LncRNA EGFR-AS1 Regulates Breast Cancer Cells Function and Sensitivity to Docetaxel Via the miR-149-5p/ELP5 Axis

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Research

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Abstract

Background: Recently, an increasing number of studies have focused on investigating long non-coding RNAs (lncRNAs) and their role in regulating the progression of various cancer types. However, the biological effects and underlying mechanisms of EGFR-AS1, a typical lncRNA, remain largely unclear in breast cancer.

Methods: Differential expression of EGFR-AS1 in breast cancer tissue was analyzed using an integrative database and verified in breast cancer tissue samples and cells via real-time PCR analysis and western blotting analysis. The tumor promoter role of EGFR-AS1 in breast cancer cells was determined through MTT, EDU analysis, colony formation and transwell assays, and the effect of EGFR-AS1 on docetaxel drug sensitivity was examined. We then performed bioinformatic analysis and the dual-luciferase reporter assay to identify the binding sites of EGFR-AS1/miR-149-5p and miR-149-5p/ELP5. Results from western blotting and biological function studies provided insights into whether the EGFR-AS1/miR-149-5p/ELP5 axis regulates breast cancer development *in vitro* and *in vivo*.

Results: EGFR-AS1 is upregulated in breast cancer tissues and cells and promotes the progression of breast cancer cells both *in vitro* and *in vivo*. Moreover, miR-149-5p is downregulated in breast cancer tissues and cell lines. Mechanistically, EGFR-AS1 regulates ELP5 levels by sponging miR-149-5p, thereby affecting cell progression and promoting epithelial-to-mesenchymal transition. Hence, the EGFR-AS1/miR-149-5p/ELP5 axis is involved in breast cancer proliferation, migration, invasion, and resistance to the chemotherapeutic drug, docetaxel, in breast cancer cells.

Conclusions: EGFR-AS1 sponges miR-149-5p to affect the expression level of ELP5 ultimately acting as a new tumor promotor in breast cancer. This study provides novel insights into diagnostic and docetaxel-related chemotherapy targets for breast cancer.

Background

Breast cancer (BC) surpassed lung cancer as the most common cancer worldwide for the first time in 2020 and is a major cause of cancer-related deaths among women worldwide [1, 2]. Although numerous advancements in surgery and chemotherapy have significantly improved the 5-year survival rate of patients with BC, 20% to 30% of patients will experience recurrence, complications from distant metastasis, and even death [3–5]. Anthracyclines and taxanes are widely used in cancer chemotherapy, especially in BC. Specifically, docetaxel, a taxane chemotherapeutic drug, is of great importance in BC chemotherapy [6, 7]. However, several patients with BC are intrinsically resistant or acquire resistance during the course of docetaxel treatment leading to cancer recurrence and metastasis. Docetaxel prevents the depolymerization of microtubules and growth arrest of tumor cells at the G2-M phase by binding to β -tubulin, a major component of microtubules [8, 9]. Therefore, a better understanding of the molecular mechanism underlying BC tumorigenesis is important to improve early diagnosis and treatment of BC.

Recent research has shown that non-coding RNAs (ncRNAs) play a vital role in regulating the initiation and development of cancers [10–12]. Specifically, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) participate in various biological and pathological processes in BC tissues [13, 14]. Moreover, increasing evidence suggests that ncRNAs serve as the hallmarks and therapeutic targets for potential BC candidates. The primary function of lncRNAs is to serve as an miRNA sponge, which functions as competing endogenous RNA (ceRNA) and participates in the regulation of cancer development. For example, Rho-associated protein kinase (ROCK)2 is upregulated by lncRNA EGFR-AS1 via miRNA-381 sponging in bladder cancer [15]. Additionally, the knockdown of the lncRNA HOTAIR suppresses cisplatin resistance, cell proliferation, migration, and invasion of DDP-resistant NSCLC cells by targeting the miR-149-5p/Doublecortin-Like Kinase 1 axis [16]. Various non-coding RNAs are involved in BC development and metastasis, however, the role and mechanism underlying these lncRNAs in BC remain unclear.

It has been reported that lncRNAs mediate cellular activities, including epithelial-to-mesenchymal transition (EMT), which is an important biological process in malignant tumor cells derived from epithelial cells and is beneficial for tumor growth, metastasis, invasion, and chemotherapy sensitivity [17–19]. Moreover, lncRNA EGFR antisense RNA 1 (EGFR-AS1) has been reported as an oncogenic molecule [15, 20, 21]. However, the clinical significance and mechanisms of EGFR-AS1 in BC remain largely unknown. EGFR-AS1 is located on human chromosome 7, in an opposite direction to that of EGFR gene transcription. Furthermore, EGFR-AS1 is predominantly localized in the cytoplasm, indicating the post-transcriptional regulatory potential of EGFR-AS1 in BC [20, 21]. The differential expression of EGFR-AS1 has been analyzed using integrative databases, and bioinformatics software (TCGA, StarBase, miRBase, and RegRNA) revealed that EGFR-AS1 predicts poor prognosis in BC patients and harbors an miR-149-5p binding site. As previously reported, miR-149-5p is robustly downregulated in BC cells, and thus inhibits tumor cell growth, invasion, and migration [14, 16]. Additionally, miR-149-5p is downregulated and exhibits tumor-suppressive effects in BC [22–24]. However, the effect of EGFR-AS1 in BC and the EGFR-AS1/miR-149-5p functional network in modulating BC cell malignant behavior remains unknown.

Using several bioinformatics databases (StarBase, miRBase, and RegRNA), we predicted that the expression of ELP5 could be regulated by EGFR-AS1 via miR-149-5p sponging. ELP5 can be characterized as an elongator subunit that controls cell motility and the tumorigenicity of melanoma cells [25–27]. However, it is not known how ELP5 affects the biological behavior of BC.

The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway can regulate cell cycles, cell growth, cell invasiveness, and migration capability. As such, it is considered a key pathway in numerous types of human tumors and has been suggested as a potential therapeutic agent [28–30]. Using the LY294002 inhibitor, which inhibits the PI3K expression and reduces AKT phosphorylation, to explore the relationship of EGFR-AS1/miR-149-5p/ELP5 axis and PI3K/AKT pathway and how the functional network ultimately inducing the EMT of BC cells.

This study aimed to explore the mechanism of the EGFR-AS1/miR-149-5p/ELP5 axis in BC tumorigenesis and to examine the effect of EGFR-AS1 on docetaxel drug sensitivity in order to make the translational improve chemotherapeutics.

Methods

Cell culture

MCF-10A, MCF-7, MDA-MB-231, MDA-MB-453, BT-549, and SK-BR-3 BC cell lines were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and all cell lines were authenticated by short tandem repeat DNA profiling. The cells were cultured and frozen in liquid nitrogen to stabilize, and experiments were performed after ten passages. The human normal breast cell line MCF-10A cells were cultured in 1:1 Dulbecco's modified eagle's medium (DMEM)/F12 (Gibco, Waltham, MA, USA) supplemented with 5% horse serum, 10 µg/mL insulin (Sigma-Aldrich Co, St. Louis, MO, USA), and 20 ng/mL epidermal growth factor (EGF). MCF7 and T47D cell lines were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS). MDA-MB-231 and MDA-MB-453 cells were cultured in L15 (Gibco) supplemented with 10% FBS. BT549 cells were cultured in Roswell Park Memorial Institute (RPMI)1640 (Gibco) supplemented with 10% FBS. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

Patients and specimens

A total of 33 paired primary tumor specimens were collected from patients who underwent surgery at the First Affiliated Hospital of China Medical University between 2005 and 2014. Follow-up data with immunohistochemically stained sections were available for 15/33 patients.

RNA preparation, treatment with RNase R, and q-RT-PCR

Total RNA was isolated from BC tissues and cells using the TRIzol Reagent Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol and were subsequently incubated with 3 U/mg RNase R (Epicentre Technologies, USA) at 37 °C for 20 min. Parts of total RNAs was reverse transcribed into cDNA (TaKaRa, RR036A, Japan). The expression of lncRNAs and gene transcription level were measured by SYBR-Green qPCR (Takara, Otsu, Shiga, Japan) (StepOne Plus; Applied Biosystems, USA). miRNA expression was assayed using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). Meanwhile, glyceraldehyde 3-phosphate (GAPDH) and U6 snRNA were used to normalize the relative amount of lncRNAs, miRNAs and mRNAs. The primers were purchased from GenePharma, primer sequences are shown in supplementary material (**Table S1**). The $2^{-\Delta\Delta CT}$ method was used to quantify gene expression.

Immunohistochemistry(IHC)

Tissue sections (4 µm thick) were deparaffinized with xylene, fixed with 10% neutral formalin and embedded in paraffin. Immunohistochemistry (IHC) staining was performed using the standard streptavidin–peroxidase complex method. Tissue sections were then incubated with ELP5 antibody **(Table S2)** at 4 °C overnight. After washing with PBS, tissues were treated with biotinylated goat anti-rabbit IgG secondary antibody, developed with a horseradish peroxidase-labeled streptavidin reagent, and stained for 3–5 min with 3,3'-diaminobenzidine(DAB). The nuclei were counterstained with hematoxylin.

ELP5 expression levels were estimated according to the percentage of positive cells (PP) and the staining intensity (SI) within the whole tissue section. ELP5 staining intensity was evaluated semi-quantitatively using the immune response score (IRS) and calculated as follows: $IRS = PP \times SI$; where PP: 0, no dye; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%; and SI: 0, no staining; 1, weak staining; 2, medium staining; and 3, strong staining. The scores for each tumor sample were multiplied to give a final score between 0–9. Tumors appeared as areas with high ELP5 expression with a score ≥ 4 . Tumor samples with scores between 1 and 3 were categorized as showing weak expression, whereas those with scores of 0 were considered to have no expression; both weak expression and no expression were defined as negative ELP5 expression.

Oligonucleotides, siRNA, plasmids, transfection and administration of PI3K/Akt inhibitors

The human BC cell lines MCF-7 and MDA-MB-231 were seeded in a 6-well plate and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ overnight. LncRNAEGFR-AS1 siRNA, siELP5 were purchased from RiboBio Co. Ltd.(Guangzhou, China). miR149-5p mimics and inhibitors were obtained from GenePharma(Shanghai, China) and were transfected with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), based on the manufacturer's instructions.

The plasmids pCMV6-ddk-myc and pCMV6-ddk-myc-ELP5 were purchased from Origene (Rockville, MD, USA). Transfection was also carried out using the Lipofectamine 3000 reagent (Invitrogen), according to the manufacturer's instructions.

To inhibit PI3K/Akt signaling, cells were treated with 10 µM LY294002 (MedChemExpress, Monmouth Junction, NJ, USA), an inhibitor that blocks the PI3K/Akt pathway. LY294002 was dissolved in dimethyl sulfoxide (DMSO) and added 12 h after a 36-h transfection, with the same volume of DMSO added to control cells.

Western blotting

Total protein was extracted using a lysis buffer (Thermo Fisher Scientific) and quantified using the Bradford method; 80 µg of the total protein samples was then separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). After blocking with 5% BSA in TBST buffer for 2 h, the membranes were incubated at 4 °C overnight with the appropriate primary antibody **(Table S2)**. The membranes were then washed four times with TTBS buffer for 5 min each and incubated with horseradish peroxidase-labeled secondary antibody at 37 °C for 2 h. After

washing with TBST buffer, the membranes were visualized with ECL (Thermo Fisher Scientific, Waltham, MA, USA) on a BioImaging System (UVP Inc., Upland, CA, USA). Protein levels were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA), with GAPDH and β -actin as internal controls for protein abundance.

Ethynyldeoxyuridine (EdU) analysis

BC cells were inoculated in 96-well plates overnight and processed using a Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China) to detect cell proliferation. The next day, EdU solution was added to the cells and incubated (50 μ M) for 2 h. The manufacturer's protocols were followed for all subsequent step and cells were assessed via fluorescence microscopy (Olympus, Tokyo, Japan) to observe cell proliferation, reflected by red (the percentage of EdU-positive cells) and blue signals (Hoechst 33342 was used for the staining of the cell nucleus), respectively.

Tetrazolium (MTT)

MCF-7 cells (3,000/well) and MDA-MB-231 (5,000/well) were plated in 96-well plates in medium containing 10% FBS 24 h after transfection; a separate blank control was also included (100 μ L of fresh medium). Cell viability was quantified via mitochondrial reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Absorbance at 490 nm (OD490) was measured after the cells had been incubated with 100 μ L sterile MTT dye (0.5 mg/mL Sigma-Aldrich) for 4 h at 37 °C and 150 μ L of DMSO for 10 min. Finally, a cell growth curve was generated using OD490 as the ordinate axis.

Colony formation assays

In the colony formation assay, MCF-7 and MDA-MB-231 cells were transfected with negative control, IncEGFR-AS1-siRNA/ELP5-siRNA pCMV6 or pCMV6-ELP5 plasmids, with or without miR-NC or miR-149-5p mimic/inhibitor for 48 h. Thereafter, we seeded MCF-7 or MDA-MB-231 cells (1,000/dish) 24 h post-transfection into 40 mm dishes and incubated for 10–15 d until visible colonies formed. Cells were then fixed with methanol, stained with hematoxylin, and manually counted.

Cell migration and invasion assays

Cell migration and invasion were assessed using similar protocols and were performed using a 24-well transwell chamber with 8 μ m pores (Costar, Cambridge, MA, USA). However, the difference between the methods lies in the type of upper chamber used. For the invasion assay, the transwell chamber was coated with 100 μ L of Matrigel (1:9 dilution; BD Biosciences); whereas for cell migration assays a normal chamber was employed. After transfection for approximately 24 h, cells were trypsinized, after which 1×10^5 cells, with 2% FBS in 100 μ L of migration medium, were seeded in the top chamber for 18 h, while 10% FBS was added to the lower chamber as a chemoattractant. After incubation at 37 °C in a humidified atmosphere containing 5% CO₂, a cotton swab was used to remove the cells, which were then

fixed with 4% paraformaldehyde and stained with hematoxylin. Finally, the cells were observed under fluorescence microscopy. All experiments were performed in triplicate.

Cellular fractionation assay of RNA

The PARISTM kit (Ambion, AM1921) was used to carry out the separation of nuclear and cytoplasmic RNA according to the manufacturer's instructions. The expression of U6 and β -actin were used as a cytoplasmic and nuclear control, respectively.

Dual-luciferase reporter assay

Wild-type or mut-lncEGFR-AS1 and wild-type or mut-ELP5 fragment of the 3'-UTR were constructed using the Dual-Luciferase Reporter System Kit (E1910, Promega, USA). Firefly and Renilla luciferase activities were measured, and Renilla luciferase was used as control. Subsequently, we co-transfected the miR-149-5p mimic with the reporter gene into MCF-7 and MDA-MB-231 cells using Lipofectamine 3000 and incubating the cells for 48 h (Invitrogen Co., Carlsbad, CA, USA).

Co-immunoprecipitation (co-IP)

BC cells were cultured with two large dishes(60mm) to full field incubated at 37 °C in a humidified atmosphere containing 5% CO₂. NP40 lysis (P0013; Beyotime Biosciences, Shanghai, China) buffer was then added. The cell solutions were centrifuged at 12000rpm for 20 minutes to extract protein lysates. After adding for 2 h using magnetic bead and serving to separate different cell populations based on the markers bound to the antibodies , the supernatant was centrifuged and TRAF2 antibody was added. The magnetic beads were then added and incubated on a 4 °C shaker at a uniform speed overnight and then shaken at the same speed for this additional 6-8 hours. The magnetic beads have already been added to the antibody-bound cells at 1000 rpm for 5 min and the cells will be pulled to the bottom of the tube. Subsequent protein co-immunoprecipitation was performed with an ELP5 antibody to detect interactions between the TRAF2 and ELP5.

Tumor xenografts in nude mice

Female BALB/C nude mice (4 weeks old) were purchased from Weitong Lihua (Beijing, China) and housed in an SPF-grade pathogen-free animal laboratory. Mice were intravenously injected on the right flank with 1×10^7 stably transfected MCF-7 cells in the logarithmic growth phase. Subcutaneous tumor volume was measured every three days beginning on Day 4 after cell injection according to the following formula: $0.5 \times \text{length} \times \text{width}^2$. On Day 21, placed in a closable box, according to the SOP plan, release carbon dioxide to suffocate the experimental animal. The tumors were resected and the final tumor volume, and weight, were determined. Tumors were snap frozen at - 80 °C for subsequent analysis.

Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD), and each experiment was performed in triplicate. The statistical software SPSS 22.0 (SPSS, Chicago, IL, USA) was used for all analyses. The Pearson's Chi-square test was used to assess possible correlations between ELP5 and clinical pathologic features of BC. Student's *t*-test was used to compare the significant differences between two groups. Differences were assessed using the log-rank test. Spearman's correlation analysis was used to identify associations among EGFR-AS1, miR-149-5p, and ELP5 mRNA. Mann-Whitney U-test was used to assess western blot images image and invasive assay results. $P < 0.05$ was considered statistically significant.

Results

Expression and correlation of EGFR-AS1, miR-149-5p and ELP5mRNA in BC patients

We first investigated several differentially expressed lncRNAs in BC and found that EGFR-AS1 was markedly upregulated. Moreover, TCGA and Starbase database analysis revealed that the level of EGFR-AS1 was upregulated in BC tissues compared with normal tissues (Fig. 1A–C). Similarly, the level of ELP5 was upregulated in BC tissues (Fig. 1 D–E). We then assessed the levels of EGFR-AS1, miR-149-5p, and ELP5 mRNA in 33 pairs of matched BC tissues by qRT-PCR, and found that the expression of EGFR-AS1 and ELP5 mRNAs in the BC tissues was upregulated, whereas that of miR-149-5p was downregulated (Figure 1 F–H). Furthermore, we investigated the relationship between EGFR-AS1, miR-149-5p, and ELP5 mRNA in BC tissues. Pearson's correlation analysis indicated negative correlations between miR-149-5p and EGFR-AS1($R^2=0.060$ $P<0.05$) and miR-149-5p and ELP5mRNA($R^2=0.090$ $P<0.05$) in the BC tissues and a positive correlation between EGFR-AS1 and ELP5 mRNA($R^2=0.204$ $P<0.01$) (Fig. 1I–K).

Effect of EGFR-AS1 knockdown on proliferation, migration, and invasion of BC cells *in vitro*

The results of qPCR analysis indicated higher levels of EGFR-AS1 in MCF-7, MDA-MB-231, MDA-MB-453, BT-549, and SK-BR-3 BC cell lines than in MCF-10A cells (Fig. 2A). Moreover, MCF-7 and MDA-MB-231 cells had the highest expression of EGFR-AS1 among the BC cell lines. Therefore, we selected MCF-7 and MDA-MB-231 cells for further analysis. To evaluate the biological functions of EGFR-AS1 in BC cell proliferation and migration, transient transfection of EGFR-AS1-expressing cell lines was performed, wherein si-EGFR-AS1-siRNA was used for knockdown functional analysis. The level of EGFR-AS1 were detected by qPCR to demonstrate the effectiveness of transfection (Fig. 2B). The growth curve of the MTT proliferation assay showed that the restraint of EGFR-AS1 significantly inhibited the growth of MCF-7 and MDA-MB-231 cells (Fig. 2C). Moreover, after transfection with si-EGFR-AS1, cell viability was more efficiently inhibited. Colony formation assay revealed that EGFR-AS1 decreased the number and size of colonies in the MCF-7 and MDA-MB-231 cells (Fig. 2D), with the same results obtained using EdU assays (Fig. 2E). Therefore, we determined that EGFR-AS1 knockdown markedly decreased cell proliferation. These results demonstrate that EGFR-AS1 promotes BC cell proliferation.

To further confirm that EGFR-AS1 facilitated the migration and invasion of BC cells, we assessed whether EGFR-AS1 knockdown affected the expression of proteins associated with cell migration and invasion,

namely, ROCK1, RhoA, RhoB, and matrix metalloproteinase (MMP)2. Result show that the expression of all of these proteins was dysregulated in EGFR-AS1 knockdown cultures (Fig. 3A).

In addition, local tumor invasion is the first step in tumor metastasis and requires significant changes in the adhesion and migration of tumor cells, which is reminiscent of the development of EMT. Therefore, we determined the levels of EMT-related proteins and found that EGFR-AS1 knockdown notably decreased the levels of EMT-related proteins, such as N-cadherin, SLUG, SNAIL, and Vimentin (Fig. 3B). Moreover, transwell assays showed that EGFR-AS1 knockdown significantly inhibited the migration and invasion of BC cells compared with those of control cells (Fig. 3 C–D). According to the literature, EGFR-AS1 knockdown also suppresses the expression of PI3K/AKT pathway markers [20], which agrees with our results (Fig. 3 E).

Collectively, these findings demonstrated that EGFR-AS1 promoted the proliferation, migration, and invasion of BC cells, consistent with our biofunction and western blot results. In addition, we evaluated patient prognosis using the TCGA database, which suggested that EGFR-AS1 expression is associated with poor prognosis in BC (Fig. 3 F–G).

Maintenance of EGFR mRNA stability by EGFR-AS1 via complementary binding

Previous studies have demonstrated that lncRNAs can be broadly divided into cis-acting lncRNAs, affecting the expression or chromatin state of nearby genes, and trans-acting lncRNAs that perform various functions in the cell [20, 21, 31, 32]. Considering that EGFR-AS1 is transcribed on the opposite strand of EGFR, we analyzed the RNA sequence base pairing between EGFR-AS1 and EGFR mRNA. A long base-pairing with a length of 194 bp was predicted by NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/>) (Fig. 4A). Next, we detected the expression of *EGFR* mRNA in the same paired 33 BC tissues by qRT-PCR, and observed an upregulation in the expression of these mRNAs (Fig. 4B). Moreover, we observed that EGFR expression was positively correlated with EGFR-AS1 in BC tissues (Fig. 4C). Previous studies have demonstrated the ability of EGFR-AS1 to cis-regulate the neighboring gene *EGFR* in hepatocellular carcinoma (HCC) tissues [33]. Thus, we investigated whether EGFR-AS1 affects *EGFR* expression in BC tissues and cells. EGFR protein abundance was significantly higher in EGFR-high samples compared with EGFR-low BC tissue samples (Fig. 4D). Furthermore, the expression of *EGFR* mRNA and abundance of EGFR protein were detected. EGFR was markedly decreased following EGFR-AS1 knockdown in BC cells (Fig. 4E and F). Thereafter, we investigated whether EGFR-AS1 knockdown suppressed *EGFR* mRNA expression following treatment with actinomycin D (ActD), a transcriptional inhibitor. The mRNA stability of EGFR were tested by qRT-PCR in 0h,2h,4h,8h,12h in BC cells. The greatest effect was observed after 4 h, indicating that *EGFR* mRNA stability decreased following EGFR-AS1 silencing (Fig. 4G). These results demonstrated that EGFR-AS1 binds to EGFR mRNA and affects its stability in BC cells. Finally, nucleoplasmic separation experiments revealed that EGFR-AS1 localizes in both the cytosol and nucleus of BC cells (Fig. 4H) and, thus, participates in target gene regulation.

Effects of ELP5 on proliferation, metastasis, and invasion of BC cells

ELP5 is a component of the RNA polymerase II elongator complex, a multiprotein complex associated with the RNA polymerase II holoenzyme, and is involved in transcriptional elongation [26]. ELP5 participates in controlling cell motility and the tumorigenicity of melanoma cells [25]. To examine the effects of ELP5 expression on tumorigenesis-related processes in BC cells, we performed several experiments. First, we analyzed the level of ELP5 in 33 paired BC and adjacent tissue samples using western blotting and determined that the expression of ELP5 was higher in BC tissues than in adjacent normal tissues (Fig. 5A and S1a). Moreover, western blotting and qPCR results indicated higher levels of ELP5 in MCF-7, MDA-MB-231, MDA-MB-453, BT-549, and SK-BR-3 BC cell lines compared to MCF-10A cells (Fig. 5B and C). Next, immunochemistry analysis of 15 paired BC and paired normal tissue samples showed higher levels of ELP5 in BC tissues than in adjacent normal tissues (Fig. 5D).

We then constructed ELP5 siRNA and overexpression plasmid constructs to explore the function of ELP5 in BC (Fig. S1b). Changes in ELP5 protein level affected the expression of proteins involved in cell migration and invasion as well as that of EMT-related markers, with ELP5 depletion resulting in decreased expression of RhoA, ROCK1, MMP2, E-cadherin, Vimentin, Snail, and Zeb and an increase in RhoB expression. However, ELP5 overexpression caused the opposite effect, suggesting that ELP5 promotes the proliferation, migration, and invasion of BC cells (Fig. 5E-F and S1c-d). ELP5 depletion inhibited the expression of PI3K/AKT pathway markers, whereas ELP5 overexpression promoted the expression of these markers (Fig. 5G and S1e). We then evaluated the effects of this pathway using LY294002, which inhibits PI3K expression and reduces phosphorylation in the MCF-7 and MDA-MB-231 cells transfected with P-ELP5. LY294002 administration reduced the induction of the various cellular functions, and the negative correlation between ELP5 expression and that of RhoA, ROCK1, P21, and cyclinD1, was reversed (Fig. 5H-I and S1f-h).

EGFR-AS1 as a miR-149-5p molecular sponge

It is widely acknowledged that lncRNAs can transcriptionally, or post-transcriptionally, activate downstream genes [33, 34]. As mentioned before, EGFR-AS1 is principally located in the cytoplasm and plays a primary role in post-transcriptional regulation [20, 21, 33]. The ceRNA network is a common post-transcriptional regulatory pattern of lncRNAs. To verify the hypothesis that EGFR-AS1 acts as a ceRNA to regulate mRNA expression, we predicted the target miRNAs of EGFR-AS1 by StarBase, miRBase, and RegRNA. We then selected the top three common miRNAs, namely hsa-miR-149-5p, hsa-miR-195-3p, and hsa-miR-661, which can be absorbed by EGFR-AS1. Moreover, qRT-PCR analysis was performed to analyze the expression of these three miRNAs. In this case, we confirmed the most evident regulatory effect of EGFR-AS1 and miR-149-5p in BC cell lines (Figure. 6A and S2a). Reports have shown that miR-149-5p plays a vital role in the suppression of cancer by targeting various oncogenes that regulate tumor-related processes [14, 16, 22–24]. Our analysis suggested that the level of miR-149-5p was markedly lowered in BC cell lines compared to MCF-10A cells (Figure. 6B).

Next, the dual-luciferase reporter assay was used to detect direct binding between EGFR-AS1 and miR-149-5p based on their complementary sequences. An EGFR-AS1 fragment was constructed and inserted

downstream of the luciferase reporter gene. We then co-transfected a miR-149-5p mimic with the reporter gene into MCF-7 and MDA-MB-231 cells. A significant reduction in luciferase reporter activity was observed relative to co-transfection with control RNA, while the co-transfection of miR-149-5p and mut-EGFR-AS1 did not induce a significant reduction in the luciferase signal (Fig. 6C–D and S2b). Therefore, a direct interaction between EGFR-AS1 and miR-149-5p was confirmed.

To investigate whether miR-149-5p was regulated by EGFR-AS1 in BC cells, we assessed the levels of ELP5, ROCK1, RhoA, and P21 via the co-transfection of cells with si-EGFR-AS1 and miR-149-5p inhibitors. Interestingly, the inhibition of ELP5, ROCK1, RhoA, and P21 protein expression induced by si-EGFR-AS1 was reversed by miR-149-5p inhibition (Fig. 6E and S2c). Thus, we sought to determine whether the biological function of EGFR-AS1 in BC cells could also be reversed by miR-149-5p inhibitors. Our analysis revealed reduced proliferation, as indicated by the MTT proliferation assay, colony formation experiments, and EdU assay. Likewise, reduced metastasis and invasion revealed by the transwell assay in BC cells and mediated by EGFR-AS1 knockdown was successfully restored by miR-149-5p inhibitors (Fig. 6F–I and S2d–g).

ELP5 is the functional target of miR-149-5p in BC

To identify miR-149-5p target genes, we screened the StarBase and TargetScan databases for the predicted targets of miR-149-5p and combined them with the genes that were significantly upregulated in the TCGA BC database. Using this strategy, we identified 12 genes (Fig. S3a).

Experiments were then performed to identify the interaction between miR-149-5p and ELP5. First, we detected the levels of miR-149-5p mimics and inhibitor in the transient transfection of cell lines by qPCR to confirm successful transfection (Fig. S3b–e). Next, the binding sequence of miR-149-5p to ELP5 3'UTR was predicted and obtained (Fig. 7A). We then examined the luciferase activity of the reporter containing wild-type ELP5 (ELP5-WT) or mutant-type ELP5 (ELP5-MUT) in cells transfected with miR-149-5p mimics. The results suggested that the luciferase activity of the ELP5-WT vector was only restrained by miR-149-5p mimics (Fig. 7B and S4a). qPCR and western blotting were then used to verify the expression of ELP5 at the mRNA and protein levels. The results showed that the mRNA expression and protein levels of ELP5 were negatively regulated by miR-149-5p (Fig. 7C–D and S4b–c).

To further investigate whether ELP5 was regulated by miR-149-5p in BC cells via the co-transfection of cells with the miR-149-5p inhibitor and si-ELP5, we measured the protein levels of ELP5, ROCK1, RhoA, and P21 by western blotting. Interestingly, inhibition of ELP5, ROCK1, RhoA, and P21 protein expression induced by the miR-149-5p inhibitor was effectively reversed by ELP5 knockdown (Fig. 7E and S4d). We then sought to determine whether the biological function of miR-149-5p inhibitors in BC cells could also be reversed by ELP5 knockdown, and observed increased proliferation, as indicated by the MTT proliferation assay, colony formation experiments, and EdU assay (Fig. 7F–H and S4e–h). Moreover, increased metastasis and invasion ability indicated by the transwell assay in BC cells mediated by miR-149-5p inhibition were successfully restored by ELP5 knockdown in BC cells (Fig. 7I).

Role of the EGFR-AS1-miR-149-5p-ELP5/PI3K/AKT axis in the metabolic alteration of BC cells and promotion of BC development

Based on these results, we hypothesized that EGFR-AS1 promotes the proliferation, migration, and invasion of BC cells by sponging miR-149-5p; thus, EGFR-AS1 regulates ELP5 expression. To test this hypothesis, we performed an immunohistochemical analysis that showed that the ELP5 protein expression in BC tissues was positively correlated with EGFR-AS1 expression (Fig. 8A). As mentioned above, qRT-PCR further confirmed the expression level of ELP5 and indicated that it was upregulated in 33 BC tissues relative to adjacent normal tissues (Fig. 8B). We then determined whether EGFR-AS1 was co-expressed with ELP5. According to the median expression of ELP5, we sorted the 33 BC tissue samples into a low ELP5 (n = 16) and high ELP5 (n = 16) expression group. We then detected the EGFR-AS1 level in the two groups and found that the level of EGFR-AS1 in the high ELP5 group was significantly higher than that in the low ELP5 group (Fig. 8C). To further analyze the regulatory role of EGFR-AS1 on ELP5, we knocked down EGFR-AS1 expression in MCF-7 and MDA-MB-231 cells using EGFR-AS1 siRNA. The results showed that EGFR-AS1 knockdown decreased ELP5 levels, as indicated by qRT-PCR and western blotting (Fig. 8D and 8E). Thus, we demonstrated the positive regulation of ceRNA ELP5 by EGFR-AS1.

According to previous studies, the EMT-related markers N-CAD and Vimentin are both regulated by EGFR-AS1, and the ELP5/PI3K/AKT pathway can also be activated by EGFR-AS1 and ELP5. Through rescue assays and based on the results of further western blotting analysis of ELP5, N-CAD, Vimentin, PI3K, P-AKT, and AKT protein abundance, we determined that ectopic ELP5 expression reversed the inhibitory effects of EGFR-AS1 suppression (Fig. 8F).

Role of EGFR-AS1 silencing in the promotion of docetaxel sensitivity in BC cells

Chemotherapy is crucial to the effective treatment of BC; however, it is limited by the development of chemoresistance [7, 8, 35]. Increasing evidence supports the idea that lncRNAs are key regulators of drug resistance [16, 17]. Therefore, we examined the ability of EGFR-AS1 to act as a mechanism of resistance to the targeted drug docetaxel. First, to test the IC₅₀ of docetaxel in MCF-7 and MDA-MB-231 cells, they were treated with a concentration gradient of docetaxel. After 48 h, the IC₅₀ values of docetaxel in MCF-7 and MDA-MB-231 cells were 9.59 nM and 4.771 nM, respectively (Fig. 9A). Next, the concentrations of docetaxel corresponding to the individual IC₅₀s and half the IC₅₀ concentration were added, and the EGFR-AS1 and miR-149-5p levels were assessed by qRT-PCR. In the two cell lines, the level of EGFR-AS1 increased with increasing drug concentration, indicating that the increase in EGFR-AS1 was docetaxel-dependent (Fig. 9B). Moreover, the level of miR-149-5p decreased with increasing drug concentration, thus, demonstrating that the decrease in miR-149-5p was also docetaxel-dependent (Fig. 9C).

Similar results were obtained via western blotting. In the two cell lines, ELP5 was increased in a docetaxel-dependent manner (Fig. 9D and 9E). These results indicated that EGFR-AS1, miR-149-5p, and ELP5 are involved in the resistance to docetaxel. After silencing EGFR-AS1, the IC₅₀s of MCF-7 and MDA-MB-231 cells decreased to 5.06 nM and 2.36 nM, respectively (Fig. 9F). Colony formation and MTT

proliferation assays were then conducted following addition of the IC50 concentration of docetaxel to MCF-7 and MDA-MB-231 cells. Cells with lower EGFR-AS1 expression had lower cell viability (Fig. 9G and 9H). These experiments confirmed our hypothesis that silencing EGFR-AS1 can promote sensitivity to docetaxel in BC cells, which provides new insights into its clinical application.

EGFR-AS1 knockdown as an *in vivo* inhibitor of BC cell tumorigenesis

We then performed an *in vivo* murine study to identify the role of EGFR-AS1 in BC growth regulation. MCF-7 cells stably transfected with sh-EGFR-AS1, or sh-NC were subcutaneously injected into BALB/c nude mice. After 21 days, the tumors were removed, measured and weighed. The results indicated that the mice injected with EGFR-AS1-silenced MCF-7 cells developed markedly smaller tumors than the control cells (Fig. 10A). Moreover, as can be seen from the tumor growth curve, EGFR-AS1 knockdown significantly suppressed tumor growth compared with the negative control group (Fig. 10B). Similar results were observed for tumor volume and tumor weight (Fig. 10C and 10D). Moreover, following harvesting of tumors qRT-PCR and western blot studies were performed, revealing that the mRNA expression of EGFR-AS1 and ELP5 was decreased (Fig. 10E and 10F), and the expression of ELP5, EMT-related proteins (N-CAM, Vimentin, Slug, Snail), and PI3K/AKT pathway-related markers was similarly reduced in the tumors of mice injected with EGFR-AS1-silenced MCF-7 cells (Fig. 10G-I). These results suggested that EGFR-AS1 promotes *in vivo* BC tumor growth and metastasis.

Discussion

BC is a significant burden on human health worldwide. Moreover, its therapeutic efficacy is limited, and complications from distant metastasis may cause severe symptoms and even death [2, 3]. Therefore, it is essential to investigate how BC is developed to advance chemotherapeutic efficacy. Recently, newly discovered lncRNAs have been reported as crucial participants in tumorigenesis and may be involved in pleiotropic modulation [36–38]. In this study, the role of lncRNA EGFR-AS1 in BC was characterized.

lncRNA EGFR antisense RNA 1 (EGFR-AS1) is a 2.8 Kb transcript that transcribes in the antisense strand of EGFR, located on chromosome 7p11.2. EGFR-AS1 has been reported to play oncogenic roles in hepatocellular carcinoma and gastric cancer [20, 33]. In the present study, we identified that lncRNA EGFR-AS1 was expressed at a high level in TCGA BC samples. Similarly, upregulated lncRNA EGFR-AS1 was identified in BC tissues and cell lines. The current *in vitro* studies also demonstrated that EGFR-AS1 knockdown inhibits the proliferation, migration, and invasion of BC cells, indicating the oncogenic role of EGFR-AS1 in BC and its potential value as a biomarker to predict the prognosis of BC patients. These results further suggest that EGFR-AS1 activates the PI3K/AKT signaling pathway, which exerts a positive effect on EMT, promoting tumor metastasis [39, 40]. An *in vivo* murine study further demonstrated that EGFR-AS1 could promote BC growth, migration, and invasion, while the intratumoral knockdown of EGFR-AS1 was effectively mediated by the PI3K/AKT signaling pathway.

It is noteworthy that EGFR-AS1 is predominantly localized in the cytoplasm, indicating its post-transcriptional regulatory potential in BC. Structurally, EGFR-AS1 has a partial sequence complementary

to that of EGFR; thus, EGFR-AS1 is able to cis-regulate the neighboring gene *EGFR* [20, 21]. Previous studies have shown that EGFR-AS1 overexpression is sufficient to induce resistance to tyrosine kinase inhibitors, while EGFR-AS1 knockdown induces sustained tumor regression of squamous cell carcinoma [41]. Moreover, EGFR-AS1 upregulation in BC cells has been reported and EGFR-AS1 was found to improve the invasion and proliferation of HCC cells *in vitro* and *in vivo* by upregulating EGFR expression [33]. In the present study, we observed that the expression of EGFR-AS1 was positively correlated with EGFR in BC. Moreover, mRNA and protein expression of EGFR were significantly decreased when EGFR-AS1 was silenced. In line with these observations, EGFR-AS1 knockdown decreased EGFR mRNA levels after treatment with actinomycin D (ActD), a transcriptional inhibitor [42]. However, as we have not explored the mechanisms by which EGFR-AS1 regulates the stability of EGFR at the protein or mRNA levels, this should be further verified.

It is widely acknowledged that lncRNAs can act as competitive endogenous RNAs (ceRNAs) that regulate the expression of cancer-related genes by competing with miRNA response elements (MREs) at the post-transcriptional level [43, 44]. Moreover, miR-149-5p is downregulated in BC tissue and plays a vital role in the suppression of cancer by targeting multiple oncogenes that regulate tumor-related processes [45, 46]. We applied bioinformatics prediction and a dual-luciferase assay to verify that miR-149-5p serves as the binding miRNA partner of EGFR-AS1, depending on their complementary binding. Moreover, Pearson's correlation analysis revealed negative correlations between miR-149-5p and the EGFR-AS1 expression in pairs of matched BC tissues by qRT-PCR analysis. EGFR-AS1 knockdown increased miR-149-5p expression, whereas overexpression of miR-149-5p decreased EGFR-AS1 expression in a sequence-dependent manner. Moreover, the EGFR-AS1/miR-149-5p functional network was confirmed to modulate BC cell malignant behavior. We then performed a rescue experiment, revealing that the EGFR-AS1 knockdown-induced suppression of tumor proliferation, migration, and invasion was reversed by treatment with an miR-149-5p inhibitor. Our results provide evidence to support the hypothesis that EGFR-AS1 functions as a miR149-5p sponge and is significant for the progression and metastasis of BC cells.

According to our experiments and bioinformatic analysis, we found that EGFR-AS1 knockdown decreased ELP5 expression by impairing the miR-149-5p level, which negatively regulated ELP5 by targeting its 3'-UTR. Moreover, we determined that ELP5 is the target of miR-149-5p at the mRNA and protein levels. ELP5 can be characterized as an elongator subunit that controls cell motility and the tumorigenicity of melanoma cells [25, 26, 47, 48]. A bioinformatics prediction and dual-luciferase assay were performed to demonstrate the interaction between miR-149-5p and ELP5. Moreover, qPCR, western blot, and Pearson's correlation analysis demonstrated that the mRNA and protein levels of ELP5 were negatively regulated by miR-149-5p. A rescue experiment was also performed, and the results revealed that the miR-149-5p inhibitor-induced suppression of tumor proliferation and migration was reversed by treatment with ELP5 knockdown, as indicated by a functional experiment, RT-qPCR, and western blot analyses.

As mentioned above, ELP5 was upregulated in BC cells and tissues. After constructing ELP5 siRNA and overexpression plasmid constructs, we observed, for the first time, that ELP5 markedly promoted the proliferation, migration, and invasion of BC cells. KEGG database analysis further revealed an interaction

between ELP5 and TRAF2(Fig. S5a-c); the TRAF family is reportedly the primary signal transducer of the TNF receptors(P55and p75) and interleukin (IL)-1 receptor/Toll-like receptor (IL-1R/TLR) superfamily. Thus, this family of proteins participates in a variety of biological processes, including immunity, inflammation, and apoptosis, thereby, providing insights for further in-depth exploration of the role played by ELP5 in BC.

Mechanistically, the PI3K/AKT pathway is involved in ELP5, which regulates the malignant progression of BC cells. Specifically, we found that ELP5 alters PI3K and p-AKT, resulting in reduced activation of the AKT pathway and stimulation of downstream gene transcription. We evaluated the effects of this pathway using the LY294002 inhibitor, which inhibits the PI3K expression and reduces AKT phosphorylation [49]. In BC cells transfected with P-ELP5, LY294002 administration reduced the induction of cell proliferation, migration, and invasion, while the negative correlation between the ELP5 expression and RhoA, ROCK1, P21, and cyclinD1 levels was reversed. Furthermore, Pearson's correlation analysis, and immunohistochemical analysis, showed that the ELP5 protein expression in BC tissues was positively correlated with EGFR-AS1 expression. EGFR-AS1 knockdown successfully blocked ELP5 expression at the mRNA and protein levels in the BC cells. Moreover, using a rescue assay, we determined that EGFR-AS1 knockdown blocked the malignant behavior of ELP5 overexpression. These results were verified by bioinformatics, RT-qPCR, and western blot analyses in the BC cell lines. Thus, a novel EGFR-AS1/miR-149-5p/ELP5 axis was found to be upregulated in BC tumorigenesis.

More importantly, an increasing amount of evidence suggests that lncRNAs are vital regulators of drug resistance and, consequently, the modulation of their activity could provide new treatment strategies for BC [50]. For example, Liu et al. (2010) reported that HOTAIR contributes to cisplatin resistance in human lung adenocarcinoma cells via the downregulation of p21 WAF1/CIP1 expression. Furthermore, they identified lncRNA EPB41L4A-AS2 as a potential biomarker to evaluate docetaxel sensitivity [51, 52]. As previously reported, docetaxel is typically used in the treatment of numerous cancer types, including BC [53]. Docetaxel is a taxane-derived antimitotic chemotherapeutic agent, and its major molecular mechanisms include antagonizing the drug-stabilizing effect on microtubules and limiting the intracellular drug concentration [6, 7]. In this study, we demonstrated that EGFR-AS1 silencing could sensitize BC cells to docetaxel; thus, EGFR-AS1 has a potentially significant role in the mechanism of docetaxel resistance.

Conclusions

In conclusion, this study provides novel insights into the upregulation of EGFR-AS1 and ELP5 in BC tissues and cell lines, as well as the role of these RNA genes in promoting the progression of BC cells. EGFR-AS1 was demonstrated to regulate the expression of ELP5 by sponging miR-149-5p, ultimately activating the PI3K/AKT pathway and inducing EMT in BC cells. Thus, a novel EGFR-AS1/miR-149-5p/ELP5 axis revealed a novel oncogenic pathway in BC tumorigenesis. The results of this study will prove essential in investigations of the molecular mechanisms associated with BC tumorigenesis, and provides novel insights into diagnostic or docetaxel-related chemotherapy targets for BC.

Abbreviations

BC: Breast cancer; lncRNAs: Long non-coding RNAs; EGFR-AS1: EGFR transcript antisense RNA; ELP5: Elongator acetyltransferase complex subunit 5; qRT-PCR: Quantitative real-time PCR; IHC: Immunohistochemistry; EMT: Epithelial-mesenchymal transformation; ceRNAs: Competitive endogenous RNAs; MREs: miRNA response elements; DMEM: Dulbecco's modified eagle medium; si-NC: Negative control siRNA; OD: Optical density; co-IP: Co-immunoprecipitation; DMSO: Dimethyl sulfoxide; FBS: Fetal bovine serum; MEM: Modified eagle medium; MMP: Matrix metalloproteinase; PBS: Phosphate-buffered saline; PI3K: Phosphatidylinositol-3 kinase; ROCK: Rho-associated protein kinase;

Declarations

Ethics approval and consent to participate

All patients provided signed informed consent. This study approved by the Institutional Research Ethics Committee (No. 2015 [LS]023, China Medical University) for the usage of the clinical materials for research purposes. Animal experiments were conducted in strict accordance with protocols approved by the animal research ethics committee of China Medical University and the principles of the declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. Further details are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

XM, MS, KD, and SL contributed conception and design of the study. SL, JZ, MH, JW, HL, BL and QC performed experiment and the statistical analysis. SL and MS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Figures

Figure 1

The expression of EGFR-AS1 in breast cancer (BC) tissues. (A) Expression of EGFR-AS1 in the TCGA cancer cohort: (B–E) Expression of EGFR-AS1 and ELP5 in BC patients from the TCGA and StarBase databases; (F–H) Expression of EGFR-AS1, miR-149-5p, and ELP5 mRNA in BC tissues detected by qRT-PCR; (I–K) Pearson's correlation analysis among EGFR-AS1, miR-149-5p, and ELP5 mRNA. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 2

Knockdown of EGFR-AS1 inhibits the proliferation of breast cancer (BC) cells in vitro. (A) The levels of EGFR-AS1 in MCF-7, MDA-MB-231, MDA-MB-453, BT-549, and SK-BR-3 BC cell lines compared with MCF-10A cells; (B) Transfection efficiency of si-EGFR-AS1 verified by qRT-PCR analysis; (C) Results of the MTT proliferation assay for detection of cell viability after FOXP4-AS1 knockdown; (D) Inhibiting ability of

EGFR-AS1 silencing on colony formation; (E) Effect of EGFR-AS1 silencing on inhibition of DNA synthesis, as determined by the EdU assay; scale bar for EdU staining: 100 μ m. *P <0.05, **P <0.01 and ***P <0.001.

Figure 3

Knockdown of EGFR-AS1 inhibits the migration and invasion of breast cancer (BC) cells in vitro. (A) Expression of proteins associated with cell migration and invasion; (B) expression of proteins associated with EMT-related markers; (C-D) Effect of EGFR-AS1 knockdown on the migration and invasion abilities of

BC cells; (E) Changes in the expression and activation of PI3K/AKT signaling proteins; (F-G) Relationship between high EGFR-AS1 expression and patient prognosis in breast adenocarcinoma (derived from the TCGA database). *P <0.05, **P <0.01 and ***P <0.001.

Figure 4

Positive correlation between the level of EGFR-AS1 and EGFR in breast cancer (BC) tissues (A) Sequence of overlap region between EGFR-AS1 and EGFR mRNA (194 bp); (B) EGFR mRNA expression in BC tissues

detected by qRT-PCR; (C) Pearson's correlation analysis of EGFR-AS1 and EGFR mRNA levels; (D) The protein level of EGFR was much higher in EGFR-high samples compared with EGFR-low samples of the BC tissues (E–F) EGFR expression in MCF-7 and MDA-MB-231 cells after transfection with si-EGFR-AS1; (G) Results of RNA stability assays performed in BC cell lines using actinomycin D to disrupt RNA synthesis. The degradation rate of EGFR mRNA was measured and calculated over 12 h. EGFR mRNA levels were measured in the EGFR-AS1 knockdown and NC groups. *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 5

ELP5 promotes proliferation, migration, and invasion in breast cancer (BC) cells. (A) Results of western blot analysis performed using the same tissues as in (Fig. 1B–E). The ELP5 expression was higher than that in the BC tissues from adjacent normal tissues. (B–C) Levels of ELP5 in MCF-7, MDA-MB-231, MDA-MB-453, BT-549, and SK-BR-3 BC cell lines compared with MCF-10A cells. (D) IHC staining of ELP5 in 15 paired BC and adjacent tissues. Scale bar: 50 μ m. (E–F) BC cells transfected with ELP5-specific (si-ELP5), control (NC) siRNA, and an ELP5 expression (p-ELP5) or vehicle (p-NC) plasmid. Western blotting was performed to evaluate the transfection and silencing efficiency. Expression changes in EMT-related proteins, and changes in cell migration, and invasion-related proteins in MCF-7 cells, respectively. (G) Changes in the expression of PI3K/Akt-related proteins in MCF-7 cells (H) Western blot analysis of cells transfected with p-ELP5 in the presence or absence of treatment with the PI3K inhibitor LY294002. (I–J) Changes in the proliferation activity of BC cells in the presence or absence of treatment with the PI3K inhibitor LY294002 and transfection with p-ELP5. *P <0.05, **P <0.01 and ***P <0.001.

Figure 6

EGFR-AS1 acted as the molecular sponge of miR-149-5p (A) Effect of EGFR-AS1 on miR-149-5p, miR-195-3p and miR-661 expression in breast cancer (BC) examined by qRT-PCR. (B) Levels of miR-149-5p in MCF-7, MDA-MB-231, MDA-MB-453, BT-549, and SK-BR-3 BC cell lines compared with MCF-10A cells. (C) Potential binding sites between EGFR-AS1 and miR-149-5p obtained from StarBase. (D) Targeting relationship between EGFR-AS1 and miR-149-5p validated using the dual-luciferase reporter assay. (E)

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ELP5 is the target of miR-149-5p in breast cancer (BC) (A) Potential binding site between miR-149-5p and ELP5 obtained from TargetScan. (B) Targeting relationship between miR-149-5p and ELP5 verified by the dual-luciferase reporter assay. (C–D) Level of ELP5 protein detected by qRT-PCR and western blotting after transfection. (E) Effect of miR-149-5p and ELP5 on expression of ELP5, ROCK1, P21, and RHOA. (F–H) Increase in BC cell proliferation mediated by miR-149-5p inhibition and successfully blocked by ELP5 knockdown. (I) Increase in metastasis and invasion of BC cells mediated by miR-149-5p inhibition and successfully blocked by ELP5 knockdown. *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 8

Positive correlation between the levels of EGFR-AS1 and ELP5 in breast cancer (BC) (A) Representative images of immunohistochemistry staining and the association between the expression of ELP5 protein and EGFR-AS1 in the analyzed BC tissues. (B–C) ELP5 upregulation in 33 BC tissues compared with adjacent normal tissues. EGFR-AS1 expression in both groups was significantly higher than that in the low ELP5 group (n = 16). (D–E) ELP5 expression examined by western blotting and qRT-PCR in MCF-7 and MDA-MB-231 cells after transfection with si-EGFR-AS1. (F) Effect of EGFR-AS1 knockdown and ELP5 overexpression on the levels of PI3K/Akt pathway molecules and EMT markers. *P <0.05, **P <0.01 and ***P <0.001.

Figure 9

EGFR-AS1 silencing promotes sensitivity of breast cancer (BC) cells to docetaxel. (A) Detection of half-lethal dose (IC₅₀) of docetaxel in MCF-7 and MDA-MB-231 cells using an appropriate concentration gradient. After a 48-h culture period, the IC₅₀s values of MCF-7 and MDA-MB-231 cells were 9.590 nM and 4.771 nM, respectively. (B–C) Concentrations of docetaxel corresponding to the appropriate IC₅₀ and half the IC₅₀ added to the two cell lines. The cells were cultured for 48 h, and RNA was extracted for

qPCR analysis. (D–E) The cells were cultured for 48 h at different drug concentrations and then subject to western blot analysis. (F) IC₅₀s values of shEGFR-AS1 MCF-7 and MDA-MB-231 cells. (G–H) The transfected cells were treated with the IC₅₀ dose of docetaxel. Cell viability was assessed by the MTT assay and colony formation assay. *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 10

EGFR-AS1 promoted breast cancer tumor growth in vivo (A) Stable expressing cells used for the in-vivo study. Nude mice carrying tumors from the respective groups and the sample tumors from each group are shown. (B) Volume of subcutaneous tumors measured every three days after injection. The tumor growth curve shows that EGFR-AS1 knockdown significantly inhibited tumor growth in the mice. (C–D) Tumor volume and weight in the different groups. (E–F) Expression of EGFR-AS1 and ELP5 detected by qRT-PCR in tumors harvested from mice. (G–I) Protein levels of ELP5, N-CA, Vimentin, Slug, Snail, PI3K, p-Akt, and Akt in the tumors harvested from mice detected by western blot. The data represent the mean $\pm$ SD from three independent experiments. *P <0.05, **P <0.01 and ***P <0.001.

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