Abstract. Accumulating evidence has indicated that aberrant expression of long non-coding RNAs (lncRNAs) is an important oncogenic factor. The aim of the present study was to investigate the role of LINC01296, an lncRNA that exerts a tumor-promoting function in many cancers, in the regulation of proliferation, metastasis and the cell cycle of osteosarcoma. The expression of LINC01296 in osteosarcoma tissues and adjacent healthy tissues of 30 patients was analyzed by quantitative real-time PCR (qRT-PCR). The relationship between LINC01296 expression and the survival of patients with osteosarcoma was also explored. The expression levels of LINC01296 in osteosarcoma cells and normal cells were compared. LINC01296 knockdown and overexpression were performed in MG63 and HOS8603 osteosarcoma cells by transfecting LINC01296 shRNA and an expression plasmid respectively, followed by investigation of the changes on cell proliferation, migration, apoptosis and cell cycle arrest. Western blotting was used to analyze the changes of cell cycle regulators. Cyclin D1 knockdown and overexpression were carried out to verify the interaction between LINC01296 and cyclin D1. LINC01296 overexpression was demonstrated as a biomarker of osteosarcoma, which was closely correlated with the poor survival of patients with osteosarcoma. A high expression of LINC01296 was observed in osteosarcoma cells, which was closely associated with enhanced proliferation, invasion, and migration of osteosarcoma cells. Cyclin D1 expression was positively correlated with the expression of LINC01296 in osteosarcoma cells. Cyclin D1 knockdown or overexpression played a deterministic role in mediating the effect of LINC01296 on osteosarcoma cells. LINC01296 is an oncogenic lncRNA in osteosarcoma. The proliferation, invasion and migration of osteosarcoma cells could be effectively retarded by inhibition of LINC01296. The cancer-promoting effect of LINC01296 on osteosarcoma was determined by cyclin D1.

Introduction

It has been recognized that less than 2% of the total genome sequence are protein coding genes, while more than 80% of the genome are non-protein coding genes (1). Apart from small amount of non-coding RNAs, such as microRNAs, a large portion of transcribed RNA constituents are long non-coding RNAs (lncRNAs), which have a length of more than 200 nucleotides with no open reading frame, and are controlled by both epigenetic and transcriptional factors (2). In recent years, thousands of lncRNAs have been found to be differentially expressed between cancers and normal tissues (3,4). Notably, the aberrant expression of lncRNAs was revealed to be an important contributor to the development of cancers and several other diseases (5,6). lncRNAs have been implicated in the regulation of various biological processes, such as chromosomal imprinting, growth, differentiation, pluripotency, apoptosis and cell cycle arrest (7). Despite the increasing studies on lncRNAs, the role of lncRNAs in cancer remains unclear. To date, lncRNA-targeted approaches have been translated to clinics. It is an important task to unravel the functions of lncRNAs in different cancers, so as to achieve optimal diagnostic and therapeutic efficacies in cancer.

Osteosarcoma is one of most common cancers in children and adolescents, comprising 20% of all primary bone cancers (9). The overall survival of osteosarcoma remains dismal and approximately 35% of patients succumb to the disease within five years, despite several improvements in the treatment approaches. Understanding the mechanisms associated with osteosarcoma cell proliferation, differentiation, invasion, and metastasis is critical in advancing the clinical management of the disease. Consequently, it is imperative to investigate the molecular mechanism of osteosarcoma and pinpoint key targets for efficient diagnosis and treatment of this disease. Recent evidence has indicated that lncRNAs are of great significance in the pathogenesis of osteosarcoma. lncRNA UCA1 (10), HULC (11) and TUG1 (12) have been reported to play an important role in promoting osteosarcoma progression, leading to poor prognosis of patients. Recently,
long intergenic non-protein-coding RNA 1296 (LINC01296) has been implicated in the regulation of a plethora of cancers. LINC01296 has been revealed to be a tumor-promoting molecule in prostate (13), colorectal (14) and gastric cancer. However, the role of LINC01296 in osteosarcoma has never been reported.

The aim of the present study was to explore the functional role of LINC01296 in osteosarcoma. The expression of LINC01296 in patients with osteosarcoma tissues was analyzed. The mechanism of action of LINC01296 in osteosarcoma invasion, migration and the cell cycle was explored.

Materials and methods

Patient samples. The present study was approved by the Research Ethics Committee of Jilin University (Changchun, China). Tissues were collected from 30 patients (17 males and 13 females from 40 to 76 years old, averaged 62.26±6.87 years old), among which 18 patients had a tumor size <6 cm and 12 patients had a tumor size ≥6 cm. Tumors of these patients were graded as well differentiated (6 patients), moderately differentiated (17 patients) and poorly differentiated (7 patients). Lymph node metastases were observed in 20 patients. Four patients were at stage II, 19 at stage III and 7 at stage IV. Informed consent was obtained from all patients.

Quantitative real-time PCR. Total RNA was extracted from human tissues and cells using TRIzol Total RNA Isolation kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Purified RNA (1 µg) was transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). Real-time PCR was performed using the SYBR Green Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.) and Mastercycler (Eppendorf, Hamburg, Germany). The following sequences were used: LINC01296 forward, AACTGGCAC CAGCCTCCT and reverse, CGGCCAAGTTTACCTACCA TC; GAPDH forward, ACTGGAACGTAAGGTTTGC and reverse, AGAGAAAGTGTTTGGTCTT; cyclin E1 forward, TGGATTGGTTAATGGAAGTGGTTG and reverse, AGC GTTGGATCTCGTTGCTTC; CDK4 forward, TAAACC TGGTTTTGGACATGTAG and reverse, GTCGGGCTTC AGAGTTTCCAGACA; CDK2 forward, TCTGCGATTCTC ATCCGGTCC and reverse, GAAATCCGGCTTGATGGA TTCC; cyclin D1 forward, GACTTACACCCGACA TCTCCAT and reverse, CGTGTGGAGCGGTAGT TAGGA. GAPDH was used as an internal control. The thermocycling conditions were based on the Tm of primer (initial denaturation was 95°C for 1 min, in 40 cycles, 95°C for 30 sec, 55-60°C for 30 sec and 68°C for 1 min, final 68°C for 10 min). Quantification of RNA levels was performed using the 2^ΔΔCq method (15). For the qRT-PCR analysis of LINC01296 expression in patient samples, the top 50% (15 cases) was divided into the high-expressing group, and the bottom 50% (15 cases) was divided into the low-expressing group.

Cell lines and cell culture. All cells used in this study, hFOB1.19, MG63 and 143B, were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). HOS-8603 was purchased from Second Military Medical University Cell Bank (Shanghai, China). It is a type of Chinese-established human osteosarcoma cell line with phenotypic characteristics of osteoblasts (16), and has been used in some studies from 1994 (17,18). The medium and atmosphere of those cell lines cultured in were following: hFOB1.19 [DMEM (cat. no. 11039-02125030149); Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 100% air], MG63 [DMEM (cat. no. 11095-080; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 95% air+5% CO2], HOS-8603 [DMEM (cat. no. 10566016; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 95% air+5% CO2] and 143B [MEM (cat. no. 11095-080B23151; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 100% air]. Due to experimental conditions and the similar pre-test results among these OS cell lines, we selected MG63 and HOS-8603 as research cell lines in functional assays.

Gene knockdown and overexpression. The following RNA sequences were used for gene knockdown: sh-LINC01296-1, CGGGATGAGATGTTAGTAGTTTTTG (forward) and AATTCGGAAGTATGCTTCT (reverse); sh-LINC01296-2, CGGGAGGGAAGTATGCTTCT (forward) and AATTCGGAAGTATGCTTCT (reverse); sh-Cyclin D1, ACCCTGGATGATGTTAGTAGTTTTTG (forward) and AATTCGGAAGTATGCTTCT (reverse); si-Cyclin D1, ACCCTGGATGATGTTAGTAGTTTTTG (forward) and AATTCGGAAGTATGCTTCT (reverse); sh-LINC01296-1, CGGGATGAGATGTTAGTAGTTTTTG (forward) and AATTCGGAAGTATGCTTCT (reverse); sh-LINC01296-2, CGGGAGGGAAGTATGCTTCT (forward) and AATTCGGAAGTATGCTTCT (reverse); sh-LINC01296-3, ACCCTGGATGATGTTAGTAGTTTTTG (forward) and AATTCGGAAGTATGCTTCT (reverse). The viability of cells was determined using the CCK-8 assay (CCK-8; Dojindo Molecular Laboratories, Tokyo, Japan) was used to monitor cell proliferation. First, cells (5x10^3) were plated in 96-well plates. Then, 10 µl of CCK-8 solution was added to the cells and incubated following at 37°C for 0.5-4 h. The absorbance was measured at 450 nm using a plate reader (Tecan Trading AG, Mannedorf, Switzerland). The viability of cells was quantified using the following equation: Viability (%) = (OD_control - OD_experiment)/OD_control x 100%.

Flow cytometry. Cell cycle progression analysis was performed using flow cytometry. Cells were harvested and fixed in 70% ethanol. Then, the cells were lysed with 0.2% Triton X-100 at 4°C for 30 min and pelleted by centrifugation (1,200 x g). Subsequently, the cells were resuspended in PBS containing RNAse (10 mg/ml), stained with propidium iodide (PI), and finally analyzed on FACSCalibur (BD Biosciences, San Jose, CA, USA). The generated histogram was used to calculate the ratio of cells in the G0/G1, S or G2/M phases.

Western blot analysis. Western blot analysis was carried out to analyze cell-cycle checkpoint protein expression. Protein extracted by RIPA buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) and was measured concentration by BCA (cat. no. 23225; Thermo Fisher Scientific, Inc.) and UV-5800H spectrophotometer (Shanghai Metash Instruments Co., Ltd.,
Shanghai, China). Cell lysates (20 µg) were used for 3-8% SDS-PAGE (cat. no. EA0375BOX; Thermo Fisher Scientific, Inc.) and then transferred to polyvinylidene fluoride (PVDF) membranes. BSA (1%) was used to block the PVDF membranes. Then, primary antibodies were applied to the membranes and incubated at room temperature for 1 h. Primary antibodies such as cyclin D1 (cat. no. ab16663), cyclin E1 (cat. no. ab133266), CDK4 (cat. no. ab108357) and CDK2 (cat. no. ab32147) were purchased from Abcam (Cambridge, MA, USA). GAPDH antibody (cat. no. 2118) was obtained from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies were diluted 1:1,000. After extensive washing, GAPDH antibody (cat. no. 2118), the secondary rabbit antibody, was added to the membrane (1:5,000). Following incubation for 1 h, the membranes were washed and ECL chemiluminescent reagents (cat. no. 6883; Cell Signaling Technology) were added for visualization of the protein bands.

Statistical analysis. All experiments were performed in triplicates unless otherwise stated. Data were expressed in the form of the mean ± standard deviation (SD). Comparisons between two groups was performed using Student’s t-test. Multiple comparisons among more than three groups were performed using one-way (ANOVA), and subsequently, SNK-q test was used additionally in the comparison between two groups. For the comparison of rates a Chi-square test was used. Correlation analysis was performed using Pearson’s correlation analysis. Kaplan-Meier analysis was used for overall survival analysis. Intergroup differences were considered statistically significant when the P-value was <0.05.

Results

Malignant osteosarcoma is characterized by high LINC01296 expression. To validate the role of LINC01296 in osteosarcoma, we performed qPCR analysis from tissues collected from patients with osteosarcoma. LINC01296 expression was revealed to be significantly higher in osteosarcoma tissues, compared to that in normal tissues (Fig. 1A). Consistently, the survival of patients with high LINC01296 expression was poorer (Fig. 1B). These data ascertained the potential of LINC01296 as a biomarker for malignant osteosarcoma. For further confirmation, LINC01296 expression in normal HFOB1.19 bone cells and several osteosarcoma cells was analyzed, respectively. As anticipated, LINC01296 was revealed to be greatly upregulated in osteosarcoma cells (Fig. 1C), which further validated the potential role of LINC01296 as an oncogenic factor in osteosarcoma. Based on this, we proceeded to analyze the role of LINC01296 in cell proliferation, migration and invasion.

Indispensable role of LINC01296 in the promotion of proliferation, migration and invasion of osteosarcoma cells. To corroborate the role of LINC01296 in promoting osteosarcoma cell proliferation, invasion, migration and cell cycle regulation, LINC01296 knockdown was carried out by shRNA transfection, followed by monitoring of the phenotypical changes. As revealed in Fig. 2A, the cells transfected with LINC01296 specific shRNAs, Kd-sh1 and Kd-sh2, demonstrated a significant downregulation of LINC01296, compared to cells transfected with non-coding shRNA (P<0.05). Proliferation of MG63 and HOS-8603 cells transfected with these shRNAs was monitored for 4 days. A significantly lower growth rate was observed in cells transfected with LINC01296 shRNAs (Fig. 2B and C). The migration and invasion of cells were decreased after LINC01296 knockdown, as revealed in Fig. 2D, E and H, respectively. Cell cycle arrest was prominent in MG63 and HOS-8603 cells transfected with LINC01296 shRNAs, as revealed by the increased ratio of cells in the G0/G1 phase (Fig. 2F and G). Fig. 2I displays representative flow cytometric graphs, from which we can summarize the findings of Fig. 2F and G. These data indicated that high LINC01296 expression plays an indispensable role in enhancing the proliferation, invasion and migration of osteosarcoma cells. Although knockdown of LINC01296 in MG63 and HOS-8603 cell lines by shRNA is presented, we also used siRNA for knockdown in the MG63, HOS-8603 and 143B cell lines in pre-tests with similar results (data not shown). Due to the special cultured condition of 143B, we only used the MG63 and HOS-8603 cells in the functional assays of this study.

To further validate the tumor-promoting role of LINC01296, ectopic overexpression of LINC01296 in osteosarcoma...
cells was carried out. The overexpression of LINC01296 was confirmed by qPCR analysis (Fig. 3A). The proliferation of MG63 (Fig. 3B) and HOS-8603 (Fig. 3C) was significantly increased after LINC01296 overexpression (P<0.05), which was consistent with our hypothesis. In addition, greater migration (Fig. 3D and H) and invasion (Fig. 3E and H) of osteosarcoma cells were observed. Notably, the reduction of MG63 (Fig. 3F) and HOS-8603 (Fig. 3G) cells at the G0/G1 phase was quite...
marked (Fig. 3I). In conclusion, this evidence points to the pivotal role of LINC01296 in promoting osteosarcoma progression. Nevertheless, further elucidation is still warranted to validate the effect of LINC01296 on the regulation of the cell cycle.
LINC01296 positively regulates cyclin D1 expression. Considering the role of LINC01296 in the regulation of the cell cycle of osteosarcoma cells, we investigated how knockdown and overexpression of LINC01296 affected the expression of cell cycle regulators, including cyclin D1, CDK4, cyclin E1 and CDK2. As revealed in Fig. 4A, in MG-63 cells, cyclin D1 expression was closely correlated with LINC01296 levels, i.e., the knockdown of LINC01296 downregulated cyclin D1 expression while the overexpression of LINC01296 upregulated cyclin D1. However, such a trend was not observed with CDK4, cyclin E1 and CDK2. In qPCR analysis of cyclin D1, CDK4, cyclin E1 and CDK2 mRNA levels, cyclin D1 was identified as a sensitive responder to LINC01296 manipulation (Fig. 4B). Spearman's analysis indicated a strong...
positive correlation between LINC01296 levels and cyclin D1 levels (Fig. 4C).

To confirm the interaction between cyclin D1 and LINC01296, a direct knockdown or overexpression of cyclin D1 was carried out to antagonize the effect of LINC01296 in promoting osteosarcoma. Western blot (Fig. 5A) and qRT-PCR analyses (Fig. 5B) demonstrated successful cyclin D1 overexpression and knockdown, respectively. In addition, as revealed in Fig. 5C and H, knockdown of LINC01296 decreased cell migration and invasion, while cyclin D1 overexpression increased cell migration and invasion. Consistently, while overexpression of LINC01296 increased cell migration and invasion, knockdown of cyclin D1 decreased cell migration and invasion in MG63 cells (Fig. 5D). Further study revealed that while knockdown of LINC01296 induced cell cycle arrest, cyclin D1 overexpression reduced cell cycle arrest (Fig. 5E and H). Consistently, while overexpression of LINC01296 reduced the number of cells at the G0/G1 phase, knockdown of cyclin D1 concurrently led to the increase in cell cycle arrest (Fig. 5D and H). This evidence demonstrated that the interaction between LINC01295 and cyclin D1 was responsible for the tumor-promoting role of LINC01296.

Discussion

Accumulating evidence has demonstrated that non-protein coding genes are important regulators of cancer. With the aim of unraveling the molecular mechanism of osteosarcoma, the tumor-promoting role of LINC01296 in osteosarcoma was investigated for the first time in the present study. The results revealed that malignant osteosarcoma was characterized by high LINC01296 expression, which was consistent with the oncogenic function of LINC01296 in other cancers (13,14,19). Furthermore, it was revealed in this study that high LINC01296 expression played an indispensable role in promoting the proliferation, migration and invasion of osteosarcoma cells. Evidently, knockdown of LINC01296 resulted in retarded proliferation, migration and invasion, and marked cell cycle arrest. To elucidate the molecular mechanism of LINC01296 in osteosarcoma, we investigated how LINC01296 expression affected cell cycle regulators. Cyclins are key regulators of CDKs (cyclin-dependent kinases). Distinct expression and degradation patterns of different cyclins lead to the temporal coordination of mitotic events. Cyclin D1 regulates CDK3 and CDK6 by forming a complex with them, thereby governing cell cycle G1/S transition. Cyclin D1 serves as a central regulator for cell cycle progression, and aberrant expression of this protein is a significant contributor to tumorigenesis (20). We demonstrated that the expression of cyclin D1 was found to be positively correlated with LINC01296 levels. Knockdown of LINC01296 led to marked downregulation of cyclin D1. We reviewed the potential pathways in which LINC01296 was involved, but there was no clear elaboration of these pathways in published research. However, we found that there were some related micro-RNAs or proteins including miR-21a, miR-122, miR-5095 and MMP9, and MMPs that were reported to play an important role in the cell cycle (19,21-23). The potential pathways included miR-21a/p27, p38MAPK/Sp-1/p21 and Skp2/p27/p21. However, the direct interaction between LINC01296 and cyclin D1 was not established in this study.

Our ongoing research aims to address the clear mechanism of LINC01296 and cyclin D1. But given the involvement of cyclin D1 in numerous oncogenic signaling pathways (24), it can be concluded that LINC01296 is a vital molecule for a large spectrum of oncogenic processes.

Diagnosis and therapy of osteosarcoma still remain a critical challenge nowadays. The upregulation of LINC01296 in osteosarcoma makes it a promising biomarker for sensitive detection of such a disease. In addition to the increased expression of IncRNAs in tumors, increased IncRNA levels can also be detected in serum, which enhances the development of IncRNA-based facile cancer detection methods (25). Validation of the diagnostic potential of serum LINC01296 in osteosarcoma diagnosis is warranted. The compromised proliferation, migration and invasion induced by LINC01296 knockdown also potentiates gene therapy strategies based on this approach. For example, in vivo delivery of siRNA for silencing tumor-promoting IncRNAs has been utilized to impede cancer progression (26-28). Our ongoing studies focus on evaluation of the therapeutic efficacy of LINC01296 knockdown in vivo. This approach could precisely control the expression of LINC01296, thereby suppressing the proliferation, migration and invasion of tumors. Moreover, this proposed approach obviates the side effects associated with conventional cancer therapies such as chemotherapy and radiation therapy (29). It would not be surprising that cancer therapeutic approaches based on IncRNA knockdown could offer substantial benefit to the clinical management of osteosarcoma.

In summary, the present study reported that high LINC01296 expression was found in osteosarcoma. LINC01296 expression was associated with the proliferation, migration, and invasion of osteosarcoma cells. In this study, positive correlation between the upregulation of cyclin D1 and high LINC01296 expression was reported, which in part accounts for the tumor-promoting role of LINC01296 in osteosarcoma. These data demonstrated that LINC01296 can be a valuable target for the diagnosis and treatment of osteosarcoma.

Acknowledgements

Not applicable.

Funding

We would like to thank the Jilin Provincial Science and Technology Department Outstanding Youth Talent Fund (no. 201705200143JH) and the National Natural Science Foundation (no. 81201382) for their financial support.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

PL, the corresponding author of this study, participated in every step of the design project and in specific experiments. XY also participated in the design of this study and most of the experiments, and was the writer of this manuscript. LP
participated in the collection of case data and cultured the cells for this study, and provided advice for the revision of the manuscript. TY helped in the experiments and modified the language of the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Jilin University (Changchun, China). Informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References