Downregulation of long noncoding RNA MALAT1 induces epithelial-to-mesenchymal transition via the PI3K-AKT pathway in breast cancer

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Abstract: The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) regulates cell motility via the transcriptional or post-transcriptional control of motility-related genes. Whether MALAT1 plays a critical role in cancer progression in breast cancer remains unclear. In this study, we found that MALAT1 was downregulated in breast tumor cell lines and cancer tissue, and showed that knockdown of MALAT1 in breast cancer cell lines induced an epithelial-to-mesenchymal transition (EMT) program via phosphatidylinositide-3 kinase-AKT pathways. Furthermore, lower expression of MALAT1 in breast cancer patients was associated with shorter relapse-free survival. Thus, our results indicate for the first time that MALAT1 is a novel regulator of EMT in breast cancer and may be a potential therapeutic target for breast cancer metastasis.

Keywords: Long noncoding RNA, MALAT1, EMT, metastasis, breast cancer

Introduction

Breast cancer is the most common malignancy among women in China and is second to lung cancer as a cause of cancer death [1]. Although advances have been made in the treatment of breast cancer over the last decade, the prognosis for patients is still poor due to the high rate of vital organ metastasis. Once organ metastasis occurs, the 5 year survival rate is less than 25% [2]. Currently, the molecular mechanism of metastasis is still unclear in breast cancer. Thus, identification of new genes related to metastasis and the molecular mechanisms underlying metastatic progression are urgently required.

The epithelial-to-mesenchymal transition (EMT) endows epithelial cells with mesenchymal properties including reduced cell-cell adhesion and increased motility [3]. In addition, EMT is central to the regulation of embryo and organ architecture during gastrulation and organogenesis [4]. The involvement of EMT has been reported in many vital processes such as cellular proliferation, cell migration and invasion in various malignant tumors [5]. EMT is essential for cancer invasion and metastasis, and is regarded as a major mechanism inducing metastasis in cancer [6].

Long non-coding RNAs (lncRNAs) are genomic transcripts longer than 200 nucleotides without protein coding function [7, 8]. Recent studies have revealed that lncRNAs are involved in transcriptional, post-transcriptional and epigenetic gene regulation [9, 10]. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is an abundantly expressed nuclear lncRNA with a length of approximately 8000 nucleotides that was identified as the first lncRNA associated with metastasis and survival in non-small cell
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MALAT1 regulates tumor cell proliferation, migration, invasion and the metastasis in various cancer entities, particularly lung cancer [11]. Aberrant expression of MALAT1 has been observed in a broad range of human malignant tumors including lung carcinoma, cervical cancer, breast cancer, bladder cancer, endocrine and ovarian cancer, osteosarcoma, colorectal cancer and hepatocellular cancer [1, 12-19]. Although various functions have been described for MALAT1 in many different cancers, the relationship between MALAT1 and EMT in breast cancer remains unclear.

In this study, we observed downregulation of MALAT1 leading to EMT and metastasis in breast cancer. We showed that levels of MALAT1 were decreased in breast cancer cell lines and tumor tissue specimens. Down-regulated MALAT1 was associated with axillary lymph node metastasis and clinical disease progression. In addition, knockdown of MALAT1 enhanced the migration and invasive capacity of breast cancer cells in vitro by activating the phosphatidylinositide-3 kinase (PI3K)-AKT pathway. Interestingly, unlike the role of MALAT1 in other cancers, we discovered a distinct regulatory mechanism underlying the function of MALAT1 in EMT and metastasis in breast cancer.

Materials and methods

Tissue specimens and patient data

Eligible patients were those with a histological diagnosis of breast cancer who had undergone surgical resection and had not received chemotherapy or radiotherapy. Breast cancer and adjacent non-cancerous specimens were obtained from patients at the Department of Breast Surgery, Harbin Medical University Cancer Hospital between 2011 and 2014. Specimens were immediately frozen in liquid nitrogen and stored in -80°C freezers until RNA extraction. This study conformed to clinical research guidelines and was approved by the research ethics committee of the hospital. We obtained written informed consent from all patients to participate in this study. To investigate the relationship between expression of MALAT1 and clinical prognosis, data from 5861 breast cancer patients in the Gene Expression Omnibus (GEO) database were used in this study [20]. Breast cancer data from The Cancer Genome Atlas (TCGA) was also utilized for validation of the relationship between MALAT1 expression and EMT in this study [21]. A list of the dataset retrieved from GEO is provided in Table S1.

Cell culture

Cell lines were provided by the Chinese Academy of Sciences (Shanghai, China). Human breast cancer cell lines MDA-MB-231 and MDA-MB-453 were cultured in Leibovitz’s L-15 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) at 37°C in 100% air without CO₂. The human ductal carcinoma cell line BT549 and adenocarcinoma cell line SK-BR-3 were incubated in RPMI-1640 medium (Hyclone) with 10% FBS at 37°C with 5% CO₂. Human MCF7 adenocarcinoma cells were cultured in Eagle’s Minimum Essential Medium (LONZA, Walkersville, MD, USA) with 0.01 mg/ml human recombinant insulin and 10% FBS at 37°C. The human breast cell line MCF10A was cultured in DMEM/F-12 medium (Hyclone) with 100 ng/ml cholera toxin (Sigma-Aldrich, St Louis, MO, USA), 5% horse serum (Gibco BRL, Grand Island, NY, USA), 2 μg/ml epidermal growth factor (Sigma-Aldrich), 5 μg/ml hydrocortisone (Sigma-Aldrich) and 10 μg/ml insulin (Sigma-Aldrich) at 37°C with 5% CO₂.

Design and cloning of short hairpin RNA constructs

MALAT1 knockdown was performed using lentivirus-encoded short hairpin RNA (shRNA), prepared by GeneChem (Shanghai, China). shRNA was cloned into pLKO.1 (GV248) lentiviral vectors. The shMALAT1 and shCtrl targeting sequences were GGGCTTCTCTTAATTTA and TTCTCCGAACGTGTCACGT, respectively. Specificity of the shRNA sequences was verified via BLAST search at www.ncbi.nih.gov. Lentivirus infection of shRNA constructs in MCF7 and MDA-MB-231 cell lines was performed in the presence of polybrene. Stable cell lines were selected after growth in 2 mg/ml puromycin for about 2 weeks. The efficiency of MALAT1 knockdown was determined by quantitative real-time PCR (qPCR).

RNA isolation, reverse transcription and qPCR

Total RNA was extracted from fresh frozen samples and cells using Trizol Reagent (Invitrogen,
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Carlsbad, CA) according to the manufacturer’s instructions. Total RNA (2 µg) was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Vilvoord, Brussel, Belgium). The relative level of MALAT1 mRNA to control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts was determined by qPCR using the ABI 7500 Fast Real-Time PCR System (Invitrogen, Carlsbad, CA, USA). The primer sequences were as follows. MALAT1: forward 5’-AAAGCAAGGTCTCCCCACAA-GGCA-3’, reverse 5’-GGTCTGTGCTAGAACAAAGGCA-3’; GAPDH: forward 5’-ACCACATGCCATTCCATCG-CATCAC-3’, reverse 5’-TCCACCCGTGTGCTGA3’. The qPCR amplification was performed in triplicate reactions beginning at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 60 s. Quantitative normalization of MALAT1 cDNA was performed in each sample using the expression of the GAPDH as an internal control. The relative level of MALAT1 transcripts to control GAPDH was determined by the 2-ΔΔCT method.

Cell Invasion assays

Transwell 24-well plates (Corning, NY) were used to measure the invasive ability of cells. A total of 5×10^4 cells were plated on top chamber inserts coated with 200 mg/ml of Matrigel (BD Biosciences, San Diego, CA, USA). Bottom chambers were filled with 600 l conditioned medium with 15% FBS. After 48 h, invading cells in the lower chamber were stained with 0.1% crystal violet, photographed and counted using an inverted microscope. Cell numbers were determined in five random fields in each chamber, and the mean value was calculated. The above assays were conducted in triplicate.

Wound healing assay

Cells were seeded in a monolayer on six-well plates in 10% FBS-containing medium. When cell confluence reached approximately 80%, the monolayer was scratched with a plastic tip and washed with PBS to remove cell debris. Medium was added, and culture plates were incubated at 37°C. Wound healing within the scrape line was observed at different time points, and representative scrape lines were photographed. Wound closure was evaluated in five random fields using an inverted microscope. Triplicate wells for each condition were examined, and each experiment was repeated in triplicate.

Immunoblotting assay

Cells were lysed in protein lysis buffer (Sigma-Aldrich) and cellular debris was cleared by centrifugation at 12,000 g for 20 min at 4°C. The BCA Protein Assay Kit (Beutime, Haimen, China) was used to measure protein concentrations. Protein was denatured at 95°C for 5 min prior to loading onto SDS-polyacrylamide gels (10% wt/vol). After gel separation, proteins were transferred to PVDF membranes and incubated with primary antibodies for 24 h at 4°C. The membranes were washed three times, 10 min per wash, and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature, followed by three washing steps. Proteins were visualized by chemiluminescence detection imaging. Binding intensities were determined using software AlphaView SA version 3.4.0 (ProteinSimple, California, USA). Antibodies used in this study were purchased from Abcam: N-Cadherin (ab18203), E-Cadherin (ab1416), vimentin (ab8978), total Akt (ab8805), pAkt Ser473 (ab81283), and β-actin (ab8229).

Statistical analysis

The significance of the difference between various groups was analyzed using Student’s t-test or the chi-square test. The correlation between parameters was analyzed using Pearson’s correlation coefficient. Survival curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparisons. All statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL). A P value < 0.05 was considered statistically significant.

Results

MALAT1 is downregulated in human breast cancer

Recent data have suggested that MALAT1 plays an important role in tumorigenesis and progression; whether it has similar functions in breast cancer has not been investigated. To evaluate the clinical relevance of MALAT1 in breast cancer, MALAT1 expression in clinical specimens was assessed by qPCR. In a collection of 135 pairs of randomly chosen samples
from breast cancer patients, the MALAT1 level was significantly lower in tumor tissue compared to adjacent non-cancerous tissue (P < 0.001) (Figure 1A). Similarly, we found that MALAT1 was downregulated in breast cancer cell lines (BT549, MCF7, MDA-MB-453, and MDA-MB-231) compared to expression in the breast epithelial cell line MCF10A (Figure 1B). These results suggest that decreased expression of MALAT1 may be important for breast tumorigenesis and cancer progression.

**Downregulated MALAT1 is correlated with axillary lymph node metastasis and clinical disease progression**

To further investigate the clinical relationship between MALAT1 expression and breast cancer characteristics, the expression of MALAT1 was examined along with other clinicopathological features in the 135 breast cancer pairs. Statistical analysis revealed that downregulation of MALAT1 was strongly correlated with axillary lymph node metastasis (P = 0.005), indicating that downregulation of MALAT1 may be associated with the progression of breast cancer (Table 1). As the follow-up period in this study was less than 5 years, the relationship between MALAT1 expression and prognosis was also examined using data retrieved from GEO [20]. Survival analysis showed that breast cancer patients with downregulated MALAT1 had shorter periods of metastatic relapse-free survival (P = 0.0420) (Figure 2).

**Downregulation of MALAT1 promoted migration and invasion in breast cancer cells**

To determine if downregulation of MALAT1 contributes to cancer progression, we investigated the effect of MALAT1 knockdown on migration and invasion of MDA-MB-231 and MCF7 breast cancer cells. Stable knockdown of MALAT1 following infection with a lentivirus encoding MALAT1 shRNA was established using 2 mg/ml puromycin for about 2 weeks in both breast cancer lines (Figure 3A and 3B). Cells with downregulated MALAT1 displayed morphologies typical of high invasiveness, with more spindle-like and fibroblastic features in MDA-MB-231-shMALAT1 and MCF7-shMALAT1 cells than in control cells (Figure 3C). In wound healing assays, both breast cancer cell lines expressing shMALAT1 almost completely filled the wound gap 48 h after injury, while an obvious gap was still present at this time point.
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Table 1. Correlation between MALAT1 expression and clinico-pathological features

<table>
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<th>No. of patients</th>
<th>MALAT1 Expression</th>
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<tr>
<td>&lt; 60</td>
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<tr>
<td>≥ 60</td>
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<td>Upregulation</td>
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ER: Estrogen receptor; PR: Progesterone receptor.

Figure 2. Kaplan-Meier survival curves for patient groups with upregulated and downregulated MALAT1 expression in Swedish cohort patients with breast cancer.

using shCtrl cells, indicating that downregulation of MALAT1 promoted cell migration in breast cancer (Figure 3D). A similar result was obtained in the Transwell invasion assay, in which invasiveness was increased approximately threefold in both cell lines infected with the MALAT1 shRNA compared to control cell lines (Figure 3E, P < 0.05). Furthermore, from analysis of the TCGA database [21], expression of some metastatic-relevant genes was also negatively associated with the expression of MALAT1 in breast cancer tissue (Figure 3F). Thus, MALAT1 may be a vital regulator of metastasis in breast cancer.

To further investigate the molecular mechanisms mediating the metastatic features of MALAT1, the levels of EMT markers were examined by qPCR. Results revealed that the mesenchymal marker Cadherin 2 (CDH2) was significantly upregulated and the epithelial marker Cadherin 1 (CDH1) was downregulated in MDA-MB-231-shMALAT1 and MCF7-shMALAT1 cells (Figure 4A and 4B). In validation of these results, similar findings were obtained from analysis of breast cancer specimens in the TCGA database (Figure 4C). Western blotting assays also revealed that mesenchymal factor N-cadherin was significantly increased and epithelial marker E-cadherin was reduced in MDA-MB-231-shMALAT1 and MCF7-shMALAT1 cells, compared to shCtrl cells (Figure 4D).

**Downregulation of MALAT1 induces EMT by regulating the PI3K-AKT pathway**

To determine the possible mechanism by which MALAT1 regulated metastasis of breast cancer cells, we performed immunoblot analysis to investigate the effects of MALAT1 knockdown on the PI3K-AKT pathway, which is often aberrantly activated in human breast cancer and contributes to metastasis. The result showed that knockdown of MALAT1 in both cancer cell lines significantly increased the levels of phosphorylated AKT (pS473) compared to control
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A

B

C

D

4886

Figure 3. Knockdown of MALAT1 enhances breast cancer migration and invasion in vitro. A & B. qPCR analysis of the efficiency of MALAT1 knockdown in MDA-MB-231 and MCF7 cell lines. C. Morphology of MDA-MB-231 and MCF7 cells infected with shCtrl and shMALAT1 lentiviral vectors. D. Wound healing assay to evaluate the effect of MALAT1 knockdown on breast cancer cell motility. E. Transwell assay to evaluate the effect of MALAT1 knockdown on breast cancer cell invasive capacity. F. Metastasis-relevant genes associated with the expression of MALAT1 in breast cancer tissue from TCGA. Each experiment was performed in triplicate (*P < 0.05 and **P < 0.01 vs. control. Original magnification ×100).
cells (Figure 4D). This result indicated that the induction of EMT by MALAT1 downregulation may be mediated by the PI3K-AKT pathway.

**Discussion**

It has been determined that up to 70% of the human genome is transcribed but only 2% of the genome serves as blueprints for proteins [22, 23]. As important members of the family of noncoding RNA, IncRNAs are transcripts longer than 200 nucleotides without protein coding potential [24-27]. The function of IncRNAs has been questioned for many years since their initial discovery via microarray tiling array methods. Due to the uncertainty regarding their role,
IncRNAs have been regarded as transcriptional noise in the genome [25]. However, accumulating evidence has shown that IncRNAs may play a critical biological role in cellular development and human diseases [17, 18].

MALAT1, also known as nuclear-enriched abundant transcript 2 (NEAT2), is a highly expressed IncRNA in mammalian species [28]. MALAT1 is localized to the nucleus and most of its functions are related to nuclear processes such as nuclear organization and architecture, gene splicing, or gene expression [29]. Aberrant expression of MALAT1 was discovered in a group of human tumor tissues and many vital biological processes including tumor cell proliferation, apoptosis, migration, invasion, and metastasis are closely associated with MALAT1 [15-19]. Studies have shown that MALAT1 is upregulated in bladder cancer and contributes to metastasis via the EMT process [15]. Furthermore, MALAT1 was recognized as an independent prognostic factor for hepatocellular carcinoma recurrence after liver transplantation [14]. Although MALAT1 functions in various cancers, its role in breast cancer remains unclear. In this study, MALAT1 levels were shown to be significantly lower in breast cancer cell lines as well as in tumor tissues compared to adjacent non-cancerous tissue, indicating that MALAT1 may act as a tumor suppressor against breast cancer and not as an oncogene as shown in other tumors.

EMT is an important step in metastasis, during which non-motile, polarized epithelial cells dissemble their cell-cell junctions and convert into individual, motile mesenchymal cells [30-32]. Reduction or loss of E-cadherin is a well-established hallmark of EMT, and E-cadherin downregulation is associated with a poor clinical prognosis for many cancers [33]. In this study, we investigated the role of MALAT1 in EMT process in breast cancer. Our results showed that downregulation of MALAT1 promoted migration and invasion of breast cancer cells in vitro and further indicated that lower MALAT1 expression was linked to metastasis in breast cancer. Mechanistically, we demonstrated that MALAT1 functions as an inducer of EMT in breast cancer by activating the PI3K-AKT pathway. Thus, this study strongly points to a close link between IncRNAs and cancer progression. Analysis of TCGA breast cancer data showed that breast cancer patients with lower expression of MALAT1 had shorter periods of relapse-free survival than patients with higher expression. These data suggest that downregulation of MALAT1 may serve as a prognostic factor in breast cancer. In contrast to its role in other cancer entities including lung cancer, bladder cancer, and liver cancer, it was downregulation of MALAT1 and not increased expression which enhanced cell migration and invasion in breast cancer [12, 13, 18]. Thus, in this study, it appeared that downregulation of MALAT1 induced EMT, suggesting that MALAT1 may utilize multiple mechanisms to carry out its specific functions in different malignant tumors.

To summarize, this study demonstrated that MALAT1 regulates metastasis in breast cancer through induction of EMT via activation of the PI3K-AKT pathway. Considering the poor prognosis for breast cancer patients with downregulated MALAT1, our finding that knockdown of MALAT1 enhanced EMT shows that MALAT1 may be a potent target for development of new therapies in breast cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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### Table S1. Datasets for prognosis analysis retrieved from GEO

| GSE2603 | GSE10510 |
| GSE1456 | GSE16391 |
| GSE1378 | GSE11264 |
| GSE2034 | GSE12093 |
| GSE2741 | GSE22133 |
| GSE3143 | GSE17907 |
| GSE4922 | GSE22219 |
| GSE8757 | GSE19615 |
| GSE7390 | GSE20711 |
| GSE5327 | GSE26971 |
| GSE7378 | GSE25055 |
| GSE6532 | GSE20685 |
| GSE7849 | GSE21653 |
| GSE9893 | GSE16987 |
| GSE9195 | GSE33926 |
| GSE11121 | GSE45255 |