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LncRNA LET function as a tumor suppressor in breast cancer development

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Abstract. – OBJECTIVE: To evaluate the effect of long-chain non-coding RNA LET (lncRNA LET) on the regulatory of human breast cancer and its underlying mechanism.

PATIENTS AND METHODS: The expression levels of lncRNA LET in breast cancer tissues, MDA-MB-231 cells and MCF-10A breast epithelial cells were detected by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The proliferation of lncRNA LET was detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Cell apoptosis was examined via flow cytometry. The invasion and migration of cells were detected by transwell and scratch assay.

RESULTS: The expression of lncRNA LET was reduced in breast cancer tissues and MDA-MB-231 cells. Overexpression of lncRNA LET resulted in the inhibition of cell proliferation, invasion and migration ability, and promotion of cell apoptosis ($p<0.05$). Up-regulation of lncRNA LET repressed epithelial mesenchymal transition (EMT) process.

CONCLUSIONS: LncRNA LET is a new type of molecule involved in the development of breast cancer, which may become a potential target for the treatment of breast cancer.

Key Words:
Breast cancer, LncRNA LET, Proliferation, Apoptosis, Invasion.

Introduction

Breast cancer is the most common malignancy in women. According to statistics, there were 232,670 newly-diagnosed cases of breast cancer in USA in 2014, and 40,000 patients died of breast cancer[1]. Although great progress has been made in the treatment of breast cancer at present, 30% patients still die from the recurrence and metastasis of breast cancer after receiving formal treatment[2]. So it is particularly important to study the mechanism of its metastasis. The long non-coding RNAs (LncRNAs) are a group of RNA molecules that are over 200 nt in length and do not encode protein. Recent investigations[3,4] have found that lncRNAs play a key role in the formation of cancer and are involved in the regulation of cell proliferation, apoptosis, invasion and metastasis. Researches[5-9] have found that lncRNA LET plays different regulatory roles in many cancers. Increased miR-548k could enhance the progression by controlling lncRNA LET in esophageal squamous cell carcinoma[10]. In gastric cancer, lncRNA LET, a tumor suppressor, might be a biomarker and a therapeutic target for gastric cancer[11]. LncRNA-LET contributes into cell proliferation and invasion of nasopharyngeal carcinoma[12]. However, few studies have investigated its regulation of cell proliferation and invasion. The primary purpose of this study was to investigate the effect of lncRNA LET on the proliferation, apoptosis, invasion and migration of breast cancer cells.

Patients and Methods

Materials and Reagents

Human breast cancer cell lines MCF-10A and MDA-MB-231 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China); fetal calf serum (FCS) was purchased from HyClone (South Logan, UT, USA); Dulbecco's Modified Eagle Medium (DMEM) high glucose medium was purchased from Gibco (Rockville, MD, USA); TRIzol reagent and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA); reverse transcription kit was purchased from Fermentas company (Burlington, Onta-

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rio, Canada). LncRNA LET overexpression vector was purchased from Shanghai Jima Pharmaceutical Technology Co., Ltd. (Shanghai, China); plasmid extraction kit was purchased from Axygen Company (Tewksbury, MA, USA); all primer synthesis and DNA sequencing by Nanjing Kingsley Biotechnology Co., Ltd. completed (Nanjing, China). A total of 70 patients with breast cancer had undergone surgery at our hospital from May 2014 and November 2017. Cancer specimen and the adjacent tissues taken from the patients were obtained. Our work was approved by the Ethical Committee and informed consent was granted by the patients.

Cell Culture

MCF-10A and MDA-MB-231 were inoculated with DMEM high glucose medium containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), placed in 37°C, 5%, saturated humidity cell culture incubator. The cells were passaged every 2 to 3 days, and all experiments used logarithmic growth phase cells.

Quantitative Real-Time Polymerase Chain Reaction (Qrt-PCR)

Detection of lncRNA LET expression: total RNA was extracted using TRIzol, followed by reverse transcription reaction according to instructions of reverse transcription reagent, and real-time polymerase chain reaction (PCR) in accordance with instructions of the fluorescence quantitative kit (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as the control.

Transfection

Cells in the logarithmic growth phase were taken, digested with 0.25% trypsin, centrifuged and prepared into the single-cell suspension. The suspension was inoculated into a 6-well plate, and the cell density reached 70-90% on the second day. 4 μg plasmids containing lncRNA LET whole genome were diluted in 250 μL serum-free medium, and 10 μL Lipofectamine 2000 were diluted in 250 μL serum-free medium and placed at room temperature for 5 min. The diluted plasmids were mixed evenly with Lipofectamine 2000, and placed at room temperature for 20 min. 1.5 mL serum-free medium was added into each well of the 6-well plate, the transfection mixture was slowly added into the 6-well plate, and the original medium was replaced with the complete medium containing 10% fetal bovine serum (FBS) after 6 h.

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide]

According to 2000 cells per well, cell line was seeded into 96-well plates with 0.5 mg/mL MTT reagent (20 μL) (R&D Systems, Minneapolis, MN, USA). The precipitate was dissolved via dimethyl sulfoxide (DMSO) (200 μL) (Sigma-Aldrich, St. Louis, MO, USA). The wavelength of 490 nm (optical density) was detected.

Flow Cytometry

Cell apoptosis was detected through Annexin-V/PI (Propidium Iodide) Apoptosis Detection Kit (KeyGEN, Nanjing, China). The early and late apoptotic cells were labeled via Annexin-V and PI, respectively. FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to assess cell apoptosis.

Wound Healing

Cells in the logarithmic growth phase were digested with trypsin, centrifuged and prepared into the single-cell suspension. The suspension was inoculated into the 6-well plate and cultured under 5% CO_2 at 37°C overnight. When 80-90% cells grew, a wound was gently made in the middle of the cell plate using a 10 μL sterile spearhead under the same strength, and the width of wound in each group should be basically the same. After the plate was washed twice with phosphate-buffered saline (PBS), cells were treated with drugs. The width of different wounds in any three parts in each group was measured under a low-power microscope at 0 and 48 h after culture.

Transwell Invasion

Cells in the logarithmic growth phase were digested with trypsin, and the density of cells in each group was adjusted to the same value using the serum-free medium. According to the experimental grouping, 100 μL cells in each group were added into the upper transwell chamber. 600 μL Dulbecco's modified Eagle medium (DMEM) containing 10% FBS were added into the lower transwell chamber, followed by incubation with 5% CO_2 at 37°C for 24 h. After the transwell chamber was taken out, the medium in the upper transwell chamber and cells not passing through the membrane were carefully wiped off with the cotton swab. Finally, after fixation with 4% paraformaldehyde for 20 min and Giemsa staining, the number of cells passing through the membrane was observed under the high-power microscope.

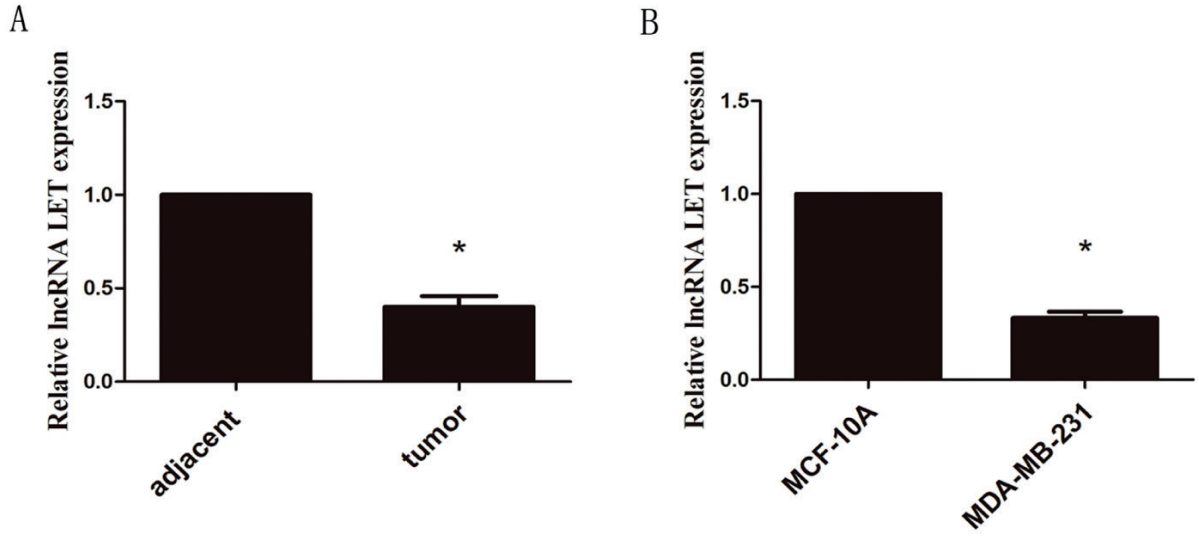

Figure 1. qRT-PCR analysis showed lncRNA LET expression was down-regulation both in tumor tissues ***(A)*** and MDA-MB-231 cells ***(B)***. $^{*}p<0.05$.

Western Blot

By using a bicinchoninic acid (BCA) Kit (Pierce, Rockford, IL, USA), the protein collected from MDA-MB-231 was detected. The antibodies against E-cadherin, N-cadherin, Vimentin and GAPDH were collected from Santa Cruz Biotechnology Company (Santa Cruz, CA, USA). The secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:5,000 (v/v) dilutions in PBS and Tween 20 (0.1%).

Statistical Analysis

Statistical analysis was performed using STATA 11.0, and presented with Graph PAD prism software (La Jolla, CA, USA). The results obtained from experiment in vitro assays are presented as mean ± SEM, and the data was analyzed by Wilcoxon rank-sum (Mann-Whitney) test. All the results were regarded as statistically significant at $p<0.05$.

Results

LncRNA LET Expression in Human Breast Cancer Samples and Cell Lines were Down-Regulated

Comparison of lncRNA LET expression between the tumor tissues and the adjacent samples was conducted by qRT-PCR. We found that lncRNA LET expression was down-regulation in human breast cancer samples compared with the adjacent tissues ($p<0.05$, Figure 1A). Furthermore, we also investigated the expression of lncRNA LET between breast cancer cell lines MDA-MB-231 and MCF-10A. The results of qRT-PCR showed that lncRNA LET was expressed in breast cancer cell lines MDA-MB-231 and MCF-10A, but LncRNA LET was lower in MDA-MB-231 cells than MCF-10A. The difference between each cell was statistically significant ($p<0.05$, Figure 1B). Therefore, MDA-MB-231 cells were used for the subsequent experiments, and lncRNA LET high expression cell lines were constructed with MDA-MB-231 cells. All the above findings turned out that lncRNA LET expression were implicated in breast cancer progression.

pcDNA-LET Up-Regulated the Expression of lncRNA LET in MDA-MB-231 Cells

To assess the role of lncRNA LET in human breast cancer cells, the pcDNA-LET vector was transfected into MDA-MB-231 cells and the empty vector group served as a control. Compared with the empty vector group, the expression level of lncRNA LET in pcDNA-LET vector group was increased, and the increase rate of pcDNA-LET was obvious ($p<0.05$, Figure 2A).

Up-Regulation of lncRNA LET Repressed Epithelial Mesenchymal Transition (EMT) Process

The EMT-related markers (E-cadherin, N-cadherin, Vimentin) were detected by Western blot. The results of Western blot showed that hi-

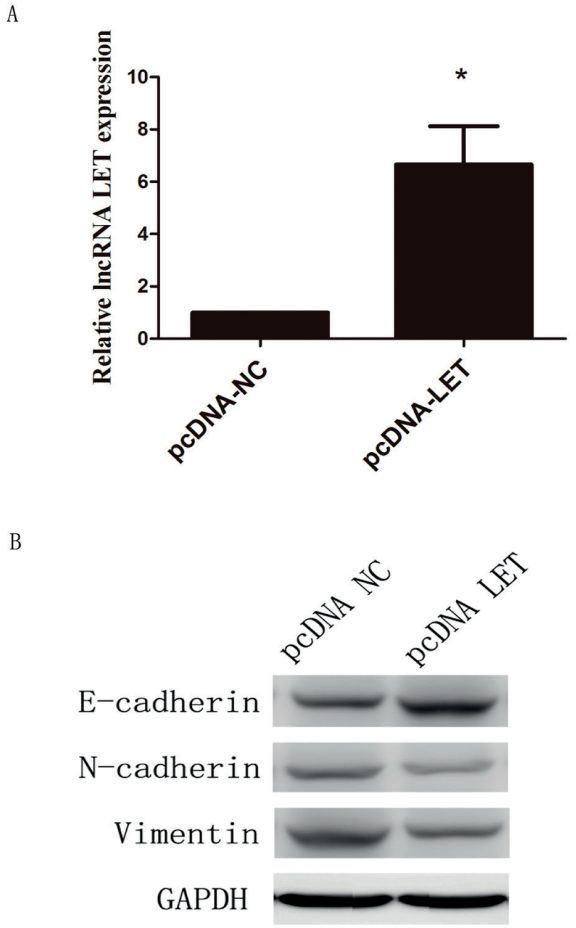

Figure 2. qRT-PCR assay confirmed that pcDNA-LET could up-regulate the expression of lncRNA LET as compared to pcDNA NC. *p<0.05 The Western blot assay showed high-expression of lncRNA LET caused in increased E-cadherin, decreased N-cadherin and Vimentin expression.

gh-expression of lncRNA LET caused in increased E-cadherin, decreased N-cadherin and Vimentin expression, which indicated up-regulation of lncRNA LET repressed EMT process (Figure 2B).

Up-regulation of lncRNA LET Repressed the Proliferation and Induced the Apoptosis of Breast Cancer Cells

To investigate the biological role of lncRNA LET in breast cancer cells, the effect of lncRNA LET on the proliferation of breast cancer cells was studied by MTT assay. The results showed that compared with pcDNA-NC group, pcDNA-LET group significantly inhibited the proliferation of cells (p<0.05, Figure 3A). In addition, we also tested the effect of lncRNA LET on the apoptosis of breast cancer cells by flow cytometry assay. The results discovered that pcDNA-LET group significantly could induce the apoptosis of cells, compared with pcDNA-NC group (p<0.05, Figure 3B)

Inhibition Effect of lncRNA LET Over-Expression on Cells Invasive and Migrate Abilities was Exhibited

Wound healing assay and transwell invasion assay were used to investigate the regulatory effect of lncRNA LET on migration and invasion of breast cancer cells. Compared with the pcDNA-NC transfection group, wound healing test results showed that the migration rate of pcDNA-LET transfection group was significantly decreased (Figure 4A). Transwell experimental results showed that the number of cells in the pc-

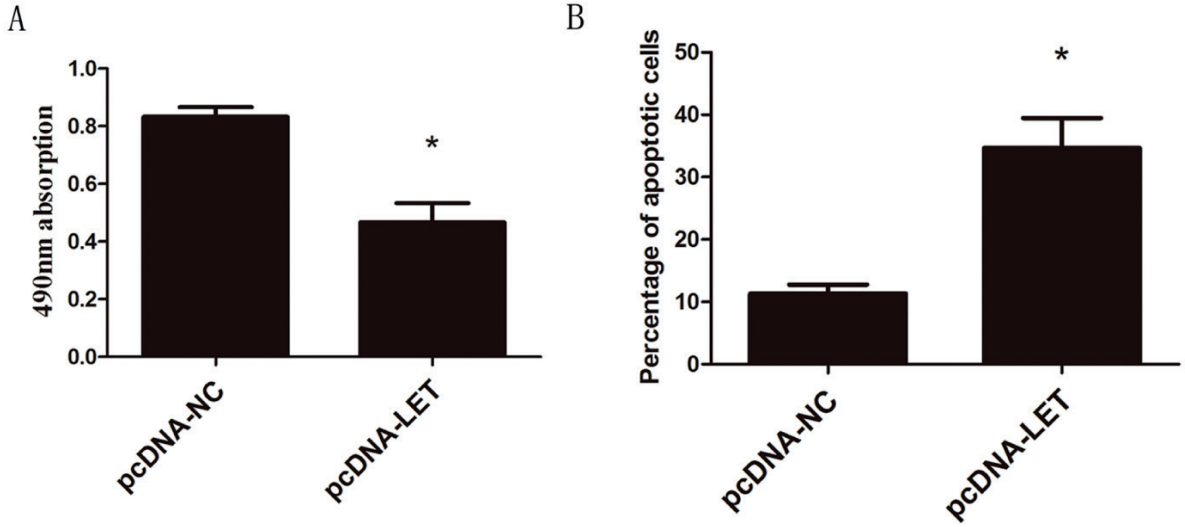

Figure 3. 490 nm absorption was assessed between pcDNA-NC and pcDNA-LET (A). Percentage of apoptotic cells was detected between pcDNA-NC and pcDNA-LET (B). *p<0.05.

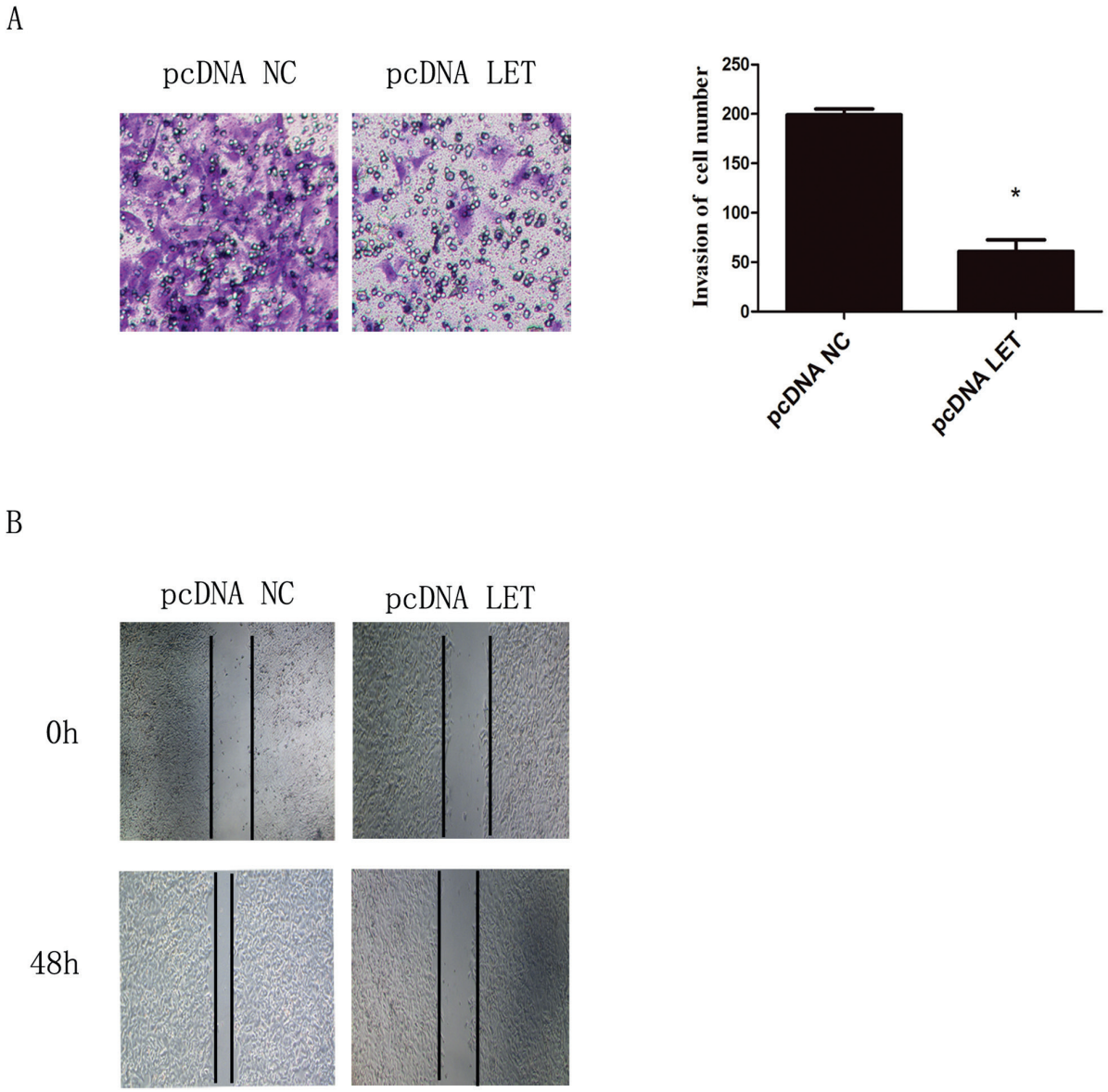

Figure 4. Cell invasive ability was evaluated by transwell invasion assay (A). *p<0.05. Cell migrate ability was detected via wound-healing method (B). *p<0.05.

DNA-LET transfection group was significantly decreased (p<0. 05, Figure 4B).

Discussion

With the continuous understanding of lncRNA, more and more studies[13] have shown that the molecular mechanism of tumor formation is not only related to the protein coding genes, but also many lncRNAs. Although some lncRNAs have been proved to play key roles in tumor development[14-17] and other diseases[18, 19], only a few lncRNAs are investigated, and there are still many important problems to be solved. Here, our work focused on the underlying function of lncRNA LET dysregulation on breast cancer. LncRNA LET plays a role of tumor suppressor gene in many tumors and regulates the occurrence and development of tumors. Although there are many studies on lncRNA LET, few studies are conducted on the role of lncRNA LET in breast cancer. In addition, there are other reports on the relationship between lncRNA LET and tumor proliferation, invasion and metastasis. However, the correlation of lncRNA LET expression with cell proliferation, apoptosis, invasion and

migration of breast cancer cells has not been reported. In order to further elucidate the mechanism of lncRNA LET in the development of breast cancer, we compared the lncRNA LET expression in breast cancer tissues and the adjacent samples. The results showed lncRNA LET was reduced in breast cancer tissues. Furthermore, we also compared lncRNA LET expression in breast cancer cell lines. The results showed lncRNA LET was lower expressed in MDA-MB-231 than MCF-10A. Therefore, lncRNA LET was overexpressed in MDA-MB-231 cells to carry out research. LncRNA LET up-regulated in MDA-MB-231 cells could inhibit the proliferation and promote the apoptosis of breast cancer cells and reduce the invasion and migration ability of breast cancer cells, indicating that lncRNA LET can affect the occurrence and development of breast cancer cells. Up-regulation of lncRNA LET repressed EMT process. These results suggest that lncRNA LET may act as a tumor suppressor in breast cancer cells, and its absence or reduced expression may lead to breast cancer.

Conclusions

lncRNA LET expression plays a key role in the proliferation, invasion and migration of breast cancer cells. LncRNA LET is a new type of molecule involved in the development of breast cancer, which may become a potential target for the treatment of breast cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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