Expression and Significance of LINC02418 in Breast Cancer

Yong-Hong Zhou, Jian-Yuan Huang

Department of General Surgery (Thyroid Gland/Blood Vessel), The First People's Hospital of Neijiang, Neijiang, 641099, People's Republic of China

Correspondence: Jian-Yuan Huang, Department of General Surgery (Thyroid Gland/Blood Vessel), The First People's Hospital of Neijiang, No. 1866, West Section of Han'an Avenue, Neijiang, 641099, People's Republic of China, Tel +86-832-2082932, Email huangjianyuan7388@163.com

Purpose: The complicated pathogenesis and poor prognosis of breast cancer have become a major difficulty in medical research. This study aims to explore new lncRNA as prognostic markers for breast cancer and explore their roles and molecular mechanisms to lay a foundation for the treatment of cancer patients.

Patients and Methods: The expression of LINC02418 and miR-766-5p in breast cancer tissues and cells was first identified using polymerase chain reaction, and Pearson was used to examine the correlation between the two. The cancer cells activities under different transfection conditions were detected using the Transwell assay and CCK8 assay. The correlation between LINC02418 and patient prognosis was analyzed using multifactor Cox regression and Kaplan-Meier.

Results: It was shown that LINC02418 expression was upregulated in breast cancer tissues and cells. There are significant differences in lymph node metastasis and TNM stage between high and low LINC02418 expression groups. The higher the expression of LINC02418, the higher the mortality rate of breast cancer patients. miR-766-5p expression was downregulated and negatively correlated with LINC02418. There are binding sites between LINC02418 and miR-766-5p; Transfection with miR-766-5p inhibitor boosted LINC02418 luciferase activity, but transfection with miR-766-5p mimic decreased it. Knockdown of LINC02418 promoted miR-766-5p expression and inhibited cancer progression, which was alleviated to some extent by transfection with miR-766-5p inhibitors.

Conclusion: LINC02418 has the potential to serve as a poor prognostic marker for breast cancer and plays a pro-oncogenic role by targeting miR-766-5p.

Keywords: LINC02418, miR-766-5p, breast cancer, prognosis, cellular processes

Introduction
Breast cancer poses a serious threat to women’s lives and health. With the change in lifestyle and dietary habits as well as the fertility rate, the incidence of breast cancer has been rising year by year, which has become a hot issue today. Breast cancer is a complex and heterogeneous disease. With the application of targeted therapy technology, the clinical efficacy of breast cancer patients has been greatly improved. However, there is still a lack of highly specific targeting markers. Therefore, actively exploring potential molecular targets for the prevention and treatment of breast cancer and improving patient prognosis are the key topics of breast cancer research today. The malignant progression of breast cancer is a complex process involving multi-factor changes, multi-gene regulation and multiple signaling pathways. In-depth exploration of the regulatory mechanism of malignant progression of breast cancer is expected to make the clinical treatment of breast cancer patients more accurate and personalized.

Long non-coding RNA (lncRNA) is a class of non-coding RNA with a length greater than 200nt, which plays an important role in histone modification, transcriptional regulation, post-transcriptional regulation, and other biological processes, and can participate in other IncRNAs, miRNAs, mRNAs, and proteins as signaling molecules, Bridges, and decoys. In the process of tumor pathogenesis, lncRNA can participate in tumor growth, migration, drug resistance and other processes by regulating genes and signaling pathways closely related to cancer or regulating its target miRNA. So far, many studies have revealed the functional characteristics of various lncRNAs in breast cancer, which can participate in the process of breast cancer invasion...
and drug resistance by regulating miRNA. For example, Kong et al. found that lncRNA-CDC6 was significantly over-expressed in breast cancer tissues and regulated the expression of CDC6 through direct adsorption of miR-215. Chemotherapy resistance in breast cancer is significantly influenced by lncRNA CBR3-AS1. In summary, there are many dysregulated lncRNAs expressed in breast cancer, which are largely involved in intracellular processes and tumor progression in breast cancer by regulating the corresponding target miRNAs. Therefore, identifying new genes associated with breast cancer and their mechanisms of action will help provide new perspectives on the prognosis and treatment of the disease.

When exploring the immune-related regulatory network of breast cancer, it has been discovered that a novel lncRNA, LINC02418, which is aberrantly expressed in breast cancer and has the potential to be a prognostic marker for breast cancer, has been identified. In colorectal cancer, it was discovered that LINC02418 significantly contributes as a critical gene in the screening of chemotherapy-related ceRNA networks. LINC02418 was found to be abnormally expressed in endometrial cancer and has the potential to be a biomarker for patient prognosis. Recent studies have found that LINC02418 can affect the expression of PD-L1 in non-small cell lung cancer and participate in CD8+ T cell infiltration. In colorectal cancer and lung cancer, LINC02418 has been found to act as a molecular sponge of miRNAs, exerting the role of endogenous competitive RNA (ceRNA) to regulate the expression of downstream target genes, which affects the development of cancer. Therefore, it is speculated that LINC02418 plays an important role as a ceRNA of miRNA in breast cancer.

We chose LINC02418 as the research target to explore the potential biomarkers for treatment and prognosis of breast cancer patients.

**Materials and Methods**

**Clinical Specimens**

This study is retrospective. From 2016 to 2018, there were 129 breast cancer patients in The First People’s Hospital of Neijiang who met the following criteria.

**Inclusion criteria:**

1. Adult female patients with pathological diagnosis of breast cancer.
2. No history of malignant tumors or chronic diseases.
3. Have not received chemotherapy, radiotherapy, targeted therapy, endocrine therapy, and other anti-tumor therapy before diagnosis.

**Exclusion criteria:**

1. Patients with breast cancer combined with other tumors.
2. Pregnant women.
3. Cognitive or communication disorders.
5. Incomplete medical records.

The resected breast cancer tissues and corresponding normal para-cancer tissue samples of 129 subjects were collected and rapidly frozen with liquid nitrogen and stored at −80°C. All samples were diagnosed as breast cancer by two professional pathologists. All subjects gave their informed consent, and our study was authorized by The First People’s Hospital of Neijiang Ethics Committee (number 201,589). The study complies with the declaration of Helsinki.

**Cell Culture**

Normal breast epithelial cell MCF-10A and breast cancer cell lines SK-BR-3, MCF-7, MDA-MB-231, and MDA-MB-468 were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. For the experiment, the cells
were first thawed and then re-suspended in DMEM medium containing 1% penicillin-streptomycin solution +10% fetal bovine serum. The cells were transferred to culture dishes and observed at 37°C and 5%CO₂.

**Cell Transfection**
The cells with logarithmic growth and good state were selected as transfection objects. Transfection complexes were obtained by mixing Lipofectamine 2000 (Invitrogen, USA) with knockdown LINC02418 fragment (si-LINC02418), negative control fragment (si-NC), miR-766-5p inhibitor (miR-inhibitor), miR-766-5p mimic (miR-mimic), miRNA negative control fragment (miR-NC), si-LINC02418 with miR-inhibitor (si+miR-inhibitor), and its negative control (si+miR-NC), respectively (the sequences was shown in Table S1). During transfection, the cell suspension and transfection complexes were added to the pore plate and thoroughly mixed. The culture was continued for 48h in the same environment for subsequent experiments.

**Dual-Luciferase Reporter Assay**
The LINC02418 sequence containing miR-766-5p binding sites and the LINC02418 sequence containing mutation sites were called WT-LINC02418 and MT-LINC02418, respectively, which were cloned into pGL3 luciferase vector, and the recombinant vector was constructed. Then the above vectors were co-transfected with miR-NC, miR-mimic, and miR-inhibitor transfection complexes into breast cancer cells, respectively. After culturing for 48h, the activity of LINC02418 luciferase in transfected cells was detected.

**Qrt-Pcr**
To detect the expression levels of LINC02418 and miR-766-5p, it is necessary to extract total RNA first by Trizol method (Invitrogen, USA), and then it was reverse transcribed to generate cDNA by the PrimeScript RT Enzyme Mix I (TaKaRa, Japan) kit, which was amplified by the TB Green Premix Ex Taq II kit (TaKaRa, Japan). The relative expression of amplified sequences was calculated using the $2^{-ΔΔCt}$ method with GAPDH and U6 as internal standards for LINC02418 and miR-766-5p, respectively. Primer sequences are shown in Table S2.

**CCK8 Assay**
A logarithm of the growth cells was taken, resuspended, and counted, and inoculated into 96-well plates and placed in incubators for further culture. CCK8 reagent was added at 24, 48, 72 and 96 hours after culture, and then continued to incubate for 2 hours. The absorbance of each group of cells at a wavelength of 450nm (OD₄₅₀) was measured by enzyme marker (Thermofisher, USA).

**Transwell Assay**
For migration experiments, the cells after transfection were digested, and then resuspended in blank DEME medium. A certain amount of cell suspension was taken and inoculated in the upper chamber of the Transwell, and a DMEM medium containing serum was added to the lower chamber, avoiding air bubbles during the entire process. The invasion experiment needs to cover the upper chamber of Transwell with matrix gel, and other steps are the same as the migration experiment. Incubate in the incubator for 24h, then take out the small chamber for fixation and staining. Finally, after washing and drying, observe and count under the microscope.

**Statistical Analysis**
SPSS 26.0 and GraphPad Prism 7.0 software were used to analyze relevant data. T-tests and Chi-square tests were employed to examine group differences and the relationship between LINC02418 and clinicopathological variables, respectively. Kaplan-Meier and multivariate Cox regression analysis evaluated the prognostic ability of LINC02418 in patients with breast cancer. The relationship between LINC02418 and miR-766-5p was analyzed by Spearman correlation analysis. All experiments in this study were conducted independently and repeated three times, expressed as mean ± standard deviation. $P < 0.05$ was considered statistically significant.
Results

Expression and Clinical Significance of LINC02418 in Breast Cancer

LINC02418 expression was upregulated in cancer tissues (Figure 1a) and cancer cells (Figure 1b), compared to normal breast tissues and cells. Based on the average LINC02418 expression in breast cancer tissues, 129 patients were divided into two groups: 61 in the low LINC02418 group, and 68 in the high LINC02418 group. As shown in Table 1, there were significant differences in TNM stage ($p=0.003$) and lymph node metastasis ($p=0.007$) between patients with high or low expression of LINC02418. LINC02418 ($r=2.951$, $p=0.012$), lymph node metastasis ($r=0.417$, $p=0.015$), and TNM stage ($r=0.408$, $p=0.031$) can be used as independent factors for the prognosis of breast cancer patients (Figure 1c). Patients in the high-LINC02418 group had lower 5-year survival than the low-LINC02418 group (log-rank $p=0.045$, Figure 1d).

![Figure 1](image-url) LINC02418 expression was upregulated in breast cancer tissues than adjacent normal tissues (a). LINC02418 expression was upregulated in breast cancer cells than normal breast cells (MCF-10A) (b). Cox multifactorial analysis of independent factors affecting patient prognosis (c). LINC02418 high expression group with low survival at 5 years (d). ***$p<0.001$, compared with normal tissue and normal cells.
Interaction of LINC02418 with miR-766-5p in Breast Cancer

miR-766-5p expression was downregulated in breast cancer tissues (Figure 2a) and cancer cells (Figure 2b), compared to normal breast tissues and cells. The correlation between the two was analyzed and found to be negatively correlated in tumor tissues ($r=-0.720$, $p<0.001$, Figure 2c). According to the luciferase activity detection experiment, it was found that miR-766-5p mimics reduced the luciferase activity of WT-LINC02418, while miR-766-5p inhibitors could increase its activity, whereas transfection of miR-766-5p mimic and inhibitor had no effect on MT-LINC02418 luciferase activity (Figure 3a). In addition, the regulatory roles between LINC02418 and miR-766-5p were explored by inhibiting their expression. It was found that si-LINC02418 could effectively reduce LINC02418 expression, while inhibition of miR-766-5p did not affect LINC02418 expression (Figure 3b), and down-regulation of LINC02418 could promote the miR-766-5p expression (Figure 3c). These results indicate that LINC02418 can target miR-766-5p and negatively regulate its expression.

Effect of LINC02418/miR-766-5p on the Activity of Breast Cancer Cells

To investigate the effects of LINC02418/miR-766-5p on breast cancer cell activities including proliferation, migration, and invasion, CCK8 and Transwell experiments were performed, respectively. It was shown that the proliferation rate of both MCF-7 and MDA-MB-468 of breast cancer cells transfected with si-LINC02418 was significantly reduced.

| Table 1 The Association of LINC02418 with patients’ clinicopathological features |
|-----------------|-----------------|-----------------|-------|
| Variant         | Cases (n=129)   | LINC02418 expression | P     |
|                 |                 | Low (n=61) | High (n=68) |     |
| Menopause       |                 |            |            | 0.105|
| No              | 58              | 32         | 26         |     |
| Yes             | 71              | 29         | 42         |     |
| Age             |                 |            |            | 0.095|
| <50             | 64              | 35         | 29         |     |
| ≥50             | 65              | 26         | 39         |     |
| Smoking history |                 |            |            | 0.998|
| No              | 74              | 35         | 39         |     |
| Yes             | 55              | 26         | 29         |     |
| HER-2 status    |                 |            |            | 0.052|
| Negative        | 52              | 30         | 22         |     |
| Positive        | 77              | 31         | 46         |     |
| PR status       |                 |            |            | 0.612|
| Negative        | 77              | 35         | 42         |     |
| Positive        | 52              | 26         | 26         |     |
| ER status       |                 |            |            | 0.108|
| Negative        | 73              | 30         | 43         |     |
| Positive        | 56              | 31         | 25         |     |
| Tumor size      |                 |            |            | 0.956|
| <5              | 68              | 32         | 36         |     |
| ≥5              | 61              | 29         | 32         |     |
| Differentiation |                 |            |            | 0.654|
| Well-moderate   | 82              | 40         | 42         |     |
| Poor            | 47              | 21         | 26         |     |
| TNM stage       |                 |            |            | 0.003|
| I-II            | 82              | 47         | 35         |     |
| III             | 47              | 14         | 33         |     |
| Lymph node metastasis | | 84 | 47 | 37 | 0.007 |
| No              | 45              | 14         | 31         |     |
| Yes             | 45              | 14         | 31         |     |
compared to the control group, which was reversed by transfection miR-766-5p inhibitor (Figure 4a). Similarly, the migration (Figure 4b) and invasion (Figure 4c) of both breast cancer cells after transfection with si-LINC02418 and miR-766-5p inhibitor were consistent with the proliferation trend of cancer cells.

**Discussion**

The high prevalence rate of breast cancer has aroused women’s great attention.\(^{17,18}\) Breast cancer has a convoluted development mechanism in addition to a high incidence rate, bad prognosis, and major effects on women’s ability to lead healthy lives.\(^{19,20}\) With the deepening of lncRNA research, more and more lncRNAs have been found to play important roles in breast
Figure 3 miR-766-5p affects LINC02418 luciferase activity (a). si-LINC02418 effectively knocks down LINC02418 expression (b). Knockdown of LINC02418 reduces miR-766-5p expression (c). **p < 0.001, compared with control group. ##p < 0.01 compared with the si-LINC02418 group.

Abbreviation: NC: negative control. si: silent, knockdown of LINC02418 expression.
IncRNA UCA1 has been reported to upregulate protein tyrosine phosphatase PTP1B in breast cancer cells through adsorption of miR-206, thereby enhancing cell proliferation. LncRNA NEAT1 promotes the expression of ZEB1, a key transcription factor in EMT, through competitive adsorption of miR-448, which in turn accelerates breast cancer cells invasion and metastasis. lncRNA TINCR can target miR-125b, promote EMT metastasis of HER2+ breast cancer cells, and increase cancer cell resistance to trastuzumab, which is a potential molecular marker and therapeutic target for breast cancer.

It can be seen that there are many lncRNAs with dysregulated expression in breast cancer. Exploring the special roles and biological functions of these lncRNAs may provide new insights into the development of targeted therapies for breast cancer.

Figure 4: Knockdown of LINC02418 inhibited cellular undesirable biological behavior, which was reversed by low expression of miR-766-5p (a-c). **p<0.01, compared with control group; ##p<0.01 compared with the si-LINC02418 group.

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functions of new lncRNAs in breast cancer and investigating their regulatory relationships and molecular mechanisms can provide a theoretical basis for the clinical targeted therapy of breast cancer.

LncRNA has multiple classification systems, among which long intergenic ncRNA (LINCRNA) is located between two protein-coding genes and can be transcribed independently. Multiple LINCRNAs have been found to be involved in the development of breast cancer. LINC00665 was found to play a pro-cancer role by negatively regulating miR-3619-5p.26 The study found that LINC01094 is upregulated in breast cancer and can be used as a marker of poor prognosis.27 Similarly, the present study found that there were significant differences in lymph node metastasis and TNM stage between the high and low LINC02418 expression groups and that the mortality rate of breast cancer patients was higher in the high LINC02418 expression group. Therefore, it is speculated that LINC02418 has the potential to be a biomarker of disease deterioration and poor prognosis. LINC02418 is highly expressed in lung adenocarcinoma cells, and knocking down its expression can inhibit the activity of cancer cells. LINC02418 promotes the expression of kinetochore scaffold 1 (KNL1) by targeting miR-4677-3p, thus promoting the development of lung cancer cells.15 It is known that the LINC02418 expression is upregulated and LINC02418 affects the BCL2 signaling pathway by sponging miR-34b-5p, thereby enhancing the life activity of colon cancer tissues.16 Similarly, LINC02418 was upregulated in both tissues and cells of non-small cell lung cancer, while overexpression of LINC02418 was found to significantly enhance the proliferative and invasive activities and inhibit apoptosis in non-small cell lung cancer.28 Based on this, we conclude that LINC02418 may be involved in the occurrence and progression of breast cancer. Further research has been carried out on it. It was found that LINC02418 can promote the activities of breast cancer cells and play the role of oncogene.

By targeting miR-4677-3p, LINC02418 causes upregulation of SEC61G expression and promotes the activity of non-small cancer cells.28 LINC02418 indirectly regulates EPHA2 by targeting miR-372-3p, which lays an important foundation for reducing the drug resistance of colorectal cancer cells and promoting the treatment of colorectal cancer.12 However, the molecular regulatory mechanisms of lncRNAs in breast cancer remain unclear, so this study delves into the molecular regulation of key lncRNAs in breast cancer progression. The luciferase activity report indicates a targeted binding site between LINC02418 and miR-766-5p. In addition, LINC02418 was found to negatively regulate miR-766-5p expression. miR-766-5p inhibitors can reverse the inhibitory effect of LINC02418 low expression on biological behavior of cancer cells. In Triple-Negative Breast Cancer (TNBC), miR-766-5p was found to regulate cancer cell development by binding to yes-associated protein 1 (YAP1).29 In addition, miR-766-5p is involved in the occurrence and development of various cancers and plays a key role. For example, miR-766-5p is involved in the molecular mechanism of lung cancer development, by targeting the oncogene kallikin-releasing peptide-associated Peptidase 12 (KLK12), and CASC15 indirectly promotes the development of cancer by negatively regulating miR-766-5p.30 miR-766-5p plays an important role in epithelial-mesenchymal transformation of papillary thyroid cells, and LCC-MPEG1-1 promotes cancer development by sponging miR-766-5p.31 Therefore, it is speculated that LINC02418 promotes the proliferative and metastatic activities of breast cancer cells by negatively regulating miR-766-5p. This conclusion will be further verified in subsequent in vivo animal experiments.

Breast cancer consists of three subtypes: ER+ Luminal, ERBB2+, and ER+ Luminal and ER-/PR-/ERBB2- triple-negative breast cancer (TNBC). The development and prognosis of patients with different subtypes of breast cancer have certain differences, so it is speculated that the expression and clinical significance of LINC02418 in different subtypes of breast cancer are different. This study did not explore the role of LINC02418 in different subtypes, which will be further deepened in future studies with the aim of advancing the use of LINC02418 in clinical practice.

**Conclusion**

In summary, LINC02418 expression is up-regulated in breast cancer and has the potential to be a biomarker of poor prognosis in breast cancer. LINC02418 may be involved in cancer cell proliferation and metastasis through negative regulation of miR-766-5p.

**Disclosure**

The authors report no conflicts of interest in this work.
References


