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# Long non-coding RNA LINC00665 promotes metastasis of breast cancer cells by triggering EMT

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**Abstract. – OBJECTIVE: In recent years, studies have found that abnormally expressed long non-coding RNA (lncRNA) is closely associated with the progression of breast cancer (BCa). Some lncRNAs have been identified to be involved in the regulation of BCa cell invasion and metastasis. In this research, LINC00665 was found to be abnormally expressed in BCa tissues and cells, and its regulatory effects on invasion and metastasis of BCa were explored. The aim of this study was to investigate the expression characteristics of LINC00665 in BCa, and its regulatory role in BCa progression.**

**PATIENTS AND METHODS: LINC00665 expressions in BCa tissue samples and normal ones were collected from GEPIA database. The expression of LINC00665 in BCa tissues and cell lines was further confirmed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). After knockdown of LINC00665 expression in BCa cells by transfection of small interfering RNA, cell migration and invasion abilities were examined by cell wound healing and transwell assay. Expressions of epithelial-mesenchymal transition (EMT)-related markers in BCa cells were examined using qRT-PCR and Western blot.**

**RESULTS: LINC00665 was highly expressed in BCa tissues and cell lines, and the high expression of LINC00665 could be used to predict a poor prognosis of BCa patients. In addition, the results of *in vitro* cell experiments showed that the migration and invasion ability of BCa cells were remarkably attenuated after downregulation of LINC00665. At the same time, qRT-PCR and Western blot results revealed that downregulation of LINC00665 was able to inhibit the expressions of EMT-related genes in BCa cells.**

**CONCLUSIONS: LINC00665 is highly expressed in BCa tissues and cell lines, which could predict poor prognosis of BCa patients. In addition, LINC00665 may promote the malignant metastasis of BCa cells by affecting the EMT process.**

*Key Words:*
BCa, LINC00665, Cell migration, Cell invasion, EMT.

## Introduction

As one of the most common malignant tumors, breast cancer (BCa) accounts for one third of the early diagnosed cancer in women, which is the second fatal cancer in the world[1,2]. In the diagnosis of BCa cases, about 6.6% of the women are under 40 years old, 2.4% are under 35 years old, and 0.65% are under 30 years old[3]. Currently, the treatment of BCa includes surgery, chemotherapy, radiotherapy, endocrine therapy, and targeted therapy, but sometimes the therapeutic effect is not ideal. Long-term continuous chemoradiotherapy will cause certain toxic side effects and induce drug resistance, while the efficacy of killing cancer cells is poor[4,5]. Therefore, it is urgent to explore new effective targets for the prediction and treatment of BCa.

Long non-coding RNA (lncRNA) is an independent transcription RNA that does not encode proteins, with a minimum length of 200 nucleotides[5]. Functionally, lncRNAs exert an important regulatory role in cell proliferation, differentiation, senescence, death, and the occurrence and development of tumor through regulating various factors and pathways[6]. At present, accumulating evidence[7] has shown the significant functions of lncRNAs in every aspect of tumor behaviors. According to their roles and expression patterns in tumor tissues, lncRNAs can be divided into oncogenes or tumor-suppressor genes. Many lncRNAs are differentially expressed in BCa tissues, which regulate malignant progression of BCa through targeting downstream genes and thus their biological functions[8]. So, H19 is the earliest-discov-

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ered tumor-related lncRNA. H19 is upregulated in primary and metastatic BCa, and plays an important role in the occurrence and development of BCa[9]. LOC554202 level is associated with stage and tumor size of advanced cases, and *in vitro* knockout of LOC554202 reduces proliferation and invasion. Meanwhile, *in vivo* silence of LOC554202 inhibits tumorigenesis[10]. However, the function and mechanism of LINC00665 in BCa are not yet clear.

The primary purpose of this study was to investigate the expression characteristics of LINC00665 in BCa tissues as well as in cell lines, and to explore its underlying mechanism in the progression of BCa.

## Patients and Methods

### GEPIA Database

Gene Expression Profiling Interactive Analysis (GEPIA) database (http://GEPIA.cancer-pku.cn/index.html) was utilized to assess the expression levels of LINC00665 in BCa tissues and prognostic information in BCa patients. The GEPIA online database was used to analyze gene sequencing data from the cancer genome atlas (TCGA) and Genotype-Tissue Expression (GTEx). As a public database, GEPIA contains 9736 tumor samples and 857 normal tissue samples. The database can be used to analyze differentially expressed genes in tumors and survival in cancer diseases. Data profiling of GEPIA in the TCGA database contains 33 types of tumors, involving data of tumor tissues and normal tissues, and corresponding clinical data.

### Sample Collection

A total of 60 pairs of triple-negative BCa tissues and corresponding paracancerous tissue samples were collected from patients who underwent BCa surgery in our hospital. All samples were confirmed by pathology as triple-negative BCa tissue or normal breast tissue. This investigation was approved by the Ethics Committee of our hospital and had obtained written informed consent from each patient. This experimental ethic was consistent with the Helsinki Declaration.

### Cell Culture

Human normal breast cell line (MCF-10A) and BCa cell lines (MCF-7, MDA-MB-231, ZR-75-30, and MDA-MB-415) were purchased from Shanghai Biocellular Research Institute (Shanghai, China). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen; Carlsbad, CA, USA) at 37°C, in a 5% $CO_2$ incubator.

### Cell Transfection

MDA-MB-231 and ZR-75-30 cells with good growth status were transfected with LINC00665 small interfering RNA (si-Linc00511) purchased from Riobobio (Guangzhou, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the instructions. Transfection efficiency was detected by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) after 24-48 h.

### RNA Extraction and qRT-PCR

According to the instructions, total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After extraction with chloroform, the aqueous phase was transferred to a new tube, precipitated by isopropanol, and the RNA precipitate was washed with 75% ethanol. After dried at room temperature, diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China) was added, and the RNA was stored at –80°C until use. RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Code No. RR036A, Otsu, Shiga, Japan). QRT-PCR was performed using SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan) according to the instructions. GAPDH and U6 were used as internal parameters, respectively, and the relative expressions of genes were calculated according to the $2^{-\Delta\Delta ct}$ method. The primer sequences were: LINC00665 Forward: 5'-TAAGCAGAGGGCTACAGAA-3', Reverse: 5'-CAGGGAGATCAGGAGGAC-3'; GAPDH Forward: 5'-TGCACCACCAACTGCTTAGC-3', Reverse: 5'-GGCATGGACTGTGGTCATGAG-3'.

### Cell Wound Healing Assay

BCa cells were seeded in 6-well plates and the density of the plated cells was determined (the majority of the number of cells plated was set to 50000 cells/well), and the confluency of the cells reached 90% or more the next day. After an artificial wound made on cells, they were cultured in medium containing 1% FBS, and rinsed gently

with phosphate-buffered saline (PBS) for 2-3 times. Wound healing was observed and photographed 24 h later.

### Cell Migration Assay

Cell migration ability of MDA-MB-231 and ZR-75-30 was tested by transwell assay 24 h after transfection. The cell suspension was first diluted with serum-free medium to a concentration of approximately $3\times10^4$ cells/ml. 100 μL of cells were seeded in a transwell chamber (Corning, Corning, NY, USA), and 600 μL of complete medium with 20% FBS (Gibco; Grand Island, NY, USA) was added to the bottom chamber. The cells were then incubated for 24 h at 37°C with 5% $CO_2$. Finally, the transwell chamber was collected and penetrating cells were stained with a 0.5% crystal violet dye for 20 min at room temperature. After PBS washing, migratory cells were photographed and observed using an optical microscope (Olympus, Tokyo, Japan).

### Cell Invasion Assay

Matrigel was diluted 1:8 in serum-free medium or PBS buffer at 4°C, then 100 mL of Matrigel was evenly applied to the surface of the polycarbonate membrane of the upper chamber, and placed at 37°C for 0.5-1 h. The cells in the logarithmic growth phase were suspended with serum-free medium at cell density to $1\text{-}10\times10^5$/mL. 500-650 μL of medium containing 5%-10% FBS or chemokine was added in the 24-well plate lower chamber. Then, the transwell chamber was placed in a 24-well plate with tweezers. 100-200 μL of the cell suspension was added into the upper chamber. Finally, cells were placed back in an incubator for 12-48 h. Subsequently, the transwell chamber was collected and invasive cells were observed and counted under the microscope.

### Western Blot

The total proteins were lysed with cell lysate containing protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Beyotime, Shanghai, China) and the total protein concentration was determined using a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). 50 μg of total protein was taken from each sample and separated in dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) gel for electrophoresis. Thereafter, the separated protein was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and the PVDF membranes were incubated with 5% skim milk-containing Tris-Buffered Saline Tween-20 (TBST). The primary antibodies were added to incubate the PVDF membranes overnight in a 4°C refrigerator. After washing the membrane three times with TBST (5 min/time), the secondary antibody was added to the membrane to incubate for 2 h at room temperature, and then the membrane was washed three times with TBST (10 min/time). The prepared enhanced chemiluminescence (ECL) solution was added to the immunoblot, and then the immunoblots were exposed in a chemiluminescent device. The experiment was repeated three times separately.

### Statistical Analysis

Data processing analysis was performed using GraphPad Prism 6 (La Jolla, CA, USA) software. All experimental results were subjected to three independent replicate experiments. Independent sample *t*-test was used to compare differences between the two groups. Survival analysis was performed using the Kaplan-Meier method and the significance was verified by the rank sum test. $p<0.05$ was considered to be statistically significant.

## Results

### LINC00665 Is Highly Expressed In BCa Tissues and Cells

GEPIA database showed that the expression of LINC00665 in BCa tissues was remarkably higher than that in normal control tissues (Figure 1A), suggesting that LINC00665 may play an important role in the progression of BCa. Subsequently, we further examined the expression of LINC00665 in BCa tissues by qRT-PCR. Consistent with the database results, we found that the expression of LINC00665 in BCa tissues was relatively higher than that in normal control tissues (Figure 1B). Further, we found that the expression level of LINC00665 was remarkably higher in BCa cell lines compared with normal control cells by qRT-PCR (Figure 1C), and the expression difference of MDA-MB-231 and ZR-75-30 cells was the most significant. Therefore, we chose these two cell lines for subsequent experiments.

### LINC00665 Is Associated With BCa Prognosis

To test whether LINC00665 was associated with the development of BCa, we grouped BCa patients by tumor stage and tumor metastasis.

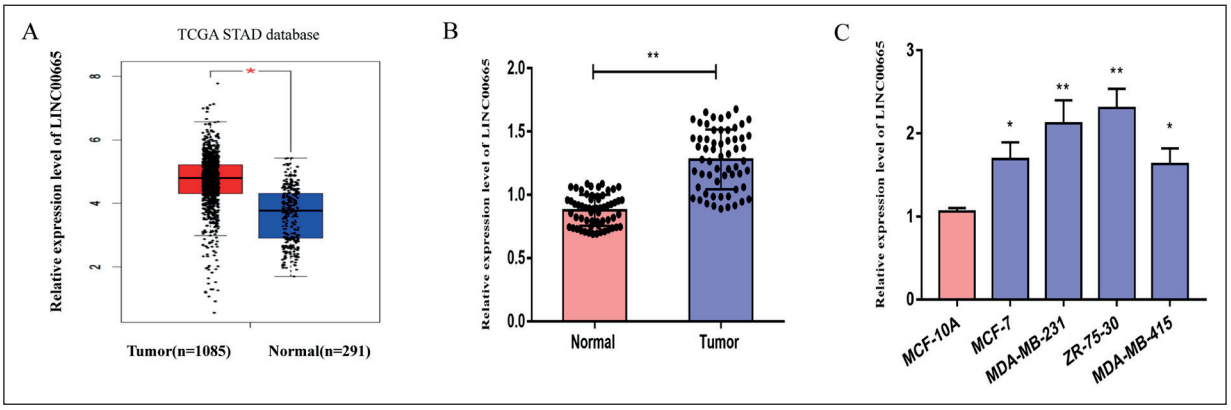


**Figure 1.** LINC00665 is highly expressed in breast cancer tissues and cells. **A,** GEPIA database showed that LINC00665 was abnormally highly expressed in breast cancer tissues. **B,** QRT-PCR showed that the expression of LINC00665 in breast cancer tissues was significantly higher than that in normal control tissues. **C,** QRT-PCR showed that the expression of LINC00665 in breast cancer cells was significantly higher than that in normal control cells. (*$p$<0.05, **$p$<0.01).

By analyzing the expression of LINC00665 in different groups, we found that the higher the expression of LINC00665 in BCa patients, the higher the tumor grade (Figure 2A). Similarly, the expression of LINC00665 in metastatic patients was also remarkably higher than that in the non-metastatic group (Figure 2B). Later, we divided BCa patients into LINC00665 high

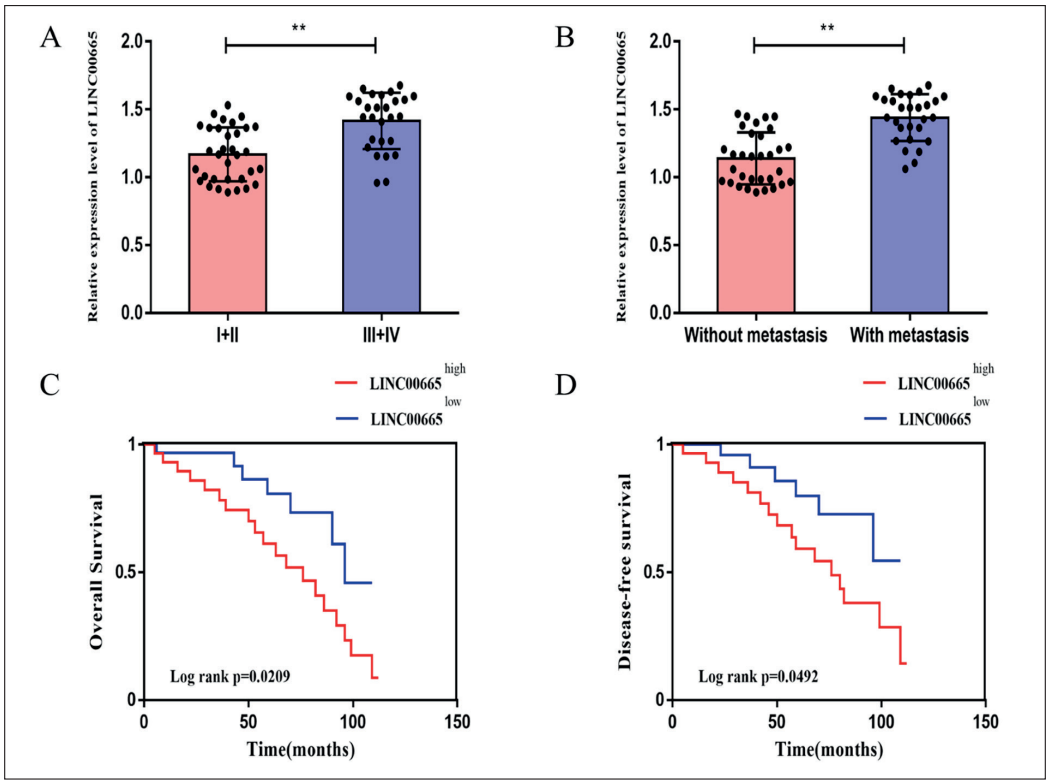


**Figure 2.** LINC00665 is associated with breast cancer prognosis. **A,** Expression of LINC00665 in stage III and IV tumor tissues was significantly higher than that in stage I and II tissues. **B,** Expression of LINC00665 in metastatic tumor tissues was significantly higher than that in non-metastatic tissues. **C,** Overall survival (OS) of breast cancer patients with high expression of LINC00665 was significantly lower than that of the low expression group. **D,** Disease-free survival (DFS) of breast cancer patients with high expression of LINC00665 was significantly lower than that of the low expression group. (**$p$<0.01).

expression group and LINC00665 low expression group according to their median level of LINC00665. By analyzing the clinical data, we found that the overall survival rate and disease-free survival rate of BCa patients with high expression of LINC00665 were remarkably lower than those of low expression group (Figures 2C, 2D). These results indicated that LINC00665 can be used as a prognostic marker for BCa patients.

### *Inhibition of LINC00665 Inhibits Migration and Invasion of BCa Cells*

To determine the biological function of LINC00665 in BCa, the expression of LINC00665 in BCa cells was silenced by transfection of a small interfering RNA, and its transfection efficiency was tested by qRT-PCR (Figure 3A). Subsequently, we further tested the effect of LINC00665 on BCa cell metastasis by wound healing assay and transwell assay. We found that

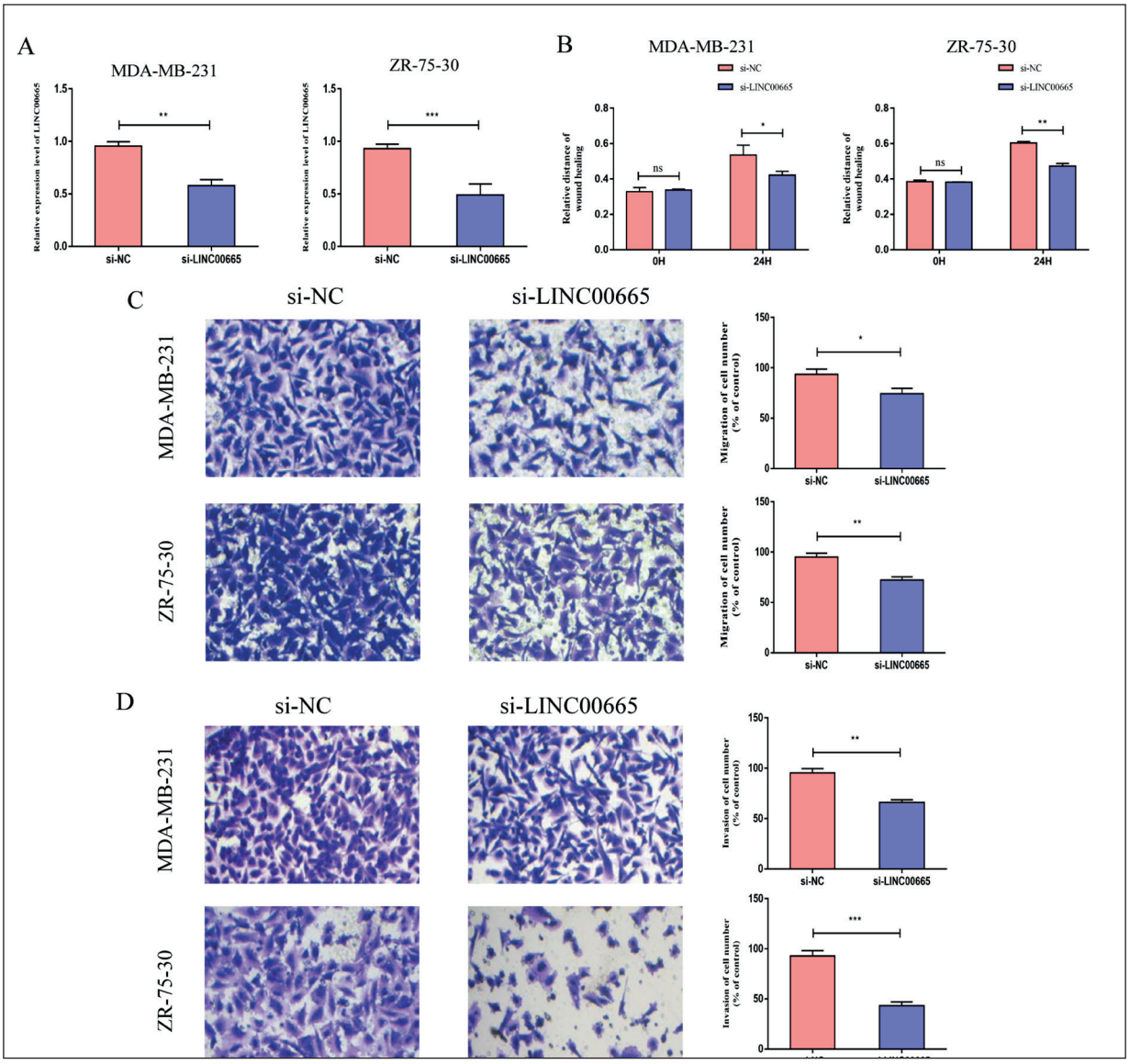


**Figure 3.** Knockdown of LINC00665 inhibits breast cancer cell migration and invasion. **A,** LINC00665 was silenced in MDA-MB-231 and ZR-75-30 cells by transfection of small interfering RNA and its efficacy was verified by qRT-PCR. **B-C,** Wound healing assay **(B)** and transwell assay **(C)** were performed to detect the effect of LINC00665 knockdown on the migration ability of breast cancer cells. **D,** Transwell invasion assay was applied to detect the effect of LINC00665 knockdown on the invasive ability of breast cancer cells. (ns, no significant difference, *$p$<0.05, **$p$<0.01, ***$p$<0.001) (Magnification: 20×).

the ability of BCa cells to heal wounds was remarkably reduced after silencing of LINC00665 in BCa cells compared with the normal control group (Figure 3B). Similarly, transwell results also demonstrated that the migration and invasion abilities of BCa cell lines MDA-MB-231 and ZR-75-30 with knockdown of LINC00665 were remarkably attenuated (Figure 3C, 3D). These results suggested that LINC00665 may contribute to the development of BCa by affecting the metastatic ability of BCa cells.

### *LINC00665 Can Affect the EMT Process*

Previous studies have shown that lncRNA can promote the invasion and metastasis of tumor cells by affecting epithelial-mesenchymal transition (EMT). Therefore, we speculated that LINC00665 may promote BCa cell metastasis by affecting the EMT process. We detected the mRNA expressions of EMT-related genes E-cadherin, N-cadherin, β-catenin, and Vimentin in BCa cells with low expression of LINC00665 by qRT-PCR. The results showed that the expressions of E-cadherin and β-catenin were remarkably elevated in BCa cells after knockdown of LINC00665, while the expressions of N-cadherin, and Vimentin were remarkably reduced (Figure 4A). Furthermore, we examined the effects of LINC00665 on the protein expressions of E-cadherin, N-cadherin, β-catenin, and Vimentin by Western blot, the results of which were found to be consistent with qRT-PCR results (Figure 4B), indicating that LINC00665 can promote EMT progression in BCa cells.

## Discussion

Although great progress has been made in the molecular mechanism of BCa occurrence, early diagnosis and treatment, BCa recurrence and metastasis still occur in 30% to 40% of patients within 5 years of onset. Invasion and metastasis are the main causes of recurrence and death of

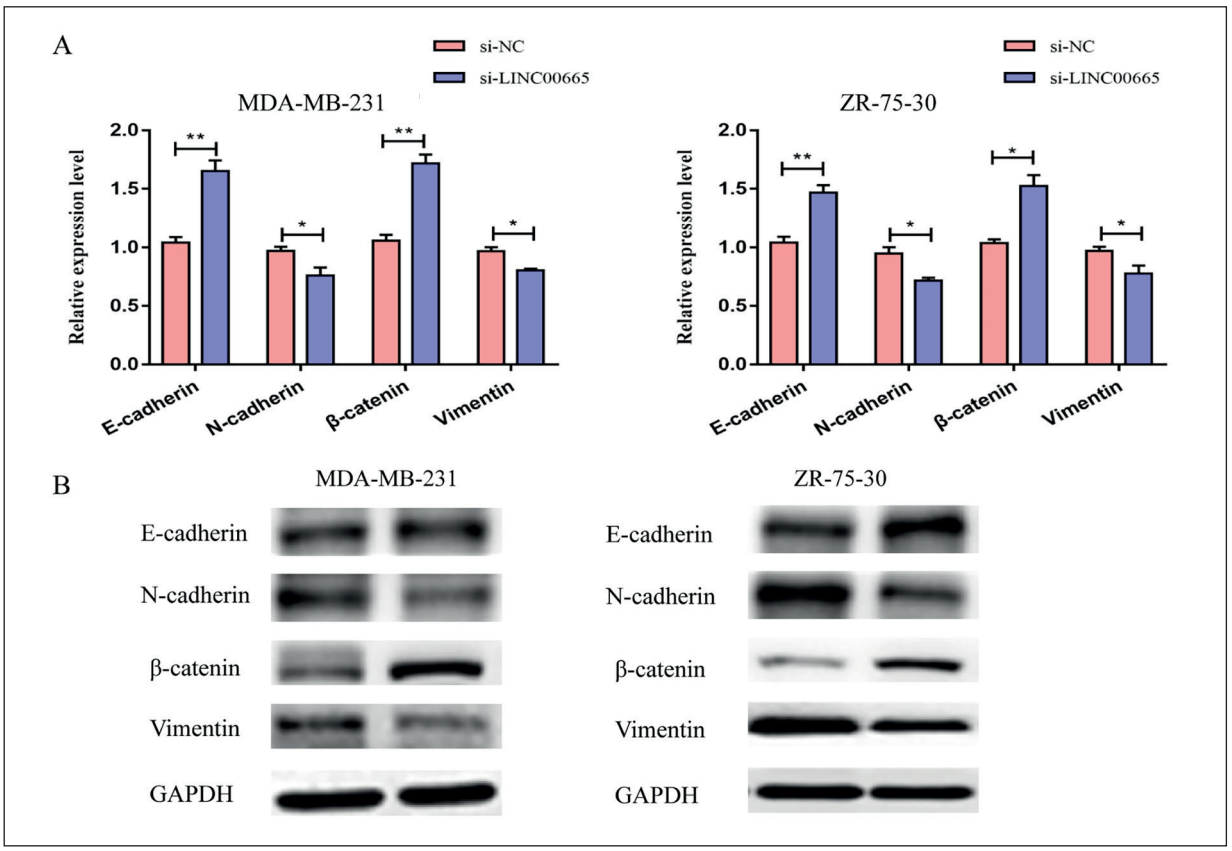


**Figure 4.** Knockdown of LINC00665 inhibits epithelial-mesenchymal transition. **A,** MRNA expressions of E-cadherin, N-cadherin, β-catenin, and Vimentin were detected by qRT-PCR after knockdown of LINC00665 in MDA-MB-231 and ZR-75-30 cells. **B,** Protein expressions of E-cadherin, N-cadherin, β-catenin, and Vimentin were detected by Western blot after the knockdown of LINC00665 in MDA-MB-231 and ZR-75-30 cells. (*$p$<0.05, **$p$<0.01).

BCa patients. Therefore, it is particularly important to study the molecular mechanism of invasion and metastasis of BCa, thus improving the prognosis.

Studies have found that protein-coding genes only account for 1.5% of total human genome. The majority is non-coding genes that can be transcribed into RNAs, that are, non-coding RNAs (ncRNAs)[11]. Among them, more than 8,000 lncRNAs have been found. Abnormally expressed lncRNAs are closely related to the occurrence and development of various malignant tumors, including BCa[12].

LncRNA expression is significantly different between various malignant tumors and normal tissues, suggesting the vital functions of lncRNAs in the occurrence and development of tumors[13]. In recent years, multiple lncRNAs have been found to be differentially expressed in breast tissues, and BCa tissues with different subtypes[14].

At present, qRT-PCR is a sensitive way to rapidly and accurately determine lncRNAs. The first oncogenic lncRNA H19 was found in chromosome llpl5.5, with a transcript length of about 2300bp[15]. It is expressed mainly in human embryonic cells, which is activated during tissue regeneration or tumorigenesis after tissue damage, suggesting that H19 is an oncogenic gene. H19 has been shown to be involved in various processes of tumor cells, especially in BCa, which can affect the proliferation, differentiation and invasion of BCa cells by the regulation of hepatocyte growth factor (HGF), transcription factor (E2F1) and ubiquitin ligase E3 family (c-cbl, cbl-b)[16,17]. In recent years, studies have found that PVT1, SOX2OT, ROR[18-20] are upregulated in BCa cells, and their high expressions are closely related to the incidence of BCa. Maternally expressed gene3 (MEG3) is the first lncRNA found to present tumor suppressive properties. It is located on the human chromosome 14q32. It belongs to an imprinted gene of DLK1-MEG3 gene and contains 12 subtypes of genes. MEG3 is expressed in various normal tissues of human testicles, ovaries, pancreas, spleen, and breast[21]. In recent years, studies[22,23] have found that LINC00628m1, LIMT, and other lncRNAs are downregulated in BCa cells, affecting the occurrence and development of BCa, and may become new molecular markers.

Epithelial cells play secretory and supportive roles in the body. In general, cell polarity and adhesion between cells limit the ability of epithelial cells to migrate arbitrarily. However, under specific physiological and pathological conditions, epithelial cells can transform into mesenchymal cells, leading to loss of cell polarity, decreased adhesion ability and changes in cytoskeleton, thus enhancing the ability of cell migration and movement. This process is called epithelial-mesenchymal transition (EMT). EMT is a complex, multi-pathway process that plays an important role in tumor metastasis and can even lead to drug resistance of tumor cell homolog[24,25]. There are many kinds of EMT markers. E-cadherin is one of the $Ca^{2+}$-dependent intercellular adhesion molecule in epithelial tissues. It can maintain cell adhesion and polarity. In addition, Vimentin is a mesenchymal cell marker molecule. E-cadherin and Vimentin are important characteristics of EMT[26].

By analyzing the dataset, LINC00665 was found to be highly expressed in BCa patients, and its expression was associated with poor prognosis of the tumor. Subsequently, we further verified its role in the process of BCa. As a potential oncogene, LINC00665 expression was closely correlated to TNM staging and lymph node metastasis in BCa patients. In addition, *in vitro* experiments confirmed that LINC00665 could promote proliferation and migration of BCa cells.

## Conclusions

In this study, we first discovered that LINC00665 is highly expressed in BCa tissues and cell lines. By analyzing clinical data, we found that high expression of LINC00665 can predict a poor prognosis of BCa patients. Further, we demonstrated that knockdown of LINC00665 remarkably inhibited proliferation and migration of BCa cells, and LINC00665 may exert its biological effects by affecting the EMT process of BCa cells.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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