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Integrated analyses of long noncoding RNAs and mRNAs in the progression of breast cancer

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Abstract

Objective: The objective was to explore the expression and potential functions of long noncoding RNA (lncRNA) and mRNAs in human breast cancer (BC).

Methods: Differentially expressed lncRNAs and mRNAs were identified and annotated in BC tissues by using the Agilent human lncRNA assay (Agilent Technologies, Santa Clara, CA, USA) and RNA sequencing. After identification of lncRNAs and mRNAs through quantitative reverse transcription polymerase chain reaction, we conducted a series of functional experiments to confirm the effects of knockdown of one lncRNA, TCONS_00029809, on the progression of BC.

Results: We discovered 238 lncRNAs and 200 mRNAs that were differentially expressed in BC tissues and para-carcinoma tissue. We showed that differentially expressed mRNAs were related to biological adhesion and biological regulation and mainly enriched in cytokine-cytokine receptor interaction, metabolic pathways, and PI3K-Akt signaling pathway. We created a protein–protein interaction network to analyze the proteins enriched in these pathways. We demonstrated that silencing of TCONS_00029809 remarkably inhibited proliferation, invasion, and migration of BC cells, and accelerated their apoptosis.

Conclusions: We identified a large number of differentially expressed lncRNAs and mRNAs, which provide data useful in understanding BC carcinogenesis. The lncRNA TCONS_00029809 may be involved in the development of BC.

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Long noncoding RNA, breast cancer, TCONS_00029809, migration and invasion, apoptosis, differential expression

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Introduction

Breast cancer (BC) has become one of the most widespread tumors among women in the world, and its incidence is increasing.[1,2] An estimated 2.1 million new cases of BC occurred worldwide in 2018.[3] BC has the highest mortality rate of tumors in women, and metastasis and recurrence are the leading causes of death.[4,5] Although a variety of treatments such as surgery, chemotherapy, radiation therapy, and endocrine therapy can suppress the growth of primary tumors, metastasis remains the greatest challenge in the treatment of BC.[6,7] About 90% of all cancer-related deaths are associated with tumor metastasis.[8] At present, the early diagnosis of BC is very difficult due to the lack of sensitive diagnostic markers.[9] In patients diagnosed with BC, most have already had lymph node metastasis or even distant metastasis in multiple organs, and the clinical treatment and prognosis are unsatisfactory.[10,11] Therefore, an in-depth study of BC metastasis-related genes and signaling pathways is of great importance for accurate and early clinical diagnosis, judgment of efficacy, and prediction of progress. This information could help identify new therapeutic targets for BC.

Long noncoding RNAs (lncRNAs) are endogenous single-stranded RNAs that do not encode proteins, with a transcription length from 200 bp to 1000 kb.[12,13] LncRNA is transcribed by RNA polymerase II without an open reading frame.[14] Studies have shown that lncRNA can regulate DNA methylation, histone modification, and chromosome remodeling via epigenetics, transcriptional regulation, and post-transcriptional regulation, among others.[15,16] Moreover, lncRNA participate in various vital biological regulatory processes, including X chromosome silencing, genomic imprinting, transcriptional activation, transcriptional interference, and intracellular transport.[17,18] Numerous studies have shown that lncRNAs can affect multiple biological activities, such as cell proliferation, apoptosis, invasion, and migration.[19–21] Currently, genome-wide association studies (GWAS) in BC have uncovered a large number of cancer-related lncRNAs.[22–24] The tissue-specific expression of lncRNAs and the GWAS results indicate that lncRNAs, as a class of new biomarkers, have numerous potential applications in cancer therapy. However, the exact functions and mechanisms of the many lncRNAs involved in the pathogenesis of BC have not been fully elucidated. Therefore, there is an urgent need to investigate the regulatory mechanism of lncRNAs in BC.

In the current study, we established the expression profiles of lncRNAs and mRNAs in BC tissues and paired adjacent normal tissues using the Agilent human lncRNA assay (Agilent Technologies, Santa Clara, Ca, USA) and RNA sequencing, respectively. We predicted the potential functions and pathways of differentially expressed mRNAs by conducting Gene Ontology (GO) and Kyoto Encyclopedia

of Genes and Genomes (KEGG) pathway analyses. We assessed the expression levels of the most promising lncRNAs and mRNAs in BC. Moreover, we demonstrated the effects of knockdown of one such promising lncRNA, TCONS_00029809, on the malignant behavior of BC cells. Our study generated a large amount of data, which might include underlying markers and therapeutic targets for the diagnosis and therapy of BC.

Materials and Methods

Clinical samples

BC tissue and paired para-carcinoma tissue samples (3 cm from the tumor) were collected from patients with BC who underwent surgical treatment in Nanfang Hospital, Southern Medical University. The patients did not receive iodine ablation or other treatment before surgery. The pathologic classification of BC was confirmed by two pathologists using a double-blind method. In the current study, we focused on breast invasive ductal carcinoma (IDC) tissues. Within 15 minutes of collection, samples were stored in liquid nitrogen to prevent secondary freezing-thawing and the samples were transported for analysis on dry ice. All patients provided written informed consent for collection of the samples. This research was approved by the Ethics Committee of the Nanfang Hospital, Southern Medical University (approval number: NFEC-202101-K14-01).

RNA extraction and purification

In accordance with the manufacturer's instructions, total RNA was extracted using the mirVana miRNA Isolation Kit (cat. no. AM1561, Ambion, Austin, TX, USA). A NanoDrop ND-1000 spectrophotometer (Nanodrop/Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the purity and concentration of total RNAs. The integrity of RNA was confirmed by electrophoresis on a denaturing agarose gel. All RNA samples were checked for purity (optical density 260/280) using a Nanodrop spectrophotometer, and only qualifying RNAs were used. The total RNA was then analyzed using an Agilent Bioanalyzer 2100 (Agilent Technologies).

Agilent human lncRNA assay

Expression profiling of lncRNAs was conducted using the Agilent human lncRNA microarray V.2.0 platform (GPL18109; Agilent Technologies). The microarray analysis was performed by Beijing Genomics Institute/HuaDa-Shenzhen (Shenzhen, China). Briefly, ribosomal RNA was depleted, and depletion was monitored using the bioanalyzer. We used the Low Input Quick Amp Labeling Kit (cat. no. 5190-2305; Agilent Technologies) to amplify and label the total RNA, following by purification using RNeasy mini kit (cat. no. 74106; Qiagen GmBH, Hilden, Germany). Hybridization was conducted using 1.65 μg of Cy3-labeled cRNA in the Gene Expression Hybridization Kit (cat. no. 5188-5242; Agilent Technologies) at 65°C for 17 hours. The slides were then scanned with the Agilent Microarray Scanner (cat. no. G2565CA; Agilent Technologies) and the raw data were then extracted and counted. Quantile normalization and subsequent data processing were performed using Agilent Gene Spring Software 11.5 (Agilent Technologies). Heat maps representing differentially regulated genes were generated using Cluster 3.0 software (Michiel de Hoon, Center for Computational Biology and Bioinformatics, Columbia University, New York, NY, USA). Exogenous RNAs developed by External RNA Controls Consortium were used as controls.

The exosomal lncRNA microarray process was performed by KangChen BioTech Co. Ltd. (Shanghai, China).

RNA sequencing

RNA sequencing was conducted by Luen Chuan Biotechnology Co. Ltd. (Hangzhou, China) using an Illumina Novaseq^{TM} 6000. The mRNA library was constructed using purified total RNA, and the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and Agilent 2100 bioanalyzer were used to determine the concentration and size of libraries. The Illumina HiSeq 2000 System (Illumina Inc., San Diego, CA, USA) was then applied to quantitatively analyze the library. Finally, mRNA expression was analyzed and differentially expressed mRNAs were identified.

Cell culture

Human breast cells (MCF10A) and two BC cell lines (MCF7 and MDA-MB-231) were obtained from ATCC (Rockville, MD, USA). MCF10A cells were grown in DMEM/F12 medium including 5% fetal bovine serum (FBS, Gibco, NY, USA), 20 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin. MCF7 and MDA-MB-231 cells were cultured in DMEM (Gibco) with 10% FBS (Gibco). All cells were incubated at 37°C in a 5% CO_2 incubator.

Cell transfection

Negative control (NC)-siRNAs and TCONS_00029809 siRNAs (si-TCONS_00029809) were acquired from GenePharma (Shanghai, China). MCF7 and MDA-MB-231 cells (1×10^5 cells/well) were evenly and gently placed into six-well plates and cultured for 12 hours. The cells were then transfected with NC and si-TCONS_00029809 using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Hierarchical clustering analysis

We conducted hierarchical clustering analysis using the R language package.[25] In the cluster diagrams, different colors were used to distinguish different information, and the clustering pattern was adopted to confirm the different experimental conditions.

GO analysis

We defined the GO enrichment of differentially expressed mRNAs between para-carcinoma and BC tissues using the bioinformatics tool DAVID, version 6.8 (https://david.ncifcrf.gov/).[26,27]

KEGG analysis

We also analyzed the KEGG enrichment pathways of differentially expressed mRNAs between para-carcinoma and BC tissues using KOBAS 2.0 software (http://kobas.cbi.pku.edu.cn/).[28]

Protein-protein interaction (PPI) network analysis

PPIs of differentially expressed mRNAs between para-carcinoma and BC tissues were predicted and analyzed using STRING (http://string-db.org/) with a comprehensive score >0.9.[29]

Quantitative reverse transcription polymerase chain reaction assay (RT-qPCR)

We isolated total RNA from BC and para-carcinoma tissues, and from treated MCF10A, MCF7, and MDA-MB-231 cells using TRIzol® reagent (Takara, Dalian, China). The total RNAs were used to synthesize cDNAs using an iScript

cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA).[30] cDNAs were then used as for quantitative analysis of genes using SYBR Green PCR Master (Takara). The obtained data were calculated using the $2-\Delta\Delta CT$ method. All primer sequences are listed in Table 1.

CCK-8 assay

MCF7 and MDA-MB-231 cells (2×10^3 cells/well) in each group were inoculated into 96-well plates and incubated for 48 hours at 37°C, followed by the addition of 10 μL CCK8 solution (Beyotime, Haimen, China) for 3 hours at 37°C. The optical density at 450 nm was then confirmed using a microplate reader (Biotek, VT, USA).

Flow cytometry

MCF7 and MDA-MB-231 cells in each group were harvested by digestion. After centrifugation ($1000 \times g$ for 5 minutes), the cell density was adjusted to 1×10^9/L using phosphate-buffered saline, followed by the addition of 5 μL Annexin V-FITC and 5 μL propidium iodide for 10 minutes in the dark. Apoptotic cells were monitored and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Transwell assay

For cell migration analysis, treated MCF7 and MDA-MB-231 cells in serum-free medium were inoculated into the upper well of an 8-μm Transwell chamber (Cat.

Table 1. Primer sequences used in quantitative reverse transcription polymerase chain reaction assay.

ID	Sequence (5′–3′)
GAPDH	Forward: TGTTCGTCATGGGTGTGAAC
GAPDH	Reverse: ATGGCATGGACTGTGGTCAT
TCONS_00028064	Forward: GAGAACCAACGAGCTGAGC
TCONS_00028064	Reverse: GTATGGTGATGCTGCTGGTG
TCONS_00028800	Forward: CAAAAGAGGCTCCCCAAACC
TCONS_00028800	Reverse: GCAAAGGAGCTGAGCAGTTT
TCONS_00029809	Forward: CGGAGCTGTGACACCTACTT
TCONS_00029809	Reverse: TTGGGATGGCTTGTTTCTGC
TCONS_00007846	Forward: GGCCGATTTGAGGACCTAGA
TCONS_00007846	Reverse: CTCTCCTCCTCCCCAACTTG
TCONS_00010076	Forward: GGCCTTTGTACCAGCTCTTT
TCONS_00010076	Reverse: ACATGAGGCAGAACGGAAGA
TCONS_l2_00028765	Forward: GTCCATCTGTCTGTCTGCCT
TCONS_l2_00028765	Reverse: GTGACCTCTCTTTGGCTCCT
PPAPDC1A	Forward: TGGCTTCACGACGTTCTACT
PPAPDC1A	Reverse: CCAGTGATGCTTGTAGTCGC
COL10A1	Forward: AAGGGAGAAAGAGGACCTGC
COL10A1	Reverse: TGGCCCTGTCTCACCTTTAG
ADAMDEC1	Forward: AAGATCCACGACCATGCTCA
ADAMDEC1	Reverse: CATCACACAACTGCCAGAGG
SPHKAP	Forward: GAGGGAGACAGAACCAAGCT
SPHKAP	Reverse: TGAAGAATCGGGCACTCTGT
CLCA4	Forward: CCAAAGCGAACCCAGAAACA
CLCA4	Reverse: CGCCTGCACCATTATCCAAA
ALDH1L1	Forward: GTTGGGGTTTGTGGCATCAT
ALDH1L1	Reverse: CCGGCCTTTAATGTCAGCTC

No. 3422; Costar, Cambridge, MA, USA), and DMEM medium supplemented with 10% FBS was added into the lower chamber. After 24 hours at 37°C, migrated cells were fixed and stained using 5% crystal violet, and photographed using an inverted optical microscope. For cell invasion analysis, the upper chambers were pretreated with Matrigel (BD Biosciences) before the addition of cells at 37°C for 30 minutes.

Statistical analysis

All experiments were repeated at least three times and all measurements are presented as means ± standard deviations. Statistical analysis was conducted using SPSS 23.0 statistical software (IBM Corp., Armonk, NY, USA). Numerical data were analyzed with χ^2 tests and differences in continuous data between groups were analyzed using Student's *t*-tests. $P < 0.05$ indicates statistical significance.

Results

Expression pattern analysis of differentially expressed lncRNAs in BC

Three BC and three adjacent normal tissues were available for study. The pathological types of BC lesions include IDC, ductal carcinoma in situ (DCIS), invasive lobular carcinoma (IIC), and invasive mucinous carcinoma (IMC). Because IDC is the most common type of pathology in BC, we focused on breast IDC tissues. Differentially expressed lncRNAs were determined using the Agilent human lncRNA assay in breast IDC and para-carcinoma tissues. Based on the data, we first applied principal component analysis (PCA) to conduct dimensionality reduction in different samples (Figure 1A). Pearson correlation coefficient analysis was used to assess biological repeatability and correlation (Figure 1B). Next, in accordance with the results of lncRNA expression, expression profiles were prepared using volcano plots and hierarchical clustering analysis. As shown in Figure 1C and 1D, there were 1855 differentially expressed lncRNAs, including 951 upregulated and 904 downregulated lncRNAs between para-carcinoma and BC tissues, with a criterion of $P < 0.05$. We also discovered 841 differentially expressed lncRNAs (452 upregulated and 389 downregulated lncRNAs) between para-carcinoma and BC tissues, based on $P < 0.05$ and fold change ≥ 2 or ≤ 0.5 (Figure 1E). Hierarchical clustering revealed 238 differentially expressed lncRNAs ($P < 0.01$, fold change ≥ 2 or ≤ 0.5) from the three patients with BC, and among the lncRNAs, 157 lncRNAs were upregulated and 81 downregulated in BC tissues (Figure 1F). Thus, our results showed numerous lncRNAs potentially involved in BC.

Analysis of differentially expressed mRNAs in BC

We applied mRNA sequencing to screen differentially expressed mRNAs in BC. PCA was also conducted to analyze dimensionality reduction in different samples (Figure 2A). Pearson correlation coefficient analysis was used to assess biological repeatability and correlation. (Figure 2B). Through mRNA sequencing analysis, we discovered 1956 differentially expressed mRNAs in BC tissues compared with para-carcinoma tissues ($P < 0.05$). The volcano plot and heat map show the expression status of mRNAs (856 upregulated and 1100 downregulated) between para-carcinoma and BC tissues ($P < 0.05$, Figure 2C and 2D). To further screen differentially expressed mRNAs, 881 mRNAs were screened according to the screening conditions $P < 0.05$ and fold change ≥ 2 or ≤ 0.5, of which 325 were upregulated and 556 were downregulated in BC tissues

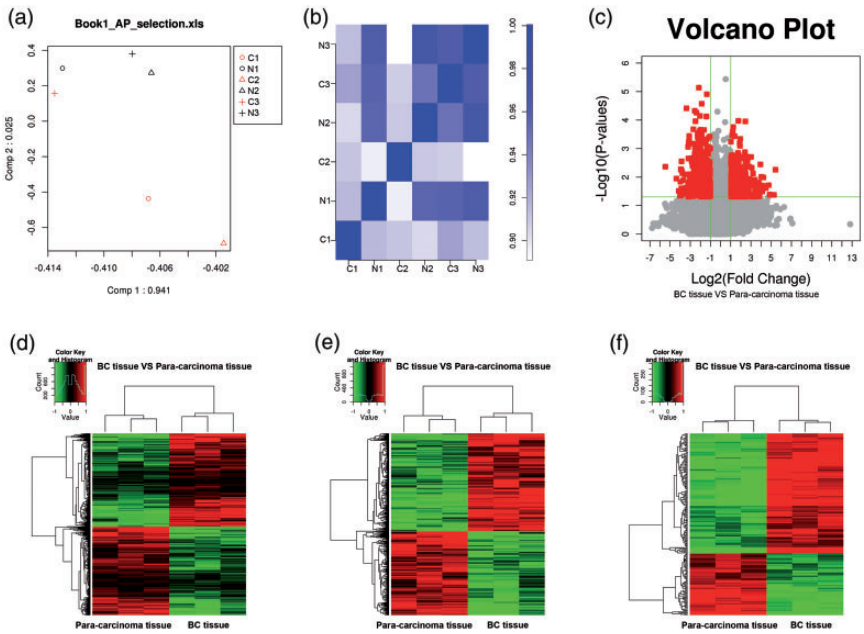

Figure 1. Expression pattern analysis of long noncoding (lnc)RNAs that were differentially expressed in breast cancer (BC). (a) Principal component analysis of each sample. (b) Pearson correlation coefficient analysis was applied to show the pairwise comparison of lncRNAs in each sample. (c) Volcano plot showing differentially expressed lncRNAs between para-carcinoma and BC tissues ($P < 0.05$). The red points indicate lncRNAs ($P < 0.05$) and the gray points indicate nondifferentially expressed lncRNAs. Hierarchical cluster analyses of lncRNAs ($P < 0.05$). Hierarchical clustering analyses of lncRNAs between para-carcinoma and BC tissues (d) with $P < 0.05$, (e) with $P < 0.05$ and fold change ≥ 2 or ≤ 0.5, and (f) with $P < 0.01$ and fold change ≥ 2 or ≤ 0.5. g1, para-carcinoma tissues; g2, BC tissues; N, normal tissue (or para-carcinoma tissue); C, BC tissue.

(Figure 2E). In addition, we discovered 200 differentially expressed mRNAs between para-carcinoma and BC tissues, which contained 47 upregulated and 153 downregulated mRNAs in BC ($P < 0.01$, fold change ≥ 2 or ≤ 0.5). The expression of specific differentially expressed mRNAs in BC is shown using a heat map (Figure 2F).

GO analyses of abnormally expressed mRNAs in BC tissues

By GO analysis, we analyzed the related functions of the differentially expressed mRNAs between para-carcinoma and BC tissues. The main enrichment function of the upregulated mRNAs included the biological process (BP) terms biological adhesion (GO:0022610), biological regulation (GO:0065007), cellular component organization or biogenesis (GO:007184); the cellular component (CC) terms cell part (GO:0044464), extracellular matrix (GO:0031012), and extracellular region (GO:0005576); and molecular function (MF) terms antioxidant activity (GO:0016209), binding (GO:0005488), catalytic activity (GO:0003824) (Figure 3A). The downregulated mRNAs were mainly enriched in BP terms biological adhesion (GO:0022621), biological regulation (GO:0065007), and cell killing (GO:0001906); the CC terms cell junction (GO:0030054), cell part (GO:0044464),

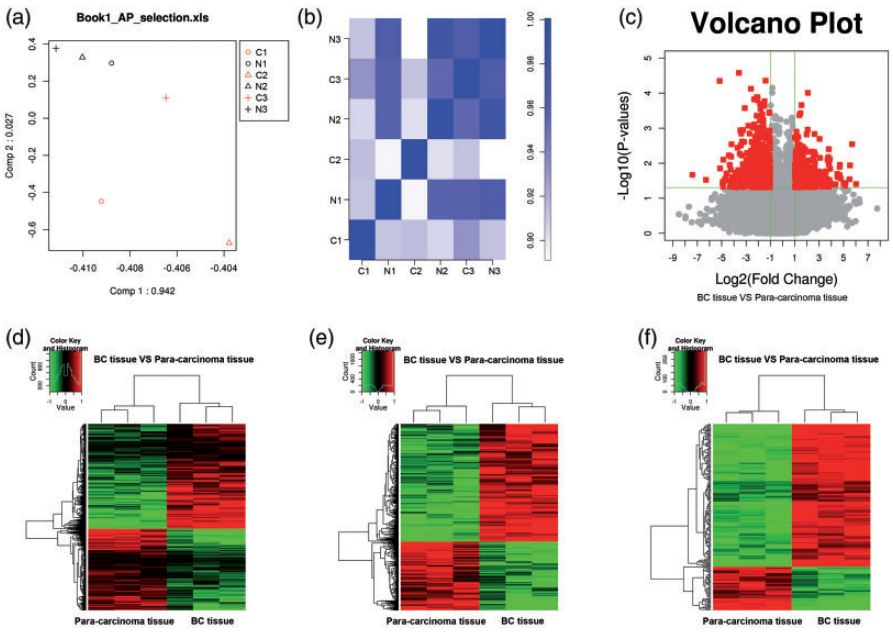

Figure 2. Expression pattern analysis of differentially expressed mRNAs in breast cancer (BC). (a) Principal component analysis of each sample. (b) Pairwise comparison of mRNAs in each sample. (c) Volcano plot showing differentially expressed mRNAs between para-carcinoma and BC tissues. The red points indicate differentially expressed mRNAs and the gray points indicate mRNAs that did not change. Hierarchical clustering analyses of mRNAs between para-carcinoma and BC tissues (d) with $P < 0.05$, (e) with $P < 0.05$ and fold change ≥ 2 or ≤ 0.5, and (f) with $P < 0.01$ and fold change ≥ 2 or ≤ 0.5. g1, para-carcinoma tissues; g2, BC tissues; N, normal tissue (or para-carcinoma tissue); C, BC tissue.

and extracellular matrix (GO:0031012); and the MF terms antioxidant activity (GO:0016209), binding (GO:0005488), and catalytic activity (GO:0003824), among others (Figure 3B).

KEGG analyses of abnormally expressed mRNAs between para-carcinoma and BC tissues

By KEGG analysis, we showed that the enriched pathways of the differentially expressed mRNAs between para-carcinoma and BC tissues included cytokine-cytokine receptor interaction, metabolic pathways, pathways in cancer, chemokine signaling pathway, cell adhesion molecules, PI3K-Akt signaling pathway, HTLVI infection, and T cell receptor signaling pathway (Table S1). Analysis suggests that the PI3K-Akt signaling pathway is closely related to BC (Figure 4). Overall, we provided a detailed functional description of differentially expressed mRNAs between para-carcinoma and BC tissues (Table S1).

Establishment of PPI network and analysis of genes in BC

To predict the underlying regulatory network of differentially expressed mRNAs between para-carcinoma and BC tissues, we identified feasible co-expressed proteins of these differentially expressed mRNAs and confirmed the connection of these proteins using a PPI. First, we evaluated the entire PPI network to identify possible

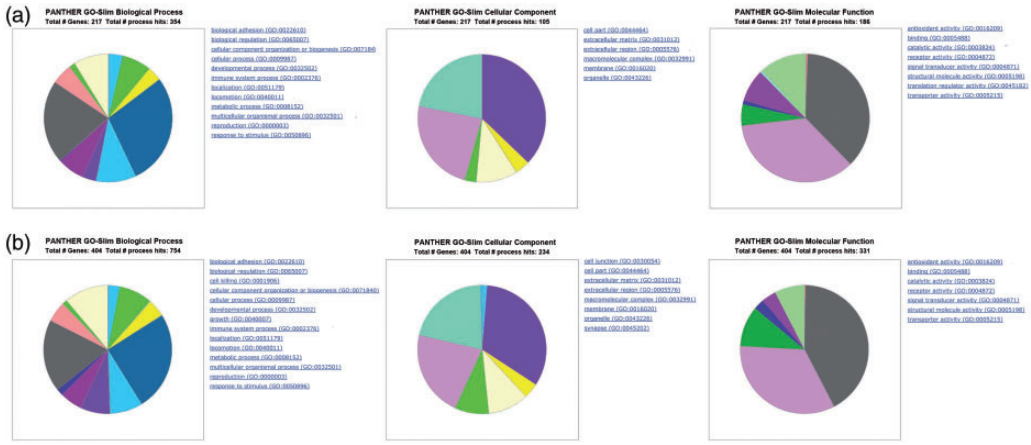

Figure 3. Gene Ontology (GO) analyses of abnormally expressed mRNAs in BC tissues. The GO analysis exhibited biological processes (left), cellular components (middle), and molecular functions (right) of (a) upregulated mRNAs in BC ($P < 0.05$) and (b) downregulated mRNAs in BC ($P < 0.05$).

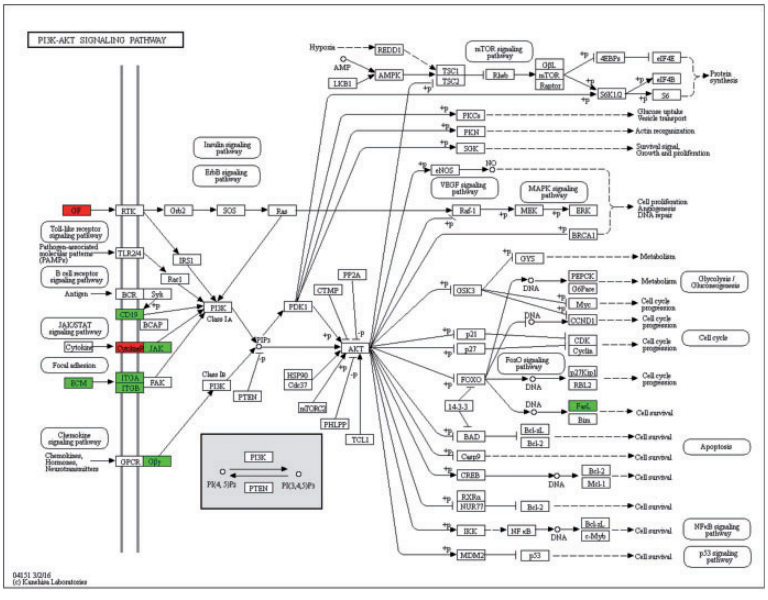

Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of abnormally expressed mRNAs in breast cancer (BC) tissues. KEGG analysis was used to analyze the signaling pathways, which were enriched by the differentially expressed mRNAs between para-carcinoma and BC tissues. We have shown the PI3K-Akt signaling pathway. Red represents upregulation, green represents downregulation.

interrelationships of proteins that might provide the basis for pathway research in BC (Figure 5A). Then, we used STRING protein interaction network analysis to predict protein networks of the PI3K-Akt signaling pathway and positive regulation of cell migration. The PI3K-Akt signaling pathway-related proteins included IL2RB,

THBS1, COL6A5, ITGB7, FIGF, IL7R, FN1, COL6A6, FASLG, IL2RG, COL4A1, ITGA8, ANGPT4, LAMB3, ITGA4, JAK3, TNXB, GNGT2, CD19, and GHR (Figure 5B and Table S2). The positive regulation of cell migration-related proteins included MYOC, UTS2, CORO1A, SELL, SOX9, CCR7, RAC2, THBS1, LEF1, CCR2, FIGF, CCL11, FBLN1, FN1, CXCL9, SASH1, F10, CCL7, INSL3, ANGPT4, CEMIP, ITGA4, PTPRC, LRRC15, SEMA4D, DMTN, HIF1A, ADAM8, and CCL5 (Figure 5C and Table S3).

Identification of differentially expressed lncRNAs and mRNAs in BC

Given the degree of difference in expression, we screened the six most significantly differentially expressed lncRNAs, including three upregulated lncRNAs (TCONS_00028064, TCONS_00028800, TCONS_00029809) and three downregulated lncRNAs (TCONS_00007846, TCONS_00010076, TCONS_l2_00028765) between para-carcinoma and BC tissues; we present detailed information of these lncRNAs in Table 2. Next, through verification by RT-qPCR, we showed that relative to para-carcinoma tissues, TCONS_00028064 and TCONS_00029809 were markedly upregulated ($P < 0.01$, $P < 0.001$, Figure 6A) and TCONS_00007846 and TCONS_l2_00028765 were downregulated ($P < 0.05$, $P < 0.01$, $P < 0.001$, Figure 6B) in BC tissues. We also showed that TCONS_00028064 and TCONS_00029809 were upregulated ($P < 0.001$, Figure 6C) and TCONS_00007846 was downregulated ($P < 0.01$, $P < 0.001$, Figure 6D) in BC cells compared with MCF10A cells.

Similarly, we screened the six most significantly differentially expressed mRNAs, including three upregulated mRNAs (*PPAPDC1A*, *COL10A1*, and *ADAMDEC1*) and 3 downregulated mRNAs (*SPHKAP*, *CLCA4*, and *ALDH1L1*) between para-carcinoma and BC tissues (detailed

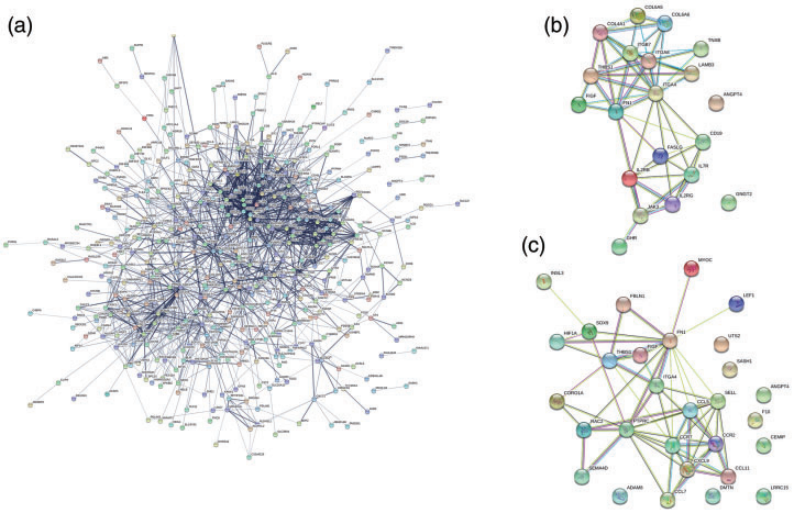

Figure 5. Protein–protein interaction (PPI) network and PI3K-Akt- or cell migration-related modules analyses of proteins in breast cancer (BC). (a) The entire PPI network in BC. Based on the functional annotation, the proteins related to the PI3K-Akt signaling pathway (b) and positive regulation of cell migration (c) were confirmed by STRING protein interaction network analysis.

Table 2. Detailed information on differentially expressed long noncoding RNAs between para-carcinoma and breast cancer tissues.

Primary accession	Gene symbol	*P* value	Fold change	Genomic coordinates	Cytoband	Result source
TCONS_00007846	XLOC_004090	≤0.01	11.28	chr4:137734865-137734806	hs|4q28.3	Agilent_HUMAN_G3V2
TCONS_00010076	XLOC_004530	≤0.01	10.96	chr5:121176363-121176422	hs|5q23.1	Agilent_HUMAN_G3V2
TCONS_l2_00028765	XLOC_l2_014820	≤0.01	10.74	chr9:67282369-67288092	hs|9q13	Agilent_HUMAN_G3V2
TCONS_00028064	XLOC_013794	≤0.01	0.07	chr20:50448398-50448339	hs|20q13.2	Agilent_HUMAN_G3V2
TCONS_00028800	XLOC_013872	≤0.01	0.06	chr21:20022616-20022675	hs|21q21.1	Agilent_HUMAN_G3V2
TCONS_00029809	XLOC_014244	≤0.01	0.05	chr22:39465900-39465959	hs|22q13.1	Agilent_HUMAN_G3V2

Fold change: LncRNA expression in para-carcinoma/LncRNA expression in BC tissues.

information of these mRNAs is given in Table 3). The RT-qPCR data showed that *ADAMDEC1* was markedly upregulated and *CLCA4* was significantly downregulated in BC tissues and cells ($P < 0.05$, $P < 0.001$ and $P < 0.01$, respectively, Figure 6E-6H).

TCONS_00029809 silencing repressed the proliferation, invasion, migration of BC cells and induced apoptosis of BC cells

By identifying lncRNAs in RT-qPCR assay, we discovered that the difference in expression of TCONS_00029809 in BC was the largest, and it was therefore the target for further study. We conducted TCONS_00029809 silencing in MCF7 and MDA-MB-231 cells to further study the roles of TCONS_00029809 on proliferation, apoptosis, invasion, and migration of BC cells. The RT-qPCR analysis showed that TCONS_00029809 was sharply decreased in MCF7 and MDA-MB-231 cells after transfection of siRNAs against TCONS_00029809 compared with the si-NC group ($P < 0.01$, $P < 0.001$, Figure 7A). Using the CCK-8 assay, we demonstrated that TCONS_00029809 silencing significantly inhibited cell proliferation in MCF7 and MDA-MB-231 cells ($P < 0.05$, $P < 0.01$, Figure 7B). Flow cytometry analysis revealed that knockdown of TCONS_00029809 facilitated apoptosis of MCF7 and MDA-MB-231 cells ($P < 0.001$, Figure 7C). Additionally, TCONS_00029809 silencing attenuated the invasive and migratory capacities of MCF7 and MDA-MB-231 cells ($P < 0.01$, $P < 0.001$, Figure 7D and 7E). Thus, silencing of TCONS_00029809 could suppress malignant behaviors of BC cells.

Discussion

BC is a common malignant tumor in clinical practice, and its main clinical manifestations include breast mass, nipple

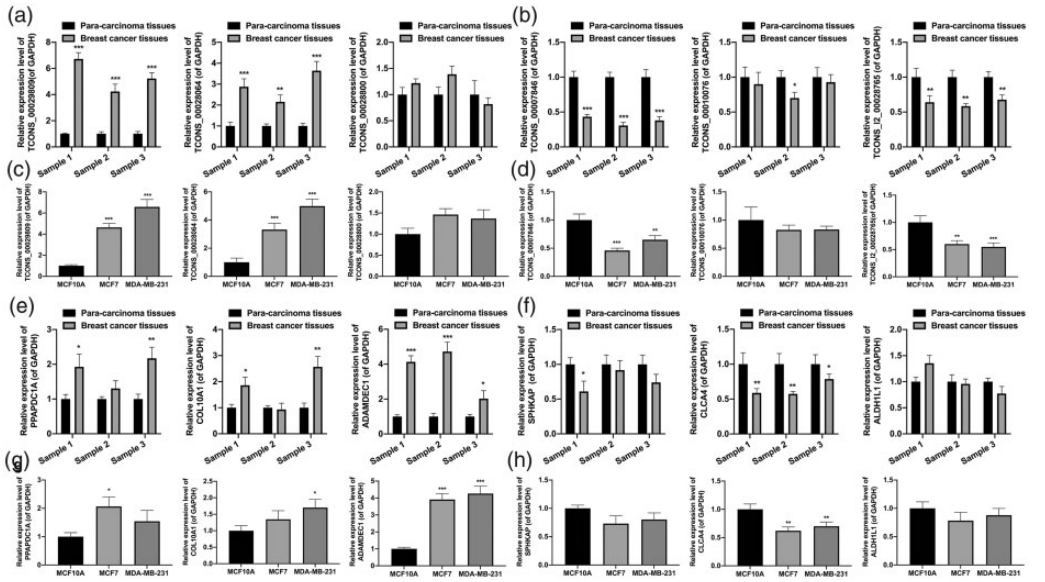

Figure 6. Expression of long noncoding (lnc)RNAs and mRNAs in breast cancer (BC). (a, b) Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis was used to assess the levels of three upregulated lncRNAs (TCONS_00028064, TCONS_00028800, TCONS_00029809) and three downregulated lncRNAs (TCONS_00007846, TCONS_00010076, TCONS_I2_00028765) between para-carcinoma and BC tissues. (c, d) The six differentially expressed lncRNAs were monitored though RT-qPCR analysis in MCF10A, MCF7, and MDA-MB-231 cells. (e, f) RT-qPCR analysis was used to assess the levels of three upregulated mRNAs (*PPAPDC1A*, *COL10A1*, and *ADAMDEC1*) and three downregulated mRNAs (*SPHKAP*, *CLCA4*, and *ALDH1L1*) between para-carcinoma and BC tissues. (g, h) The six differentially expressed mRNAs were assessed by RT-qPCR analysis in MCF10A, MCF7, and MDA-MB-231 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

discharge, skin changes, abnormalities of the areola, and axillary lymphadenoma.[31,32] At present, the prevention and treatment of BC are crucial areas of research in the study of malignant tumors. However, the cause of BC is not fully understood. BC develops in a complex, multi-stage, multi-step process that can be influenced by various molecules and factors.[11,33] LncRNAs are associated with numerous biological functions, including epigenetics, cell differentiation, and cell cycle.[34,35] With the development of next-generation sequencing and computer technology, lncRNAs have been widely studied in the pathogenesis, diagnosis, and drug screening of various diseases.[36,37] Numerous studies have shown that lncRNAs participate in the development, progression, invasion, and metastasis of BC in a variety of ways. For example, lncRNA SNHG14 induces resistance to trastuzumab in BC by H3K27 acetylation-mediated regulation of PABPC1 expression;[38] lncRNA MALAT1 inhibits BC metastasis;[39] lncRNA GHSROS has significant promoting effects on the growth and migration of BC;[40] and lncRNA NONHSAT101069 induces epirubicin resistance and promotes the migration and invasion of BC cells by targeting the microRNA miR-129-5p to regulate Twist1.[41]

The expression profile of lncRNA has been analyzed in previous studies.[42,43] However, because of tissue specificity, numerous lncRNAs screened through microarray analysis have not yet been

Table 3. Detailed information of differentially expressed mRNAs between para-carcinoma and breast cancer tissues.

Gene symbol	Gene name	*P* value	Fold change	Genomic coordinates	Cytoband	Result source	Sources
SPHKAP	SPHK1 interactor, AKAP domain containing	≤0.01	14.94	chr2:228846461-228846402	hs|2q36.3	Agilent_HUMAN_G3V2	NM_001142644
CLCA4	chloride channel accessory 4	≤0.01	22.68	chr1:87045163-87045222	hs|1p22.3	Agilent_HUMAN_G3V2	NM_012128
ALDH1L1	aldehyde dehydrogenase 1 family, member L1	≤0.01	20.26	chr3:125826021-125824747	hs|3q21.3	Agilent_HUMAN_G3V2	NM_012190
PPAPDC1A	phosphatidic acid phosphatase type 2 domain containing 1A	≤0.01	0.03	chr10:122349102-122349161	hs|10q26.12	Agilent_HUMAN_G3V2	NM_001030059
COL10A1	collagen, type X, alpha 1	≤0.01	0.03	chr6:116441120-116441061	hs|6q22.1	Agilent_HUMAN_G3V2	NM_000493
ADAMDEC1	ADAM-like, decysin 1	≤0.01	0.01	chr8:24259534-24259593	hs|8p21.2	Agilent_HUMAN_G3V2	NM_001145271

Fold change: mRNA expression in para-carcinoma/mRNA expression in BC tissues.

identified. In our study, we discovered 238 differently expressed lncRNAs (157 upregulated and 81 downregulated) that might be associated with BC. We showed that TCONS_00028064 and TCONS_00029809 were upregulated and TCONS_00007846 was downregulated in BC tissues and cells. Our functional experiments showed that knockdown of TCONS_00029809 could prevent the proliferation, invasion, and migration of BC cells, and accelerate apoptosis of BC cells. Therefore, inhibition of TCONS_00029809 might have potential value in the treatment of BC.

RNA sequencing has been extensively applied to the identification of differentially expressed genes in cells and tissues of multiple diseases, and has significant applications in genetics and epigenetics.[44,45] In our study, we screened 200 differentially expressed mRNAs, including 47 upregulated and 153 downregulated mRNAs in BC tissues relative to that in para-carcinoma tissues. Through GO analysis, we found that the differentially expressed mRNAs might be involved in biological adhesion, biological regulation, cellular component organization or biogenesis, and cell killing. In KEGG pathway analysis, we found that the differentially expressed mRNAs were abundant in cytokine-cytokine receptor interaction, metabolic pathways, pathways in cancer, chemokine signaling pathway, and PI3K-Akt signaling pathway. Several studies have shown that the cytokine-cytokine receptor interaction pathway might be associated with multiple cancers, including renal cell carcinoma,[46,47] oral squamous cell carcinoma,[48] glioma,[49] and colorectal cancer.[50] Metabolic pathways have also been shown to contribute to cancer progression.[51,52] Research has shown that the chemokine signaling pathway participates in cancer metastasis.[53] The PI3K-Akt signaling pathway can be activated by G-protein-coupled receptors and has a significant effect in

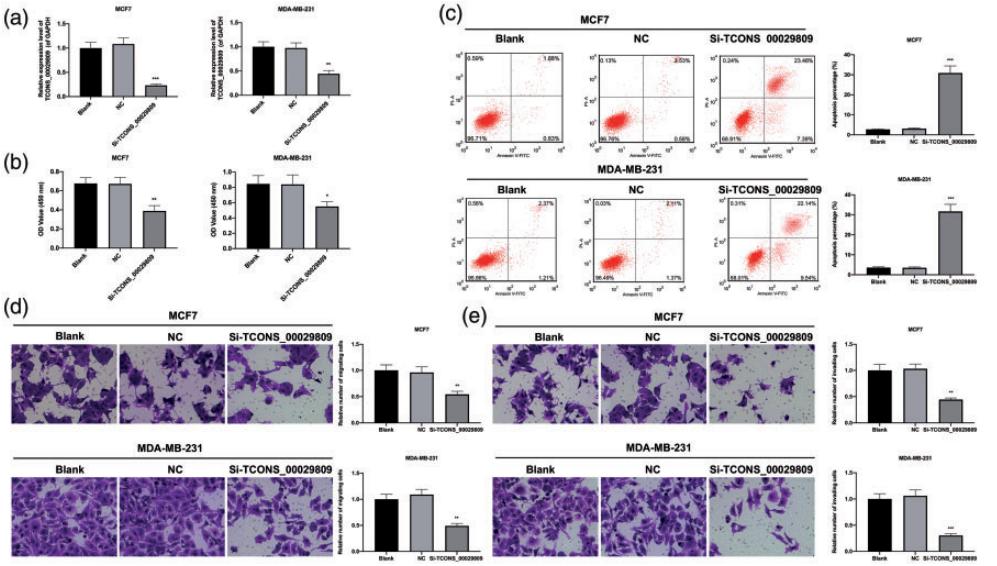

Figure 7. Silencing of the long noncoding (lnc)RNA TCONS_00029809 repressed breast cancer (BC) cell proliferation, invasion, and migration, and induced apoptosis. (a) Quantitative reverse transcription polymerase chain reaction (RT-qPCR) assessment of TCONS_00029809 expression in MCF7 and MDA-MB-231 cells transfected with short interfering negative control (si-NC), and short interfering (si)RNAs against TCONS_00029809. (b) Proliferation of MCF7 and MDA-MB-231 cells was estimated by CCK-8 cell counting assay in MCF7 and MDA-MB-231 cells after TCONS_00029809 knockdown. (c) Cell apoptosis was analyzed by flow cytometry in transfected MCF7 and MDA-MB-231 cells. (d, e) Invasive and migratory abilities of MCF7 and MDA-MB-231 cells were evaluated by Transwell assay after TCONS_00029809 knockdown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NC group.

malignant proliferation, angiogenesis, and drug resistance of tumor cells.[54,55] We suggest that differentially expressed mRNAs have an important contribution in the processes of BC development. We further analyzed and predicted the network of proteins related to the PI3K-Akt signaling pathway and positive regulation of cell migration, based on the differentially expressed mRNAs. We verified for the first time that the mRNAs *ADAMDEC1* and *CLCA4* were differentially expressed in BC. ADAMDEC1 is part of a group of secretory glycoproteins with various functions;[56] it has been reported to be closely related to inflammation and tumor progression.[57] CLCA4 is a class of calcium-activated chloride channel regulator.[58] Research has shown that CLCA1 is connected with the development of colorectal cancer.[59] However, the function and mechanism of ADAMDEC1 and CLCA1 in BC are not fully understood. ADAMDEC1 and CLCA1 might be potential targets for the treatment of BC.

This study had several limitations. First, the sample size was small. Furthermore, the mechanism by which TCONS_00029809 silencing prevents the progression of BC, and the regulatory relationship between differentially expressed lncRNAs and genes in BC remain unclear. In addition to TCONS_00029809, the effects and mechanisms of other verified differentially expressed lncRNAs and genes on BC progression also need to be confirmed. Further studies

with more samples are therefore needed to verify these potential lncRNAs and mRNAs.

In conclusion, we used high-throughput sequencing to identify 238 candidate lncRNAs and 200 potential mRNAs that might be related to BC. The functions of the differentially expressed mRNAs were annotated through GO and KEGG analyses. The lncRNAs TCONS_00028064, TCONS_00029809, and TCONS_00007846 and mRNAs *ADAMDEC1* and *CLCA4* were identified as the most-differentially expressed in BC. We showed, for the first time, that TCONS_00029809 knockdown could inhibit malignant processes of BC cells, suggesting that TCONS_00029809 might be a potential disease-causing lncRNA and a diagnostic and therapeutic target for BC. However, further *in vitro* and *in vivo* experiments are needed to verify the functions and mechanisms of these potential lncRNAs and mRNAs in different types of BC.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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Supplemental material

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References

1. Harbeck N and Gnant M. Breast cancer. *Lancet* 2017; 389: 1134–1150.
2. Tung MC, Chang FW, Fan TP, et al. Acidity is one of the main mechanism for hypoxia triggering chemoresistance to mitoxanthrone (MX) in the human breast cancer MCF-7 cell line. *Eur J Gynaecol Oncol* 2019; 40: 994–999.
3. Anastasiadi Z, Lianos GD, Ignatiadou E, et al. Breast cancer in young women: an overview. *Updates Surg* 2017; 69: 313–317.
4. Kimbung S, Loman N and Hedenfalk I. Clinical and molecular complexity of breast cancer metastases. *Semin Cancer Biol* 2015; 35: 85–95.
5. Dittmer J. Mechanisms governing metastatic dormancy in breast cancer. *Semin Cancer Biol* 2017; 44: 72–82.
6. Narod SA and Sopik V. Is invasion a necessary step for metastases in breast cancer? *Breast Cancer Res Treat* 2018; 169: 9–23.
7. Medeiros B and Allan AL. Molecular mechanisms of breast cancer metastasis to the lung: clinical and experimental perspectives. *Int J Mol Sci* 2019; 20: 2272.
8. Peart O. Metastatic breast cancer. *Radiol Technol* 2017; 88: 519m–539m.
9. Jafari SH, Saadatpour Z, Salmaninejad A, et al. Breast cancer diagnosis: imaging techniques and biochemical markers. *J Cell Physiol* 2018; 233: 5200–5213.
10. Burke EE, Kodumudi K, Ramamoorthi G, et al. Vaccine therapies for breast cancer. *Surg Oncol Clin N Am* 2019; 28: 353–367.
11. Winters S, Martin C, Murphy D, et al. Breast cancer epidemiology, prevention, and screening. *Prog Mol Biol Transl Sci* 2017; 151: 1–32.
12. Quinn JJ and Chang HY. Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet* 2016; 17: 47–62.
13. Jarroux J, Morillon A and Pinskaya M. History, discovery, and classification of lncRNAs. *Adv Exp Med Biol* 2017; 1008: 1–46.
14. Dahariya S, Paddibhatla I, Kumar S, et al. Long non-coding RNA: classification, biogenesis and functions in blood cells. *Mol Immunol* 2019; 112: 82–92.
15. Dykes IM and Emanueli C. Transcriptional and post-transcriptional gene regulation by long non-coding RNA. *Genomics*

Proteomics Bioinformatics 2017; 15: 177–186.
16. Mathy NW and Chen XM. Long non-coding RNAs (lncRNAs) and their transcriptional control of inflammatory responses. *J Biol Chem* 2017; 292: 12375–12382.
17. Kondo Y, Shinjo K and Katsushima K. Long non-coding RNAs as an epigenetic regulator in human cancers. *Cancer Sci* 2017; 108: 1927–1933.
18. Ransohoff JD, Wei Y and Khavari PA. The functions and unique features of long intergenic non-coding RNA. *Nat Rev Mol Cell Biol* 2018; 19: 143–157.
19. Chi Y, Wang D, Wang J, et al. Long non-coding RNA in the pathogenesis of cancers. *Cells* 2019; 8: 1015.
20. Fang Y and Fullwood MJ. Roles, functions, and mechanisms of long non-coding RNAs in Cancer. *Genomics Proteomics Bioinformatics* 2016; 14: 42–54.
21. Misawa A, Takayama KI and Inoue S. Long non-coding RNAs and prostate cancer. *Cancer Sci* 2017; 108: 2107–2114.
22. Li N, Deng Y, Zhou L, et al. Global burden of breast cancer and attributable risk factors in 195 countries and territories, from 1990 to 2017: results from the Global Burden of Disease Study 2017. *J Hematol Oncol* 2019; 12: 140.
23. Tian T, Gong Z, Wang M, et al. Identification of long non-coding RNA signatures in triple-negative breast cancer. *Cancer Cell Int* 2018; 18: 103.
24. Tian T, Wang M, Lin S, et al. The impact of lncRNA dysregulation on clinicopathology and survival of breast cancer: a systematic review and meta-analysis. *Mol Ther Nucleic Acids* 2018; 12: 359–369.
25. Li M, Zhao LM, Li SL, et al. Differentially expressed lncRNAs and mRNAs identified by NGS analysis in colorectal cancer patients. *Cancer Med* 2018; 7: 4650–4664.
26. Shen S, Kong J, Qiu Y, et al. Identification of core genes and outcomes in hepatocellular carcinoma by bioinformatics analysis. *J Cell Biochem* 2019; 120: 10069–10081.
27. Klopfenstein D, Zhang L, Pedersen BS, et al. GOATOOLS: A Python library for Gene Ontology analyses. *Sci Rep* 2018; 8: 10872.
28. Wang Z, Shang P, Li Q, et al: iTRAQ-based proteomic analysis reveals key proteins affecting muscle growth and lipid deposition in pigs. *Sci Rep* 2017; 7: 46717.
29. von Mering C, Huynen M, Jaeggi D, et al. STRING: a database of predicted functional associations between proteins. *Nucleic Acids Res* 2003; 31: 258–261.
30. Lin S, Zhang R, Xu L, et al. LncRNA Hoxaas3 promotes lung fibroblast activation and fibrosis by targeting miR-450b-5p to regulate Runx1. *Cell Death Dis*, 2020; 11: 706.
31. Li T, Mello-Thoms C and Brennan PC. Descriptive epidemiology of breast cancer in China: incidence, mortality, survival and prevalence. *Breast Cancer Res Treat* 2016; 159: 395–406.
32. Tan AR. Cutaneous manifestations of breast cancer. *Semin Oncol* 2016; 43: 331–334.
33. Nagini S. Breast cancer: current molecular therapeutic targets and new players. *Anticancer Agents Med Chem* 2017; 17: 152–163.
34. Charles Richard JL and Eichhorn PJA. Platforms for investigating lncRNA functions. *SLAS Technol* 2018; 23: 493–506.
35. Jathar S, Kumar V, Srivastava J, et al. Technological developments in lncRNA biology. *Adv Exp Med Biol* 2017; 1008: 283–323.
36. Kumar MM and Goyal R. LncRNA as a therapeutic target for angiogenesis. *Curr Top Med Chem* 2017; 17: 1750–1757.
37. Peng WX, Koirala P and Mo YY. LncRNA-mediated regulation of cell signaling in cancer. *Oncogene* 2017; 36: 5661–5667.
38. Dong H, Wang W, Mo S, et al. Long non-coding RNA SNHG14 induces trastuzumab resistance of breast cancer via regulating PABPC1 expression through H3K27 acetylation. *J Cell Mol Med* 2018; 22: 4935–4947.
39. Kim J, Piao HL, Kim BJ, et al. Long non-coding RNA MALAT1 suppresses breast cancer metastasis. *Nat Genet* 2018; 50: 1705–1715.
40. Thomas PB, Seim I, Jeffery PL, et al. The long non-coding RNA GHSROS facilitates breast cancer cell migration and orthotopic

xenograft tumour growth. *Int J Oncol* 2019; 55: 1223–1236.
41. Yao N, Fu Y, Chen L, et al. Long non-coding RNA NONHSAT101069 promotes epirubicin resistance, migration, and invasion of breast cancer cells through NONHSAT101069/miR-129-5p/Twist1 axis. *Oncogene* 2019; 38: 7216–7233.
42. Li J, Gao C, Liu C, et al. Four lncRNAs associated with breast cancer prognosis identified by coexpression network analysis. *J Cell Physiol* 2019; 234: 14019–14030.
43. Deva Magendhra Rao AK, Patel K, Korivi Jyothiraj S, et al. Identification of lncRNAs associated with early-stage breast cancer and their prognostic implications. *Mol Oncol* 2019; 13: 1342–1355.
44. Stark R, Grzelak M and Hadfield J. RNA sequencing: the teenage years. *Nat Rev Genet* 2019; 20: 631–656.
45. Hedlund E and Deng Q. Single-cell RNA sequencing: technical advancements and biological applications. *Mol Aspects Med* 2018; 59: 36–46.
46. Li F, Guo P, Dong K, et al. Identification of key biomarkers and potential molecular mechanisms in renal cell carcinoma by bioinformatics analysis. *J Comput Biol* 2019; 26: 1278–1295.
47. Wan B, Huang Y, Liu B, et al. AURKB: a promising biomarker in clear cell renal cell carcinoma. *PeerJ* 2019; 7: e7718.
48. Li S, Chen X, Liu X, et al. Complex integrated analysis of lncRNAs-miRNAs-mRNAs in oral squamous cell carcinoma. *Oral Oncol* 2017; 73: 1–9.
49. Li X and Meng Y. Survival analysis of immune-related lncRNA in low-grade glioma. *BMC Cancer* 2019; 19: 813.
50. Liu J, Li H, Sun L, et al. Aberrantly methylated-differentially expressed genes and pathways in colorectal cancer. *Cancer Cell Int* 2017; 17: 75.
51. Boroughs LK and DeBerardinis RJ. Metabolic pathways promoting cancer cell survival and growth. *Nat Cell Biol* 2015; 17: 351–359.
52. Singh SR, Tan M and Rameshwar P. Cancer metabolism: targeting metabolic pathways in cancer therapy. *Cancer Lett* 2015; 356: 147–148.
53. Lim SY, Yuzhalin AE, Gordon-Weeks AN, et al. Targeting the CCL2-CCR2 signaling axis in cancer metastasis. *Oncotarget* 2016; 7: 28697–28710.
54. Murugan AK. Special issue: PI3K/Akt signaling in human cancer. *Semin Cancer Biol* 2019; 59: 1–2.
55. Carnero A, Blanco-Aparicio C, Renner O, et al. The PTEN/PI3K/AKT signalling pathway in cancer, therapeutic implications. *Curr Cancer Drug Targets* 2008; 8: 187–198.
56. Jimenez-Pascual A, Hale JS, Kordowski A, et al. ADAMDEC1 maintains a growth factor signaling loop in cancer stem cells. *Cancer Discov* 2019; 9: 1574–1589.
57. Liu T, Deng Z, Xie H, et al. ADAMDEC1 promotes skin inflammation in rosacea via modulating the polarization of M1 macrophages. *Biochem Biophys Res Commun* 2020; 521: 64–71.
58. Liu CL and Shi GP. Calcium-activated chloride channel regulator 1 (CLCA1): more than a regulator of chloride transport and mucus production. *World Allergy Organ J* 2019; 12: 100077.
59. Li X, Hu W, Zhou J, et al. CLCA1 suppresses colorectal cancer aggressiveness via inhibition of the Wnt/beta-catenin signaling pathway. *Cell Commun Signal* 2017; 15: 38.