LINC00467 enhanced the proliferative, migratory and invasive ability of breast cancer cells by targeting miR-18a/b-5p/MAPK4 axis

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**ABSTRACT**

Breast cancer (BC) is a common gynaecological malignancy worldwide. Long noncoding RNAs (lncRNAs) were identified to take part in the regulation of the occurrence and development of tumors. lncRNA The role of LINC00467 in lung adenocarcinoma has been reported, but its mechanism remains unclear in BC. To explore the role of LINC00467 deeply, we designed and performed a series of experiments. According to the result, it was discovered that LINC00467 was overexpressed in BC tissues and cells, and then the knockdown of LINC00467 resulted in a decline in cell growth and metastasis. Mechanistically, miR-18a/b-5p was screened out and validated to bind with LINC00467. Additionally, LINC00467 was negatively correlated with miR-18a/b-5p. Hereafter, there is evidence that miR-18a/b-5p targets MAPK4. Rescue assays suggested that MAPK4 amplification recovered the inhibitive effect of LINC00467 knockdown on cell growth and metastasis. In a word, LINC00467 enhanced BC cell growth and metastasis by targeting miR-18a/b-5p/MAPK4, which implies a potential revelation for exploring the therapeutic tactic of BC.

**Introduction**

Breast cancer (BC) is one of the most common gynaecological malignancies worldwide, which has caused a multitude of cancer-related death in women (1,2). Currently, surgical resection, radiotherapy and chemotherapy are widely adopted to treat BC (3). However, the overall survival rate in 5 years remained low due to the distant metastasis and high recurrence (4). Unfortunately, the tumorigenesis of BC was still unclear (5). Hence, the molecular mechanism of BC occurrence and development is required to be deeply explored.

Long non-coding RNAs (lncRNAs) belong to a class of non-coding RNAs (ncRNAs) with no less than 200bp in length (6). A large number of studies claimed that the aberrant lncRNA expression was closely associated with various cellular activities including proliferation, differentiation and metastasis (7-9). SNHG16 was reported to promote BC cell migration by sponging miR-98 and regulating E2F5 expression (10). PANDAR inhibition limited cell proliferation and invasion, and suppressed the EMT process in BC (11). Additionally, LINC00467 was supposed to improve cell survival (12). Yet, the function of LINC00467 remained uncharacterized in BC. In mechanism, lncRNAs were capable of regulating gene expression at several levels, including the epigenetic, transcriptional and post-transcriptional levels (13). More importantly, lncRNA could sponge microRNAs (miRNAs) to release messenger RNAs (mRNA) by functioning as ceRNA (14-16). For instance, The BRAF pseudogene induced lymphoma by functioning as ceRNA in vivo (17). Long noncoding RNA NORAD, a novel ceRNA regulated the protein level of the small GTP binding protein RhoA through competitively binding with miR-125a-3p in pancreatic cancer (18). Recently, long intergenic non-coding RNA 467 (LINC00467) was proven to promote the proliferation and metastasis of lung adenocarcinoma cells by interacting with EZH2 and HTRA3 (19). In BC, however, the biological role and mechanism of LINC00467 remain unknown.

In this research, we proved that LINC00467 was overexpressed in BC tissues and cells, and then the knockdown of LINC00467 resulted in a decline in cell growth and metastasis. Mechanistically, miR-18a/b-5p was screened out and validated to bind with LINC00467. Additionally, LINC00467 was negatively correlated with miR-18a/b-5p. Hereafter, there is evidence that miR-18a/b-5p targets MAPK4. Rescue assays suggested that MAPK4 amplification recovered the inhibitive effect of LINC00467 knockdown on cell growth and metastasis. In a word, LINC00467 enhanced BC cell growth and metastasis by targeting miR-18a/b-5p/MAPK4, which implies a potential revelation for exploring the therapeutic tactic of BC.

**Materials and Methods**

**Clinical specimens**

54 matched BC tissue and adjacent non-cancerous tissue samples were acquired in 54 patients of BC between 2013 and 2018, after obtaining ethical approval from the Committee of Union Hospital, Tongji Medical College. All subjects had offered written informed consent for participation in this study. Instantly after resection, specimens were kept at -80°C in liquid nitrogen for further analysis.

**Cell lines**

Human BC cell lines (MCF7, MDA-MB-468, MDA-MB-231, SK-BR-3) and MCF10A (both; ATCC, Rockville, Maryland), were routinely cultured at 37°C with 5% CO2 in DMEM. 10% FBS-containing antibiotics were acquired for supplementing DMEM.

**qRT-PCR**

According to the protocol, 2 ug of total RNA was extracted with Trizol reagent and then treated for cDNA
synthesis. SYBR Green PCR Master Mix (Takara) was employed for qPCR. Gene level was following a 2-AACt method. Controls for loading were GAPDH and U6 in relevant assays.

Transfection
The triplicate LINC00467-specific shRNAs, termed sh-LINC00467#1/2/3, and sh-NC, were all produced by GenePharma (Shanghai, China). The overexpression of miR-18a-5p or MAPK4, the plasmids miR-18b-5p mimics or pcDNA3.1/MAPK4, and NC mimics and pcDNA3.1 vector, all designed at Genechem Company (Shanghai, China). Transfections were conducted with Lipofectamine 2000 (Invitrogen).

CCK-8 assay
CCK-8 analysis was used to determine cell viability. A cell suspension of 100 liters was prepared in 96-well plates, and they were then pre-cultivated. After 24 hours, 48 hours, or 72 hours of 12 incubation, 10L of CCK-8 was added to each sample. Finally, the absorbance at 450nm was measured via a microplate reader after 24, 48, or 72 hours.

Colony formation
Cells after transfection were prepared for the 14 days of culture in the 6-well plates (500 cells per well). Subsequently, colonies were incubated with 0.5% crystal violet to determine colony formation.

Wound-healing assay
Marked lines were drawn through the diameter of the wells on the back of the 6-well plates. A total of 5×10^6 well-grown cells were inoculated in the 6-well plates and cultivated in an incubator at37°Covernight until they formed a monolayer. Next, the bottom of the 6-well plates was scored with a sterile gun in the center perpendicular to the marker lines. Following this, the cells were washed three times using PBS and added to a 1% serum-supplemented medium for growth. Photographs of cell migration were taken under the microscope at 0 h and 24 h, and wound healing rates were measured by the ImageJ software.

Transwell assays
24-well transwell inserts (Costar, MA, USA) with or without coating Matrigel were conducted for invasion or migration assay. 1 × 10^6 cells per well in serum-free medium were transferred to the upper compartment, while the lower compartment was supplied with the complete DMEM. Invasion or migration ability of cells was treated with 0.1% crystal violet for observation.

Subcellular fraction assay
1 × 10^6 cells were centrifuged for 5 min at 4oC after rinsing in phosphate buffer saline (PBS). The supernatant (cell cytoplasm) was removed and the rinsing step was conducted twice. Nuclear and cytoplasmic RNAs were separated from cultured cells by applying PARIS™ Kit (Invitrogen) as per the manual. qRT-PCR was followed to determine the level of LINC00467 and corresponding controls (GAPDH, U2).

RIP
A RIP assay was taken in cells using the Imprint® RNA Immunoprecipitation Kit and an anti-IgG control antibody. Cell lysates were incubated with antibodies against AGO2 or IgG. In the end, RT-qPCR was used to analyze the precipitated RNAs.

Dual-luciferase reporter gene analysis
Dual-luciferase analysis was conducted via the 48 h of co-transfection of indicated reporter vectors and transfection plasmids (miR-18a/b-5p mimics and NC mimics), followed by exposure to Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Western blot
RIPA buffer was used to extract total proteins from cells, and after SDS-PAGE, in 10% skim milk, proteins were transferred to PVDF membranes. Following the blockade, samples were cultured with primary antibodies specific to MAPK4 (ab211501; Abcam, Cambridge, MA) and GAPDH (ab181603; Abcam), as well as the corresponding secondary antibodies conjugated to HRP. Signals of membranes were monitored through ECL Substrate (Pierce, Rockford, IL).

Statistical analysis
Results from all assays containing more than two repeats were given as the mean value ± SD. Statistical Package was employed for data analyses with Student’s t-test or one-way ANOVA. P<0.05 seems statistically significant.

Results
Up-regulated LINC00467 accelerated tumor development by inducing cell proliferation, migration and invasion
According to GEPIA, LINC00467 levels were higher in BRCA (breast invasive carcinoma) tissue (n=1085) than in ANT (adjacent normal tissue) (n=291). (Figure 1A). Additionally, qRT-PCR assay indicated that LINC00467 was up-regulated not only in BC tissues but also in BC cells compared to matched ANT and human breast epithelial cell lines (Figure 1B-C). Then, LINC00467 was markedly reduced by transfecting sh-LINC00467#1, sh-LINC00467#2 or sh-LINC00467#3 into MDA-MB-468 and SK-BR-3 cells (Figure 1D). Especially, sh-LINC00467#1 and sh-LINC00467#2 possessed a better knockdown efficacy in comparison with sh-LINC00467#3, so sh-LINC00467#1 and sh-LINC00467#2 were chosen for loss-of-function assay. CCK-8 assay demonstrated that LINC00467 repression inhibited the proliferation of BC cells (Figure 1E). Likewise, Colony formation studies showed a reduction in the number of colonies after transfection with sh-LINC00467#1 or sh-LINC00467#2. (Figure 1F). Additionally, wound healing studies showed that the percent of wound closure was decreased by LINC00467 silence (Figure 1G). At last, transwell studies suggested that the capability of cell migration and invasion was reduced by the knockdown of LINC00467 (Figure 1H-I). Collectively, Up-regulated LINC00467 accelerated BC development by inducing cell growth and metastasis.
LINC00467 sponged miR-18a/b-5p in BC

In the mechanism aspect, numerous studies claimed that IncRNA was able to modulate gene expressions at the post-transcriptional level by acting as a ceRNA. According to this theory, we conjectured that LINC00467 also functioned in this pattern. Based on nuclear-cytoplasmic separation assays, the majority of LINC00467 was found in the cytoplasm (Figure 2A). As Figure 2B, 3 miRNAs containing LINC00467 binding sequences were screened after starBase prediction (conditions: moderate stringency of CLIP data and 4 cancer types of Pan-Cancer). To specify the potential miRNAs interacted with LINC00467 in BC, several experiments were carried out. qRT-PCR assay uncovered that the miR-18a/b-5p level was down-regulated BC cells and miR-217 presented no difference (Figure 2C). Similarly, compared with matched normal tissues, miR-18a/b-5p was lower in tumor tissues (Figure 2D). Subsequently, the expression of miR-18a/b-5p was markedly enhanced by transfection of miR-18a/b-5p mimics (Figure 2E). Importantly, qRT-PCR disclosed that miR-18a/b-5p overexpression reduced LINC00467 level and LINC00467 restraint raised miR-18a/b-5p levels (Figure 2F-G). Moreover, according to starBase data, LINC00467 and miR-18a/b-5p are negatively correlated (Figure 2F). RIP assay manifested that LINC00467 and miR-18a/b-5p were co-immunoprecipitated by Ago2 antibody (the main component of RISC), suggesting that they co-existed (Figure 2I). Afterward, StarBase hypothesized that LINC00467 binds miR-18a/b-5p (Figure 2J). Luciferase reporter assay using BC cells delineated that the transfection of miR-18a/b-5p mimics provoked an evident depletion of the luciferase activity of the LINC00467-WT vector whereas no alteration was detected in the LINC00467-Mut vector (Figure 2K). In conclusion, LINC00467 sponged miR-18a/b-5p in BC.

MAPK4 was targeted by miR-18a/b-5p in BC

To seek potential targets of miR-18a/b-5p, bioinformatics analysis was employed. Venn diagrams described the possible target gene of miR-18a-5p (right) and miR-18b-5p (left) according to starBase (Figure 3A). In addition, MAPK2 and ADAT2 were predicted to serve the target gene of both miR-18a-5p and miR-18b-5p. qRT-PCR suggested that MAPK4 was up-regulated in BC cells and tissues but ADAT2 indicated no differential expression between tumor tissues and normal tissues (Figure 3B-C). Besides, miR-18a/b-5p overexpression caused repression of mRNA and protein levels of MAPK4 (Figure 3D-F). And RIP assay unveiled that MAPK4 and miR-18a/b-5p expressions were enriched in Ago2 antibody (Figure 3G). Based on starBase, we discovered that miR-18a-5p and miR-18b-5p contained the same binding sequences on 3'UTR of MAPK4 (Figure 3H). Based on luciferase reporter assays, miR-18a/b-5p amplification weakened LINC00467 sponged miR-18a/b-5p in BC.
miRGLO-MAPK4-WT luciferase activity (Figure 3I). Taken together, MAPK4 was targeted by miR-18a/b-5p in BC.

**LINC00467 accelerated cell growth by regulating MAPK4 in BC**

Various cancers have been found to express MAPK4 as an oncogene. To confirm whether MAPK4 mediated the role of LINC00467 in BC, rescue assays were conducted. In the beginning, the inhibitive effect of LINC00467 deficiency on MAPK4 mRNA and protein levels was reduced by MAPK4 overexpression (Figure 4A-B). Moreover, MAPK4 amplification restored the inhibitory effect of LINC00467 knockdown on proliferation (Figure 4C-D). Over and above that, the inhibited impact on the migratory and invasive capability of BC cells resulting from LINC00467 suppression was restored by transfection of pcDNA3.1/MAPK4 (Figure 4E-G). In summary, LINC00467 accelerated tumorigenesis and progression by regulating MAPK4 in BC.

**Discussion**

Originating from mammary gland epithelial tissue, females are most likely to develop BC, ranking it second among malignant tumors worldwide (20). Thousands of women are diagnosed with BC every year (20,21). The typical characteristics of BC were distant metastasis and high invasiveness whereas the underlying mechanism of tumor initiation and progression remained unknown (22). Consequently, it is essential to clarify the potential molecular markers of BC.

LncRNAs can be categorized into 5 types: intergenic lncRNA, bidirectional lncRNA, intronic lncRNA, sense lncRNA and antisense lncRNA (6,23). Dysregulation of lncRNA has been recognized to be associated with tumorigenesis and progression (24). Functionally, lncRNA participated in the modulation of biological physiological activities. For instance, lncRNA HOTAIR modulated cell growth and metastasis of BC by miR-20a-5p/HMGA2 axis (25). LncRNA DANCR accelerated invasion by targeting TIMP2/3in prostate cancer cells (26). Similarly, in the present research, LINC00467 was highly expressed in BC tissues and cells. In the functional aspect, LINC00467 inhibition limited cell proliferation and metastasis. Those data suggested the carcinogenic property of LINC00467 in BC.

Mechanistically, increasing researches have proposed a ceRNA model that lncRNA sponged miRNA to modulate the expression of target gene, and thereby modulate tumor development in a direct or indirect way (27). LncRNA-UCA1 improved cell proliferation in esophageal cancer as a ceRNA of Sox4 (28). SNHG5 regulated the proliferation and migration of gastric cancer cells via miR-32-KLF4 axis (29-32). Based on this theory, we hypothesized that LINC00467 may also function as ceRNA in BC. A series of assays were implemented to validate our prediction. We found that LINC00467 is located in the cytoplasm, and after prediction and screening, we confirmed that miR-18a/b-5p binds to LINC00467. Especially, LINC00467 was negatively correlated with miR-18a/b-5p. Hereafter, mitogen-activated protein kinase 4 (MAPK4) was pre-
dicted and screened out. Then, we verified the combination between miR-18a/b-5p and MAPK4 in BC. On top of that, rescue assays illustrated that overexpression of MAPK4 recovered the inhibitive effect of downregulated LINC00467 on the proliferative, migratory and invasive capabilities.

To conclude, we testified that LINC00467 enhanced the proliferative, migratory and invasive ability of BC cells by targeting miR-18a/b-5p/MAPK4 axis, implying a potential tactic for BC treatment. Nevertheless, this is only a preliminary exploration of LINC00467, and the mechanism of LINC00467 deserves further investigation.

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Conflicts of interest
The authors declare that there are no competing interests in this study.

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