IncRNA JPX modulates malignant progress of osteosarcoma through targeting miR-33a-5p and PNMA1 regulatory loop

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ABSTRACT

Osteosarcoma (OS) is a common type of bone tumor, present worldwide, that has distal metastasis ability. Although continuous development in cancer therapy has taken place, there are still no effective metastasis-curbing strategies for OS available. Hence, a better understanding of the biological characteristics and molecular mechanisms of OS carcinogenesis is urgently needed. Long noncoding RNAs (lncRNAs) have captured great interest among cancer scientists with considerable potential implications for cancer treatment. In this study, we found that lncRNA JPX was up-regulated in OS tissues and cells. We subsequently examined the functional role of JPX in OS cells through knocked-down JPX by using siRNA. JPX down-regulation was observed to suppress OS cell proliferation, migration and invasion. Furthermore, it was verified that JPX acts as a sponge for miR-33a-5p, and that JPX regulated OS cell proliferation, migration and invasion through miR-33a-5p. Moreover, down-regulation of miR-33a-5p in OS contributed to PNMA1 upregulation, and PNMA1 depletion inhibited OS cell proliferation, migration and invasion in vitro. Taken together, our data support an important role of JPX in regulating OS cell proliferation, invasion and migration that highlights JPX may be a potential therapeutic target for OS.

Introduction

Osteosarcoma (OS) is characterized by high malignancy and a high metastasis rate, resulting in high mortality and disability rates [1]. Due to micrometastatic spread, radical surgery alone rarely results in cure, and chemotheraphy is limited due to the side effects and development of drug resistance. Hence, a better understanding of the biological characteristics and molecular mechanisms of OS carcinogenesis is urgently needed. Long noncoding RNAs (lncRNAs) are defined as a class of transcribed RNA molecules longer than 200 nucleotides in length [2]. Recently, the lncRNAs have captured great interest among cancer scientists with considerable potential to drive cancer development.

Abnormally expressed IncRNAs have been found to be involved in a variety of physiological and pathological processes, especially in cancers [3–6]. For instance, LncRNA DLEU1 has been shown to contribute to colorectal cancer progression via activation of KPNA3 [7]. Recent studies also reported that IncRNA DANC1 promotes migration and invasion through suppression of IncRNA-LET in gastric cancer cells [8]. LncRNA BCRT1 has been reported to promote breast cancer progression by targeting the miR-1303/PTBP3 axis [9]. However, the function and mechanism of most aberrantly expressed IncRNAs in OS remain largely unknown.

Mechanically, IncRNAs have been reported to regulate their target genes by acting with microRNA (miRNA) as sponges, thereby affecting the growth, proliferation, migration, and invasion of cancer cells [10, 11]. MicroRNAs (miRNAs) are small noncoding RNAs 22–24 nt in length. By binding the 3′-untranslated region (3′-UTR) of target genes, miRNAs degrade them or suppress their translation, thus down-regulating target gene expression [12]. Abrantly expressed miRNAs are involved in tumorigenesis [13]. Recently, the IncRNA CRNDE has been reported to act as an oncogene in cervical cancer through sponging by miR-183 to regulate CCNB1 expression [14]. LncRNA DANC1 has also been verified to promote proliferation and metastasis in pancreatic cancer by regulating miRNA-33b [15]. However, the specific functional roles of lncRNAs acting with miRNAs in OS and their potential implications for OS progression remain unclear.

In this study, we found that IncRNA JPX was up-regulated in OS tissues and cells and that higher expression of JPX was significantly
associated with down-regulated miR-33a-5p. In addition, we found that JPX regulated OS cell proliferation, migration and invasion in through miR-33a-5p. Furthermore, down-regulation of miR-33a-5p in OS contributed to PNMA1 upregulation, and PNMA1 depletion inhibited OS cell proliferation, migration and invasion in vitro. It can thus be seen that lncRNA JPX modulates malignant progress of OS by targeting miR-33a-5p and PNMA1 regulatory loop.

Materials and methods

Sample collection/ patients and clinical specimens

We collected fresh OS specimens and adjacent nontumorous tissues from 20 patients at Wuhan No.1 Hospital. None of the samples had received chemotherapy or radiotherapy before samples were obtained. All specimens were frozen in liquid nitrogen immediately after collection and stored at –80 °C until use.

Cell culture

This human cell line, hFOB1.19, has been a widely used model system for studying normal human osteoblast differentiation, osteoblast physiology, and so on. SAOS-2 and U2OS are the most frequently studied osteosarcoma cell lines. The normal osteoblast cell line hFOB1.19 and human OS cell lines (Saos-2, U2OS) were commercially available from the Chinese Academy of Medical Sciences (Beijing, China) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma–Aldrich, St. Louis, MO, USA), penicillin (100 U/ml, Sigma–Aldrich, St. Louis, MO, USA), and streptomycin (100 μg/ml, Sigma–Aldrich, St. Louis, MO, USA). All cells were incubated at 37 °C in air containing 5% CO2 and 95% relative humidity.

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA extraction was the first step in the study of gene isolation and expression. The total RNA was isolated from the tissue samples and cells using the mirVana™ miRNA isolation kit (Ambion, Austin, TX) according to manufacturer-recommended protocols. RNA concentration and purity were measured on the QIAxpert (Qiagen, Germantown, MD, USA). After removal of the residual DNA by DNase I (Invitrogen, Carlsbad, CA, USA), RNAs were reverse transcribed into cDNA using the high-capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Waltham, MA, USA). The specific gene expression was detected by Taqman quantitative real-time PCR (qRT-PCR) which was performed on a QuantStudio 6 Flex system (Life technologies, Gaithersburg, MD, USA). GAPDH was used as a housekeeping gene in comparisons of gene expression data.

For the mature miRNA quantification, total RNA was reverse-transcribed using the Taqman advanced miRNA cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. U6 small nuclear RNA (snRNA) was used as the internal control in comparisons of miR-33a-5p expression data and was reverse-transcribed by Taqman™ microRNA reverse transcription kit following the manufacturer’s protocol (Thermo Fisher Scientific, Waltham, MA, USA).

All the specific primers for gene and miRNA expression were commercially available from Thermofisher. The expression of each gene or miRNA relative to the internal control was calculated as previously described [16].

Transfection

JPX small interfering RNA (si-JPX) and siRNA with the corresponding non-coding RNA (si-NC), miR-33a-5p mimics (miR-33a-5P-OE), miR-33a-5p inhibitor (antimiR-33a-5p) and relative scramble (miR-NC), or lentiviral vectors carrying PNMA1 shRNA (sh-PNMA1) and control plasmids (sh-NC) were all commercially available from GenePharma (Shanghai, China). The transfection was performed using the Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol in logarithmic growth phase.

RNA-binding protein immunoprecipitation (RIP) assay

RIP assays were carried out to determine the interaction between JPX and miR-33a-5p as previously described [17]. We used the EZMag-a Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Chengdu, Sichuan, China) following the manufacturer’s protocol. Cells were lysed using RNA lysis buffer with 1X protease inhibitor cocktail after subjected to different treatments. The cell lysate was incubated with magnetic beads conjugated with human Argonaute-2 antibody (Ago2, Millipore, Chengdu, Sichuan, China) or negative control IgG (Abcam, Beijing, China) to immunoprecipitate the RNA-induced silencing complex (RISC). The co-precipitated RNAs were reverse-transcribed and analyzed by qRT-PCR after overnight incubation at 4 °C.

Cyquant assay

The cell growth was carried out by Cyquant assay (Thermo Fisher Scientific, Boston, MA). Cells were seeded in the 96-well plate (BD Biosciences, Bedford, MA, USA) at a density of 5000 cells per well. Plates were frozen after following incubation for the indicated time (24, 48, 72, and 96 h). 100ul of fresh prepared Cyquant solution was added to the wells and incubated in the dark for 45 min at room temperature. We determined the cell growth rate by measuring their optical density (OD) value at excitation at 497 nm and emission at 520 nm.

Colony formation assay

The soft agar colony formation assay is a traditional method to monitor anchorage-independent growth in vitro, which is considered the most stringent assay for detecting the malignance of cells. 1.4% (v/v) agarose (Sigma–Aldrich, St. Louis, MO, USA) was melted in a microwave and cooled to room temperature. Then, an equal volume of the 1.4% melted agarose was mixed with the complete cell culture medium. After that, 2 ml of 0.7% (v/v) low melt agar was added into each well of the 6-well plate (BD Biosciences, Bedford, MA, USA) and set aside to allow agarose to solidify. Cells (5000/well) were mixed with 1.4% agarose in complete culture medium, plated on top of the solidified layer to form colonies in 1–3 weeks. Cells were fed with new complete culture medium every 3 d after removal of old medium.

The colony is defined to consist of at least 50 cells [18]. After the cells formed sufficiently large colonies, the medium above the cells was removed and rinsed carefully with PBS. Then, colonies were fixed with 4% paraformaldehyde (Shanghai Chemical Co., Ltd, Shanghai, China) for 10 min and stained with crystal violet solution (0.5%, v/v) (Sigma–Aldrich, St. Louis, MO, USA) for at least 30 min. After removal of the crystal violet solution and carefully washing with tap water until excess dye was removed, the plates were left to dry at room temperature. Colonies were determined by light microscopy.

Transwell invasion assay

Cell invasion was investigated by Boyden chamber assay with a 24-well collagen-based cell invasion assay kit (Sigma–Aldrich, St. Louis, MO, USA). 200ul of serum-free medium containing 0.5 M cells for the invasion assay were added to the filter. The bottom chamber was prepared with 750ul of complete cell culture medium containing the FBS as a chemoattractant. After being incubated for the indicated time, the non-invasive cells were cleaned by scrubbing with a cotton swab. The cells
that adhered to the outside of the membrane were fixed and stained with crystal violet solution.

**Wound healing assay**

Cell migration was measured using a scratch assay. A cell suspension (1 M/mL) was plated in a 6-well plate and cultured overnight until a monolayer was formed. A straight scratch was introduced on the cell monolayer with a 10-μL sterile pipette tip. The detached cells were washed 3 times with PBS and microscopic images were recorded at 0 h. Subsequently, serum-free medium was added and cells were cultured for another 24 h, followed by microscopic imaging.

**Western blotting**

Total protein lysis was separated on 4–15% precast gels (Bio-Rad, Richmond, CA, USA) and then blotted onto nitrocellulose paper (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% dried non-fat milk powder in TBST (Sigma–Aldrich, St. Louis, MO, USA) for 1 h and incubated with primary antibody (anti-PNMA1,1:500, Sigma–Aldrich, St. Louis, MO, USA) overnight at 4 °C. After being washed with TBST (Sigma–Aldrich, St. Louis, MO, USA) 3 times, the membranes were incubated with the goat anti-rabbit IgG (H+L)-HRP conjugate secondary antibody (1:3000, Bio-Rad, Bio-Rad, Hercules, CA, USA) for 2 h at room temperature. The band signals of target proteins were visualized using an enhanced chemiluminescence kit (Pierce, Minneapolis, MN, USA). GAPDH was used as a loading control (anti-GAPDH, 1:500, Abcam, Cambridge, MA, USA) with the goat anti-rabbit IgG (H+L)-HRP conjugate secondary antibody (1:3000, Bio-Rad, Bio-Rad, Hercules, CA, USA).

In order to avoid possible problems related to incomplete stripping, all the results are from separate blots.

**Immunohistochemistry (IHC)**

IHC was done by our core facility. In brief, paraffin sections were cut to a thickness of 4 μm, the slides were deparaffinized in xylene and rehydrated with ethanol, and the endogenous peroxidase was inactivated with 0.3% hydrogen peroxide. All of the steps were performed using an UltraSensitive™ S-P kit (Maixinbio, China) according to the manufacturer’s protocol.

**Dual luciferase reporter activity assay**

The binding sites of PNMA1 was commercially available at GeneCopoeia (Rockville, MD, USA). Saos-2 and U2OS cells were seeded in a 6-well plate (BD Biosciences, Bedford, MA, USA) at a density of 0.25 M cells/well with the complete media. The cells were co-transfected with 1 μg PNMA3′-UTR luciferase reporter construct with 20 nM miR-33a-5p mimic or miR-NC using lipofectamine 2000 (Sigma–Aldrich, St. Louis, MO, USA) with Opti-MEN (Gibco, Carlsbad, CA) for 48 h. Luciferase assays were performed using the dual-luciferase reporter assay reagent from GeneCopoeia (Rockville, MD, USA). Data are expressed as the ratio of firefly luciferase activity to Renilla luciferase activity.

**Statistics**

The student’s t-test for two groups or one-way analysis of variance (ANOVA) test, followed by Tukey’s multiple comparison for multiple groups were used to compare the significance of differences between the mean of different groups. All the experiments were repeated 3 times, and results are shown as means ± standard errors (SE). SPSS (SPSS Inc. Chicago, IL) was used to calculate the significance. A p-value of less than 0.05 generally indicates a statistical significance.

**Results**

**JPX was upregulated in OS tissues and cells**

Based on our preliminary lncRNA microarray data, JPX is one of the upregulated lncRNAs in OS tissues compared with the adjacent non-tumorous tissues. JPX has been reported to be upregulated in lung cancer [19]. However, it is still unknown whether JPX also upregulated in OS. We subsequently determined the JPX expression level in OS tissues and compared it to the JPX expression level of the adjacent non-tumorous tissues by qRT-PCR. As shown in Fig. 1A, the JPX expression level was significantly upregulated in OS tissues when compared to the adjacent nontumorous tissue. After that, we also detected JPX expression levels in normal osteoblast cell line hFOB1.19 and human OS cell lines (Saos-2, U2OS). The JPX expression levels were also higher in both Saos-2 and U2OS than hFOB1.19 by around 1.7-fold (Fig. 1B).

**Downregulation of JPX inhibited OS cell proliferation, migration and invasion in vitro**

In order to validate the biological function of JPX on OS malignancy, we suppressed JPX in both Saos-2 and U2OS. As shown in Fig. 2A, JPX expression levels were remarkably downregulated to half of original levels after si-JPX transfection when compared with si-NC. The effect of JPX downregulation on the growth of Saos-2 and U2OS cells was determined by Cyquant assay. We found that downregulation of JPX significantly inhibited Saos-2 and U2OS cell proliferation compared with the wild type without transfection and si-NC groups by cyquant assay (Fig. 2B). The colony formation assays also revealed that depletion of JPX inhibited the growth and proliferation of Saos-2 and U2OS cells (Fig. 2C). To assess the effect of JPX on the migration of Saos-2 and U2OS cells, transwell assays were performed, since enhanced migration and invasion is another key feature across the metastatic cascade. As shown in Fig. 2D and E, a remarkably decreased cell migration and invasion rate in Saos-2 and U2OS cells with JPX downregulation was observed compared with the parent cells by transwell assay. These results suggest that MTDH overexpression significantly enhanced cell migration and invasion in vitro in MPM cells.

**JPX acted as a sponge for miR-33a-5p**

MiR-33a-5p has been reported to be downregulated in OS [20] and JPX has acted as a sponge for miR-33a-5p in lung cancer [19]. We further detected whether JPX also works as a sponge for miR-33a-5p in OS. We first confirmed that miR-33a-5p was downregulated in OS tissues (Fig. 3A) and Saos-2, U2OS compared to normal osteoblast cell line hFOB1.19 by 3-fold (Fig. 3B). After then, we determined the expression level of miR-33a-5p in Saos-2 and U2OS cells after si-JPX transfection. As shown in Fig. 4A, downregulation of JPX significantly increased the expression level of miR-33a-5p in Saos-2 and U2OS cells after si-JPX transfection. As shown in Fig. 4A, downregulation of JPX significantly increased the expression level of miR-33a-5p in Saos-2 and U2OS cells. Meanwhile, RNA immunoprecipitation experiments also confirmed that both miR-33a-5p and JPX were present in the Ago2-pulled down pellet (Fig. 3C). JPX was increased by 7-fold and 6-fold in Sao-2 and U2OS cells after Ago2-pulled down, respectively (Fig. 3C). Thus, these results suggested that miR-33a-5p was negatively correlated with JPX expression.

**JPX regulated cell proliferation, migration, and invasion by regulating miR-33a-5p**

To explore the biological function of the JPX/miR-33a-5p regulatory loop in OS cancer cells, we enhanced miR-33a-5p in Sao-2 and U2OS cells with JPX knockdown. The enhancement of miR-33a-5p was confirmed by qRT-PCR by around 2-fold (Fig. 4A). After then, we added antimiR-33a-5p to inhibit miR-33a-5p after JPX knockdown by around
4-fold and 6.3-fold compared with JPX knockdown in Sao-2 and U2OS cells, respectively (Fig. 4A). To determine the effect of JPX/miR-33a-5p on the cell proliferation, migration and invasion of OS cancer cells, the cyquant assay, colony formation, transwell and wound healing assays were performed in Sao-2 and U2OS cells. The cyquant assay results showed that inhibition of miR-33a-5p after JPX knockdown rescued the cell proliferation (Fig. 4B). In addition, the decrease in cell colony number due to JPX knockdown was restored by miR-33a-5p inhibition in the two OS cancer cell lines (Fig. 4C). Similarly, the transwell assay showed that miR-33a-5p inhibition significantly enhanced the migration and invasion of Sao-2 and U2OS cells (Fig. 4D and E). These results indicated that JPX regulated the cell proliferation, migration, and invasion via miR-33a-5p in OS cancer cells.

*Downregulation of miR-33a-5p in OS contributed to PNMA1 upregulation*

To decipher the underlying molecular mechanism of the JPX/miR-33a-5p regulatory network, we investigated the related downstream genes of miR-33a-5p. PNMA1 has been showed to be regulated by miR-33a-5p, and promoted proliferation and EMT in hepatocellular carcinoma by activating the Wnt/β-catenin pathway [21]. However, it is still unclear whether PNMA1 also contributed in the JPX/miR-33a-5p regulatory network in OS. We conducted IHC staining of PNMA1 in OS tissue samples and adjacent nontumorous tissues. As shown in Fig. 5A, PNMA1 is highly expressed in OS tissue samples compared to adjacent nontumorous tissues. To further confirm that PNMA1 was a target for miR-33a-5p, the luciferase reporter assay was performed to reveal that the upregulation of miR-33a-5p significantly reduced the luciferase activity of PNMA 3’UTR luciferase vector by around 3-fold but no effect on miR-NC (Fig. 5B). Additionally, PNMA protein expression level was also detected to be decreased after miR-33a-5p overexpression (Fig. 5C).

*PNMA1 depletion inhibited OS cell proliferation, migration and invasion in vitro*

To explore the potential biological function of PNMA1, we
suppressed PNMA1 in Sao-2 and U2OS cells after miR-33a-5p inhibition. The PNMA1 depletion was verified by qRT-PCR by around 6.7-fold and 4.5-fold compared with JPX knockdown in Sao-2 and U2OS cells, respectively (Fig. 6A), while PNMA1 suppression was confirmed by western blotting (Fig. 6B). The cyquant assay showed that knockdown of PNMA1 in cells with antimiR-33a-5p significantly reduced proliferation of both Sao-2 and U2OS cells in vitro (Fig. 6C). Meanwhile, the knockdown of PNMA1 in cells with antimiR-33a-5p resulted in fewer colonies when compared with parental cells (Fig. 6D). Moreover, the Transwell assay showed that PNMA1 knockdown also reduced migratory and invasive capacities of the cells (Fig. 6E).

Fig. 3. MiR-33a-5p was negatively correlated with JPX expression. (A) Expression level of miR-33a-5p in adjacent nontumorous tissues and OS tissue. *p<0.05 vs. adjacent nontumorous tissues (B) Expression level of miR-33a-5p in hFOB1.19, Saos-2, and U2OS cells. *p<0.05 vs. hFOB1.19 cells. (C) Both miR-33a-5p and JPX were present in the Ago2-pulled down pellet by RNA immunoprecipitation experiments. *p<0.05 vs. Anti-IgG.

Fig. 4. JPX regulated cell proliferation, migration, and invasion through miR-33a-5p. (A) MiR-33a-5p expression in different groups as shown by qRT-PCR. *p<0.05 vs. non-treatment. Saos-2 and U2OS cells were transfected with si-JPX, si-JPX + miR-NC, or si-JPX + antimiR-33a-5p, respectively. The proliferation, migration and invasion were measured by cyquant assay. *p<0.05 vs. si-JPX. *p<0.05 vs. si-JPX + miR-NC. (B), colony formation (C), wound healing (D), and transwell (E) assays.
invasive ability both in Sao-2 and U2OS cells (Fig. 6E). These findings strongly suggest that PNMA1 participated in the JPX/miR-33a-5p regulatory network.

Discussion

Molecular mechanisms of the malignancy of OS still remain complex and largely unknown. In this study, we mainly investigated the biological function of lncRNA JPX, miR-33a-5p and PNMA1 regulatory loop in the progression of OS. Recent studies have shown that abnormally expressed lncRNAs play important roles in cancer occurrence and development [22, 23]. For instance, lncRNA KCNQ1OT1 has been found to sponge miR-34c-5p to promote OS growth via ALDOA enhanced aerobic glycolysis [24]. It has also been verified that lncRNA RP11-361F15.2 promoted OS tumorigenesis by inhibiting M2-like polarization of tumor-associated macrophages of CPEB4 [25]. In addition, lncRNA MALAT1 has been reported to promote cancer metastasis in OS via activation of the PI3K-Akt signaling pathway [26]. In this study, we specifically found that lncRNA JPX was upregulated in OS tissues and cells. In addition, downregulation of JPX inhibited OS cell proliferation, migration and invasion after PNMA1 suppression were measured by cryquant assay. \( ^{*}p<0.05 \) vs. non-treatment. (B) The protein level of PNMA1 was assessed by western blotting in different groups of Sao-2 and U2OS cells. The proliferation, migration and invasion after PNMA1 suppression were measured by cryquant assay. \( ^{*}p<0.05 \) vs. antimir-33a-5p. \( ^{\wedge}p<0.05 \) vs. antimir-33a-5p + sh-NC. (C), colony formation (D), wound healing (E), and transwell (F) assays.
PNMA1 expression, more altered downstream signaling pathways PI3K/AKT and MAPK/ERK pathways [32]. PNMA1 has also been reported to be downregulated in OS [20]. However, it still remains unclear how miR-33a-5p serves as a modulator for OS malignancy, especially through regulating its downstream targets. PNMA1 is a member of the paraneoplastic Ma family (PNMA), consisting of at least fifteen target and provide a new research direction for OS treatment.

Data availability

All data produced during the current study are included in the article or uploaded as supplementary information.

Ethics approval and consent to participate

The experiment was approved by ethics Committee of Wuhan No.1 hospital. This paper has not been published elsewhere in whole or in part. All authors have read and approved the content, and agree to submit it for publication. Informed consent was obtained from all individual participants included in the study.

CRedit authorship contribution statement

Wei Xiong: Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. Dan Liu: Data curation, Formal analysis, Writing – review & editing. Xi Chen: Data curation, Formal analysis. Leiting Liu: Data curation, Formal analysis. Weihong Xiao: Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft.

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