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LncRNA BACE1-AS Promotes the Progression of Osteosarcoma Through miR-762/SOX7 Axis

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Abstract

Background: Osteosarcoma (OS) is a rare malignant primary tumor of mesenchymal origin affecting bone that occurs in adolescents and children. LncRNAs are important regulators of tumorigenesis and development. This study aimed to explore the role and molecular basis of LncRNA BACE1-AS in OS.

Methods and results: Through the analysis of differential expressed lncRNAs in OS tissues by GEO database, LncRNA BACE1-AS displayed a remarkably lower expression. This found could also be observed in both OS tissues and cell lines by qRT-PCR. Furthermore, using Cell counting kit-8 (CCK-8), transwell, wound healing and western blot assays, overexpression LncRNA BACE1-AS remarkably reduced cell proliferation, migration and invasion abilities in OS. In addition, LncRNA

BACE1-AS was validated as a sponge of miR-762 through the prediction of lncRNASNP. Further, luciferase reporter and RIP assays were conducted to confirm the binding sites between lncRNA BACE1-AS and miR-762. SOX7 was a target of miR-762 and could be regulated by lncRNA BACE1-AS. Moreover, inhibition of miR-762 could attenuate the role of sh-lncRNA BACE1-AS in OS cells, at meanwhile reduced the expression of SOX7.

Conclusion: In this study, lncRNA BACE1-AS regulated proliferation, migration, invasion and apoptosis of OS cells by miR-762/SOX7 axis, implying that lncRNA BACE1-AS was a potential target for OS therapy.

Key word: lncRNA BACE1-AS; miR-762; SOX7; Osteosarcoma; proliferation; invasion

Introduction

Osteosarcoma is the most common primary osseous sarcoma, with a high morbidity and poor prognosis. Osteosarcoma often starting with the distal femur and proximal tibia as the site of invasion, after which extensive metastases, mainly to the lungs. 60-80% of patients have varying degrees of neurological damage, including limb numbness and weakness, difficulty in mobility, sphincter dysfunction, and even paraplegia^{1,2}. Due to advances in treatment strategies such as radiotherapy, adjuvant chemotherapy, and surgery, overall survival has improved in most surgical patients, but patients with distant metastases still have a poor survival³. To further explore the biomarkers and potential therapeutic targets, it is essential to find the molecular mechanisms behind them.

Long non-coding RNA (lncRNA) is a kind of non-coding RNAs greater than 200 nt in length⁴. A growing number of studies have shown that lncRNAs are involved in large amount of biological processes, including signal transduction, cell cycle, chromatin remodeling, and epigenetic gene regulation, and their aberrant regulation is closely related to the pathogenesis and progression of most diseases^{5,6}. lncRNAs act as important molecular markers with diverse mechanisms of action, which can be mediated by adsorption of microRNAs (miRNAs), thus regulating the expression of

relevant target genes, and also interacting with different RNA-binding proteins^{7,8}. lncRNAs play an important role in regulating the biological processes of many tumors. For example, lncRNA SNHG5 accelerates OS progression by spongy protein miR-212-3p and regulates serum and glucocorticoid-regulated kinase 3⁹. LncRNA PCAT6 has been reported to bind to miR-143-3p and promote osteosarcoma metastasis¹⁰. Emerging evidence suggests that lncRNA BACE1-AS is involved in various types of cancers as a tumor-promoting gene^{11,12}. LncRNA BACE1-AS could promote hepatocellular carcinoma (HCC) through regulate miR-377-3p¹³. However, the biological function and molecular mechanism of LncRNA BACE1-AS in OS is still unclear.

In the present study, we aimed to detect the function of LncRNA BACE1-AS in OS. We found that LncRNA BACE1-AS promoted process of osteosarcoma by acting as a ceRNA for miR-762 . Collectively, this study reveals the mechanism of LncRNA BACE1-AS and provides a potential target for the treatment of OS progression.

Materials and methods

Tissue specimen.

The surgical specimens of 20 paired OS and adjacent normal tissues were collected from Department of Orthopedics, The Third Affiliated Hospital of Harbin Medical University. The experiment was permitted by the Ethics Review Committee and the patients signed informed consent.

Cell culture

The human normal osteoblast Hfob1.19 cell line and human OS cell lines (Saos2, U2OS, HOS, MG63) were purchased from Shanghai CAS Cell Bank (Shanghai, China). The cells were cultured by 1640 with 10% FBS(Beit-Haemek, Israel), and incubated with 37 °C, 5% CO₂.

Cell transfection

Full-length BACE1-AS was used to overexpression LncRNA BACE1-AS. pcDNA3.1 empty vector was used as a negative control. For BACE1-AS knockdown,

shRNA targeting BACE1-AS were synthesized, and scrambled shRNA was used as a negative control. All plasmids were isolated using AxyPrep DNA Miniprep Kit (Shanghai, China). The miR-762 mimics, miR-762 inhibitors were purchased from GenePharma (Shanghai, China).

The U2OS and HOS cells were transfected with Lipofectamine 2000 while cell density reached about 80% (Invitrogen, Carlsbad, CA).

Quantitative RT-PCR (qRT-PCR)

Total RNA extraction of OS tissues and cell lines were separated by TRIzol (Invitrogen, Carlsbad, CA) and RNA concentration was estimated by NanoDrop spectrophotometer. Reverse transcription kit (Thermo Fisher Scientific, USA) was used to RNA reverse transcription. SYBR Green PCR Kit (Qiagen, USA) for qRT-PCR to determine the relative mRNA levels. Data processing method according to $2^{-\Delta\Delta CT}$. The primer sequences for qPCR analysis are as follows:

LncRNA BACE1-AS	F	5'-GGCCACCAGTTGGATTCAGCTT-3'
	R	5'-GGAAACAGGGAAGGTGCGGTT-3'
U6	F	5'-GCTTCGGCAGCACATATACTAA-3'
	R	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	F	5'-CGCTGAGTACGTCGTGGAGT-3'
	R	5'-GTCGCTGTTGAAGTCAGAGG-3'
miR-762	F	ACACGGGGCUGGGGCCGGGGCCGAGCGCC
	R	CTCAGGGGCUGGGGCCGGGGCCGAGCCAGA
SOX7	F	5'-ACCAACGGGTCCCACAGA-3'
	R	5'-CCACTCAAGGCACAAGAAGG-3'

Cell proliferation assay

Use the CCK-8 kit according to the manufacturer's instructions. After incubate

treated OS cells for 24 h in 96-well plates. Add 10 μ L of CCK8 solution to each well and then incubate for 2 h at 37°C. Read the value at 450nm with a microplate reader.

Wound-healing assay

U2OS and HOS cells were seeded in 6-well plates, then scratched by 200 μ L pipette tip after transfected. Images were obtained by light microscopy at 0, 48h.

Western blot

After rinsing in ice-cold PBS, cells were lysed in RIPA contained 1% protease inhibitor. BCA was used to measure protein concentration. SDS-PAGE used to separate protein sample and NC membrane to transferred. The proteins were blocked in 5% milk(BD Biosciences) for 2h. Primary antibodies treating overnight at 4°C, which against E-cadherin (Cat No. 20874-1-AP; dilution, 1:1,000; proteintech), vimentin(Cat No. 10366-1-AP; dilution, 1:1000; proteintech), slug(Cat No. 12129-1-AP; dilution, 1:1000; proteintech) and actin(Cat No. 23660-1-AP; dilution, 1:1000; proteintech). Subsequently with secondary antibodies anti-rabbit IgG horseradish peroxidase-linked antibody(Cat No. 7074; dilution, 1:3000; Cell Signaling Technology)

Transwell assay

Transwell filters were added to 24-well plates. Following transfection, 2×10^5 U2OS and HOS cells were incubated for 24 h and resuspended in DMEM with 5% fetal bovine serum. Medium include 10% FBS was used to stimulate cells invasion. After 24 h, cells on the membrane surface were fixed with 4% paraformaldehyde for 15 min. 0.1% crystal violet solution was used to stained the invasion cells.

RNA immunoprecipitation (RIP)

U2OS and HOS cells were lysed by RNA immunoprecipitation (RIP) lysis buffer. Cell extract incubate with magnetic beads conjugated combined antiArgonaute 2 (AGO2) or anti-immunoglobulin G(IgG) antibody for 8 h at 4°C. Then, the RNA binding with antibody was isolated and its CT value was measured by qRT-PCR.

Statistical analysis

The data are expressed as the mean $\pm$ SEM. Student's t-test or one-way

ANOVA was used to compare multiple experimental groups. GraphPad Prism 8.0 was used for statistical analyses. $P < 0.05$ was considered as a statistically significant difference.

Results

LncRNA BACE1-AS expression in OS tissues and cells.

To find the lncRNAs that play a key role in osteosarcoma, we analyzed the dataset of GSE113916 and GSE126209, which included tumor tissues from 16 human patients and 11 adjacent normal tissues. There were 89 lncRNAs down-regulated lncRNAs and 344 up-regulated lncRNAs, among which lncRNA BACE1-AS was significantly down-regulated in osteosarcoma (Figure 1A).

The expression of lncRNA BACE1-AS was detected by qRT-PCR analysis. lncRNA BACE1-AS was reduced in the osteosarcoma compared with paired normal tissues (Fig. 1B). Consistently with the findings in tissues, lncRNA BACE1-AS was revealed to be downregulated in four osteosarcoma cancer cell lines (Saos2、MG63、U2OS and HOS) compared with the normal osteoblast Hfob1.19(Fig. 1C). All these data suggest a negative correlation between lncRNA BACE1-AS and osteosarcoma progression.

LncRNA BACE1-AS inhibited the proliferation, invasion and migration of OS cells.

Considering the lower expression of lncRNA BACE1-AS in U2OS and HOS cell lines, these cells were selected for subsequent experiments. After verified the transfection efficiency of lncRNA BACE1-AS by qRT-PCR.(Fig. 2A). Subsequently, CCK8 analysis was used to detect the effect of lncRNA BACE1-AS on cell proliferation. Overexpression lncRNA BACE1-AS significantly decreased the viability of OS cells compared with control (Fig. 2B). Wound healing experiments showed that lncRNA BACE1-AS inhibited the migration ability of OS cells. (Fig. 2C). In addition, transwell results showed that lncRNA BACE1-AS significantly suppressed the invasive ability of OS cells (Fig. 2D). Western blot results showed that

LncRNA BACE1-AS inhibited the EMT process in osteosarcoma cell lines. Overall, these findings suggested that LncRNA BACE1-AS inhibition restrained OS progression by inhibiting cell viability, migration and invasion.

LncRNA BACE1-AS acts as a sponge for miR-762 in osteosarcoma cell.

Bioinformatics analysis using lncRNASNP showed that miR-762 is a potential downstream target of LncRNA BACE1-AS (Fig. 3A). qRT-PCR analysis also showed that miR-762 was highly expressed in osteosarcoma tissues and cells compared to normal samples and cells (Fig. 3B-C). Therefore, we hypothesized that there is a direct binding between miR-762 and LncRNA BACE1-AS. After verified the transfection efficiency of miR-762 mimics (Fig. 3D), Luciferase reporter gene analysis was used to further confirm our hypothesis. A significant decreased relative luciferase activity in OS cells cotransfected with the BACE1-AS WT plasmid and miR-762 mimics, indicating a direct binding relationship between BACE1-AS and miR-762 (Fig. 3E). Meanwhile, we overexpressed LncRNA BACE1-AS in U2OS and HOS cell lines and found that miR-762 expression was significantly downregulated (Fig. 3F). Anti-AGO2 was used for RIP assay, after transfected with miR-762, miRNPs containing AGO2 expressed higher LncRNA BACE1-AS compared with anti-IgG (Fig. 3G).

LncRNA BACE1-AS modulates SOX7 by competitive binding to miR-762.

SOX7 was identified as a downstream target gene of miR-762 by starBasev2.0, and the potential binding sequence was shown (Fig.4A). Luciferase reporter assay showed that the relative luciferase activity was significantly decreased after cotransfected with SOX7-WT plasmid and miR-762 mimic, which confirmed that SOX7 is a downstream target gene of miR-762 (Fig.4B). The expression level of SOX7 was markedly impeded by miR-762 overexpression(Fig.4C). miR-762-induced inhibitory effects on SOX7 transcription could be partially restored by overexpression of LncRNA BACE1-AS, indicating that LncRNA BACE1-AS competes with CTGF for miR-762(Fig.4D). Finally, qRT-PCR and western blotting

showed that overexpression of LncRNA BACE1-AS also significantly improved the expression level of SOX7 (Fig.4E-F).

LncRNA BACE1-AS/miR-762/SOX7 promotes the EMT process in osteosarcoma cells.

To further investigate the role of the LncRNA BACE1-AS/miR-762/SOX7 axis in osteosarcoma, a rescue assay was performed. After confirmed the transfection efficiency of sh-LncRNA BACE1-AS and miR-762 inhibitor(Fig.5A-B). Wound healing analysis and transwell analysis showed that knockdown LncRNA BACE1-AS promoted cell migration and invasion, while re-expression miR-762 inhibitor reversed this effect (Fig.5C-D). Western blot results showed that knockdown LncRNA BACE1-AS promoted the EMT process in osteosarcoma cell lines, while this effect could be reversed by re-expression of miR-762 inhibitor .

Discussion

Osteosarcoma is a common kind of bone tumor that occurs frequently in adolescents , and is the most common among pediatric bone malignancies, accounting for about 5% of pediatric tumors^{14,15}. Dysregulation of lncrna has been shown to strongly influence OS progression by acting as a tumor suppressor gene or oncogenic genes^{16,17}.

Long noncoding RNAs (lncRNAs) are a type of non-coding RNA defined as transcripts >200 nucleotides that lack the potential to encode proteins and are involved in the regulation of a variety of tumors^{18,19}. In addition, some lncRNAs are closely associated with drug resistance in osteosarcoma cells²⁰. Faghihi et al have found lncRNA BACE1-AS could influence β -secretase-1 (BACE1) mRNA and protein expression in AD mouse brain²¹. It has also been shown that Anisomycin inhibits the proliferation and invasion ability in human OCSCs by increasing lncRNA BACE1-AS levels²². Targeting the expression of lncRNA BACE1-AS may be a potential therapeutic approach for cancer²³.

In this study, we show that lncRNA BACE1-AS is significantly downregulated in osteosarcoma tissues and cells. Functionally, some lncRNAs contain miRNA binding

elements and act as ceRNAs that inhibit miRNA activity²⁴. In the present study, using bioinformatics databases, it was shown that miR-762 may be a target of lncRNA BACE1-AS. miR-762 has been reported several times as a pro-oncogene in a variety of tumors through regulating the Wnt/ β -catenin signaling pathway and mitochondrial function²⁵. In present study, our results showed miR-762 was upregulated in osteosarcoma tissues and cells. Here we found that miR-762 can reverse the effects of lncRNA BACE1-AS on osteosarcoma cells by acting on SOX7, which has been reported to be a potential suppressor gene in cancer²⁶. SOX7 is frequently downregulated in many human cancers and its reduced expression is associated with poor prognosis in several cancers²⁷. Regarding the present study, SOX7 was found to be a direct target of miR-762. In addition, lncRNA BACE1-AS was found to significantly reduce SOX7 expression levels. Inhibition of miR-762 could reverse the effect of knockdown lncRNA BACE1-AS on osteosarcoma cells. Thus, SOX7 mediates the oncogenic role of lncRNA BACE1-AS in the development of bladder cancer.

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Competing Interests: The authors declare that there are no competing interests associated with the manuscript.

Availability of data and material: All data generated or analysed during this study are included in this published article [and its supplementary information files]

Author Contribution: Chunlei Wang performed the majority of experiments and analyzed the data; Tao Zhang, Lin Yang performed cellular experiments; Xinyu Na performed bioinformatic analysis; Yanlong Qu wrote the paper.

Ethics approval: The experimental protocol was established, according to the ethical

guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Harbin Medical University.

Consent to participate: Not applicable.

Consent for publication: Not applicable.

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Figure legend

Figure 1. LncRNA BACE1-AS is downregulated in OS tissues and cells. (A) lncRNAs expression profiles of 16 patients with osteosarcoma obtained from Gene Expression Omnibus (series accession: GSE113916 and GSE126209). (B) qRT-PCR was used to detect

the expression levels of LncRNA BACE1-AS in patient samples (n=20) and adjacent normal tissues (n=10). (C) four OS cell lines (Saos2, U2OS, HOS and MG63) and normal osteoblast cells (hFOB1.19). n=4; ** $p<0.01$ vs normal tissues or hFOB1.19 OS, osteosarcoma.

Figure 2. LncRNA BACE1-AS inhibits osteosarcoma cell proliferation, migration and invasion. (A) qRT-PCR was used to confirm the overexpression ability of LncRNA BACE1-AS in OS cells. (B) Cell proliferative ability of each group were respectively detected by CCK8. (C) Wound healing and (D) transwell assay to detect the ability of cell migration and invasion. (E) Expression levels of EMT associated proteins were examined by western blot analysis. Bar=200 μ M. n=5, * $p<0.05$, ** $p<0.01$ vs pcDNA3.1.

Figure 3. LncRNA BACE1-AS serves as a molecular sponge for miR-762 in OS.

(A) Binding sites of LncRNA BACE1-AS and miR-762. (B-C) qRT-PCR were performed to validate the expression of LncRNA BACE1-AS in OS tissues and cells. * $p<0.05$, ** $p<0.01$ vs normal tissues or hFOB1.19. (D) PCR was used to examine the efficiency of miR-762 mimic and in OS cells. n=5; ** $p<0.01$ vs NC mimic. (E) Luciferase reporter assay in OS cells of LncRNA BACE1-AS and miR-762. n=4; ** $p<0.01$ vs NC mimic. (F) qRT-PCR was implemented to detect the correlation between LncRNA BACE1-AS and miR-762 expression. n=5; ** $p<0.01$ vs pcDNA3.1. (G) AGO2- RNA immunoprecipitation (RIP) assays for correlation between LncRNA BACE1-AS and miR-762. n=3; * $p<0.05$, ** $p<0.01$ vs IgG.

Figure 4. SOX7 is a downstream target gene of miR-762. (A) The starBase website predicted the potential mRNAs miR-762. (B) Luciferase reporter assay in OS cells of SOX7 and miR-762. n=4; ** $p<0.01$ vs NC mimic. (C) qRT-PCR were performed to validate the expression of SOX7 in OS cells. * $p<0.05$, ** $p<0.01$ vs NC-mimic. (D) Luciferase reporter assays revealed the interaction between LncRNA BACE1-AS, miR-762 and SOX7. ** $p<0.01$ vs. NC mimic; # $p<0.05$ vs miR-762 mimic. (E-F) qRT-PCR and western blot was implemented to measure the expression of SOX7. n=5; ** $p<0.01$ vs pcDNA3.1.

Figure 5. LncRNA BACE1-AS modulates osteosarcoma process via miR-762/SOX7.

(A-B) qRT-PCR was used to examine the knockdown efficiency of LncRNA BACE1-AS and miR-762 in OS cells. n=4; * $p<0.05$, ** $p<0.01$ vs sh-scramble or NC inhibitor. (C-D) Wound healing and transwell assay to cell migration and invasion. Bar=200 μ M. (E) Expression levels of EMT relative proteins were examined by western blot analysis.

n=5, * $p<0.05$, ** $p<0.01$ vs sh-scramble; # $P<0.05$, ## $P<0.01$ vs sh-LncRNA BACE1-AS;
& $P<0.05$, && $P<0.01$ vs sh-LncRNA BACE1-AS+miR-762.

Figures

Figure 1

LncRNA BACE1-AS is downregulated in OS tissues and cells. (A) LncRNAs expression profiles of 16 patients with osteosarcoma obtained from Gene Expression Omnibus (series accession: GSE113916 and GSE126209). (B) qRT-PCR was used to detect the expression levels of LncRNA BACE1-AS in patient samples (n=20) and adjacent normal tissues (n=10). (C) four OS cell lines (Saos2, U2OS , HOS and MG63) and normal osteoblast cells (hFOB1.19). n=4; **p<0.01 vs normal tissues or hFOB1.19 OS, osteosarcoma.

Figure 2

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LncRNA BACE1-AS serves as a molecular sponge for miR-762 in OS. (A) Binding sites of LncRNA BACE1-AS and miR-762. (B-C) qRT-PCR were performed to validate the expression of LncRNA BACE1-AS in OS tissues and cells. * $p < 0.05$, ** $p < 0.01$ vs normal tissues or hFOB1.19. (D) PCR was used to examine the efficiency of miR-762 mimic and in OS cells. $n = 5$; ** $p < 0.01$ vs NC mimic. (E) Luciferase reporter assay in OS cells of LncRNA BACE1-AS and miR-762. $n = 4$; ** $p < 0.01$ vs NC mimic. (F) qRT-PCR was implemented to detect the correlation between LncRNA BACE1-AS and miR-762 expression. $n = 5$; ** $p < 0.01$ vs pcDNA3.1. (G) AGO2- RNA immunoprecipitation (RIP) assays for correlation between LncRNA BACE1-AS and miR-762. $n = 3$; * $p < 0.05$, ** $p < 0.01$ vs IgG.

Figure 4

SOX7 is a downstream target gene of miR-762. (A) The starBase website predicted the potential mRNAs miR-762. (B) Luciferase reporter assay in OS cells of SOX7 and miR-762. $n=4$; $^{**}p<0.01$ vs NC mimic. (C) qRT-PCR were performed to validate the expression of SOX7 in OS cells. $^{*}p<0.05$, $^{**}p<0.01$ vs NC-mimic. (D) Luciferase reporter assays revealed the interaction between LncRNA BACE1-AS, miR-762 and SOX7. $^{**}p<0.01$ vs. NC mimic; $^{#}p<0.05$ vs miR-762 mimic. (E-F) qRT-PCR and western blot was implemented to measure the expression of SOX7. $n=5$; $^{**}p<0.01$ vs pcDNA3.1.

Figure 5

LncRNA BACE1-AS modulates osteosarcoma process via miR-762/SOX7. (A-B) qRT-PCR was used to examine the knockdown efficiency of LncRNA BACE1-AS and miR-762 in OS cells. $n=4$; * $p<0.05$, ** $p<0.01$ vs sh-scramble or NC inhibitor. (C-D) Wound healing and transwell assay to cell migration and invasion. Bar=200 μ m. (E) Expression levels of EMT relative proteins were examined by western blot analysis.

n=5,*p<0.05,**p<0.01 vs sh-scramble; #P<0.05, ##P<0.01 vs sh-LncRNA BACE1-AS; &P<0.05, &&P<0.01 vs sh-LncRNA BACE1-AS+miR-762.