Long non-coding RNA LINC00628 suppresses the growth and metastasis and promotes cell apoptosis in breast cancer


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Abstract. – OBJECTIVE: Breast cancer is the most common malignant tumor in women. However, the detailed mechanisms of its tumorigenesis remain largely unknown. Evidence and data have shown that abnormality in expression of Long non-coding RNA (LncRNA) is closely related to tumorigenesis. The aim of this study is to identify the detailed mechanisms of LncRNA LINC00628 in breast cancer.

PATIENTS AND METHODS: The expression of LINC00628 in breast cancer tissues, adjacent non-cancerous tissues and cell lines were detected by qRT-PCR. Kaplan-Meier method and log rank-test were applied to analyze the overall survival of patients with low and high expression level of LINC00628 respectively. The LCC2 and MCF-7 cells were transfected with LINC00628 and the proliferation, invasion and migration were examined. The cell cycle distribution and cell apoptosis rate in LCC2 and MCF-7 cells after transfection with LINC00628 were explored by flow cytometry. The relative expression level of Bcl-2, Bax and Caspase-3 protein in LCC2 and MCF-7 cells after transfection with LINC00628 was detected by Western blotting.

RESULTS: The relative expression level of LINC00628 in breast cancer tissues and cell lines were significantly decreased and the low expression level of LINC00628 has a poorer prognosis and lower overall survival rate. The overexpression of LINC00628 suppressed breast cancer cells proliferation, invasion and migration. Further, with the overexpression of LINC00628, cell cycle was arrested in G0/G1 phase in breast cancer cells and cell apoptosis was promoted. The relative expression of Caspase-3 and Bax protein were significantly increased and the relative expression of Bcl-2 protein was significantly decreased after transfection with LINC00628.

CONCLUSIONS: The expression of LINC00628 was decreased in breast cancer. The overexpression of LINC00628 suppressed the proliferation, invasion and migration of breast cancer cells and promoted cell apoptosis associated with the regulation of Bcl-2/Bax/Caspase-3 signal pathway.

Key Words: LncRNA LINC00628, Proliferation, Invasion, Migration, Apoptosis, Bcl-2, Bax, Caspase-3.

Introduction

Breast cancer is the most common malignant tumor in women1,2. Breast cancer is closely related to abnormality in differentiation induced by epigenetic alterations and progressive genetic abnormalities3,4. The epigenetic events regulate chromatin structure and result in the expression change of genes involved in cellular proliferation, invasion and migration5,6. Current treatments include chemotherapy, surgery, and sometimes radiotherapy all together, but the prognosis remains poor3,8, despite studies9,10 have identified many carcinogens and tumor suppressor genes, which are believed to account for the growth of breast cancer. The detailed mechanisms of its tumorigenesis still remain largely unknown.

Long non-coding RNAs (LncRNAs) are a class of RNAs that have more than 200 nucleotides and do not have the ability to code proteins in plants and animals11,12. Accumulating evidence has shown that LncRNAs are involved in multiple gene regulatory networks, including gene transcription13-15. Decades of studies have found that proliferation, apoptosis, metastasis, and invasion are closely associated with abnormal expression of LncRNAs in human cancer cells, including breast cancer16,17. LncRNA UCA1 was shown to confer tamoxifen resistance to breast cancer18,19. LncRNA UCA1 suppressed breast cancer growth
and invasion by regulating SATB120. Various lncRNAs have been demonstrated to play significant roles in breast cancer21,22. However, the detailed mechanisms underlying these regulatory networks need to be further identified.

LncRNA LINC00628 locates in the second intron of PLEKHA6 (pleckstrin homology domain containing A6) in chromosome 1q32.1, the mature RNA which is 1290bp has a poly A tail and is discovered in many tumor formations23. It was shown to be down-regulated in human gastric cancer and worked as a tumor suppressor imposing long-range modulation on the expression of cell cycle-related genes23. However, the underlying mechanisms of LINC00628 expression abnormality remain to be uncovered.

In this study, the role of LINC00628 in breast cancer was elucidated. A total of 60 paired cancer-adjacent control tissues were collected from breast cancer patients and expression of LINC00628 in breast cancer tissues, adjacent non-cancerous tissues and cell lines were detected by qRT-PCR. The detailed role of LINC00628 expression abnormality remain to be uncovered.

In this study, the role of LINC00628 in breast cancer was elucidated. A total of 60 paired cancer-adjacent control tissues were collected from breast cancer patients and expression of LINC00628 in breast cancer tissues, adjacent non-cancerous tissues and cell lines were detected by qRT-PCR. The detailed role of LINC00628 in breast cancer was explored by cell proliferation, invasion migration assay, cell cycle assay and cell apoptosis assay. Our results showed the overexpression of LINC00628 suppressed breast cancer cells proliferation, invasion and migration and promoted cell apoptosis associated with regulation of Bcl-2/Bax/Caspase-3 signal pathway.

Patients and Methods

Human Tissues

This study was approved by the Ethics Committee of Chongqing Cancer Institute/Hospital. Sixty human tissue samples of breast cancer and sixty adjacent non-cancerous tissues samples from breast cancer patients who underwent clinical surgeries were collected at the Department of Breast Surgery, Chongqing Cancer Institute/Hospital from 2010 to 2013. All patients showed their full intentions to participate in this research and signed a consent form, and had not accepted preoperative neoadjuvant chemotherapy and radiotherapy. 38 breast cancer patients were with lymph node metastasis, and 22 breast cancer patients without lymph node metastasis. The tumor tissues and their adjacent non-tumor tissues were frozen in liquid nitrogen once dissected from the patients, and those tissues were used for RNA extraction and RT-PCR analysis.

Cell Culture and Transfection

Normal human breast cell line HCC1937 and breast cancer cell lines LCC9, MDA-MB-231, LCC2 and MCF-7 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All of the cells were cultured in the RPMI 1640 medium (Life Technologies, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA) in a 5% CO2 container at 37°C. Cell transfection was conducted with lipofectamine 2000 reagent according to the manufacturer’s protocol.

Real-time qPCR

Total RNAs from tissues and cultured cells were extracted by TriZol reagent, which was purchased from TaKaRa (Tokyo, Japan). RNA quantification was performed by collecting the absorbance at 260 nm and 280 nm. cDNAs were reversely transcribed from RNAs using a Reverse Transcription Kit (TaKaRa, Tokyo, Japan). RT-PCR analysis was performed with the SybrGreen reagent in the ABI 7900 machine (ABI Biotechnology, La Jolla, CA, USA). GAPDH was used as an internal control for all the tissues and cultured cells. The primers were synthesized by Quanshijin (Co., Wuhan, Hubei, China). All operations in experiments above were conducted three times.

Cell Proliferation Assay

Cell proliferation was assessed by the CCK-8 assay. Briefly, LCC2 and MCF-7 cells were cultured in 96-well plates (3000 cells/well) and transfected with LncRNA NC or LncRNA LINC00628 for 48h. Afterwards, 10 μL CCK-8 solution (Dojindo Laboratories, Shanghai, China) was added to each well and then cells were incubated for additional 10 min before proceeding to detection of absorbance at 405 nm. Air bubbles were strictly avoided during the process. Each treatment was repeated for at least three times.

Cell Invasion and Migration Assays

3 × 10^4 cells/ml of LCC2 and MCF-7 cells were respectively prepared after being transfected with LncRNA NC or LncRNA LINC00628 for 48h. Transwell insert chambers were used to determine assays of cell migration and invasion. 4% polyoxymethylene was applied to fix cells, and 0.2% crystal violet was used as a stain. Inverted microscope (Olympus, Tokyo, Japan) was used to image and count the cells.
Cell Cycle Analysis and Apoptosis Analysis

Each group of LCC2 and MCF-7 cells was seeded into six-well plates at a concentration of 3×10⁵ cells/well after being transfected with LncRNA NC or LncRNA LINC00628 for 48h. Afterward, cells were collected by low-speed centrifugation (1000 rpm, 5 min) at 4°C and cell pellets were re-suspended in 1 ml of PBS solution, fixed with 75% of ice-cold ethanol and stored at -20°C for two days. Prior to flowcytometry (FCM, BD Biosciences, Franklin Lakes, NJ, USA) analysis, cells were lysed, centrifuged and re-suspended in propidium iodide (PI, JingMei Biotech, Beijing, China) staining buffer containing 50 μl/ml of PI and 250 μl/ml of RNase A. Finally, the cell mixture was incubated at 4°C for 30 min in the dark environment to detect cell cycle and stained with 5 μl of annexin V-FITC (JingMei Biotech, Beijing, China) to detect apoptosis by fluorescence activated cell sorting (FACS) technique (BD Biosciences, Franklin Lakes, NJ, USA). The flowcytometry assay was repeated three times.

Western Blot Assay

Proteins from cells were lysed in a lysis buffer containing 10 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% deoxycholic acid. Then, 30 μg of protein samples were subjected to sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). After blocked with 5% skim milk, antibodies against Bcl-2, Bax, Caspase-3 or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied to probe the membranes. After all those procedure, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies.

Statistical Analysis

Data were presented as mean ± standard deviation (SD). SPSS 21.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Two-tail Student’s t-test was applied to test the results. Kaplan-Meier method and log rank test were applied to determine patient survival and their differences. Any value that \( p < 0.05 \) was considered statistically significant.

Results

The Expression Level of LINC00628 in Breast Cancer Tissues

The relative expression level of LINC00628 in 60 breast cancer tissues of patients and adjacent non-cancerous tissues were detected by qRT-PCR. We found the expression level of LINC00628 in tumor tissues was significantly decreased compared with the adjacent non-cancerous tissues (Figure 1A). Additionally, 38 breast cancer patients with lymph node metastasis and 22 breast cancer patients without lymph node metastasis were classified on the basis of clinical stage. The expression level of LINC00628 in breast cancer tissues of patients with lymph node metastasis was significantly lower than that in those without lymph node metastasis (Figure 1B).

Figure 1. The relative expression level of LINC00628 in breast cancer tissues. (A) The relative expression level of LINC00628 in breast cancer tissues and adjacent non-cancerous tissues were detected by qRT-PCR. (B) The relative expression level of LINC00628 in primary breast cancer without or with metastasis. \( p < 0.05 \).
The Expression Level of LINC00628 in Breast Cancer Cell Lines and the Prognosis in Breast Cancer According to the Expression Level of LINC00628

HCC1937 cells were derived from healthy breast tissue and were included here as controls. The expression level of LINC00628 in all of the four breast cancer cell lines, including LCC9, MDA-MB-231, LCC2 and MCF-7, were detected by qRT-PCR. The expression level of LINC00628 in all breast cancer cell lines was notably decreased compared with that in HCC1937 cells (Figure 2A). LCC2 and MCF-7 showed the lowest expression level of LINC00628 and were used for the subsequent analysis. Moreover, the correlations between the prognosis of the patient with breast cancer and the different expression level of LINC00628 were analyzed. Kaplan-Meier method and log rank test were used, which showed the lower expression of LINC00628 led to a significantly shorter overall survival of patients in comparison with those who with higher LINC00628 expression (Figure 2B).

The Overexpression of LINC00628 Suppressed Breast Cancer Cells Invasion and Migration

The further experiments were aimed at confirming the inhibitory function of LINC00628 in breast cancer. The invasion and migration capacity were detected after LCC2 and MCF-7 cells transfected with LINC00628. The results demonstrated that LINC00628 has inhibitory effects on the invasion (Figure 4A) and migration (Figure 4B) of LCC2 and MCF-7 cells. These results indicated that the mobility of breast cancer cells was suppressed by ectopic expression of LINC00628.

The Overexpression of LINC00628 Caused Cell Cycle Arrested in G0/G1 Phase Breast Cancer Cells and Promoted Cell Apoptosis

To explore the functional mechanism of LINC00628 in breast cancer, the cell cycle distribution and cell apoptosis rate in LCC2 and MCF-7 cells, after transfection with LINC00628, were examined by flowcytometry. When LCC2 and MCF-7 cells were transfected with LINC00628, cell cycle was prominently shifted from S phase and G2/M phase to G0/G1 phase. Cell percentage...
in G0/G1 phase was significantly increased while cell percentage in S phase was significantly decreased and cell apoptosis was promoted (Figure 5A-B). These data revealed that overexpression of LINC00628 arrested cell cycle in G0/G1 phase to induce cell apoptosis.

The Overexpression of LINC00628 on the Effects of Bcl-2/Bax/Caspase-3 Signal Pathway

The Bcl-2 and Caspase family play an important role in the regulation of cell apoptosis. In order to characterize the mechanism of LINC00628, which induced breast cancer cell apoptosis, the protein level of Bcl-2, Bax and Caspase-3 were detected by Western blot. The results showed that the relative expression of Caspase-3 and Bax protein was significantly increased while the relative expression of Bcl-2 protein was significantly decreased in LCC2 and MCF-7 cells after transfection with LINC00628 (Figure 6A-B). These data revealed that overexpression of LINC00628 promoted cell apoptosis, and it is associated with regulation of Bcl-2/Bax/Caspase-3 signal pathway.

Discussion

In this study, the expression level of LINC00628 was significantly decreased in LCC9, MDA-MB-231, LCC2 and MCF-7 cells compared with normal human breast cell line HCC1937. LINC00628 expression level was also significantly lowered in breast cancer tissues in comparison with the adjacent non-cancerous tissues and patients with a lower expression level of LINC00628 had a poorer prognosis and lower overall survival rate, which indicated lowered expression of LINC00628 could promote the development and metastasis in breast cancer patients. Meanwhile, the cell proliferation, invasion and migration in LCC2 and MCF-7 cells were inhibited by the
overexpression of LINC00628. The further experiments were used to confirm the inhibitory function of LINC00628 in breast cancer. With the overexpression of LINC00628 cell cycle was arrested in G0/G1 phase in breast cancer cells, and cell apoptosis was promoted. The relative expression of Caspase-3 and Bax protein was significantly increased while the relative expression of Bcl-2 protein was significantly decreased after transfection with LINC00628. Together, LINC00628 might serve as a tumor suppressor in breast cancer and provide a novel target for the diagnosis and treatment.

More evidence reveals that the LncRNAs play a crucial role in regulating genes in cancer development, progression, and metastasis. Decades of studies have found that proliferation, apoptosis, metastasis, and invasion are tightly associated with abnormal expression of LncRNAs in human cancer cells, including breast cancer. LncRNA UCA1 was shown to confer tamoxifen resistance to breast cancer. LncRNA UCA1 suppressed breast cancer growth and invasion by regulating SATB1. Various LncRNAs have been demonstrated to play significant roles in breast cancer. LncRNA LINC00628 is a newly discovered LncRNA and its role in human gastric cancer was shown in previous literature. LINC00628 negatively regulated its neighboring gene LRRN2 expression; however, its inhibitory effects on gastric cancer progression mainly depended on regulating cell cycle-related genes. In this work, the expression of LINC00628 was confirmed to be lower in tumor tissues. Overexpres-

![Figure 4](image_url)

Figure 4. The overexpression of LINC00628 suppressed breast cancer cells invasion and migration. (A) The invasion assays were performed in LCC2 and MCF-7 cells after transfection with LINC00628. (B) The migration assays were performed in LCC2 and MCF-7 cells after transfection with LINC00628. *p<0.05.

![Figure 5](image_url)

Figure 5. The overexpression of LINC00628 caused cell cycle arrested in G0/G1 phase breast cancer cells and promoted cell apoptosis. (A) The cell cycle distribution and cell apoptosis rate were explored in LCC2 cells after transfection with LINC00628. (B) The cell cycle distribution and cell apoptosis rate were explored in MCF-7 cells after transfection with LINC00628. *p<0.05.
LncRNA LINC00628 and breast cancer

The overexpression of LINC00628 inhibited cell proliferation, invasion, and migration. The results indicated that LINC00628 can affect the growth and mobility of breast cancer to some degree.

Apoptosis in multicellular organisms is one of the most effective ways to eliminate the harmful or unnecessary cells, the abnormal regulations of which might be associated with tumorigenesis. Apoptosis can be initiated mainly by two pathways: intrinsic pathway where cells suicide upon stress stimulation and extrinsic pathway, in which cells kill themselves due to signals from other cells. The Bcl-2 family (Bcl-2, Bax) and Caspase family play important roles in the regulation of cell apoptosis. Bcl-2 inhibits cell apoptosis and prevents Bax gene expression. Bax is an essential protein to determine cell apoptosis. The proapoptotic mechanism of Bax is performed by promoting the release of cytochrome c, which can activate caspase-3 and form a dipolymer with bcl-2 in the mitochondrial apoptosis signaling pathway. Meanwhile, researches showed that the changes of Bax/Bcl-2 ratio are important to regulate the activity of Caspase proteins and promote apoptosis. In this study, the overexpression of LINC00628 caused cell cycle to be arrested in G0/G1 phase breast cancer cells and promoted cell apoptosis. The results showed the relative expression of Caspase-3 and Bax protein were significantly increased while the relative expression of Bcl-2 protein was significantly decreased in LCC2 and MCF-7 cells after transfection with LINC00628. These data revealed that overexpression of LINC00628 promoted cell apoptosis by modulating Bcl-2/Bax/Caspase-3 signal pathway.

**Conclusions**

Our study showed the inhibitory effects of LINC00628 in breast cancer progression by regulating cell proliferation, invasion, migration, cell cycle, and cell apoptosis. Meanwhile, we demonstrated that LINC00628 serve as a tumor suppressor in human breast cancer associated with regulation of Bcl-2/Bax/Caspase-3 signal pathway.
Conflict of interest
The authors declare no conflicts of interest.

References


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