**IncRNA NEAT1 is closely related with progression of breast cancer via promoting proliferation and EMT**

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**Abstract.** – **OBJECTIVE:** Breast cancer (BC) is one of the most common cancers in females. Abnormal proliferation and metastasis are key reasons that cause mortality in BC patients. More and more evidence showed that lncRNA played an important role in the BC development, but the mechanism was not well established.

**PATIENTS AND METHODS:** The expression of IncRNA NEAT1 was detected in 40 BC patients by qRT-PCR technology. MTT and Wound Healing assays were applied to detect the effect of IncRNA NEAT1 in proliferation and metastasis in BC. Western blot was used to detect possible protein, which was regulated by IncRNA NEAT1.

**RESULTS:** IncRNA NEAT1 was highly expressed in BC tissue, and the expression was also closely related to the tumor size and lymph node metastasis. Survival study also showed that the expression of IncRNA NEAT1 was closely related with prognosis of BC patients. MTT and Wound Healing assays showed that suppression of IncRNA NEAT1 could lead to decreased proliferation and metastasis in BC cell lines. Western blot also showed that β-catenin and N-cad were decreased while E-cad was increased after IncRNA NEAT1 being suppressed.

**CONCLUSIONS:** IncRNA NEAT1 may act as an oncogene in BC, which can promote proliferation and metastasis of BC.

**Key Words:** Breast cancer, IncRNA NEAT1, Epithelial-mesenchymal transition (EMT).

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**Introduction**

Breast cancer (BC) is one of the most common tumors in the world, and there are about 1,676,600 new cases of breast cancer were diagnosed, while 51,900 patients died of breast cancer. The five-year survival rate of BC patients remained low, though advances in the diagnosis and therapy were made during the past decades. The abnormal proliferation and invasion of BC cells are major reasons that promote mortality in BC patients. Thus, further investigation into the malignant activity of BC cells is important for the clinical treatment.

Gene regulation is important in regulating activity of cancer cells. IncRNA can regulate bioactivity at both transcriptional and post-transcriptional level, especially in cancer. Yuan et al. found that IncRNA DANCR associating with expression of CTNNB1 could promote stemness features of hepatocellular carcinoma to increase tumorigenesis and intra-/extra-hepatic tumor colonization, by blocking the repressing effect of miR-214, miR-320a and miR-199a. They also found that IncRNA MVIH was associated with microvascular invasion and tumor node metastasis, by inhibiting secretion of phosphoglycerate kinase 1. Arab et al. reported that IncRNA TARID could activate TCF21 expression by inducing promoter demethylation, via interacting TCF21 promoter and GADD45A. What’s more, Qu et al. also found that IncRNA ARSR played an important role in the regulation of Sunitinib resistance in renal cancer by binding miR-34/miR-449 to facilitate AXL and c-MET expression in renal cancer cells. These studies indicated that IncRNA was important in carcinoma development, exploring the effect and the regulatory mechanism was important for the treatment of cancer. IncRNA NEAT1 (nuclear enriched abundant transcript 1) has two isoforms (3.7 kb NEAT1-1 and 23 kb NEAT1-2), which are components of nuclear paraspeckle. Recent studies showed that IncRNA NEAT1 was highly expressed in human malignancies. Wang et al. reported that IncRNA NEAT1 was highly expressed in laryngeal squamous cancer cells, promoting the proliferation and metastasis of laryngeal squamous cancer cell by regulating miR-107/CDK6 pathway. Carmen et al. reported that IncRNA NEAT1 promoted ATR signaling resulting
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in a negative feedback loop that attenuated activation of p53, suggesting that IncRNA NEAT1 might be a promising target to enhance sensitivity of chemotherapy. Cao et al. also showed that IncRNA NEAT1 could sponge miR-335-5p to up-regulate expression of c-Met in pancreatic cancer, thus regulating the proliferation, metastasis, and apoptosis. These studies indicated that the function of IncRNA NEAT1 was important in human malignancies. In this work, we examined the expression of IncRNA NEAT1 in the BC tissue and adjacent tissue using qRT-PCR. We detected the effect of IncRNA NEAT1 on the proliferation and metastasis ability of BC cells. The possible mechanism that IncRNA NEAT1 may involve in the regulation of BC cells was also detected.

Patients and Methods

Clinical Samples
40 cases of breast cancer tissue and matched adjacent normal tissue samples were collected from patients in Huzhou Central Hospital, Huzhou, Zhejiang Province, China, from September 2012 to October 2013. These patients had not received any radiotherapy or chemotherapy before the surgery. All tissues were divided into equal size and frozen in liquid nitrogen immediately after surgery until RNA extraction. Clinicopathological characteristics of those patients were also collected at the same time. Informed consent was obtained from all subjects in the study. The Ethical Committee of the Huzhou Central Hospital for Clinical Research approved the protocol of the study.

Cell Culture
Five breast cell lines (MDA-MB-231, MDA-MB-468, MCF-7, T47D and BT-547) and one normal breast cell line (MCF-10A) were obtained from Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 cells were cultured in F15 medium (Hyclone, Logan, UT, USA). MDA-MB-468, MCF-7, T47D and BT-547 cells were cultured in DMEM medium (Hyclone, Logan, UT, USA). MCF-10A cells were cultured in F12/DMEM 1:1 medium (Hyclone, Logan, UT, USA). All cells were cultured in 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin at 37°C with 5% CO2.

siRNA Transfection
Cell lines were transfected with siRNA (si-NEAT1 or Control) (2 μg/ml) using Lip2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. The siRNAs were obtained from GenePharma (GenePharma, Shanghai, China). The sequence of siRNAs was listed as follows: si-NEAT1-1 5’-UGGUAUGGUG-GAGGAAGA-3’; si-NEAT1-2 5’-GUGAGAA-GUUGCUCUGAA-3’.

MTT Assay
2000 cells were seeded in 96-well plates with 200 μl medium. Three replicates wells were set in each group. Cell proliferation was measured using an MTT assay (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer’s instructions. Before analysis, 20 μl of MTT (2.5 mg/ml) were added to each well and incubated for 4 h at 37°C. Then the medium was removed, and the cells were solubilized in 150 μl of dimethylsulfoxide for analysis at 490 nm on a microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA). The data was collected for 5 days.

Apoptosis Assay
The activity of Caspase 3 was chosen as an indicator to measure apoptosis in this study. Apoptosis level of target cells was measured by Caspase-3 Colorimetric Activity assay Kit (Millipore, Billerica, MA, USA). Each sample was measured under 405 nm by microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Wound Healing Assay
In 6-well plates, when cells grew to 90% confluent, cell monolayers were scraped with a sterile micropipette tip. Then, wounded monolayers were washed with phosphate buffer saline (PBS). The distance between two edges of wound was calculated from three different positions immediately. Then, the cells were cultured in medium with 1% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) to avoid the affection of proliferation. The distance was measured again 24 h later.

RNA Extraction and qRT-PCR Analysis
Total RNA was isolated from cells and tissues using RNAiso Plus (TAKARA, Dalian, China) and Trizol LS Reagent (TAKARA, Dalian, China) separately. cDNA synthesis was performed by the PrimeScript™ RT reagent Kit (TAKARA, Dalian, China) according to the manufacturer’s protocol. qRT-PCR reactions were carried out using SYBR Premix Ex Taq (TaKaRa, Dalian, China) and ABI StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA,
USA). The housekeeping gene (GAPDH) levels were used as controls. The mean cycle threshold value (Ct) of each sample was normalized to the Ct value of GAPDH to calculate gene expression values. The primer was listed as follows: Neat1 Forward-5’-TGGCTAGCTCAGGGCTTCAG-3’, Neat1 Reverse-5’-TCTCCCTGGCCAAGCTTCTTCCTC-3’, GAPDH Forward-5’-TGAACGGGAAGCTCAGTGG-3’, GAPDH Reverse-5’-TCACCACCTGTTGCTGTA-3’.

**Western Blot Analysis**

Total proteins in cell were obtained using RIPA buffer containing phenylmethylsulfonyl fluoride (PMSF) (Beyotime Biotechnology, Beijing, China) on ice. Equal amounts of protein (50 μg protein per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% denaturing gel and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The immunoblots were blocked with 5% skim milk in tris buffered saline (TBS)-Tween 20 (0.05%, v/v) for 1 h at room temperature. TBST was used to wash these membranes. Then the membranes were probed with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with respective secondary antibody for 1 h at room temperature. The immunobLOTS were then exposed and detected using an enhanced chemiluminescence Western blotting detection system (Bio-Rad, Hercules, CA, USA).

**Statistical Analysis**

Each experiment was repeated at least three times. Data are represented as mean ± SD. A two-tailed Student’s t-test was used for comparisons of continuous variables between two groups. One-way ANOVA with Tukey, Dunnett post tests or two-way ANOVA was used when three or more groups were compared. The data were presented as the means±standard deviation (SD), and p < 0.05 was considered to indicate a significant difference. All the statistical analyses were performed with GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA).

**Results**

**NEAT1 was Highly Expressed in the BC Tissue and cell Lines**

To detect the roles of lncRNA NEAT1 in breast cancer cells, we first examined the expression of lncRNA NEAT1 in 40 breast cancer tissues and adjacent tissues by qRT-PCR (Figure 1A). The result showed that lncRNA NEAT1 was highly expressed in the BC tissue, compared with the paired adjacent tissue. Moreover, we detected the expression of lncRNA NEAT1 in normal breast epithelial cell lines and BC cell lines (Figure 1B). Taken together, we could know that lncRNA NEAT1 was highly expressed in both BC tissue and cell lines.

**Figure 1.** NEAT1 was highly expressed in the BC tissue and cell lines. (A) The expression of lncRNA NEAT1 was measured by qRT-PCR technology in 40 BC tissues and paired adjacent tissues. (B) The expression of lncRNA NEAT1 in five BC cell lines and one normal breast epithelial cell line was detected by qRT-PCR. ***p<0.001, **p<0.01.
Clinical Features of lncRNA NEAT1 in BC Patients

To further detect the clinical features of lncRNA NEAT1, we then analyzed the relationship between lncRNA NEAT1 and clinical features. We found that the expression of lncRNA NEAT1 was closely related with the tumor size and lymph node metastasis (Figure 2A and Figure 2B). What’s more, results also showed that BC patients with higher expression of lncRNA NEAT1 (>2 folds of increase, n=26) had a shorter overall survival compared with those with lower NEAT1 expression levels (<2 folds of increase, n=14) (Figure 2C). These results showed that lncRNA NEAT1 was related to poor prognosis in BC.

Inhibition of NEAT1 could Suppress Proliferation and Metastasis of BC Cells and Promote the Apoptosis of BC Cells

As it was shown, lncRNA NEAT1 was highly expressed in BC tissue and closely related with the lymph node metastasis and tumor size. We then explored the effect of lncRNA NEAT1 on the proliferation of BC cells. We used siRNA to reduce the expression of lncRNA NEAT1 in BC cell lines (MDA-MB-468 and MCF-7) (Figure 3A). Then MTT assay was applied to detect the proliferation ability of BC cells, after the expression of lncRNA NEAT1 was suppressed (Figure 3B). We found that suppression of lncRNA NEAT1 could reduce the proliferation ability of BC cells. Wound healing assay also demonstrated that suppression of NEAT1 inhibited migration of BC cells (Figure 3C and D). Also, we used Caspase 3 as the indicator of apoptosis in our study. We found that suppression of lncRNA NEAT1 could increase the activity of Caspase 3 (Figure 3E). These experiments showed that lncRNA NEAT1 could promote proliferation and metastasis of BC and suppression of lncRNA NEAT1 might increase the apoptosis of BC cells.

IncRNA NEAT1 could Promote the Expression of β-catenin and EMT in BC Cells

We found that IncRNA NEAT1 could promote the proliferation and metastasis of BC, but the possible mechanism remained unclear. A recent study\textsuperscript{14} has shown that over-expression of IncRNA NEAT1 could up-regulate the expression of Wnt-4 expression, thus promoting tumor development. According to this work, we would like...
to explore whether lncRNA NEAT1 could affect Wnt/β-catenin in BC cells. We found that suppression of lncRNA NEAT1 in BC cell lines could lead to the decrease of β-catenin in BC cell lines (Figure 4A). Studies have shown that β-catenin was an important translation factor in tumorigenesis, such as proliferation and metastasis. We then detected whether lncRNA NEAT1 could affect the expression of EMT-related proteins. Notably, we found that suppression of lncRNA NEAT1 could inhibit EMT of BC cells (Figure 4B and Figure 4C). According to these results, lncRNA NEAT1 could promote the proliferation and metastasis by affecting β-catenin and EMT in BC cells.

**Discussion**

BC is the most common cancer in females, which is the leading cause of cancer-related death in females. Dysregulation of proliferation and metastasis of BC cells are the main reasons that lead to poor prognosis in BC patients. Thus, exploring the regulatory mechanism in BC is important for clinical practice. Several studies have shown that lncRNA may be the key regulatory factors in BC. Zhang et al\(^{15}\) reported that lncRNA LINP1 could serve as a scaffold, which linked Ku80 and DNA-PKcs in non-homologous end joining pathway, reducing the sensitivity to radiotherapy in triple-negative breast cancer. Lin et al\(^{16}\) found another lncRNA, named Link-A, which could promote breast cancer and reprogram tumorigenesis. They observed that Link-A could mediate HIF1α stabilization, and activate HIF1α transcriptional programs under normoxic conditions in triple-negative breast cancer. What’s more, lncRNA UCA1 has been reported to promote EMT, proliferation and tamoxifen resistance in breast cancer\(^{17-19}\). Moreover, Liu et al\(^{20}\) also revealed that some specific lncRNA could act as a suppressor gene in breast cancer. Liu et al\(^{20}\) showed that lncRNA NKILA could interact with NF-κB/IκB to form a stable complex, preventing over-activation of NF-κB pathway in breast epithelial cells. Their study also found that lncRNA NKILA was negatively correlated with breast cancer metastasis, suggesting that lncRNA NKILA could be used as NF-κB modulators to suppress cancer.
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These researches suggested that lncRNA was involved in breast cancer progression, which could be used as a target for treating breast cancer. In this work, we found that lncRNA NEAT1 was highly expressed in BC tissues, compared with adjacent tissues. Notably, results also showed that the expression of lncRNA NEAT1 was remarkably higher in larger tumors and lymph node metastasis, suggesting that lncRNA NEAT1 may be involved in BC progression. We also demonstrated that lncRNA NEAT1 was closely related with prognosis of BC patients. The expression of lncRNA NEAT1 was negatively related with the survival time of BC patients, which was confirmed in 40 BC patients. To further explore the effect of lncRNA NEAT1 in BC, we then detected the expression of lncRNA NEAT1 in BC cell lines. We, then, used siRNA to suppress the expression of lncRNA NEAT1 in BC cell line MD-MBA-468 and MCF-7. MTT assay and Wound Healing assay showed that suppression of lncRNA NEAT1 leads to decrease in proliferation and migration in BC cells. These findings suggested that lncRNA NEAT1 may promote BC development, but the mechanism remained unclear. lncRNA NEAT1 was up-regulated in various cancers, but its mechanism was not fully understood. Scholars have shown that lncRNA NEAT1 could promote progression of non-small cell lung cancer, and lncRNA NEAT1 accelerated NSCLC cell growth and metastasis via different pathways. Sun et al showed that IncRNA NEAT1 could sponge miR-377-3p to regulate expression of E2F3, which was a core oncogene in progressing non-small cell lung cancer. Another study also showed that NEAT1 might act as competing for endogenous IncRNA to sponge hsa-mir-98-5p to upregulate CTR1 in lung cancer. Interestingly, recent investigations also showed that lncRNA NEAT1 might sponge several of miRNAs to regulate cancer progression. Wang et al demonstrated that lncRNA NEAT1 promoted proliferation of laryngeal squamous cancer by sponging miR-107 to regulate the expression of CDK6. Cao et al showed that lncRNA NEAT1 could regulate proliferation and metastasis in pancreatic cancer through miR-335-5p/c-met. These studies suggested that lncRNA NEAT1 could regulate the progression of cancer through different mechanism. Epithelial-mesenchymal transition (EMT) was important in the process of invasion and metastasis in tumor development. Thus, clarifying the role of NEAT1 in EMT of breast cancer was crucial to understanding its regulatory mechanism. We found that suppression of lncRNA NEAT1 led to increased expression of E-cad, while N-cad was decreased, suggesting that lncRNA NEAT1 could regulate EMT in BC. This finding was consistent with Lu et al work. They found that lncRNA NEAT1 could regulate ZEB1 by sponging miR-204 and promote EMT phenotype in nasopharyngeal carcinoma. There are also some drawbacks in our study. We didn’t detect the expression of lncRNA NEAT1 in the serum of BC patients. In vitro experiments were also absent, which could have further identified the role of IncRNA NEAT1.

Conclusions

Our research demonstrated that lncRNA NEAT1 could act as oncogene in BC progress. LncRNA NEAT1 was highly expressed in BC tissues and cell lines, and its expression was negatively correlated with the survival time of BC patients, suggesting that it could be a potential biomarker in diagnosis of BC. Also, IncRNA NEAT1 was related to the abnormal proliferation and migration of BC, indicating that IncRNA NEAT1 might be a promising target in treating BC.

Conflict of interest

The authors declare no conflicts of interest.
References


