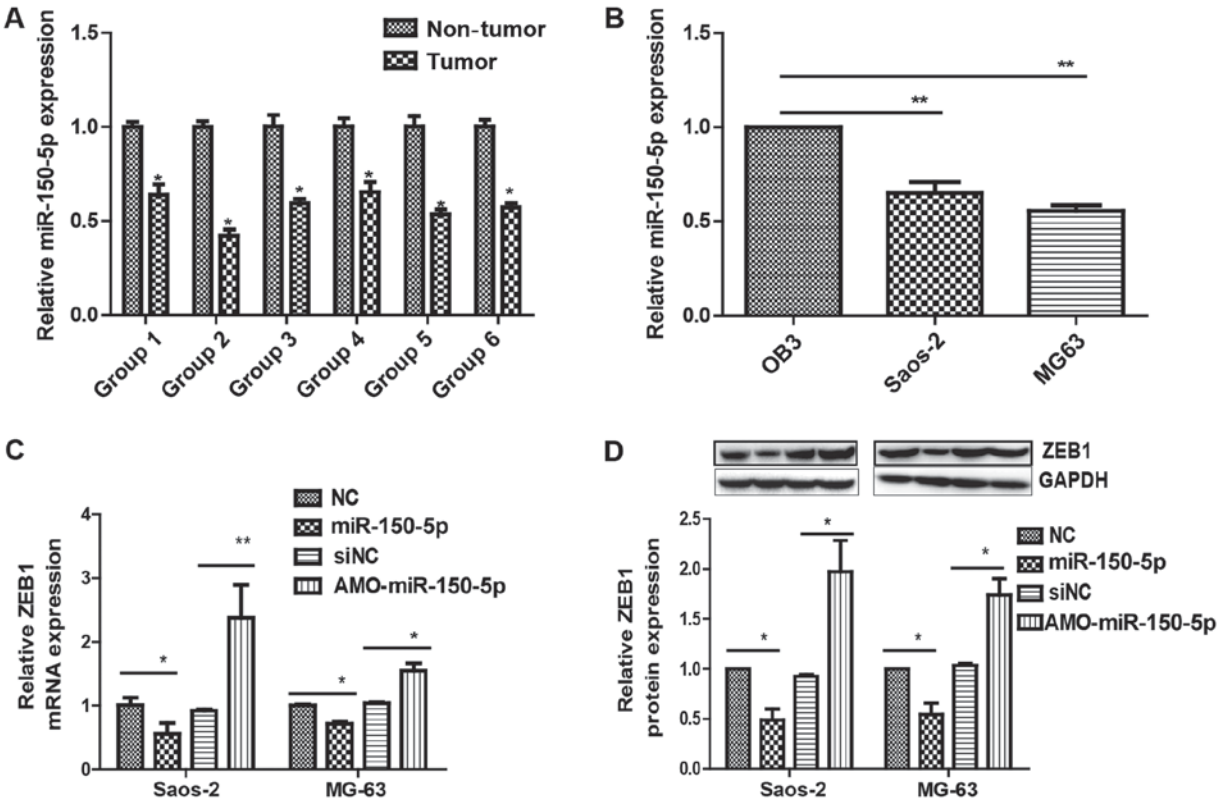

Figure 4. ZEB1 is a downstream target of miR-150-5p. (A) miR-150-5p levels were decreased in six groups of osteosarcoma tissues compared to adjacent non-tumor tissues. (B) miR-150-5p levels were decreased in Saos-2 and MG-63 osteosarcoma cells compared to OB3 osteosarcoma cells. ZEB1 (C) mRNA and (D) protein levels were decreased when Saos-2 and MG-63 osteosarcoma cells were transfected with miR-150-5p and increased when transfected with miR-150-5p inhibitor. Data are expressed as the mean ± the standard error of the mean. n=3. *P<0.05, **P<0.01 compared with the control group. MIAT, myocardial infarction associated transcript; ZEB1, zinc finger E-box binding homeobox 1.

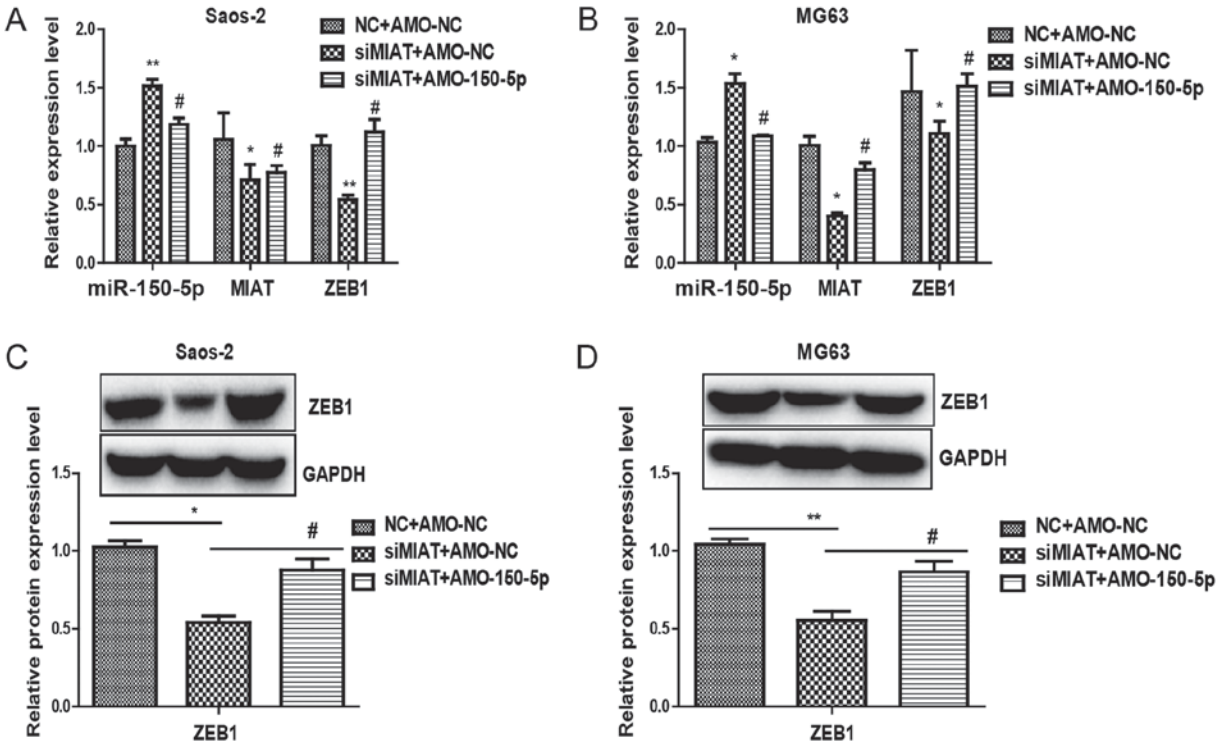

Figure 5. MIAT regulates ZEB1 levels through miR-150-5p. miR-150-5p expression was visibly elevated in (A) Saos-2 and (B) MG63 cells transfected with MIAT-siRNA. ZEB1 mRNA expression levels are reduced by inhibition of MIAT and reversed by a miR-150-5p inhibitor. ZEB1 protein expression levels are in accordance with mRNA expression levels in (C) Saos-2 and (D) MG63 cells. Data are expressed as the mean ± the standard error of the mean. n=3. *P<0.05, **P<0.01 compared with the control group, $^{\#}$P<0.05 vs. siMIAT groups. MIAT, myocardial infarction associated transcript; ZEB1, zinc finger E-box binding homeobox 1.

miR-150-5p was first considered as the main miRNA in immune and hematopoietic cells (27). Research has recently shown that specific cellular functions in diverse tumors also involve miR-150-5p (28-30). A previous study demonstrated that expression of miR-150-5p was decreased in osteosarcoma cells compared to analogous human normal osteoblasts and cells from normal tissues (31).

MIAT was identified as a target of miR-150-5p with both MIAT and miR-150-5p having an inhibitory effect. These results demonstrate that MIAT may improve tumor progression in osteosarcoma since it can inhibit miR-150-5p and activate ZEB1. The present study demonstrated that in osteosarcoma tissues, MIAT expression was increased compared with the adjacent normal tissues. The present study also identifies that MIAT is important in the development and progression of osteosarcoma. However, the mechanisms associated with MIAT-mediated gene expression in tumorigenesis need to be clarified.

Research has revealed that MIAT acts as a molecular sponge by managing microRNAs in the progression of breast cancer (32). It has been reported that during specific cellular processes, lncRNAs are able to compete with endogenous RNAs to manage microRNAs (33). MIAT may therefore be considered as an endogenous miRNA which controls miR-150-5p-5p and manages its role. Although several potential miRNA binding partners were identified, the present study focused on miR-150-5p, as it has been demonstrated to be important in numerous cancers, including lung (34) and liver cancer (35). The present study revealed that miR-150-5p levels were decreased in the osteosarcoma cell lines MG63 and Saos-2 cells. An opposing association was observed between miR-150-5p and MIAT levels in Saos-2 and MG63 cells, suggesting that there may be an association between miR-150-5p and MIAT to control osteosarcoma cell proliferation and invasion. In addition, miR-150-5p expression decreased ZEB1 levels in Saos-2 cells and MG63. Furthermore, the present data suggested that the MIAT sequence had miR-150-5p sites for binding and implied that miR-150-5p reduced MIAT level by directly binding MIAT. ZEB1 is a master regulator of the EMT phenotype within the progression of cancer (36). The present study demonstrates that by the miR-150-5p/ZEB1 pathway, MIAT may induce EMT phenotype in osteosarcoma cells.

To conclude, the present study revealed that MIAT may be a biomarker for patients with osteosarcoma. It is hypothesized that the MIAT-miR-150-5p-ZEB1 axis may be a potential therapeutic target in osteosarcoma.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

HJ performed the flow cytometric analysis and drafted the manuscript. XJ performed cell culture and viral preparation experiments. WC and FD contributed to statistical analyses. ZY, YL and WW designed the study.

Ethics approval and consent to participate

Written informed consent was obtained from all patients. The study was approved by the Research Ethics Committee at Harbin Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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