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Effect of lncRNA LET on proliferation and invasion of osteosarcoma cells

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Abstract. – OBJECTIVE: To investigate the expression of long non-coding RNA (LncRNA) LET in osteosarcoma and its effect on the proliferation, apoptosis, migration and invasion of osteosarcoma cells.

PATIENTS AND METHODS: The expression of lncRNA LET was detected in osteosarcoma tissues and cell lines (MG63 and hFOB1.19). MG63 cells stably overexpressing lncRNA LET were constructed by lentiviral. The effects of lncRNA LET overexpression on the proliferation, apoptosis, migration and invasion of osteosarcoma cells were detected by cell counting kit-8 (CCK-8), flow cytometry and transwell chamber assay.

RESULTS: The expression of lncRNA LET in osteosarcoma tissues and MG63 cells was significantly down-regulated. Overexpression of lncRNA LET significantly inhibited the proliferation, migration, invasion, and induced apoptosis of MG63 cells.

CONCLUSIONS: LncRNA LET was participated in the development of osteosarcoma, and may be used as a potential molecular target for the treatment of osteosarcoma.

Key Words:
Osteosarcoma, LncRNA LET, Proliferation, Migration, Invasion.

Introduction

Osteosarcoma is a kind of common and highly aggressive skeletal system tumor, whose morbidity and mortality rates rank first in primary bone tumors[1]. Malignant proliferation, metastasis and invasion of tumor cells have become the main causes of death of patients with osteosarcoma[2]. Therefore, exploring the new target interfering in malignant biological phenotype of osteosarcoma has become a research hotspot currently. Long non-coding ribonucleic acid (lncRNA) is a kind of non-coding RNA with 200 nucleotide in length, which plays important regulatory roles in the proliferation, apoptosis, migration and invasion of tumor cells[3]. LncRNA LET was repressed by EZH2, and could repress cell proliferation and induced apoptosis in nasopharyngeal carcinoma[4]. LncRNA LET could exhibit tumor-suppressive activity and acted as a prognostic factor in gallbladder cancer[5]. LncRNA LET suppressed tumor growth and EMT in lung adenocarcinoma[6], gastric cancer[7] and cervical cancer[8]. However, its role in osteosarcoma has not been studied yet.

In this work, Real-time fluorescence quantitative polymerase chain reaction (PCR) was used to detect the expression levels of lncRNA LET in osteosarcoma tissues and MG63 cells. The effects of lncRNA LET on the proliferation, apoptosis, migration and invasion capacities of osteosarcoma cells were detected via cell counting kit-8 (CCK-8), flow cytometry and transwell chamber assay, respectively. A series of *in-vitro* experiments aim at exploring the mechanism of action of lncRNA LET, so as to find the potential molecular target of osteosarcoma.

Patients and Methods

Specimen Source and the Main Reagents

Osteosarcoma tissues and para-carcinoma normal tissues were collected from patients in the surgery of our hospital. Patients received radical resection and underwent no radiotherapy, chemotherapy and other intervention therapies before operation. Patients were informed of specimen collection before operation and signed the informed consent. The research methods were approved by the Ethics Committee of Yantai Yuhuangding Hospital (Yantai, China). Osteosarcoma cell lines MG63, human osteoblast cell line hFOB1.19 were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle Medium

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(DMEM), DMEM/F12 cell culture medium, and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA), RT-PCR reverse transcription kit and SYBR Green fluorescence quantitative kit were purchased from TaKaRa (Otsu, Shiga, Japan). TRIzol was purchased from Invitrogen (Carlsbad, CA, USA). Primers used for qRT-PCR were synthesized by Shanghai Genma Biological Co., Ltd. (Shanghai, China). CCK-8 kit was purchased from Beyotime (Shanghai, China), transwell chamber was purchased from Corning Company (Corning, NY, USA), lncRNA LET lentiviral vector containing luciferase gene and luciferase substrate were purchased from Shanghai Ji Man Co., (Shanghai, China).

Cell culture and Lentiviral Vector Infection of Osteosarcoma Cells

hFOB1.19 cells were cultured in the Dulbecco's modified Eagle medium (DMEM)/F12 containing 10% fetal bovine serum (FBS). Osteosarcoma MG63 cell was cultured in DMEM containing 10% FBS in an incubator with 5% CO_2 at 37°C. MG63 cells in the logarithmic growth phase were taken and transfected with the lentiviral vector containing lncRNA LET with luciferase, and the lentiviral vector carrying nonsense sequence was used as the negative control. Lentiviral particles wrapped were cultured and collected, and osteosarcoma MG63 cells were infected at [multiplicity of infection (MOI) =30] and cultured using the puromycin-containing medium. The total RNA was extracted after 4 weeks for subsequent experiments.

qRT-PCR

Tissues (100 mg) or cells (1×10^6) were taken to extract the total RNA using TRIzol. The purity and concentration of RNA were measured using a microplate spectrophotometer. After the RNA concentration was adjusted to 300-500 ng/µL, RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) using the RNA reverse transcription kit. The reaction system was prepared according to instructions of the SYBR Green fluorescence quantitative kit. Three repeated wells were set for each system with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

Cell Counting kit-8 (CCK-8)

MG63 cells in the logarithmic growth phase were taken and inoculated into a 96-well plate (1×10^5/well). 200 µL DMEM containing 10% FBS were added into each well. Cells were divided into Lv-lncRNA LET group and Lv-NC group, and 6 repeated wells were set for each group. The cell proliferation was detected at 0, 24, 48 and 96 h after culture. During detection, 20 µL CCK-8 reagent were added into each well in the 96-well plate for incubation at 37°C for 2 h. The optical density (OD) value of each well was detected at a wavelength of 450 nm using a spectrophotometer. Cells continued to be incubated with 5% CO_2 at 37°C for 14 d, followed by 0.1% crystal violet staining.

Flow Cytometry

According to the instructions, Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were used. Annexin V assay kit (Miltenyi Biotech., Bergisch Gladbach, Germany) was used to detect cells apoptosis.

Transwell Assay

MG63 cells in the logarithmic growth phase in Lv-lncRNA LET group and Lv-NC group were taken, and prepared into the single-cell suspension at a density of 2.5×10^5/mL using the serum-free DMEM. 200 µL suspension were added into the upper transwell chamber, while DMEM containing 10% FBS were added into the lower chamber. After 24 h, cells were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet.

Statistical Analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by least significant difference (LSD). p-values < 0.05 were considered statistically significant.

Results

LncRNA LET was Low Expression in Osteosarcoma Tissues and MG6 Cell

We performed Real-time quantitative PCR method to investigate the expression of lncRNA LET in osteosarcoma tissues and MG6 cell. The results showed that the expression of lncRNA LET in osteosarcoma tissues was significantly lower than that in adjacent tissues ($p < 0.05$; Figure 1). The expression level of lncRNA LET in osteosarcoma MG63 cells was significantly lower

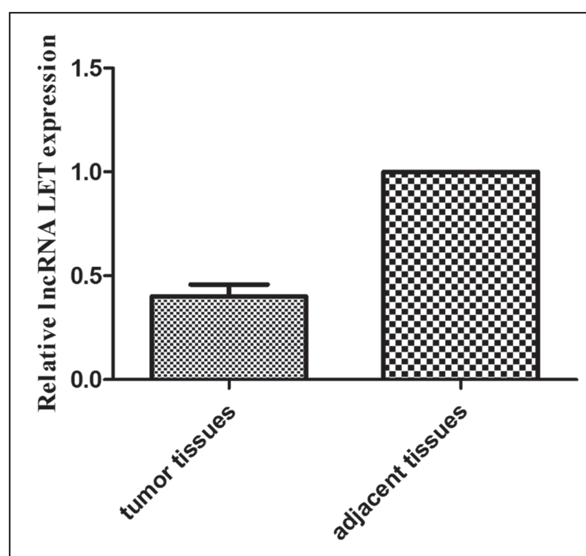

Figure 1. LncRNA LET expression was reduced in tumor tissues relative to the adjacent tissues by qRT-PCR. *$p < 0.05$.

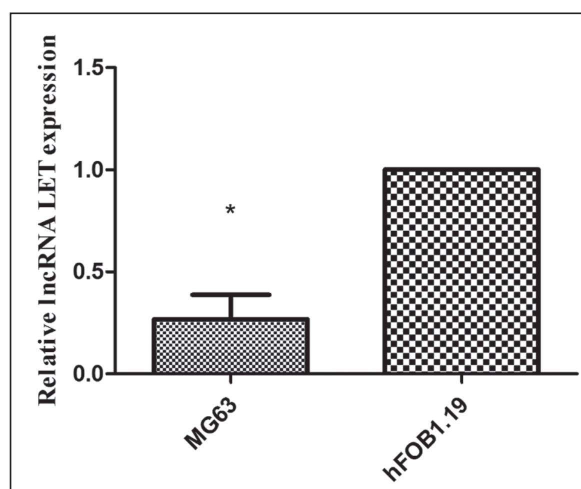

Figure 2. LncRNA LET expression in MG63 cell was lower than that in hFOB1.19 cell by qRT-PCR. *$p < 0.05$.

than that in hFOB1.19 cells ($p < 0.05$; Figure 2). Furthermore, we found that the expression of lncRNA LET was not significantly correlated with age or gender, but was significantly related to disease stage and metastasis (Table I).

Overexpression of lncRNA LET Inhibited Osteosarcoma Cell Proliferation and Induced Cell Apoptosis

Firstly, we significantly up-regulated the expression of lncRNA LET in osteosarcoma MG63 cells by lentiviral ($p < 0.05$; Figure 3A). Furthermore, cell proliferation and apoptosis abilities were evaluated via CCK-8 and flow cytometry. The results of CCK-8 assay (Figure 3B) showed that lncRNA LET overexpression significantly inhibited the proliferation of osteosarcoma MG63 cells. Meanwhile, the flow cytometry assay showed that lncRNA LET overexpression significantly induced the apoptosis of osteosarcoma MG63 cells ($p < 0.05$; Figure 3C).

Overexpression of lncRNA LET Reduced Cell Migration and Invasion

In addition, to explore cell migration and invasion, transwell chamber test was performed. The test results (Figure 4) showed that the migration and invasion ability of cells transfected with over-expression lncRNA LET were significantly lower than the number of cells in the control group. This result indicated that up-regulation of lncRNA LET reduced cell migration and invasion.

Table I. lncRNA LET expression and clinic characteristics.

Characteristics	All patients	lncRNA LET low expression	lncRNA LET high expression	*p*
No.	52	26	26	
Age (year)				0.569
≤ 60	20	11	9	
> 60	32	15	17	
Gender				0.578
Male	24	13	11	
Female	28	13	15	
Disease stage				0.011
T1-2	31	11	20	
T3-4	21	15	6	
Metastasis				0.020
No	34	13	21	
Yes	18	13	5	

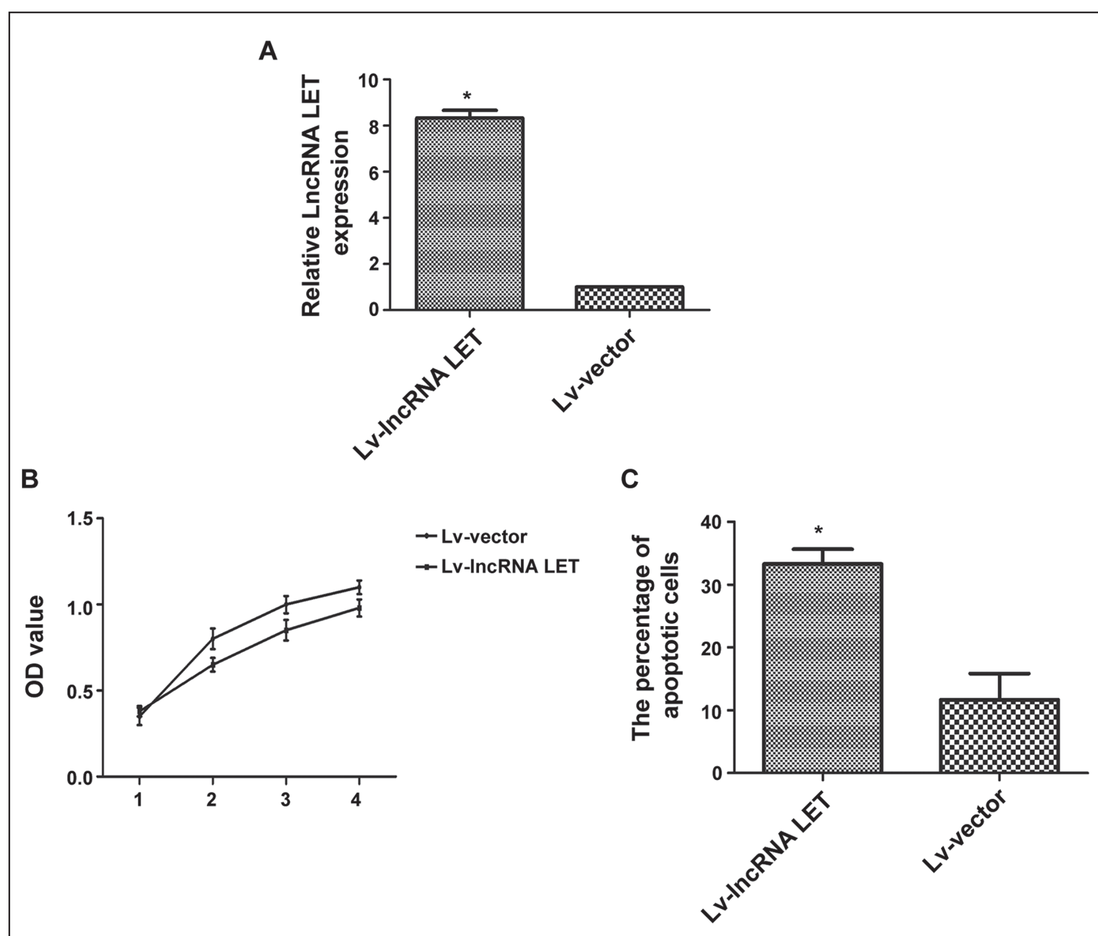

Figure 3. ***A,*** qRT-PCR method showed lncRNA LET was up-regulation by lentiviral. ***B,*** OD value was examined by CCK-8 between Lv-lncRNA LET and Lv vector. 1 refers to 24 h, 2 refers to 48 h, 3 refers to 72 h, and 4 refers to 96 h. ***C,*** Flow cytometry analysis showed the percentage of apoptotic cells between Lv-lncRNA LET and Lv-vector. *$p < 0.05$.

Discussion

Non-coding RNA plays an important biological function in regulating biological process such as cell growth, differentiation and metabolism, and is closely related to the occurrence and development of various diseases including tumors[3]. In recent years, lots of lncRNAs such as lncRNA HOTAIR, lncRNAH19, lncRNA DANCR, lncRNA MEG3 and so on, have been reported in pancreatic cancer, liver cancer, prostate cancer, nasopharyngeal cancer, cervical cancer and other tumors[9-12]. This study focused on the expression of lncRNA LET in osteosarcoma and its role in the regulation of malignant biological phenotype in order to provide a new molecular target for the treatment of osteosarcoma.

LncRNAs that are abnormally expressed in tumors often play an important role in the regulation of malignant biological phenotypes such as proliferation, migration and invasion of tumor cells[13]. The expression of lncRNA H19 is significantly upregulated in pancreatic cancer and promotes the proliferation of pancreatic cancer cells[14]. The expression of lncRNA GAS5 is significantly downregulated in prostate cancer cells and has the inhibitor effect on the proliferation of prostate cancer cells[15]. In this study, low expression of lncRNA LET was observed in osteosarcoma tissues and cell lines, suggesting that lncRNA LET may play an important role in the development of osteosarcoma.

In order to investigate the role of lncRNA LET in the development of osteosarcoma, we constructed a cell line stably overexpressing lncRNA LET and observed the effect of lncRNA LET on the proliferation, apoptosis, migration and invasion of osteosarcoma cells. We found that overexpression of lncRNA LET could inhibit the proliferation, migration, invasion and induce

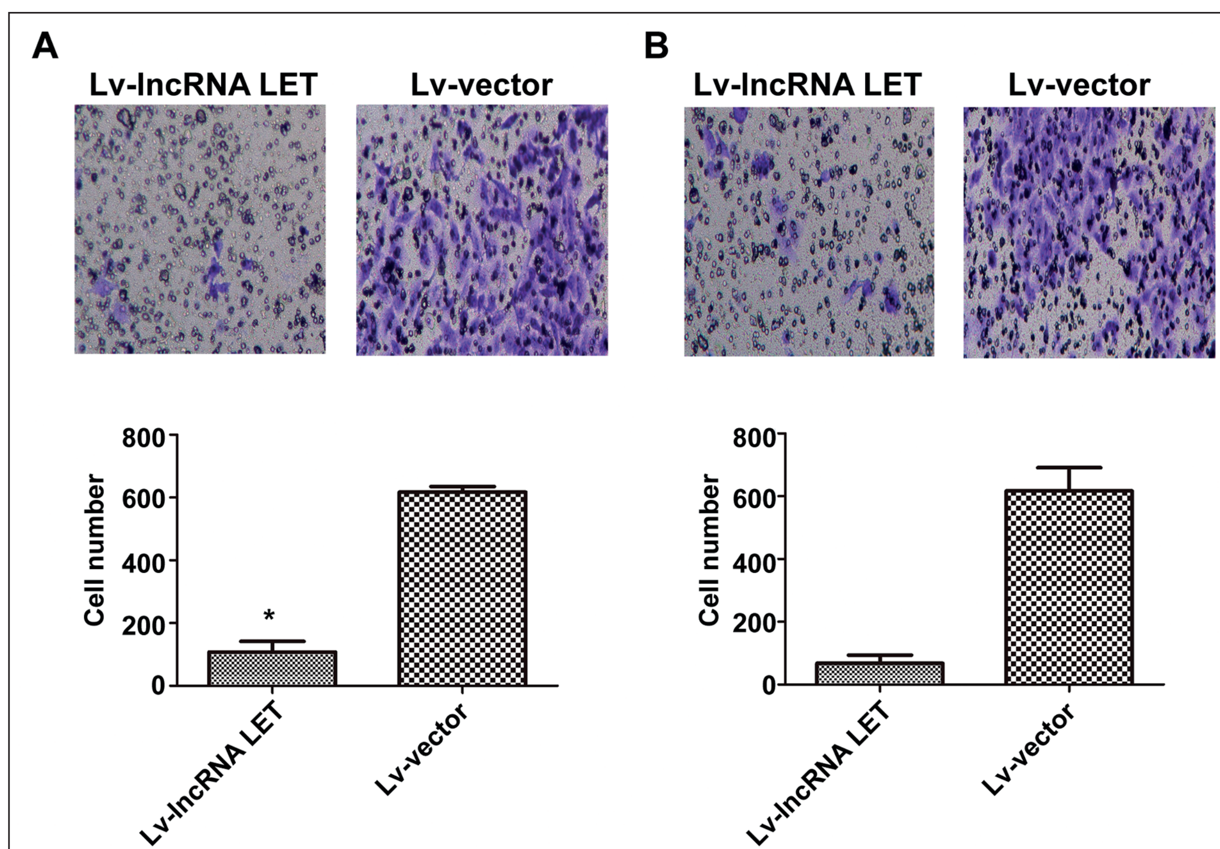

Figure 4. A, Up-regulation of lncRNA LET repressed cell migration via transwell method. ***B,*** Up-regulation of lncRNA LET also suppressed cell invasive ability by transwell invasion assay. $*p < 0.05$.

apoptosis of osteosarcoma cells. The molecular mechanism by which lncRNA plays a role in oncology is complex and not yet known. However, there are three main molecular mechanisms by which lncRNAs play a role in tumors: (1) LncRNA can regulate the expressions of cancer-associated genes through binding to endogenous micro RNA (miRNA). (2) LncRNA can bind to transcription factor proteins to regulate the expressions of downstream relevant genes by activating or inhibiting transcription factors. (3) LncRNA can bind to chromosome spatial conformation or mRNA to regulate the expressions of cancer-associated genes[16-18].

Conclusions

We found that lncRNA LET was significantly down-regulated in osteosarcoma and inhibited the development of osteosarcoma. Therefore, lncRNA LET may be a potential molecular target for the treatment of osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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