LINC01614 Promotes Osteosarcoma Progression as ceRNA to Target SNX3 by Sponging hsa-miR-520a-3p

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Research

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Abstract

Long noncoding RNAs (lncRNAs) have been reported as significant biomarkers for diagnosis and prognosis in osteosarcoma (OS), the most malignant bone tumor usually observed in children and adolescents. In the present study, we detected differentially expressed lncRNAs in three OS and paired adjacent tissues through RNA-seq technology. By comprehensively analyzing the expression profile and bioinformatics, we determined the relationship between noncoding RNA and OS-related functions, signal pathway, regulatory network, patient survival information and selected LINC01614 as the study object. Through related *in vitro* experiments, we confirmed that *LINC01614* knockdown could inhibit the proliferation, invasion, and metastasis of OS cells. Furthermore, we used the luciferase reporter assay and qRT-PCR to confirm the regulatory relationship between LINC01614/hsa-miR-520a-3p/SNX3. We performed relevant *in vitro* experiments to prove that LINC01614 promotes the proliferation, invasion, and metastasis of OS cells through the LINC01614/hsa-miR-520a-3p/SNX3 axis. In conclusion, we identified that lncRNAs participate in various malignant behaviors in OS. We also proved that LINC01614 could function as competing endogenous RNAs and promote the proliferation, invasion, and metastasis of OS cells, and thus acts as a novel prognostic marker for OS in clinic.

Introduction

Osteosarcoma (OS) is the most frequent malignant primary bone tumor observed in children and adolescents and is associated with a limited overall survival and early metastasis[1]. Despite improvements in treatment strategies, the 5-year survival rate of OS patients is less than 70%. The early clinical symptoms of OS are not specific, but the disease progresses rapidly. Once lung metastasis occurs, the 5-year survival rate drops to less than 30%[2]. Therefore, exploring and identifying novel and effective prognostic biomarkers and therapeutic targets for OS patients is critical.

Long noncoding RNAs (lncRNAs) are a class of transcripts composed of more than 200 nucleotides long that have no protein-coding capacity[3]. However, some recent studies have shown that lncRNAs participate in several important biological processes such as cell signaling transduction[4], cell cycle regulation[5], chromatin remodeling[6], and epigenetic gene regulation[7]. They can act as a competitor to diverse proteins and a binder to many miRNAs, and can interact with different RNA-binding proteins[8]. Abnormal expression of lncRNA greatly affects the metastatic process, drug resistance, and cancer development in many different types of tumors such as hepatocellular carcinoma, non-small cell lung cancer, and ovarian cancer[9–11]. Several studies have shown that the aberrant regulation of lncRNA is related to OS pathogenesis and metastasis[12, 13]. However, the role of lncRNA in OS is still diverse and unclear. Therefore, summarizing the lncRNA expression profiles of OS is helpful in improving the accuracy of diagnosis of OS through lncRNA. In addition, some lncRNAs (e.g., SNHG12[14], CCAT1[15], DANCR[16], GAS5[17], and lncRNA-p21[18]) and lncRNA-miRNA-mRNA axes related to OS (e.g., MALAT1-miR-205-SMAD4[19], HOXA11-AS-miR-124-3p-ROCK1[20], APTR↑-miR-132-3p-YAP1[21], NEAT1-miR-34c-BCL-2[22], XIST-miR-21-5p-PDCD4[23]) have been gradually discovered in recent years. However, the role of LINC01614, a lncRNA in OS, and its regulatory pathway have not been reported.

In this study, we performed RNA sequencing to compare OS tumor and paired normal tissues to identify differentially expressed lncRNAs. We also identified the functions of lncRNAs in OS biological processes.

Materials And Methods

Clinical samples

Tissues from 10 patients with OS at the Second Hospital of Jilin University (age: 10–63 years, 4 males/6 females) were obtained through surgical resection between May 2017 and December 2018. These patients had histologically diagnosed OS but had not received any radiotherapy and/or chemotherapy prior to surgery. Three pairs of OS (C) and paracancerous (N) tissues were collected, transported into liquid nitrogen after resection, and stored at – 80 °C until analysis. We received informed consent from all participants, and the study was approved by the hospital's ethics committee (2016.169). The clinical and demographical characteristics of the patients are summarized in Table 1.

Table 1
Clinical characteristics of the subjects.

No.	Age	Sex	Anatomic location	Stage
1	14	M	Femur	ⅡA
2	35	F	Femur	ⅡB
3	12	F	Femur	ⅡA
4	15	M	Femur	ⅡB
5	63	F	Femur	ⅡB
6	10	F	Femur	ⅡA
7	59	M	Femur	ⅡB
8	37	F	Femur	ⅡB
9	54	F	Tibia	ⅡA
10	42	M	Scapula	ⅡB

RNA isolation, library preparation, and sequencing

RNA isolation

The RNA isolated in total was from three pairs of OS and paracancerous tissues from the patients with OS by making use of TRIzol reagent (Invitrogen, USA).

RNA quantification and qualification

RNA contamination and degradation were determined using 1% agarose gels as per the protocol. The verification for the purity of RNA was done through the Nano Photometer® (IMPLEN, CA, USA), while the integrity of RNA was evaluated using the RNA Nano 6000 Assay Kit and Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The concentration of RNA determined using the Qubit® RNA Assay Kit and Qubit® 2.0 Fluorometer (Life Technologies, CA, USA).

Library preparation for lncRNA sequencing

Sequencing libraries were prepared according to the standard protocol. Briefly, 3 µg of RNA was inputted, and ribosomal RNA was separated using the Ribo-zero™ rRNA Removal Kit (Epicentre, USA) and cleansed through ethanol precipitation. The rRNA-free RNA was then processed using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina® (NEB, USA) regarding the protocol. Performance of fragmentation was brief and at considerably higher temperatures using divalent positive ions and First Strand Synthesis Reaction Buffer (5×). First-strand complementary DNA (cDNA) was produced using a hexamer primer which was random and M-MuLV reverse transcriptase (RNase H). Second-strand cDNA was then produced by DNA Polymerase I, RNase H, and dNTPs; dTTP was interchanged by dUTP in the buffer where reactions are carried out. The AMPure XP system was used (Beckman Coulter, Beverly, USA) to generate 150–200 bp fragments of cDNA in a preferential manner. Before the polymerase chain reaction (PCR), the cDNA selected was put together with 3-µL USER Enzyme (NEB, USA) at 37 °C for 15 minutes and then at 95 °C for 5 min. Performance of PCR was conducted using Phusion High-Fidelity DNA polymerase, universal PCR primers, and an index (X) primer. Purification of the products was done using the AMPure XP system, and the quality of the library was evaluated using the Bioanalyzer 2100 system.

Clustering and sequencing

Index-coded samples were clustered was using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) as per the protocol. After generation of clusters, processing of the libraries was done using an Illumina Hiseq 4000 platform, and 150 bp paired-end reads were produced.

GO and KEGG enrichment analysis

Gene Ontology (GO) analysis of enrichment of genes which are differently expressed or lncRNA target genes was carried out using GOSeq. Correction of bias of the gene length was performed and GO terms which had a P value of < 0.05 already corrected were rendered enriched in a significant manner[24]. Drawing from molecular databases which are large-scale and produced using genome sequencing along with other experimental technologies which were highly throughout. KEGG (<http://www.genome.jp/kegg/>) is a crucial source used for comprehension of functions which are at a high level and biological system utilities[25]. KOBAS software was used to assess the enrichment statistics of those same genes in KEGG pathways[26].

Cell lines and culture conditions

Cell lines (143B, MG63, HOS, V2OS, and Saos2) of Human OS and the osteoblast cell line (hFOB 1.19) of the human being were obtained from American Type Culture Collection. Culturing of the hFOB 1.19 cells

was done in Dulbecco's modified Eagle's medium (DMEM)/F12 (Corning, Armonk, NY, USA), and culturing of Saos-2 cells was done in McCoy's 5a medium which was modified (Gibco, NY, USA). Culturing of other cell lines was done in the Eagle's minimum essential medium in conjunction with 1% antibiotic (Sigma, St. Louis, MO, USA) and 10% fetal bovine serum (FBS, BD Biosciences, San Jose, CA, USA) at 37 °C in an atmosphere which is humidified using 5% Carbon(iv)oxide and air which is 95%. Passaged of the cells was done after every 2 or 3 days.

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNAs were obtained with the use of TRIzol reagent (Invitrogen, USA) the same was as the protocol of the manufacturer. Afterwards, the cDNAs were produced through usage of the PrimeScript RT Reagents Kit (TaKaRa) according to the instructions of the manufacturer. Performance of the quantitative real-time reverse transcription (qRT)-PCR was done in a 20 µL reaction mixture including 1 µL PCR forward primer (10 µM), 1 µL PCR reverse primer (10 µM), 4 µL cDNA, 10 µL 2 × Master Mix (TaKaRa, Otsu, Japan), and 4 µL double distilled water. The protocol was run at for 10 minutes at 95 °C, 95 °C (10 s) followed suit, and then 60 °C (60 s) for 40 cycles. The reference gene used was β-Actin/U6. Calculation of the relative expression level was done using the $2^{-\Delta\Delta C_t}$ method. A < 0.05 P value was rendered significantly statistical. The following are the primers which were used: LINC01614: F, 5'-GTCAACCAAGAGCGAAGCCA-3'; R, 5'-GGGCTTGGACACAGACCCTA-3' MALAT1: F, 5'-TCTCTCCCCTCCCTTGGTCT-3'; R, 5'-ACTGGGTCTGGCTTCTCTGG-3'. hsa-miR-520a-3p: CGCGAAAGTGCTTCCCTTTGGACTGT. SNX3: F, 5'-TCGATGTGAGCAACCCGCAAAC-3'; R, 5'-CTTCGCAGCCATTCAAAGTCACTG-3'.

Survival analysis

Plotting of the overall survival curves were done in accordance with the method of the Kaplan–Meier, with 66 OS cases in the datasets of “TARGET” (<https://ocg.cancer.gov/programs/target>).

Protein–protein interaction analysis

Protein–protein interaction (PPI) analysis of differentially expressed genes was carried out on the basis of the information from the STRING database (<https://string-db.org/>), which stores PPIs that are known and can also be predicted. For the existing species, networks were constructed by obtaining their target gene lists from the database. For the remaining species, Blastx (v2.2.28) was performed basically for the alignment of the target gene sequences to corresponding protein sequences of reference, after which building of networks was done in accordance with the known interactions of sequences which were selected.

Plasmids construction and transfection

All the plasmids were purchased from PPL (Genebio Technology, Inc, Nanjing, China). Insertion of cDNA oligonucleotides which target LINC01614 into the vector pLKO.1, termed LINC01614 short hairpin RNA (shRNA) was done. The shRNA-LINC01614 sequence was as follows 5'-

CCGGGGATTGATAGTGTCTTAACCTCGAGTTAAAGAACACTATCAATCCTTTTTTG-3'; the shRNA-NC sequence was as follows 5'-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTTG-3'. The sequence of hsa-miR-520a-3p mimics was AAAGUGCUUCCCUUUGGACUGU, while that of mimic NC was UUGUACUACACAAAAGUACUG. The sequence of the hsa-miR-520a-3p inhibitor was ACUUUCACGAAGGGAAACCUGA, while that of the inhibitor-NC was CAGUACUUUUGUGUAGUACAA. The shRNA-SNX3 sequence was as follows 5'-GCGUCAGCUUCCUUUUAGA-3'; the shRNA-NC sequence was as follows 5'-UUCUCCGAACGUGUCACGUTT-3'. Performance of all transfections were done using the X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) in accordance with the user's guide. After 48 h, the cells were reaped for the subsequent assays.

Cell counting kit-8 assay

Fifteen hundred cells were seeded in each well, and measurement of the rate of proliferation rate was done by cell counting kit-8 ((Beytime, ShangHai, China) at 24 h, 48 h, and 72 h. Measurement of the optical density was done at a 450 nm wavelength. Performance of the assay was done in accordance with the protocols of the manufacturer.

Transwell assay

Analysis of the ability of cell migration ability was done using the transwell assay performed in transwell chambers of 24-well(Corning, USA). The incubated chamber was taken out, liquid in the upper chamber was sucked out, and 200 μ L single cell suspension was added to the upper chamber gently along the sides of the wall. Three holes were made in each well. Then, addition of a 600 μ L medium which was complete and had in it 10% FBS was done to the 24-well plate under the chamber and maintained at 37°C for 24 h for routine cultivation. After incubation, removal of cells which were located in the inner membrane was done. 700 μ L methanol was used for fixation of the outer membrane and it was stained with 1% of a solution which was crystal violate in color. Observation of the cells was done under a microscope after which the fields were selected randomly for photography purposes.

Wound healing assay

After transfection, when cell proliferation reached 80–90% of the 6-well plate, the culture medium was discarded and 1 mL of mitomycin solution was added. It was allowed to stand for 1 h at 37°C; the liquid in the hole was sucked out and 1 mL phosphate buffered saline (PBS) was added. Then, a vertical line was drawn using a steel ruler as the standard, and a vertical line with 10 μ L gun head perpendicular to the plate surface and the back side was marked. The liquid in the hole was discarded and rinsed with PBS gently for three times. Then, 600 μ L complete medium was added and placed in an incubator at 37°C for routine culture. At 0 and 48 h after the scratch, the film was placed under the microscope and the photo coordinates were recorded. The position of the parallel line on the back side was marked to ensure that the shooting position was consistent each time. The scratch area of each group was analyzed using ImageJ software, the healing percentage of 48-h scratch was calculated, and the experiment was repeated three times.

Western blotting assay

Lysing of cells was conducted with the help of Radio Immunoprecipitation Assay cell lysis buffer and protease inhibitor (Beyotime, Jiangsu, China) was used for supplementation. Concentration of proteins was determined using the BCA protein detection kit (Beyotime, Jiangsu, China). The same amount of protein was separated by 15% SDS-PAGE and later moved to the PVDF membrane. After being sealed with skimmed milk, incubation of the membranes was done using primary antibodies whose dilution was in the TBST buffer the whole night at a temperature of 4 °C and later the HRP-conjugated secondary antibody at room temperature for 2 hours. Bands which were specific were detected through electrochemiluminescence and quantified using the density method. Rabbit anti-SNX3 antibody was purchased from the BIOS company (Beijing, China).

Dual-luciferase reporter assay

In a pGL3 control luciferase reporter vector (Promega), the cDNA fragment containing a wild-type or mutant LINC01614 fragment and 3' untranslated region (UTR) of *snx3* were subcloned in the luciferase gene's downstream. Confirmation of all constructs nucleotide sequences was done through DNA sequencing. Luciferase reporter plasmids plus hsa-miR-520a-3p mimics or miR-NC were cotransfected into human embryonic kidney 293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Collection of the cells was done after 2 days within which transfection was done and activity of the luciferase was found using the Dual-Luciferase Reporter Assay System (Promega).

Statistical Analysis

Differences existing between all the two groups were found via Student's t-test and were rendered significantly statistical at a < 0.05 P value. The data were analyzed using SPSS 19.0 and GraphPad Prism 7.0.

Results

1. Expression profiling in OS and normal tissues

To explore expression alternations in OS, we performed lncRNA, mRNA, and miRNA sequencing to compare the expression profiles between the collected form and the discarded OS specimens (Fig. 1A). The Pearson correlation analysis revealed that the expression similarity of each group was strong (Fig. 1B). The levels of 67 lncRNAs were significantly upregulated, while those of 96 lncRNAs were downregulated in OS tissues compared with adjacent tissues ($|\text{fold change}| > 1.5$, $P < 0.05$, Fig. 1C). A total of 207 miRNAs and 4247 mRNAs were significantly differentially expressed in OS ($|\text{fold change}| > 1.5$, $P < 0.05$, Fig. 1D). These results showed that OS tissues have extensive transcription alternations compared with paracancerous tissues.

2. Functional annotation of differentially expressed lncRNAs in OS

Because analyzing the function of lncRNAs directly is not possible, we performed the functional analysis of mRNA that is targeted by lncRNA. We performed the GO analysis and KEGG enrichment to explore the functions of differentially expressed lncRNAs. The GO analysis revealed that the differentially expressed lncRNAs are widely involved in biological processes including the ubiquitin-dependent protein catabolic process (GO: 0006511) and regulation of protein binding (GO: 0043393) (Fig. 2A, 2B, 2C). On the other hand, KEGG enrichment revealed that lncRNAs are involved in signaling pathways including the PI3K-Akt signaling pathway and AMPK pathways that have been reported to promote OS malignant behaviors (Fig. 2D). These results confirmed the oncogenic role of differentially expressed lncRNAs in OS.

3. LINC01614 and MALAT1 are involved in competing endogenous RNA networks in OS

Through further evaluations of lncRNAs, we found the top five upregulated lncRNAs in OS: LINC01614, MALAT1, RP11-343B18.2, RP11-229O3.1, and MIR181A1HG (Fig. 2E). Further, we found LINC01614 had the most distinct overexpressed level and was firstly reported in OS. Moreover, compared with normal tissues, we observed significant overexpression of MALAT1 in OS, which is the most frequently reported lncRNA in OS. Studies have shown that lncRNAs could regulate target gene expression by functioning as competing endogenous RNAs (ceRNAs). To identify LINC01614- and MALAT1-associated miRNAs and mRNAs, we constructed the lncRNA-miRNA-mRNA network of LINC01614 and MALAT1 (Fig. 3A, 3B). We found five miRNAs negatively correlated with LINC01614 and six miRNAs negatively correlated with MALAT1, which were proven to participate widely in the regulation of tumor biological processes. By overlapping the miRNA-targeted mRNAs with LINC01614- and MALAT1-positive-associated genes, the lncRNA-miRNA-mRNA networks of LINC01614 and MALAT1 were constructed. Furthermore, by performing GO analysis of the genes from the network, we found the genes associated with LINC01614, which were involved in the negative regulation of cell growth (GO: 0030308), cell migration (GO: 0016477), and other biological processes (Table 2, 3). Moreover, we analyzed the PPI networks of LINC01614 and MALAT1 to detect functional genes (Fig. 3C, 3D). These results indicated that LINC01614 plays a crucial role in regulating gene expression by functioning as ceRNAs.

Table 2

Go analysis and KEGG pathway enrichment of mRNAs in LINC01614-associated LncRNA-miRNA-mRNA network.

Term	Genes	PValue
GO biological process		
Carnitine metabolic process, CoA-linked(GO:0019254)	ACADM, CRAT	0.0042
Negative regulation of cell growth(GO:0030308)	INHBA, DACT3, BMPR2, BCL6, APBB2, CAPRIN2, SERTAD2, SLIT3	0.0067
Ubiquitin-dependent protein catabolic process(GO:0006511)	PSMA2, USP3, USP38, PSMD3, FBXO32, TCEB1, USP24, USP13, USP31	0.0076
Intracellular protein transport(GO:0006886)	KIF13A, AP2A1, TOM1L2, ANKRD50, IPO4, TIMM17A, SNX8, TIMM17B, TOM1, DSCR3, SAR1B, CLTCL1	0.0084
NLS-bearing protein import into nucleus(GO:0006607)	IPO4, DAG1, KPNA5, KPNA4	0.0118
Negative regulation of phagocytosis(GO:0050765)	SNX3, ADIPOQ	0.014
Regulation of GTPase activity(GO:0043087)	BVES, SYDE1, BCL6, EFNA5, RICTOR	0.0142
Chromatin remodeling(GO:0006338)	HDAC4, KAT2B, RUVBL2, INO80C, MYSM1, SUV39H2	0.0162
Regulation of cell shape(GO:0008360)	FGD1, FMNL3, BRWD1, BVES, CDC42EP1, STRIP2, MYH9, BAMBI	0.0169
Cell migration(GO:0016477)	PTPRK, FMNL3, CCDC88A, STRIP2, VAV2, BAMBI, MYO18A, USP24	0.0237
Protein ubiquitination involved in ubiquitin-dependent protein catabolic process(GO:0042787)	CUL5, RNF115, RMND5B, ANKIB1, NEDD4L, TCEB1, KLHL20, SPOP	0.0248
Regulation of protein catabolic process(GO:0042176)	XPO1, PSMD3, CHEK2	0.0284
KEGG pathway		
Huntington's disease	NDUFV3, NDUFB5, NDUF54, NDUF3A, AP2A1, CREB3L2, CLTCL1, NDUF51, SOD2, HIP1, UQCRB	0.0174
Lysine degradation	KMT2E, KMT2A, KMT2C, AASS, SUV39H2	0.0297

Table 3

Go analysis and KEGG pathway enrichment of mRNAs in MALAT1-associated LncRNA-miRNA-mRNA network.

Term	Genes	PValue
GO biological process		
Osteoblast differentiation(GO:0001649)	BMP3, BMP2, RRBP1, CREB3L1, GJA1, SYNCRIP, CLTC, GPNMB, RBMX, TPM4, ADAR	0.0001
Myo-inositol transport(GO:0015798)	SLC5A3, SLC2A13, PGAP1	0.0015
Ubiquitin-dependent protein catabolic process(GO:0006511)	CUL2, CUL5, UBE4A, NUB1, PSMA3, USP38, PSMD3, FBXO32, TCEB1, RLIM, TTC3, USP13	0.0026
Ephrin receptor signaling pathway(GO:0048013)	ACTB, AP2B1, ADAM10, AP2A1, EFNB2, EFNA4, YES1, RASA1	0.0031
Negative regulation of cell proliferation(GO:0008285)	DLC1, BMP2, KAT2B, KMT2A, NF2, PTPN2, PTPN14, SOX4, CDK6, ZBTB16, SLIT3, NOTCH2, CUL2, CUL5, ETS1, SPEG, PRKRA, GPNMB, RAPGEF2	0.0035
Microtubule cytoskeleton organization(GO:0000226)	EML3, PCNT, MAP1A, OBSL1, DST, TACC1, EML4	0.005
Protein transport(GO:0015031)	RAB2B, RRBP1, PGAP1, SNX25, KTN1, SNX4, MYH9, COG4, DGKD, IPO4, NUP210, ARF3, ARCN1, TOM1, RAB15, SEC22C, SERP1, PIK3R2	0.0076
Myoblast fusion(GO:0007520)	CDON, ADAM12, MYH9, PITX2	0.0081
Actin cytoskeleton organization(GO:0030036)	CORO1C, DLC1, PFN2, NPHP4, NF2, FSCN1, AMOT, CAP1, TRIP10	0.0087
Generation of neurons(GO:0048699)	SIX1, CDK6, THOC2	0.0096
Cytoskeleton organization(GO:0007010)	ACTB, AJUBA, BRWD3, ABLIM1, DES, OBSL1, MSN, DST, PSTPIP2, SPTB	0.0101
Membrane protein ectodomain proteolysis(GO:0006509)	ADAM10, DAG1, MYH9, RBMX	0.0123
KEGG pathway		
Hypertrophic cardiomyopathy (HCM)	ACTB, DES, ITGA5, ITGB6, DAG1, CACNB1, ITGA4, TPM4, TPM3	0.0001
Dilated cardiomyopathy	ACTB, DES, ITGA5, ITGB6, DAG1, CACNB1, ITGA4, TPM4, TPM3	0.0014

Term	Genes	PValue
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	ACTB, DES, ITGA5, ITGB6, DAG1, CACNB1, GJA1, ITGA4	0.0016
Lysine degradation	KMT2E, KMT2A, KMT2C, WHSC1L1, ALDH2, AASS, SUV39H2	0.002
Bacterial invasion of epithelial cells	ACTB, ITGA5, CBL, MET, CLTC, CLTCL1, FN1, PIK3R2	0.0038
Huntington's disease	AP2B1, NDUFB5, NDUFS4, SLC25A4, GNAQ, AP2A1, CYCS, CREB3L1, ATP5A1, CLTC, CLTCL1, UQCRB	0.0104
Endocytosis	CBL, PSD3, ASAP1, SNX4, CLTC, AP2B1, SH3GLB1, AP2A1, ARF3, PDGFRA, NEDD4L, RAB10, CLTCL1	0.0213
Renal cell carcinoma	CUL2, ETS1, MET, RAP1B, TCEB1, PIK3R2	0.0269
Endocrine and other factor-regulated calcium reabsorption	AP2B1, GNAQ, AP2A1, CLTC, CLTCL1	0.0278
Transcriptional misregulation in cancer	KMT2A, SIX1, MET, TSPAN7, GRIA3, BCL2L1, ZBTB16, ETV6, MEIS1, CDK14	0.028

4. LINC01614 and MALAT1 levels are high in OS cell lines and are associated with poor prognosis in OS patients

We validated the findings of LINC01614 and MALAT1 levels *in vitro*. We designed primers for LINC01614 and MALAT1 and performed qRT-PCR assays to evaluate their levels in five OS cell lines compared with a normal human osteoblast cell line and cancer and paracancerous tissues (Fig. 4A,4B,4C,4D). We found that LINC01614 was significantly overexpressed in OS cells and cancer tissues compared with normal osteoblast cells and adjacent tissues. To understand the effect of LINC01614 and MALAT1 in OS patients, we analyzed overall survival in these patients. The results demonstrated that the high expression levels of LINC01614 and MALAT1 were negatively associated with overall survival in OS patients (LINC01614: $P = 0.0450$, MALAT1: $P = 0.0195$ in Fig. 4E,4F). In addition, LINC01614 might function as a proto-oncogene lncRNA in OS.

5. LINC01614 knockdown inhibits proliferation, invasion, and metastasis in Saos2 and MG63 cells

Rapid proliferation, invasion, and migration of cells are the main factors affecting tumor progression. In our studies, LINC01614 and MALAT1 were found to be highly expressed in OS cells, and the role of MALAT1 in OS cells was reported. However, the effect of LINC01614 on the proliferation, migration, and invasion of OS cells was not determined. We transfected Saos2 and MG63 cells with shRNA-NC and shRNA-LINC01614, and successful transfection was confirmed by real-time PCR (Fig. 5A,5B). The CCK8 assay was performed to detect the proliferation ability of Saos2 and MG63 cells. Compared with the

empty vector control group, the proliferation of Saos2 and MG63 cells was significantly inhibited after *LINC01614* knockdown (Fig. 5C,5D).

To verify the effect of *LINC01614* on the invasion ability of OS cells, we performed cell invasion experiments. The MG63 and Saos2 cells were seeded into chambers for 24 h. Cells that migrated through the matrigel-coated chambers were stained with crystal violet. Representative images were photographed, and the cells were counted in three independent experiments. The results showed that *LINC01614* could significantly inhibit the invasion of Saos2 and MG63 cells (Fig. 5E,5F,5G).

Metastasis of OS is associated with the survival rate of patients with OS. To explore the effect of *LINC01614* on the migration of OS cells, we designed a wound healing assay. The lateral migration ability of OS cells was measured by the wound healing assay. Representative images of wound were photographed at 0 and 48 h, and the healed rate is shown in Fig. 5H,5I,5J. In conclusion, *LINC01614* knockdown can significantly inhibit the proliferation, invasion, and migration of OS cells.

6. *LINC01614* regulates *SNX3* expression in an hsa-miR-520a-3p-dependent manner

To further explore the mechanism of *LINC01614* participation in OS progression, we focused on searching for a *LINC01614*-regulated miRNA. In our previous bioinformatics analysis, *Linc0614* was found to regulate hsa-miR-520a-3p. Therefore, we performed a dual-luciferase reporter assay to verify the previous results. The *LINC01614* fragment cloned into the luciferase construct was used for the assay. As shown in Fig. 6A, the relative luciferase activity in the cells cotransfected with *LINC01614*-WT and hsa-miR-520a-3p was significantly inhibited compared with that in the cells cotransfected with *LINC01614*-WT and miR-NC. This effect was abolished when the putative *LINC01614* binding site was mutated. We performed real-time PCR to detect hsa-miR-520a-3p in OS cells and normal human osteoblast cells and in cancer and adjacent tissues. We also performed real-time PCR to detect hsa-miR-520a-3p expression in OS cells after *LINC01614* knockdown. The real-time PCR results were consistent with our bioinformatics analysis results. We found that the hsa-miR-520a-3p level was significantly low in OS cells and cancer tissues compared with normal human osteoblast cells and adjacent tissues (Fig. 6B). *LINC01614* knockdown increased hsa-miR-520a-3p expression Fig. 6C. These results suggested that *LINC01614* inhibits hsa-miR-520a-3p expression by direct binding.

Similarly, we validated the target gene *SNX3* of hsa-miR-520a-3p by the bioinformatics analysis, dual-luciferase reporter assay, real-time PCR, and western blotting. The results of verification were also satisfactory. As shown in Fig. 6D, *SNX3* 3'UTR-WT reduced luciferase activity after transfection with hsa-miR-520a-3p compared with miRNA; however, it failed to reduce luciferase activity when the putative hsa-miR-520a-3p-binding site was mutated (*SNX3* 3'UTR-Mut), indicating that hsa-miR-520a-3p specifically targets the binding sites in the *SNX3* 3'UTR region. Real-time PCR and western blotting results showed that *SNX3* mRNA and protein were significantly overexpressed in OS cells and cancer tissues compared with normal human osteoblast cells and adjacent tissues (Fig. 6E,6F), whereas *SNX3* mRNA and protein expression was significantly decreased and increased after overexpression and suppression respectively

of hsa-miR-520a-3p (Fig. 6G,6F). These results indicated that hsa-miR-520a-3p regulates SNX3 expression by directly binding to the 3'UTR region of SNX3 mRNA. To confirm the interaction of LINC01614 with hsa-miR-520a-3p involved in the regulation of SNX3-3'UTR, we measured SNX3 levels through qRT-PCR and western blotting. *LINC01614* knockdown significantly reduced SNX3 expression (Fig. 6I,6J), whereas the inhibition of hsa-miR-520a-3p significantly increased the expression. These results strongly suggested that LINC01614 regulates SNX3 expression in an hsa-miR-520a-3p-dependent manner.

7. LINC01614 could function as ceRNA by directly sponging hsa-miR-520a-3p

We proved hsa-miR-520a-3p is the direct target of LINC01614 in the previous experiment. To further explore the function and mechanism of hsa-miR-520a-3p in OS cells, MG63 and Saos2 cells were transfected with mimic-NC, hsa-miR-520a-3p-mimic, inhibitor-NC, or hsa-miR-520a-3p-inhibitor, and then, CCK8, transwell, and wound healing assays were performed. Successful transfection was confirmed by real-time PCR (Fig. 7A,7B). As shown in Fig. 7C-7J, our results showed that hsa-miR-520a-3p-mimic can inhibit the proliferation, invasion, and migration of OS cells, whereas hsa-miR-520a-3p-inhibitor can promote the proliferation, invasion, and migration of OS cells. The effect of hsa-miR-520a-3p-mimic on OS cells was consistent with that of shRNA-LINC01614, while that of hsa-miR-520a-3p-inhibitor was opposite to that of shRNA-LINC01614. Moreover, hsa-miR-520a-3p was confirmed as a functional target of LINC01614. Taken together, our results proved that LINC01614 acts through the LINC01614/hsa-miR-520a-3p axis.

8. LINC01614 promotes the proliferation, invasion, and lateral migration of OS cells via the LINC01614/hsa-miR-520a-3p/SNX3 axis

We proved that LINC01614 regulates SNX3 expression in an hsa-miR-520a-3p-dependent manner and hsa-miR-520a-3p is a functional target of LINC01614. To determine the functions of SNX3, *SNX3* was knocked down in two types of OS cells, and then, CCK8, transwell, and wound healing assays were performed. Successful transfection was confirmed through real-time PCR (Fig. 8A,8B). As shown in Fig. 8C-8J, our results showed that shRNA-SNX3 can inhibit the proliferation, invasion, and migration of OS cells. The effect of shRNA-SNX3 on OS cells was consistent with that of shRNA-LINC01614 and hsa-miR-520a-3p-mimic. Taken together, our results proved that LINC01614 promotes the proliferation, invasion, and lateral migration of OS cells through the LINC01614/hsa-miR-520a-3p/SNX3 axis.

Discussion

In recent years, combined treatment of OS has improved progression-free survival from 10–65%[27]. Unfortunately, 20–25% patients with newly diagnosed OS have metastatic lesions at the time of initial diagnosis. These patients have a poor prognosis with a 5-year survival rate of less than 30%[2]. Studies have shown that lncRNAs play a critical role in malignant biological processes in OS. However, aberrant lncRNA expression profiling in OS requires further exploration. Expression profiles of OS and adjacent

tissues help reveal the roles of critical lncRNAs in the OS disease course. In this study, we collected three paired OS and adjacent tissues from surgical wastes and detected alternations in transcriptome variations by RNA-seq. The expression levels of 163 lncRNAs were significantly altered (67 high, 96 low, |fold change| > 1.5, $P < 0.05$) in OS tissues compared with normal tissues.

lncRNAs play an important role in cell growth and proliferation, regulation of signaling pathways, apoptosis, invasion, and metastasis in OS[12, 28, 29]. For example, MALAT-1 acts through the PI3K-AKT signaling pathway to promote the malignant biological phenotype in OS[30]. Differentially expressed lncRNAs have been reported to be closely related to various biological processes including the protein binding and ubiquitin-dependent protein catabolic process, which are essential for malignant progression of OS[31].

lncRNAs can act as predictive biomarkers of prognosis and as therapeutic targets against drug resistance in OS[32]. A two-stage case-control study showed that patients with the lncRNA HOTAIR rs7958904 CC genotype had significantly lower HOTAIR expression levels and OS risk than those with other genotypes[33]. Another report showed that lncRNA HOTTIP overexpression could promote cisplatin resistance by activating the Wnt/ β -catenin pathway[34]. Here, we found that LINC01614, MALAT1, RP11-343B18.2, RP11-229O3.1, and MIR181A1HG were the top five overexpressed lncRNAs and that LINC01614 had the most distinct expression level and has been reported for the first time in OS. Based on the hypothesis that lncRNAs could regulate biological processes through ceRNAs[8], we constructed LINC01614 and MALAT1 lncRNA-miRNA-mRNA ceRNA networks and found the endogenous RNA regulation mechanism among lncRNAs, miRNAs, and mRNAs. *In vitro* real-time PCR confirmed LINC01614 overexpression in OS cells compared with normal osteoblast cells. We also showed that higher expression levels of LINC01614 and MALAT1 were negatively associated with overall survival in patients with OS.

In recent years, with an increase in annotated lncRNAs (<http://www.lncrnadb.org/>), various regulatory functions of lncRNAs in the emergence and progress of OS have been discovered. For example, lncRNA HOXA11-AS promoted the proliferation, invasion, and migration of OS cells[20], whereas WWOXAS1 inhibited the proliferation, invasion, and migration of OS cells[35]. Tay[8] reported that lncRNAs and miRNAs can mutual adjustment and interactions with each other through natural miRNA sponges or ceRNAs. A study on the lncRNA-miRNA interaction and cross regulation in OS showed that down-regulated expression of SNHG12, which is a kind of lncRNAs suppressed OS cell proliferation and regulated the cell cycle through the regulation of Notch2 by alleviating miR-195-5p *in vitro*[14]. Overexpression of lncRNA-ATB heightened OS cell proliferation, invasion, and migration and affected tumor expansion *in vivo* by upregulating and downregulating miR-200 s target genes such as ZEB2 and ZEB1[36]. Although these findings are still in the basic research stage, from a long-term perspective, these findings will play a crucial role and promote the early diagnosis and targeted treatment of OS. However, many lncRNAs and related mediation networks are undetected. Therefore, exploring the unknown function of lncRNAs and improving the existing lncRNA database are necessary.

In this study, we identified LINC01614, which is highly expressed in OS, through full transcriptome sequencing analysis of clinical samples and explored its function and mechanism in OS. Our study demonstrated that *LINC01614* knockdown can significantly inhibit the proliferation, migration, and invasion of OS cells. To date, the role of lncRNA in OS has not been reported, and in-depth studies on LINC01614 in other tumors are also rare. Some studies have shown *LINC0614* knockdown can inhibit the progress of lung cancer[37], LINC0614 can be used as a tumor marker of breast cancer[38], and LINC0614 can promote the progress of glioma. Until now, studies have reported that LINC01614 can promote the occurrence and development of tumors, which is consistent with our results. We predicted the LINC01614-hsa-mir-520a-3p-snx3 regulatory network through bioinformatics analysis of our sequencing results. The luciferase reporter gene and qRT-PCR experiments proved that the three types of RNAs are indeed related, which is consistent with our prediction. To further prove that LINC0614 plays a crucial role in OS cells through LINC0614-hsa-mir-520a-3p-snx3, we performed relevant cell experiments on hsa-mir-520a-3p and snx3, which confirmed our prediction. Hsa-mir-520a-3p has not been reported and snx3 has not been studied in OS. Studies have shown that snx3 can regulate Wnt secretion[30, 39–41]. Wnt pathway is closely related to various physiological processes, and deregulation of Wnt pathways can affect various diseases including cancer[42]. These results suggest that LINC01614 may indirectly regulate snx3, thus affecting the Wnt signaling pathway and the function and development of OS. This provides direction and ideas for our future research. In conclusion, our study identified differentially expressed lncRNAs between OS and normal adjacent tissues, and the functions of lncRNAs in the OS biological process. Our findings showed that LINC01614 acts as an oncogene in OS development by sponging hsa-miR-520a-3p, thereby releasing and upregulating SNX3 (Fig. 9). These results revealed that LINC01614 could serve as a potential biomarker in OS diagnosis, although its clinical value should be further consolidated in intensive studies.

Declarations

Ethics approval and consent to participate

Tissue specimens were acquired from patients consent and approved by the Clinical Research Ethics Committee of the Second Hospital of Jilin University.

Consent for publication

All the authors agreed to publish the article.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

WJC and LF co-designed and co-edited this study. CQX completed most of the experiments in this study, searched and extracted the data, and was a major contributor in writing the manuscript. ZX and WYB analyzed the data and collected the papers. LS cultured the cells and performed qRT-PCR. All authors approved the final manuscript and Consented for publication.

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Availability of data and materials

All data generated or analysed during this study are included in our own sequencing results or its supplementary information files.

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Figures

Figure 1

The expression profiling in osteosarcoma and normal tissues. (A) Flow chart for obtaining three paired osteosarcoma and adjacent normal tissues samples and RNA-seq. (B) The heatmap of the correlation coefficient between samples. R^2 , the square of the Pearson correlation coefficient. (C-E) Volcanic map and Heatmap of differentially expressed lncRNAs(C), mRNAs(D) and microRNAs(E). The filter threshold

in volcanic map was set to q value < 0.05. The heatmap was clustered with log10 (FPKM+1) values, with red indicating high expression genes and green indicating low expression genes.

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Figure 2

Functional Annotation of differentially expressed LncRNA in osteosarcoma. (A,B,C) GO analysis results of differentially expressed LncRNAs(BPCC,MF). (D) KEGG pathway enrichment results of different LncRNA target genes. (E) Heatmap of top 10 upregulated LncRNAs.

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Figure 3

LINC01614, MALAT1 involved in the competing endogenous RNAs networks in osteosarcoma. (A) The LINC01614-associated lncRNA-miRNA-mRNA network. (B) The MALAT1-associated lncRNA-miRNA-mRNA network. (C) Protein-protein interaction (PPI) networks of LINC01614 related genes. (D) Protein-protein interaction (PPI) networks of MALAT1 related genes.

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Figure 4

LINC01614 and MALAT1 were overexpressed in osteosarcoma cell lines. (A) q-PCR results of LINC01614 in osteosarcoma cells compared to osteoblast cells—student t-test. (B) q-PCR results of MALAT1 in osteosarcoma cells compared to osteoblast cells, student t-test. (C) q-PCR results of LINC01614 in cancer tissues compared to paracancerous tissues student t-test. (D) q-PCR results of MALAT1 in cancer tissues compared to paracancerous tissues student t-test* $P < 0.05$, ** $P < 0.01$. (E) Kaplan-Meier survival analysis

of patient overall survival between patients with high LINC01614 or low LINC01614 expression. (F) Kaplan-Meier survival analysis of patient overall survival between patients with high MALAT1 or low MALAT1 expression.

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Figure 5

LINC01614 knockdown inhibits proliferation, invasion, and metastasis in Saos2 and MG63 cells. (A,B) Verified the efficiency of LINC01614 knockdown. (C,D) Effect of LINC01614 knockdown on cell proliferation. (E,F,G) Effect of LINC01614 knockdown on invasion ability of cells. (H,I,J) Wound healing assay examined effect of LINC01614 knockdown on cell migration. Results were mean $\pm$ SD for three individual experiments which, for each condition, were performed in triplicate. * $p < 0.05$, ** $p < 0.01$.

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Figure 6

LINC01614 regulates SNX3 expression in an hsa-miR-520a-3p-dependent manner (A) The luciferase reporter plasmid containing WT (wild type) or Mut (mutant) LINC01614 was cotransfected into Human Embryonic Kidney 293 cells with hsa-miR-520a-3p in parallel with an empty plasmid vector. (B) quantitative real time polymerase chain reaction analysis results of hsa-miR-520a-3p in osteosarcoma cells compared to osteoblast cells and cancer and paracancerous tissues. (C) q-PCR analysis of hsa-miR-520a-3p expression in MG63 cells and Saos2 cells transfected with shRNA-NC or shRNA- LINC01614. (D) Luciferase activity assay in Human Embryonic Kidney 293 cells transfected with luciferase report plasmids containing SNX3 3' untranslated region (UTR; WT [wild type] or Mut [mutant]) and miR-NC or hsa-miR-520a-3p. (E,F) Relative mRNA and protein levels of SNX3 in osteosarcoma cells compared to osteoblast cells and cancer and paracancerous tissues. (G,H) Relative mRNA and protein levels of SNX3 in MG63 cells and Saos2 cells transfected with mimic-NC, hsa-miR-520a-3p-mimic, inhibitor-NC or hsa-miR-520a-3p-inhibitor. (I,J) Relative mRNA and protein levels of SNX3 in MG63 cells and Saos2 cells transfected with shRNA-NC or shRNA- LINC01614, student t-test, * $p < 0.05$, ** $p < 0.01$.

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(D) Luciferase activity assay in Human Embryonic Kidney 293 cells transfected with luciferase report plasmids containing SNX3 3' untranslated region (UTR; WT [wild type] or Mut [mutant]) and miR-NC or hsa-miR-520a-3p. (E,F) Relative mRNA and protein levels of SNX3 in osteosarcoma cells compared to osteoblast cells and cancer and paracancerous tissues. (G,H) Relative mRNA and protein levels of SNX3 in MG63 cells and Saos2 cells transfected with mimic-NC, hsa-miR-520a-3p-mimic, inhibitor-NC or hsa-miR-520a-3p-inhibitor. (I,J) Relative mRNA and protein levels of SNX3 in MG63 cells and Saos2 cells transfected with shRNA-NC or shRNA- LINC01614, student t-test, * $p < 0.05$, ** $p < 0.01$.

Figure 7

LINC01614 could function as ceRNA by directly sponging hsa-miR-520a-3p (A,B) Verified the efficiency of hsa-miR-520a-3p-mimic and hsa-miR-520a-3p-inhibitor. (C,D) Effect of hsa-miR-520a-3p-mimic and hsa-miR-520a-3p-inhibitor on cell proliferation. (E,F,G) Effect of hsa-miR-520a-3p-mimic and hsa-miR-520a-3p-inhibitor on invasion ability of cells. (H,I,J) Wound healing assay examined effect of hsa-miR-520a-3p-mimic and hsa-miR-520a-3p-inhibitor on cell migration. Results were mean $\pm$ SD for three individual experiments which, for each condition, were performed in triplicate. * $p < 0.05$, ** $p < 0.01$.

Figure 7

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Figure 8

LINC01614 promotes the proliferation, invasion, and lateral migration of OS cells via the LINC01614/hsa-miR-520a-3p/SNX3 axis (A,B) Verified the efficiency of SNX3 knockdown. (C,D) Effect of SNX3 knockdown on cell proliferation. (E,F,G) Effect of SNX3 knockdown on invasion ability of cells. (H,I,J) Wound healing assay examined effect of SNX3 knockdown on cell migration. Results were mean $\pm$ SD for three individual experiments which, for each condition, were performed in triplicate. * $p < 0.05$, ** $p < 0.01$.

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Figure 9

LINC01614 participates in the process of osteosarcoma by sponging hsa-miR-520a-3p to upregulate the expression of SNX3 and promote the proliferation, invasion, and metastasis of OS cells.

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