Long non-coding RNA PANDAR enhances osteosarcoma cell proliferation and invasion through induction of epithelial-to-mesenchymal transition

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Received April 24, 2017; Accepted November 14, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: Objective: Long non-coding RNA (lncRNA) PANDAR has been identified as an oncogenic gene in multiple human malignancies, and dysregulation of PANDAR was tightly linked to carcinogenesis and cancer progression. Herein, we aimed at elucidating the expression profile and biological function of PANDAR in osteosarcoma (OS). Methods: PANDAR expression in OS tissues and cell lines was investigated using qRT-PCR, and the association between PANDAR expression and clinicopathological factors of OS patients was also analyzed. Cell proliferation and transwell assays were conducted by using MG63 and U2OS cells transfected with si-PANDAR. Expression of EMT markers (E-cadherin, N-cadherin and vimentin) was validated using western blot assay. OS xenograft tumor model was set up to investigate the biological functions of PANDAR in vivo. Results: Our findings demonstrated that PANDAR levels were evidently increased in OS tissues and cell lines. Higher expression of PANDAR was associated with aggressive phenotypes of OS, and led to a more unfavorable five-year survival of OS patients. Knock-down of PANDAR impaired the proliferation, migration and invasion of MG63 and U2OS cells in vitro, suppressed in vivo OS xenograft tumor growth, and led to elevated E-cadherin protein expression and reduced N-cadherin and vimentin protein expression. Conclusions: Collectively, the present study provided evidence that overexpression of PANDAR promoted proliferation and EMT-mediated OS migration and invasion. PANDAR may serve as a potential diagnostic indicator and therapeutic target for OS patients.

Keywords: Osteosarcoma, long noncoding RNA, PANDAR, prognosis, epithelial-to-mesenchymal transition

Introduction

Worldwide, osteosarcoma (OS) is one of the most common malignant bone cancers in children, adolescents and young adults, with a peak incidence at the age of 15-19 years [1, 2]. Malignant OS cells produce osteoid matrix and fibillary stroma [3]. Pain and swelling in the affected bone are the most common symptom of OS. OS has a high metastatic rate of about 20%. The lung and other bones are the most common targets [4]. Although the standard treatment, including adjuvant chemotherapy and tumor resection, has evidently improved the five-year survival rate of OS patients to nearly 70% [5], there has been no remarkable progress in improving the prognosis of those with lung or bone metastases [6, 7]. Unfortunately, most of the current strategies have limited efficacy in the treatment of metastatic OS, which remains a major challenge in bone cancer fields. Thus, it is highly desired to develop novel sensitive markers of diagnostic and therapeutic targets for OS. Meanwhile, the underlying mechanism of OS progression remains largely dismal, which needs to be further elucidated.

The long non-coding RNAs (lncRNAs), defined as a subgroup of non-protein-coding RNA molecules that are longer than 200 nucleotides in length, serve pivotal roles in various biological processes through functionally regulating epigenetic, transcriptional and post-transcriptional gene expression [8, 9]. They are often transcribed by RNA polymerase II but have no open reading frame, and thus cannot be translated into proteins. Recent studies have verified that nearly 80% of mammalian genomic transcripts are lncRNAs [10]. Overwhelming studies have
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clarified that lncRNAs may function as oncogenes or tumor suppressors in various types of cancers [11, 12].

The gene lncRNA PANDAR (promoter of CDKN1A antisense DNA damage-activated RNA), which is located at chromosome 6p21.2, has been documented to be involved in tumor progression in several human cancers. Such as, increased PANDAR expression was strongly associated with progression and unfavorable prognosis of patients with hepatocellular carcinoma [13]. Ma et al. reported that the up-regulation of PANDAR could serve as an independent prognostic role in gastric cancer [14]. Another literature showed that PANDAR plays oncogenic role in breast cancer through regulating the G1/S transition [15]. However, to the best of our knowledge, the role of PANDAR in OS and the underlying mechanisms of its effects remain elusive.

In the current research, the correlation between the expression profile of PANDAR and clinical characteristics and prognosis of OS patients was analyzing through detecting the expression profile of PANDAR in OS tissues. The expression levels of PANDAR were determined by qRT-PCR. The knockdown of PANDAR was achieved by RNA interference. siRNA specifically targeting PANDAR and a scrambled negative control (si-NC) were synthesized by GenePharma, Shanghai, China. The siRNA sequence for PANDAR was si-PANDAR, 5'-GCAAUCUACAACCUGUUC-3'. Transfection was conducted using the Lipofectamine 2000 kit (Invitrogen) according to the manufacturer’s protocols. Specificity and efficacy of the si-PANDAR were validated by qRT-PCR after transfection.

Materials and methods

Patients and clinical specimens

Matched fresh OS specimens and corresponding non-tumorous (NT) tissues were acquired from 96 patients at Laiyang Central Hospital of Shandong, China. All tissue samples were evaluated by two professional pathologists. None of the patients recruited to this study had undergone chemotherapy or radiotherapy prior to surgery. All specimens were frozen in liquid nitrogen immediately after collection and stored at -80°C until further use. The clinical and pathological characteristics of the patients were recorded in Table 1. The follow-up data were available and complete for each patient. Overall survival was calculated from the day of primary surgery to death or last follow-up. The study methodology complied with the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of Laiyang Central Hospital. All patients provided written informed consent.

Cell culture

The normal osteoblast cell line (hFOB) and five human OS cell lines (HOS, G293, SAOS2, MG-63, U2OS) were purchased from the Institute of Cell Research, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Invitrogen, Camarillo, CA, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified incubator at 37°C in 5% CO₂.

RNA interference

Knockdown of PANDAR was achieved by RNA interference. siRNA specifically targeting PANDAR and a scrambled negative control (si-NC) were synthesized by GenePharma, Shanghai, China. The siRNA sequence for PANDAR was si-PANDAR, 5'-GCAAUCUACAACCUGUUC-3'. Transfection was conducted using the Lipofectamine 2000 kit (Invitrogen) according to the manufacturer’s protocols. Specificity and efficacy of the si-PANDAR were validated by qRT-PCR after transfection.

RNA extraction and qRT-PCR

Total RNA was isolated from tissues and cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of the total RNA were determined spectrophotometrically. The first-strand cDNA was synthesized from 1 μg of total RNA using the Reverse Transcription System Kit (Takara, Dalian, China). The qRT-PCR was carried out using SYBR Premix Ex Taq kit (Takara, Dalian, China) on an ABI 7500 Fluorescent Quantitative PCR system (Applied Biosystems, Bedford, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene. The sequences of the primers were as follows: PANDAR, 5'-TGCACACATTTAACCCGAAG-3' (forward) and 5'-CCCCAAAGCTACATCTATGACA-3' (reverse); GAPDH, 5'-GCAAUCUACAACCUGUUC-3' (forward) and 5'-GCCAACACATTTAACCCGAAG-3' (reverse); MCM4, 5'-GGCTTCTTCTCGAGGCAATG-3' (forward) and 5'-GGCTTCTTCTCGAGGCAATG-3' (reverse). Relative expression fold change of mRNAs was calculated by the 2-ΔΔCt method [16]. Each experiment was performed three times independently.
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**Cell proliferation assay**

The cells were seeded in 96-well plates at the density of 5 × 10^3 cells per well after transfection. At the indicated time (24 h, 48 h and 72 h), cell proliferation was investigated using Cell Counting Kit-8 (CCK-8; Beyotime, China) according to the manufacturer’s protocols. The absorbance of each well at 450 nm was measured using a microplate reader (ELx800NB; BioTek Instruments, Inc., Winooski, VT, USA). All of the experiments were performed in triplicate.

**Cell migration and invasion assays**

Cell migration and invasion were investigated using non-Matrigel-coated or Matrigel-coated transwell cell culture chambers (BD Matrigel Invasion Chamber; BD Biosciences, San Jose, CA, USA) of 8 μm pore size. 1 × 10^5 cells were seeded in the upper chamber of these transwell inserts supplemented with 100 μL serum-free medium, and 600 μL of medium containing 20% FBS, a chemoattractant, was added to the lower chamber. After incubation for 24 hours, cellson the surface of the upper chamber were gently removed, and migrated or invaded cells were fixed in ethanol and stained with 2% crystal violet. The number of migrated or invaded cells was calculated and photographed under a microscope in five random fields and presented as the average per field.

**Western blot analysis**

Total proteins were extracted using sodium dodecyl sulfate lysis buffer (Beyotime, Jiangsu, China) with protease inhibitor cocktail (Merk, Germany), and BCA assay was applied to determine the total protein concentration of the cell lysates. Total proteins (20 μg) were separated by 8% SDS-PAGE, and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blockage at room temperature with 5% skim milk for 2 h, membranes were incubated overnight at 4°C with a 1:1000 dilution of anti-GAPDH (Sigma), E-cadherin, vimentin, or N-cadherin (Cell Signaling Technology, Beverly, MA). After additional incubation with a 1:1000 dilution of anti-immunoglobulin horseradish peroxidase-linked secondary antibodies for 1 h, protein bands were developed using the enhanced chemiluminescence (Cell Signaling Technology), and the band intensity was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA). Protein levels of GAPDH were used as loading controls.

**Tumor growth assay in vivo**

A total of 10 female BALB/c nude mice (4–6 weeks old) were brought from Shanghai Laboratory Animals Center (Shanghai, China), and maintained under specific pathogen-free (SPF) conditions. Animal studies were performed according to Institutional Animal Care and Use Committee guidelines of Laiyang Central Hospital. MG63 cells (2 × 10^6) transfected with si-PANDAR or si-NC were resuspended in 150 μl of medium, and were injected into the flanks of nude mice. To maintain the knockdown efficacy, the siRNAs in PBS were injected to the xenografts every three days throughout the study. Tumor volume was measured every five days, and was calculated using the following formula: Volume (mm^3) = length × width^2 × 0.5. 30 days after inoculation, the mice were sacrificed, and tumors were dissected and weighted.

**Statistical analysis**

All statistical analyses were performed using the SPSS 17.0 software package (SPSS, Chicago, IL, USA). All data on continuous variables were expressed as mean ± standard deviation.
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Table 1. Correlation between PANDAR expression and clinicopathological features in OS patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total number</th>
<th>PANDAR expression</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Low (n=53)</td>
<td>High (n=43)</td>
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</tr>
<tr>
<td>Age (years)</td>
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<tr>
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<td>≥18</td>
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<tr>
<td>Gender</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Female</td>
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<td>22</td>
<td>19</td>
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<tr>
<td>Tumor size (cm)</td>
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<tr>
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<tr>
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<tr>
<td>Tumor site</td>
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<tr>
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<tr>
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<tr>
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Results

PANDAR expression was increased in OS tissues and cell lines

PANDAR expression in OS tissues and cell lines was detected by qRT-PCR. The results showed that PANDAR expression in OS samples was evidently higher than in adjacent NT tissues (Figure 1A, *P* < 0.001). In addition, PANDAR expression was markedly elevated in five OS cell lines compared to hFOB cells, which is a normal osteoblast cell line (Figure 1B, all *P* < 0.001). Since MG63 and U2OS cells exhibited the relatively highest PANDAR expression among the five OS cell lines, these two cell lines were chosen for the transfection of si-PANDAR in further validations.

PANDAR expression correlates with clinicopathologic features and prognosis of OS patients

To assess the correlation of PANDAR expression with clinicopathologic features of OS patients, the OS samples were stratified into high PANDAR expression group (n=43) and low PANDAR expression group (n=53) according to the median PANDAR expression level of all samples. As recorded in Table 1, high expression of PANDAR was closely correlated with advanced Enneking stage (*P*=0.010) and positive distant metastasis (*P*=0.014).

Using the Kaplan-Meier method and log-rank test, we found that OS patients with lower PANDAR expression had better 5-year overall survival than those with higher PANDAR expression (*P* < 0.001, Figure 2). In other words, BANCR expression was noticeably associated with OS patients’ prognosis.

PANDAR promotes OS cell proliferation, migration and invasion in vitro

To investigate the biological effect of PANDAR on OS cells, MG63 and U2OS cells were transfected with si-PANDAR or si-NC. Transfection
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Efficiency was verified by qRT-PCR analysis. As demonstrated in Figure 3A, compared to NC, PANDAR was strongly reduced in MG63 and U2OS cells after transfection with si-PANDAR (all $P<0.001$).

Subsequently we evaluated the role of PANDAR in cell proliferation, migration and invasion in OS. CCK-8 assay revealed that inhibition of PANDAR noticeably suppressed the proliferation of MG63 and U2OS cells compared to NC.
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Then, migration and invasion assays were conducted to analyze the effects of PANDAR on migration and invasion abilities of OS cells. We observed that down-regulating of PANDAR expression induced a significant decrease in migration and invasion abilities of MG63 and U2OS cells (Figure 3C, all \( P < 0.001 \)). All these data demonstrated the tumor-promoting roles of PANDAR in OS.

**PANDAR induces epithelial-to-mesenchymal transition (EMT) in OS cells**

Epithelial-to-mesenchymal transition (EMT) process is modulated by developmental transcriptional factors, which repress epithelial marker expression, but induce the expression of mesenchymal markers. To explore whether aberrant PANDAR expression might be involved in the EMT process, the epithelial marker E-cadherin, and the mesenchymal markers N-cadherin and vimentin were investigated by western blotting. As shown in Figure 4, down-regulation of PANDAR in MG63 and U2OS cells resulted in increased E-cadherin protein expression and decreased N-cadherin and vimentin protein expression, which indicates that PANDAR contributes to the regulation of EMT marker expression in OS cell lines.

(Figure 3B). Then, migration and invasion assays were conducted to analyze the effects of PANDAR on migration and invasion abilities of OS cells. We observed that down-regulating of PANDAR expression induced a significant decrease in migration and invasion abilities of MG63 and U2OS cells (Figure 3C, all \( P < 0.001 \)). All these data demonstrated the tumor-promoting roles of PANDAR in OS.

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To investigate the effects of PANDAR on OS growth in vivo, MG63 cells transfected with si-PANDAR or si-NC were injected into the flanks of nude mice, tumor volume was measured every five days until 30 days. We observed that tumor growth was obviously slower in MG63/si-PANDAR group than that of MG63/si-NC group (Figure 5A). 30 days after inoculation, the mice were killed, and tumors were dissected and weighted, and observed that average tumor weight was also remarkably decreased in MG63/si-PANDAR group compared with that of MG63/si-NC group (Figure 5B, P<0.001). These data indicated that PANDAR promotes OS growth in vivo.

Discussion

During the post-genome period, IncRNAhas attracted increasing attention. As a kind of non-coding RNAs, IncRNAs are mRNA-like transcripts ranging in length from 200 nt to ~100 kb that lack coding protein function [17]. More and more data have exhibited the important function of IncRNAs in a variety of human cancers, which might be helpful for improving the diagnosis, prevention, and treatment of these malignancies [18]. Up to date, abnormal expression of multiple IncRNAs has been found to be correlated with OS progression [19]. For example, studies from Wang et al. have revealed that LINC00161 sensitizes OS cells to cisplatin-induced apoptosis through modulating the miR-645-IFIT2 signaling axis [20]. Many researchers reported that PANDAR acted as a robust oncogene and is often overexpressed during tumorigenesis. However, little is understood about the function of PANDAR in OS patients.

Herein, we observed a high PANDAR expression in OS specimens and cell lines, providing the first evidence that OS overexpression was strongly associated with OS carcinogenesis. Then, we found that elevated PANDAR expression was correlated with aggressive clinicopathological characteristics of OS patients. These findings revealed that PANDAR might be involved in OS progression. In addition, our research showed that OS patients with high PANDAR levels tended to have shorter overall survival than patients with lower levels. PANDAR overexpression was also associated with unfavorable prognosis in patients with other cancer types, such as bladder cancer [21] and colorectal cancer [22]. To our knowledge, this might be the first study to evaluate the expression profile and clinical significance of PANDAR in OS.

Intriguingly, PANDAR expression varies from one cancer to another and therefore its role is diverse in different malignancies. The functional role of PANDAR is extremely complicated and PANDAR can hold not only oncogenic, but also tumor suppressive roles in different tumor types. Han et al. reported that PANDAR was significantly decreased in NSCLC, and PANDAR overexpression evidently repressed NSCLC cell proliferation in vitro and in vivo [23]. Herein, to determine the functional role of PANDAR in OS, loss-of-function experiment was performed to investigate the effect of PANDAR on OS cell lines. We observed that PANDAR silencing in OS cells would reduce cell proliferation, impair cell invasion and migration capacities, and inhibit OS tumor growth. To further explore the molecular mechanism responsible of PANDAR, we examined protein levels of EMT-associated markers in OS cells with knockdown of PANDAR expression.

Featured by a loss of cell polarity and intracellular junctions and acquisition of mesenchymal characteristics, EMT is one of the key processes for primary tumor cells to obtain migratory and invasive capacities [24]. Loss of E-cadherin expression, and increased N-cadherin and vimentin expression are hallmarks of EMT [25, 26]. Growing evidence demonstrates that IncRNAs, including PANDAR, may regulate EMT processes in various human carcinomas [22, 27-29]. In the present study, inhibition of PANDAR resulted in upregulated E-cadherin and downregulated N-cadherin protein levels. These results suggested that PANDAR might be involved in the regulation of EMT in OS, providing a possible explanation for PANDAR-associated high cell migration and invasion.

The data of our study are compelling; however, because we obtained clinical data from Chinese patients only, PANDAR expression levels as a diagnostic and prognostic indicator should be verified in different ethnic populations before clinical application. Accordingly, a multicenter trial that include a larger cohort of patients collected from several hospitals and diverse eth-
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nic populations are required to confirm whether PANDAR can be incorporated into routine clinical practice in the future.

Collectively, PANDAR expression was up-regulated in OS tissues and cell lines. Our data revealed that PANDAR may affect the OS cell proliferation, migration and invasion, thereby affecting OS occurrence and progression. Although the precise function of PANDAR in OS development remains largely elusive, the results from our study strongly support that PANDAR might function as an oncogene in OS, and PANDAR could be considered as a potential prognostic marker and therapeutic target for OS in the near future.

Disclosure of conflict of interest

None.

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References

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