Long non-coding RNAs lnc-ANGPTL1-3:3 and lnc-GJA10-12:1 present as regulators of sentinel lymph node metastasis in breast cancer

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Abstract. Long non-coding RNAs (lncRNAs) participate in various biological processed involved in tumorigenesis, metastasis and proliferation. The aim of the present study was to identify candidate long non-coding RNAs (lncRNAs) involved in sentinel lymph node (SLN) metastasis in breast cancer. Specimens of SLNs were collected from patients with SLN metastasis via punch biopsy. Total RNA was extracted and RNA sequencing (RNA-seq) was conducted. Differential expression profiles of mRNAs and lncRNAs were obtained via bioinformatics analysis, and Gene Oncology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed on differentially expressed mRNAs. The expression levels of lncRNAs were analyzed via reverse transcription-quantitative PCR (RT-qPCR), and the regulation network of the lncRNAs to downstream microRNAs (miRs) and mRNAs was predicted. Based on RNA-seq results, six differentially expressed candidate lncRNAs were identified in patients with and without SLN metastasis: lnc-ANGPTL1-3:3, lnc-GJA10-12:1, lnc-ACAN-2:1, lnc-ZPB2-4:1, lnc-GATA3-16:1 and lnc-ACOX3-5:1. KEGG and GO analysis identified that the mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling pathways were the most enriched pathways. After RT-qPCR analysis, lnc-ANGPTL1-3:3 and lnc-GJA10-12:1 exhibited expression patterns that were consistent with those from RNA-seq. Moreover, receiver operating characteristic curve analysis demonstrated that lnc-ANGPTL1-3:3 and lnc-GJA10-12:1 expression levels had high sensitivity and specificity in the diagnosis of SLN metastasis, and that their expression levels were upregulated in patients with axillary lymph node metastasis. Further analysis revealed that lnc-GJA10-12:1 and lnc-ANGPTL1-3:3 were commonly involved in regulating the miR-302 family, including miR-302d-3p and miR-302c-3p, which together targeted AKT1. Additionally, lnc-ANGPTL1-3:3 was predicted to target miR-520b to regulate MAP3K2 expression. lnc-GJA10-12:1 was also predicted to target miR-34a-5p to regulate MAP2K1 and MAP3K9 expression levels, as well as miR-449a to regulate MAP2K1 expression. The results of the present study suggested that lnc-ANGPTL1-3:3 and lnc-GJA10-12:1 may potentially serve a role in SLN metastasis of breast cancer by regulating the PI3K/Akt and MAPK signaling pathways via targeting the miR-302 family, miR-520b-3p, miR-34a-5p and miR-449a. Thus, lnc-ANGPTL1-3:3 and lnc-GJA10-12:1 in SLN may serve as potential markers of breast cancer metastasis.

Introduction

In the Global Cancer Statistical Analysis of 2018, the number of breast cancer cases was 2,088,849, which was only lower than the number of lung cancer cases, however, due to the relatively good prognosis the number of deaths from breast cancer was much lower compared with those from lung cancer (1). The disease status of the axillary lymph nodes is the most important prognostic factor for patients with early-stage breast cancer (2). The most common predictors of node metastasis include lymphovascular invasion, age, tumor size and tumor grade; additionally, the predictive value is influenced by casting-type calcifications on mammography, receptor states, tumor location and the method of detection (3-8). However, no combination of these predictors of axillary lymph node status can currently replace histopathology and the surgical resection of lymph nodes (9). Moreover, the histopathological diagnosis of removed lymph nodes via axillary lymph node dissection is thought to be the most effective method to assess the disease (10). Unfortunately, the anatomical disruption caused by axillary lymph node dissection can result in side effects, such as nerve injury, lymphedema and other complications (11).

It has been demonstrated that breast cancer usually spreads to one or a few lymph nodes, known as the sentinel lymph nodes (SLNs), before spreading to other axillary nodes (12).
Therefore, the use of SLN identification and sampling procedures, referred to as SLN biopsies, can be a reliable treatment strategy in patients with early-stage breast cancer, as it reduces the need for axillary lymph node dissection and avoids the associated morbidity (13-17). Based on the aforementioned principles, SLN metastasis detection may be a key method for assessing the spread of breast cancer to the axillary lymph nodes. However, the underlying molecular mechanism of SLN metastasis in breast cancer remains unknown.

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts of >200 nucleotides in length. Previous studies have reported that lncRNAs participate in various biological processes involved in tumorigenesis, metastasis and proliferation (18-21). Therefore, lncRNAs may be considered as diagnostic biomarkers for numerous types of cancer, such as gastric, bladder, colorectum and prostate cancer (22-25). For example, lncRNA-BANCR has been associated with lymph node metastasis in colorectal cancer (26). The function of lncRNAs in breast cancer has also been studied. For instance, lncRNA-SNHG15 regulates microRNA (miRNA/miR)-211-3p and promotes cell proliferation, migration and invasion of breast cancer (27). Additionally, lncRNA-MAPT-AS1 inhibits cell proliferation and migration by regulating MAPT expression in breast cancer (28). Therefore, the aforementioned studies have indicated that lncRNAs may be important for regulating breast cancer processes and may be potential biomarkers of the disease. However, the role served by lncRNAs in SLN metastasis of breast cancer is yet to be elucidated.

To screen for markers that can be used to identify whether SLN has metastasized in breast cancer, in the present study, the SLNs of patients with breast cancer were collected and RNA sequencing (RNA-seq) was used to identify the key lncRNAs involved in SLN metastasis. Furthermore, reverse transcription-quantitative PCR (RT-qPCR) was conducted to analyze the expression levels of lncRNAs among specimens with or without SLN metastasis.

Materials and methods

Patient review and specimen collection. The database of the Peking University Shenzhen Hospital (Shenzhen, China) was reviewed between January 2018 and December 2018 in breast cancer, and patients in the early stages (I and II) of breast cancer were included in the present study. The patients were >18 years old, had not received surgical contraindication, chemotherapy or endocrine therapy, and had no other malignancy or immune diseases. Samples were obtained from 46 patients with an age range of 26-72 years. The mean age of 26 patients with SLN metastasis was 47±2.726 years and that of 20 patients without SLN metastasis was 47±3.674 years. All the patients were female. Patients were studied prospectively and data were collected with regards to age, metastasis-relevant parameters including disease history, tumor position, lymph node metastasis by sentinel lymph node biopsy (SLNB) and pattern of the axillary lymph nodes; and TNM stage according to the guidelines from the American Joint Committee on Cancer (29). Mammary areola injection was performed for the ultrasound contrast to search for SLNs. Subsequently, punch biopsy was conducted in order to collect the specimens. The patients underwent breast cancer resection and intraoperative SLN biopsy within 48 h of puncture. The present study was approved by the Institute Research Medical Ethics Committee of the Peking University Shenzhen Hospital, and informed consent was provided orally and in writing by all patients.

RNA-seq. Total RNA from the tissues was purified using an RNeasy Mini kit (Qiagen GmbH). RNA integrity was evaluated based on the RNA integrity number (RIN) value using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). RNA clean-up was performed using an RNA Clean XP kit (Beckman Coulter, Inc.) and the DNA residue was removed with an RNase-free DNase Set (Qiagen GmbH). The quality and concentration of the RNA were determined using NanoDrop 2000 (Thermo Fisher Scientific, Inc.). The ribosomal RNA was removed using a NEBNext rRNA Depletion kit (New England BioLabs, Inc.). Subsequently, 1 μg total RNA was used for library preparation using a VAHTSTM mRNA-seq v2 library Prep kit (Vazyme Biotech Co., Ltd.), according to the manufacturer's protocol. Briefly, the RNA was fragmented and the double strand cDNA was synthesized. Subsequently, end-polishing was performed and the cDNA fragments were ligated with adapters. The ligated cDNA was amplified using 15 cycles of PCR for 10 sec at 98°C, 30 sec at 60°C and 30 s at 72°C using a PCR master mixture (Illumina, Inc.) and subjected to universal PCR amplification using DNA polymerase I (New England BioLabs, Inc.) in order to obtain a library sufficient for sequencing. The Agilent Bioanalyzer 2100 was used to evaluate the quality of the library and an Illumina Hiseq 4000 (Illumina, Inc.) was used for RNA-seq. SOAP (http://soap.genomics.org.cn/) was used to calculate lncRNAs and mRNAs expression. Differentially expressed lncRNAs and mRNAs were screened using R software version 3.1 (30) according to the following criteria: False discovery rate (FDR) ≤0.001 and fold-change ≥2.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov/) was used to annotate the potential functions in various signaling pathways of the differentially expressed mRNAs. Subsequently, the functional annotation of parental genes was predicted via GO functional annotation (http://geneontology.org/page/go-enrichment-analyses). KEGG pathway annotation (http://www.genome.jp/kegg/pathway.html) was also used to identify the relevant pathways of the differentially expressed mRNAs.

RT-qPCR. Six lncRNAs with lengths of 500-3,000 bp were selected for assessment based on information from the IncRNASNP2 database (http://bioinfo.life.hust.edu.cn/IncrNASNP2/) and their predicted association with breast cancer. lncRNA expression levels were verified by RT-qPCR. RNA was extracted according to the above RNA-seq method. Gene expression was analyzed via RT-qPCR. RNA was reverse-transcribed into cDNA using the cDNA Synthesis Kit system (Promega Corporation) according to the manufacturer's protocol at 42°C for 15 min and then 95°C for 3 min. The qPCR reaction was performed using GoTaq qPCR Master mix (Promega Corporation), and qPCR amplification was performed using an ABI 7500 system (Applied Biosystems;
Thermo Fisher Scientific, Inc.). Thermocycling conditions for qPCR were as follows: 40 cycles of 20 sec at 95˚C followed by 30 sed at 60˚C and 30 sec at 72˚C. The relative mRNA expression levels were calculated by $2^{-\Delta\Delta Cq}$ method (31). Primers for qPCR are presented in Table I. GAPDH was used as an internal control.

Statistical analysis. The data from three experimental repeats was shown as mean ± standard deviation. Data comparisons were performed using unpaired Student’s t-tests and $\chi^2$ tests. Receiver operating characteristic (ROC) curve was drawn to analyze the specificity and sensitivity of lncRNA as a disease diagnosis. P<0.05 was considered to indicate a statistically significant difference. SPSS version 19.0 (IBM Corp.) and GraphPad Prism 8 (GraphPad Software, Inc.) were used to conduct the statistical analyses.

Results

Characteristics of the patients involved in the present study. The inclusion criteria for the present study were patients diagnosed with breast cancer and those suitable for axillary lymph node dissection. The reagent for contrast-enhanced ultrasound was subcutaneously injected near the mammary areola to search for the position of the SLN biopsy. The patients were categorized into SLN(+) or SLN(‑) metastasis groups and their characteristics are summarized in Table II. Analysis of the data identified that the positive rate of SLN metastasis was associated with the pattern of the axillary lymph nodes (P=0.0001), but was not associated with age, disease history, tumor position, molecular subtyping of breast cancer or TNM stage (all P>0.05; Table II).

Analysis of differentially expressed lncRNAs and mRNAs associated with SLN(+) and SLN(‑) metastasis. The differentially expressed lncRNAs and mRNAs in patients with SLN(+) metastasis and SLN(‑) metastasis are presented in Figs. 1 and 2 as volcano plots, scatter plots and heatmaps. A total of 2,335 differentially expressed lncRNAs were identified between patients with SLN(+/‑) metastasis; of these, 1,120 were upregulated and 1,215 were downregulated (Fig. 1A‑C; Table SI). Furthermore, the expression levels of lncRNAs on different chromosomes were both upregulated and downregulated (Fig. 1D). A total of 2,335 differentially expressed mRNAs were found between patients with SLN(+/‑) metastasis; of these, 1,120 were upregulated and 1,215 were downregulated (Fig. 2A‑C and Table SII). The Reads Per Kilobase of transcript per Million mapped reads profiles for lncRNAs and mRNAs were summarized and a fold-change >2 was used as a selection criterion to screen significantly differentially expressed lncRNAs and mRNAs. The top ten upregulated and downregulated lncRNAs and mRNAs are summarized in Tables III and IV, respectively.

Functional analysis of differentially expressed genes. GO annotation analysis was used to examine the processes in which the differentially expressed mRNAs were involved. The majority of these mRNAs were involved in ‘cell differentiation’, ‘cell proliferation’, ‘immune response’, ‘cell death’, ‘cell migration’ and ‘mitogen-activated protein kinase (MAPK) cascade’, but a few were also involved in the ‘Wnt signaling pathway’ and the ‘apoptotic signaling pathway’ (Fig. 2D). In order to assess the pathways in which the mRNAs were involved, KEGG pathway annotation analysis was conducted; the results revealed that the ‘PI3K/Akt signaling pathway’, ‘Rhoptry-associated protein1 (Rap1) signaling pathway’ and ‘MAPK signaling pathway’ were the main enriched pathways (Fig. 2E), suggesting that these were the primary signaling pathways in which mRNAs were involved.

Validation of lncRNA expression levels in patients with SLN(+) and SLN(‑) metastasis. In the present

Table I. Primers used for quantitative PCR.

| Gene         | Sequence, 5’→3’ | Product length, bp |
|--------------|-----------------|-------------------|
| Inc-ACAN-2:1 | F: CAAAGGGAGACCAAGGTAGG, R: GGGTGACGTTCAGATTCA | 149 |
| Inc-ZPBP2-4:1| F: CCTAGACGGCAGCTTAGGAC, R: TTGTGGGCAAGGTGAAACCC | 100 |
| Inc-GATA3-16:1| F: CGAGGGACCGAGTGTAAGCAA, R: CTCTAGGAAGTGGAGGCACC | 178 |
| Inc-ACOX3-5:1| F: TTCATCATCTCAGGGGAC, R: GTGTCAGCCTATGTCGACC | 96 |
| Inc-ANGPTL1-3:3| F: AGTTGGGACGCTAAGATGC, R: TGTTGCTTATCTCCGCTGTT | 109 |
| Inc-GJA10-12:1| F: TCCAAGCTGTCCTGTACGAAG, R: GCTGTATGCAAGCTGAAA | 99 |
| GAPDH       | F: GTCTCCCTGACTCTCAACGCG, R: ACCACCTCTGCTCTGTAGCCA | 235 |

F, forward; R, reverse; lnc, long non-coding RNA.
study, the differentially expressed lncRNAs, including lnc-ANGPTL1-3:3, lnc-GJA10-12:1, lnc-ACAN-2:1, lnc-ZPBP2-4:1, lnc-GATA3-16:1 and lnc-AOX3-5:1 were analyzed by qPCR. As shown in Fig. 3, the expression levels of lnc-ANGPTL1-3:3, lnc-GJA10-12:1, lnc-ACAN-2:1 and lnc-ZPBP2-4:1 were significantly downregulated in the SLN (+) group compared with the SLN (-) groups. However, only the expression results of lnc-ANGPTL1-3:3 and lnc-GJA10-12:1 were confirmed using RNA-seq, while the results for lnc-ACAN-2:1 and lnc-ZPBP2-4:1 expression were the opposite to those obtained via RNA-seq (Table SI). It was also found that lnc-GATA3-16:1 and lnc-AOX3-5:1 did not exhibit significant differential expression levels according to the qPCR results (Fig. 3).

Receiver operating characteristic (ROC) curve analysis identified that both lnc-ANGPTL1-3:3 and lnc-GJA10-12-1 had high area under the curve values (>0.8), which indicated that both lncRNAs were closely associated with SLN metastasis and may be suitable biomarkers for SLN diagnosis (Fig. 4). According to associations of lnc-ANGPTL1-3:3 and lnc-GJA10-12-1 expression levels with pathological features, lnc-ANGPTL1-3:3 and lnc-GJA10-12-1 expression levels were highly associated with patients with axillary lymph node metastasis or SLN metastasis diagnosis (Table V).

### Table II. Clinicopathological parameters of patients with (n=26) and without (n=20) SLN metastasis.

| Parameter                                      | SLN(+) | SLN(+) NA | SLN(‑) | SLN(‑) NA | P-value |
|------------------------------------------------|--------|-----------|--------|-----------|---------|
| Cases                                          | 26     | -         | 20     | -         |         |
| Mean age ± SD, years                           | 47.27±2.726 | -          | 47.60±3.674 | -          | 0.9414  |
| Disease history, year                          |        |           |        |           |         |
| ≤1                                              | 19     | -         | 15     | -         | 0.8211  |
| >1                                              | 7      | -         | 5      | -         |         |
| Tumor position                                 |        |           |        |           |         |
| Left breast                                    | 17     | -         | 13     | -         | 0.9783  |
| Right breast                                   | 9      | -         | 7      | -         |         |
| Lymph node metastasis by SLN biopsy            |        |           |        |           |         |
| +                                               | 26     | -         | 1      | -         | <0.0001 |
| -                                               | 0      | -         | 19     | -         |         |
| Pattern of the axillary lymph nodes            |        |           |        |           |         |
| Non-suspicious                                 | 1      | -         | 12     | -         |         |
| Suspicious                                     | 25     | -         | 8      | -         |         |
| Human epidermal growth factor receptor 2       |        |           |        |           |         |
| +                                               | 3      | 9         | 1      | 1         | 0.5162  |
| -                                               | 14     | 18        |        |           |         |
| Triple negative breast cancer                  |        |           |        |           |         |
| +                                               | 0      | 9         | 3      | 1         | 0.2310  |
| -                                               | 17     | 16        |        |           |         |
| Luminal A                                       |        |           |        |           |         |
| +                                               | 0      | 9         | 2      | 1         | 0.4873  |
| -                                               | 17     | 17        |        |           |         |
| Luminal B                                       |        |           |        |           |         |
| +                                               | 14     | 9         | 13     | 1         | 0.5631  |
| -                                               | 3      | 6         |        |           |         |
| Tumor size, cm                                 |        |           |        |           |         |
| <2                                              | 1      | -         | 5      | -         | 0.0949  |
| ≥2                                              | 25     | -         | 15     | -         | 0.0531  |
| N stage                                         |        |           |        |           |         |
| N0                                              | 7      | -         | 11     | -         | 0.0531  |
| N1                                              | 19     | -         | 9      | -         |         |
| M stage                                         |        |           |        |           |         |
| M0                                              | 25     | -         | 20     | -         | 1.0000  |
| M1                                              | 1      | -         | 0      | -         |         |

T, tumor; N, node; M, metastasis; SLN, sentinel lymph node; NA, Not available.
Co-expression network analysis of lncRNAs and mRNAs. After ROC analysis and verification using qPCR, Inc-ANGPTL1-3:3 and Inc-GJA10-12:1 were selected for functional prediction analysis, in which the targets of these lncRNAs were predicted based on the competing endogenous RNA (ceRNA) principle (32). The target genes of lncRNAs, which were significantly downregulated in the SLN (+) group in mRNA sequencing compared with the SLN (−) group, in the top five pathways in the KEGG pathway analysis based on the ceRNA principle were used to establish network regulatory maps. The miRNAs that interacted with lncRNAs were predicted based on the sequences of lncRNAs, and the targets of miRNAs were thus also predicted. Under this principle, lncRNA-miRNA-mRNA cascades were identified. The top ten miRNAs and their corresponding targeted mRNAs associated with either Inc-ANGPTL1-3:3 or Inc-GJA10-12:1 are presented in Tables VI and VII, respectively. Moreover, the interactions are summarized in the regulation network illustrated in Fig. 5. The results demonstrated that the miRNAs associated with Inc-GJA10-12:1 and Inc-ANGPTL1-3:3 were commonly involved in regulating the miR-302 family, including miR-302d-3p and miR-302c-3p, which together targeted AKT1. Specifically, Inc-ANGPTL1-3:3 was predicted to target miR-520b to regulate MAP3K2 expression. In addition, Inc-GJA10-12:1 was predicted to target miR-34a-5p to regulate MAP2K1 and MAP3K9 expression, as well as miR-449a to regulate MAP2K1 expression. Therefore, the results indicated that Inc-GJA10-12:1 and Inc-ANGPTL1-3:3 may be involved in signal conditioning networks to control SLN metastasis in breast cancer.

Discussion
In the present study, RNA-seq was performed to identify the lncRNAs involved in the SLN metastasis processes in breast cancer. According to the qPCR results, Inc-ANGPTL1-3:3
and lnc-GJA10-12:1 were downregulated in SLN metastasis specimens. The first identified lncRNA, lnc-ANGPTL1-3:3, is derived from the angiopoietin-like 1 (ANGPTL1) gene, which is a potent regulator of angiogenesis and can interact with integrin α1β1 to suppress hepatocellular carcinoma angiogenesis and metastasis via inhibition of Janus kinase 2/STAT3 signaling (33). Furthermore, the second lncRNA, lnc-GJA10-12:1, is derived from the gap junction protein...
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Thus, the results of the current study indicated that both lncRNAs may potentially serve important roles in the regulation of SLN metastasis in patients with breast cancer.

Table III. Top ten differentially expressed lncRNAs.

| lncRNAs            | Length, bp | SLN(-), RPKM | SLN(+), RPKM | log₂ ratio, SLN(+)/SLN(-) | Upregulation/downregulation (SLN+/SLN-) | FDR   |
|--------------------|------------|---------------|---------------|---------------------------|----------------------------------------|-------|
| lnc-DDX47-3:1      | 2,740      | 0.391338128   | 225.814620700 | 9.172507504               | Up                                     | 5.90x10⁻¹⁷⁰ |
| LINC01087:1        | 3,516      | 2.744709397   | 779.663719700 | 8.150054784               | Up                                     | 0.00x10⁹  |
| lnc-SLC39A11-10:48 | 5,836      | 0.183733117   | 50.494217380  | 8.102362680               | Up                                     | 3.28x10⁻⁷⁹ |
| TBILA:3            | 1,937      | 0.553570713   | 133.185239200 | 7.910450865               | Up                                     | 5.70x10⁻⁶⁹ |
| lnc-CCDC74A-8:1    | 1,521      | 3.524873343   | 789.454094100 | 7.807140148               | Up                                     | 0.00x10⁹  |
| lnc-DTNB1P1-16:4   | 73,231     | 0.014642248   | 2.978646596   | 7.668376078               | Up                                     | 6.65x10⁻⁵⁸ |
| lnc-DHCR24-1:1     | 503        | 4.263484974   | 748.474609300 | 7.455776394               | Up                                     | 4.34x10⁻¹⁰⁰ |
| lnc-CCDC74A-11:1   | 5,658      | 3.411240099   | 562.345523100 | 7.365016729               | Up                                     | 0.00x10⁹  |
| lnc-ADPRHL1-5:1    | 509        | 2.106613892   | 309.046682100 | 7.196755050               | Up                                     | 4.72x10⁻⁴¹ |
| lnc-IDNK-10:1      | 5,970      | 0.179609124   | 23.011640110  | 7.001359361               | Up                                     | 1.47x10⁻³⁵ |
| lnc-MB-6:1         | 1,733      | 69.298237020  | 0.605134684   | -6.839418562              | Down                                   | 3.73x10⁻⁵¹ |
| lnc-P2RX3-4:1      | 2,727      | 35.388332380  | 0.384561206   | -5.523916736              | Down                                   | 1.18x10⁻²⁴ |
| lnc-RANBP3L-4:2    | 3,613      | 21.071386510  | 0.290256963   | -6.181810760              | Down                                   | 4.35x10⁻¹⁹ |
| lnc-TFF3-1:1       | 1,328      | 184.093492300 | 3.158730444   | -5.864953654              | Down                                   | 4.06x10⁻⁶¹ |
| lnc-TNFRSF13C-1:1  | 1,078      | 53.712791690  | 0.972818560   | -5.786951142              | Down                                   | 3.67x10⁻¹⁴ |
| lnc-UUDTL2-11:1    | 549        | 99.609453600  | 1.910197464   | -5.704488892              | Down                                   | 2.58x10⁻¹³ |
| lnc-N4BP2-3:4      | 530        | 103.180358500 | 1.978676241   | -5.704488892              | Down                                   | 2.58x10⁻¹³ |
| lnc-MMP23B-1:1     | 1,808      | 27.874183710  | 0.580032305   | -5.586652492              | Down                                   | 3.63x10⁻¹² |
| lnc-NDUFA10-7:1    | 1,024      | 49.215355610  | 1.024119539   | -5.586652492              | Down                                   | 3.63x10⁻¹² |
| lnc-HACL1-2:1      | 1,778      | 25.932203740  | 0.589819127   | -5.458328395              | Down                                   | 4.94x10⁻¹¹ |

RPKM, Reads Per Kilobase of transcript per Million mapped reads; lncRNA, long non-coding RNA; FDR, false discovery rate; SLN, sentinel lymph node.

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**α10 (GJA10) gene** (34). Thus, the results of the current study indicated that both lncRNAs may potentially serve important roles in the regulation of SLN metastasis in patients with breast cancer.

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Figure 3. Quantitative PCR analysis of the six candidate lncRNAs between the SLN(+) and SLN(-) metastasis specimens. Relative expression levels of (A) lnc-GJA10-12:1, (B) lnc-ANGPTL1-3:3, (C) lnc-GATA3-16:1, (D) lnc-ACOX3-5:1, (E) lnc-ACAN-2:1 and (F) lnc-ZPBP2-4:1. *P<0.05; **P<0.01; ns, not significant. SLN, sentinel lymph node; lncRNA, long non-coding RNA.
LncRNAs interact with miRNAs and can activate or suppress their functions, resulting in increased or decreased expression of their downstream targeted mRNAs (35). The GO and KEGG analyses in the present study revealed that the MAPK and PI3K/Akt signaling pathways may serve critical roles in SLN metastasis in breast cancer. Additionally, bioinformatics analysis predicted the targeted miRNAs, as well as the mRNAs. It was found that miR-302c-3p and miR-302d-3p were each targeted by both candidate lncRNAs, lnc-ANGPTL1-3:3 and lnc-GJA10-12:1, whereas miR-302a-3p and miR-302b-3p...
were targeted by lnc-ANGPTL1-3:3 alone to regulate the SLN metastasis processes of breast cancer; therefore, the miR-302 family may serve an important role in SLN metastasis of lncRNA-regulated breast cancer. A previous study reported that miR-302c-3p suppressed tumorigenesis and development in hepatocellular carcinoma by targeting tumor necrosis factor receptor-associated factor 4 (36). Furthermore, AKT1 serves a crucial role as a regulator of cell invasion and proliferation in breast cancer (37-39). It has also been shown that the inhibition of AKT1 induces breast cancer metastasis via β-catenin nuclear accumulation mediated by the epidermal growth factor receptor (40). Based on the present results, it was speculated that Inc-ANGPTL1-3:3 and Inc-GJA10-12:1 may regulate two AKT1-targeting miRNAs, miR-302c-3p and miR-302d-3p, and thus control SLN metastasis in breast cancer.

MAPK, which is an important transmitter of signals from the cell surface to the nucleus, serves an important role in the development and metastasis of cancer, and it has been reported that chemokine ligand 28 promotes the growth and metastasis of breast cancer via MAPK-mediated pro-metastatic and anti-apoptotic mechanisms (41). A previous study revealed that miR-34c-3p regulated cancerous development and epithelial-mesenchymal transition of triple-negative breast cancer cells by regulating MAP3K2 signaling (42). Furthermore, in

Table V. Association of Inc-ANGPTL1-3:3 and Inc-GJA10-12:1 with patient clinicopathological features.

| Groups                      | Inc-ANGPTL1-3:3 expression | χ²-value (P-value) | Inc-GJA10-12:1 expression | χ²-value (P-value) |
|-----------------------------|----------------------------|-------------------|---------------------------|-------------------|
| Age, years                  |                            |                   |                           |                   |
| <45                         | 22                         | 69.65±37.93 (0.8166 | 0.4185)                   | 42.19±14.43 (0.1345) |
| ≥45                         | 24                         | 40.52±15.60 (0.1345) | 19.23±5.765 (0.0074)      |                   |
| SLN metastasis              |                            |                   |                           |                   |
| +                           | 26                         | 21.32±5.312 (0.0009) | 19.61±4.827 (0.0074)      |                   |
| -                           | 20                         | 188.7±93.27 (0.0074) | 71.16±31.90 (0.0074)      |                   |
| Pattern of the axillary lymph nodes |                   |                   |                           |                   |
| Non-suspicious              | 13                         | 149.2±67.41 (2.944)     | 0.0052)                   | 77.07±23.62 (0.0052) |
| Suspicious                 | 33                         | 21.16±10.50 (2.944)     | 0.0052)                   | 13.92±3.269 (0.0052) |
| Tumor size, cm              |                            |                   |                           |                   |
| <2                          | 6                          | 24.51±11.61 (0.5866)     | 0.5605)                   | 12.47±5.894 (0.5866) |
| ≥2                          | 40                         | 60.29±23.34 (0.5866)     | 0.5605)                   | 32.87±8.677 (0.5866) |

Inc, long non-coding RNA; SLN, sentinel lymph node.

Table VI. Top ten miRNAs and corresponding targeted mRNAs associated with Inc-ANGPTL1-3:3.

| miRNA       | Specific binding sites predicted | mRNA |
|-------------|----------------------------------|------|
| hsa-miR-302c-3p | 306, 1284, 1993, 2215          | ESR1, CCND1, BMPR2, AKT1, MICA |
| hsa-miR-548d-5p | 960, 1053, 1491, 2264          | PPARA |
| hsa-miR-302a-3p | 306, 1281, 2215                | CCND1, CCND1, CDK4, CDKN1A, LEFTY1, LEFTY2, DAZAP2, SLAIN1, TOB2, NR2F2, AKT1, TAC1, CDK2, BM11, CDK1, MBD2, NANO |
| hsa-miR-302b-3p | 307, 1284, 2215                | CCND2, BMI1, TGFBR2, RHOC, AKT1, HDAC4, EGFR, ERBB4, AKT2 |
| hsa-miR-373-3p | 304, 1281, 2215                | RAD52, RAD23B, XPA, MRE11A, CD44, CD44, LATS2, LATS2, LATS2, LATS2, RECK, VEGFA, TXNIP, RABEP1, MYC, MBD2, RASSF1, MTOR, SIRT1, NFIB, DKK1, TGFBR2, BTG1, LEFTY1, TNFAIP1, LEFTY2 |
| hsa-miR-520c-3p | 307, 1282, 2218                | TRPS1, KLF13, VEGFA, MBNL2, NR4A2, ERBB4, CDK2, CCND2, LEFTY1, LEFTY2, AKT1 |
| hsa-miR-520d-3p | 306, 1284, 2218                | CDKN1A, MICA, LAMTOR5, IL8, MAP3K2, CCND1, CD46, PFKP |
| hsa-miR-520b   | 308, 1283, 2219                | CDKN1A, MICA, LAMTOR5, IL8, MAP3K2, CCND1, CD46, PFKP |
| hsa-miR-520a-3p | 304, 1282, 2216                | CDKN1A, PFKP |
| hsa-miR-520d-3p | 304, 1282, 2216                | EPFA2, EPFA2 |

miRNA/miR, microRNA.
non-small cell lung cancer, miR-520a-3p suppresses cell proliferation, metastasis and apoptosis by targeting MAP3K2 (43), and miR-449a inhibits cell invasion by suppressing MAP2K1 (44). Based on the aforementioned studies and the present results, it was indicated that in SLN metastasis of breast cancer Inc-ANGPTL1-3:3 may target miR-520b to regulate MAP3K2 expression, and that Inc-GJA10-12:1 may target miR-449a to regulate MAP2K1 expression.

At present, there are no mature animal models in vivo and cell models in vitro for the study of SLN metastasis. Therefore, the specific mechanism of Inc-ANGPTL1-3:3 and Inc-GJA10-12:1 regulating SLN metastasis were not investigated in the present study. In addition, the expression levels of Inc-ANGPTL1-3:3 and Inc-GJA10-12:1 in breast cancer in situ were not investigated in the present study. The aforementioned limitations should be investigated by future studies.

In conclusion, Inc-ANGPTL1-3:3 and Inc-GJA10-12:1 may be important regulators of SLN metastasis in breast cancer via their downregulated expression. Moreover, the present results suggested that Inc-ANGPTL1-3:3 and Inc-GJA10-12:1 may regulate the PI3K/Akt and MAPK signaling pathways by targeting the miR-302 family, miR-520a-3p, miR-34a-5p and miR-449a, which may serve a crucial role in SLN metastasis of breast cancer. It was also demonstrated that Inc-ANGPTL1-3:3
and Inc-GJA10-12:1 in SLN may serve as potential markers of breast cancer metastasis. Hence, future studies should further investigate Inc-ANGPTL1-3:3 and Inc-GJA10-12:1 expression in breast cancer tissues to provide a basis for the surgical treatment of breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

DS and JZ conceived and designed the experiments. DS, JZ, and WW performed the experiments. LL, JL, and XL analyzed the experimental data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institute Research Medical Ethics Committee of the Peking University Shenzhen Hospital (Shenzhen, China), and informed consent was provided orally and in writing by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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