

Long non-coding RNA UCA1 confers tamoxifen resistance in breast cancer endocrinotherapy through regulation of the EZH2/p21 axis and the PI3K/AKT signaling pathway

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Abstract. Tamoxifen is the gold standard for breast cancer endocrinotherapy. However, drug resistance remains a major limiting factor of tamoxifen treatment. Long non-coding (lnc) RNA serves an important role in drug resistance; however, the molecular mechanisms of tamoxifen resistance in breast cancer endocrinotherapy are largely unclear. lncRNA urothelial cancer associated 1 (lncRNA UCA1, UCA1) has been proven to be dysregulated in human breast cancer and promotes cancer progression. In the present study, it was demonstrated that UCA1 was significantly upregulated in breast cancer tissues compared with healthy tissues. Furthermore, the expression level of UCA1 was significantly greater in tamoxifen-resistant breast cancer cells (LCC2 and LCC9) when compared with those in the tamoxifen-sensitive breast cancer cells (MCF-7 and T47D). UCA1 silencing in LCC2 and LCC9 cells increased tamoxifen drug sensitivity by promoting cell apoptosis and arresting the cell cycle at the G₂/M phase. Notably, the induced overexpression of UCA1 in MCF-7 and T47D cells decreased the drug sensitivity of tamoxifen. The molecular mechanism involved in UCA1-induced tamoxifen-resistance was also investigated. It was identified that UCA1 was physically associated with the enhancer of zeste homolog 2 (EZH2), which suppressed the expression of p21 through histone methylation (H3K27me3) on the p21 promoter. In addition, it was demonstrated that UCA1 expression was paralleled to the phosphorylation of CAMP responsive element binding protein (CREB) and AKT. When LCC2 cells were treated with the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway

inhibitor LY294002, the phosphorylation levels of CREB and AKT were significantly downregulated. Taken together, it was concluded that UCA1 regulates the EZH2/p21 axis and the PI3K/AKT signaling pathway in breast cancer, and may be a potential therapeutic target for solving tamoxifen resistance.

Introduction

Breast cancer is the most common female malignancy and the second most common cause of cancer-associated fatality in the world (1). Approximately 70% of patients with breast cancer are estrogen receptor-positive (ER⁺) (2). Apart from surgery, endocrine therapy (including tamoxifen, fulvestrant and letrozole) has improved the overall survival and quality of life for patients with breast cancer (2-4). Among all endocrine therapies, tamoxifen is the most extensively used hormone therapy and functions as an estrogen antagonist in breast cancer (5,6). Although the majority of patients with ER⁺ breast cancer benefit from tamoxifen therapy, many tumors eventually recur because of tamoxifen resistance (7,8). Tamoxifen resistance can arise via several mechanisms, including loss of ER α , induction of abnormal estradiol levels and alterations of coregulatory proteins, including amplified in breast cancer 1 and histone deacetylase (9-11).

An increasing number of long non-coding (lnc)RNAs in the human genome have been identified, and have provided new directions in cancer research (12). lncRNA, a class of non-protein coding transcripts with >200 nucleotides, regulates protein-coding genes during transcription and post-transcription in a sequence-specific manner (13-15). Importantly, lncRNAs in cancer cells are associated with the formation of tamoxifen resistance (16-18). However, only a few lncRNAs have been proposed to be clinically relevant biomarkers for tamoxifen resistance, such as H19 and homeobox antisense intergenic RNA (19-21). Searching for appropriate lncRNAs is valuable for the management of tamoxifen-resistance.

Out of the numerous cancer-associated lncRNAs, lncRNA urothelial carcinoma-associated 1 (UCA1) serves an important oncogenic role in several cancer types, including bladder cancer, colorectal cancer and gastric cancer (22). UCA1 has three exons that encode a 1.4-kb isoform and a 2.2-kb isoform (23). It was originally identified as a urine marker (the 1.4-kb isoform) in

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bladder cancer (24). Tuo *et al* (25) demonstrated that UCA1 can modulate breast cancer cell growth and apoptosis through downregulation of the tumor suppressor microRNA (miR)-143. Huang *et al* (23) reported that UCA1 can promote breast tumor growth by suppressing the level of p27. UCA1 is also associated with the poor prognosis of cancer. Bian *et al* (26) demonstrated that patients with colorectal cancer and higher UCA1 expression had a significantly poorer prognosis. Furthermore, it was reported that UCA1 expression was correlated with a reduction in recurrence-free survival in breast cancer (27). These findings highlight the important role of UCA1 in cancer development.

The polycomb group protein enhancer of zeste homolog 2 (EZH2) is a critical regulator of tumorigenesis (28,29). It has been demonstrated that the level of EZH2 is elevated in human bladder cancer, breast cancer, colon cancer and prostate cancer (30). Furthermore, the expression and mutation of EZH2 can regulate the level of H3K27me3 (31). In hepatocellular carcinoma, UCA1 repressed p27 expression through its association with EZH2, which suppresses p27Kip1 through H3K27me3 on the p27Kip1 promoter (32). However, the effects of UCA1 on EZH2 expression and the underlying molecular mechanisms in breast cancer are not fully understood.

The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway is the most frequently altered pathway in human cancer, and previous studies have demonstrated that UCA1 regulates the cell cycle progression of bladder carcinoma cells via PI3K/AKT-dependent signaling (33,34). Notably, the knockdown of UCA1 inhibits AKT phosphorylation in breast cancer cells (35). Additionally, activation of the PI3K/AKT signaling pathway has been demonstrated to confer resistance to antiestrogens in tamoxifen-resistant breast cancer cells (36). Therefore, it would be useful to determine whether UCA1 is involved in the PI3K/AKT signaling pathway and if it induces tamoxifen resistance in breast cancer cells.

In the present study, the level of UCA1 expression was investigated in tamoxifen-resistant cells and compared with tamoxifen-sensitive cells. Induction of UCA1 overexpression in MCF-7 and T47D breast cancer cells and silencing of UCA1 in LCC2 and LCC9 breast cancer cells was performed to assess the drug sensitivity of the cells to tamoxifen. Furthermore, it was explored whether UCA1 was physically associated with EZH2. In addition, it was investigated whether UCA1 regulates tamoxifen resistance through a EZH2/p21 axis and the PI3K/AKT signaling pathway in breast cancer.

Materials and methods

Patients and specimens. A total of 10 hormone receptor-positive breast cancer specimens and 10 non-tumor specimens were randomly selected from the First Hospital of Jilin University (Changchun, China) between April 2015 and April 2017. All these participants were female. The breast cancer specimens were histologically diagnosed as breast carcinoma using ultrasound-guided core needle biopsy of the breast. In the 10 breast cancer specimens, 1 was at stage I, 5 were at stage II and 4 were at stage III. The age range of the 10 patients was from 37-68 years old, with a median age of 51. Evidence of bilateral disease and pregnancy concomitant with the diagnosis of breast cancer resulted in exclusion from the study. All

samples were collected prior to tamoxifen therapy and stored in liquid nitrogen (-196°C) until use. Permission to use the clinical samples for research purposes was obtained and approved by the Ethics Committee of the First Hospital of Jilin University. Informed consents were obtained from all patients.

Cell culture. Human breast cancer cell lines MCF-7 (tamoxifen-sensitive), T47D (tamoxifen-sensitive), LCC2 (tamoxifen-resistant) and LCC9 (tamoxifen-resistant), were purchased from American Type Culture Collection (Manassas, VA, USA). All cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) 2 mM glutamine (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (HyClone). Cells were cultured at 37°C in an incubator with a humidified atmosphere containing 5% CO₂.

Overexpression and knockdown of UCA1 in breast cancer cells. To induce the overexpression of UCA1 in breast cancer cells, the cDNA encoding UCA1 was polymerase chain reaction (PCR)-amplified. The primer sequences were as follows: UCA1, forward 5'-CGCGGATCCTTTATCAGGCATATTAGCTTTAA-3' (*Bam*HI) and reverse 5'-GCGAATTCTGACATTC TTCTGGACAATG-3' (*Eco*RI). Following this, the PCR product was subcloned into the pGreen.puro lentivirus vector (SBI, Palo Alto, CA, USA) with *Bam*HI and *Eco*RI restriction sites (Takara Biotechnology Co. Ltd., Dalian, China). Viral particles were harvested at 48 h post-cotransfection of the pGreen-UCA1-puro constructs with the packaging plasmid ps-PAX2 and the envelope plasmid pMD2G (SBI) into 293T cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). The empty vector was used as the control (lv-NC). MCF-7 and T47D cells were infected with the lentiviral particles (5x10⁷ TU/ml; lv-UCA1 or lv-NC) plus 6 µg/ml polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The virus titers in the control and experimental groups were nearly the same as above (~5x10⁷ TU/ml).

For the knockdown of UCA1, the small interfering (si)RNA targeting UCA1 (si-UCA1) and the scramble non-target control siRNA (si-NC) were synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China). si-UCA1 and si-NC sequences were as follows (37): si-UCA1, 5'-GTTAATCCAGGAGACAAAGA-3'; and si-NC, 5'-TTCTCCGAACGTGTCACGT-3'. LCC2 and LCC9 cells were transfected with equal amounts (100 nM) of si-UCA1 and si-NC using Lipofectamine 3000. All the following cellular or molecular experiments were carried out at 48 h post-transfection (38,39).

RNA extraction, reverse transcription-PCR (RT-PCR) and RT-quantitative PCR (RT-qPCR). Total RNA was extracted from breast cancer tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was reverse transcribed using 1 µg of total RNA and the SuperScript III First-Stand Synthesis Kit (Invitrogen; Thermo Fisher Scientific, Inc.). The expression level of UCA1 were determined on a PCR thermal cycler (T100, Bio-Rad Laboratories, Inc., Hercules, CA, USA) using 2X Taq PCR StarMix buffer (GeneStar, Beijing, China)

or on a real-time PCR thermal cycler (ABI PRISM 7500, Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The endogenous control gene was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The RT-PCR amplification process was as follows: 1 cycle at 98°C for 2 min and 32 cycles at 95°C for 20 sec, 62°C for 15 sec, followed by 72°C for 15 sec; ending with an extension cycle at 72°C for 5 min. The qPCR amplification process consisted of 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec and 58°C for 30 sec. The results of RT-PCR were visualized using a 3% agarose gel and qPCR was performed using the $2^{-\Delta\Delta C_q}$ method. All the oligonucleotide primers were synthesized by Takara. The primer sequences used were as follows (33): UCA1, forward 5'-CTTCTGCATAGGATCTGCAATCAG-3' and reverse 5'-TTTTGTCCCCATTTTCCATCATACG-3'; GAPDH, forward 5'-AGGTCGGAGTCAACGGATTTG-3' and reverse 5'-GTGATGGCATGGACTGTGGT-3'.

WST-1 assay to assess cell viability. Tamoxifen was purchased from Sigma-Aldrich; Merck KGaA. The stock solution of tamoxifen (500 μ M) was prepared in 100% MeOH and maintained at 4°C. Working standard solutions at different concentrations were prepared by dilution in DMEM supplemented with 10% FBS. Solutions were added into the 96-well plate at a final concentration of 0, 0.01, 0.1, 1, 10 or 100 μ M and incubated with cells for 24 h. Following this, 10 μ l WST-1 (Roche Diagnostics, Shanghai, China) was added into each well and the cells were incubated at 37°C in the dark for 2 h. The absorbances of 450 and 630 nm were monitored. The relative cell viability percentage in each group was calculated by comparison to that of the control group.

Flow cytometry for cell cycle and apoptosis analysis. LCC2 cells transfected with si-UCA1 (si-UCA1 LCC2) were treated with 10 μ M tamoxifen for 24 h, trypsinized, collected and washed with PBS. For cell cycle analysis, cells were fixed in pre-cold 70% ethanol for 20 min and stored at -20°C. Following this, cells were washed with PBS and stained with a solution containing 3.5 μ M Tris-HCl (pH 7.6), 10 mM NaCl, 50 μ g/ml propidium iodide (PI) (Sigma-Aldrich; Merck KGaA), 20 μ g/ml RNase and 0.1% igeal CA-630 (Sigma-Aldrich; Merck KGaA) for 20 min on ice to label DNA. Subsequently, LCC2 cells were analyzed using a FACSCaliber flow cytometer with a FlowJo software (version 10.0, BD Biosciences, Franklin Lakes, NJ, USA). The percentages of cells at different phases were calculated from three independent experiments.

For cell apoptosis analysis, cells were stained using an Annexin V-FITC/PI double staining apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions, and analyzed with a FACSCaliber flow cytometer. The cells in the different portions represented the different cell states as follows: The late-apoptotic cells were present in the upper right portion, the viable cells were present in the lower left portion and the early apoptotic cells were present in the lower right portion.

RNA immunoprecipitation (RIP) assay. The RIP experiment was performed in LCC2 cells using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit

(EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. EZH2 antibody for the RIP assay was purchased from Abcam (1:500; #ab186006; Shanghai, China). Samples were treated with proteinase K (Thermo Fisher Scientific, Inc.) to digest the protein for 1 h at 37°C and the immunoprecipitated RNA was isolated. Final analysis of co-precipitated RNA was performed using qPCR and demonstrated as fold enrichment of UCA1.

Chromatin immunoprecipitation (ChIP) assay. The ChIP experiment was performed in LCC2 cells using the EZ ChIP Chromatin Immunoprecipitation Kit (#17-371, EMD Millipore) according to the manufacturer's instructions. Briefly, LCC2 cells were incubated with formaldehyde for 10 min to generate DNA-protein cross-links; the crosslinked chromatin DNAs were sonicated into 200 to 1,000-bp-sized fragments. Subsequently, immunoprecipitation was performed using anti-EZH2 antibody (1:1,000; #07-689, EMD Millipore) and anti-H3K27me3 antibody (1:1,000; #17-622, EMD Millipore), or normal IgG (1:200, EZ ChIP Chromatin Immunoprecipitation Kit) as control. Precipitated chromatin DNA was recovered and analyzed by qPCR. The primer sequences of the p21 promoter were as follows: Forward (40), 5'-GAGGCAAAAGTCCTGTGTTCCAAC-3', and reverse, 5'-AAGAAATCCCTGTGGTTGCAGCAGCT-3'.

Western blot analysis. Cell samples were lysed using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing protease inhibitor. The total protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). A total of 20 μ g of total protein was loaded per lane and separated by SDS-PAGE (10 or 12% gels) and transferred to polyvinylidene fluoride membrane (Roche). The membranes were blocked in 5% skimmed milk diluted with Tris-buffered saline/Tween-20 (Tris-HCl 20 mmol/l, NaCl 150 mmol/l, 0.1% Tween-20, pH 7.5) at room temperature for 1 h and subsequently incubated overnight at 4°C with primary antibodies: Anti-AKT (1:1,000, #ab8805, Abcam), anti-phospho(p)-AKT (1:2,000, #ab8933, Abcam), CAMP responsive element binding protein (CREB, 1:2,000, #ab178322, Abcam), anti-p-CREB (1:1,000, #ab10564, Abcam), anti-GAPDH (1:2,000, #ab181603, Abcam), anti-B cell lymphoma/leukemia-2 (Bcl-2, 1:2,000, #ab196495, Abcam), anti-cleaved caspase-3 (1:1,000, #9661, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-cleaved caspase-9 (1:1,000, #52873, Cell Signaling Technology, Inc.), anti-cyclin D1 (1:1,000, #2978, Cell Signaling Technology, Inc.) and anti-p21 (1:1,000, #2947, Cell Signaling Technology, Inc.). Subsequently, the membranes were incubated with anti-mouse (1:5,000, SAB3701214, Sigma-Aldrich; Merck KGaA) or rabbit (1:5,000, SAB3700852, Sigma-Aldrich; Merck KGaA) horseradish peroxidase-conjugated secondary antibodies at 37°C for 1 h. The immunoreactive bands were visualized using the ECL western blot substrate (Promega Corporation, Madison, WI, USA) and the relative band density was analyzed by Quantity-one software (version 4.6, Bio-Rad Laboratories, Inc.).

Suppression of the PI3K signaling pathway. The PI3K signaling pathway was suppressed by the PI3K inhibitor LY294002 (Cell Signaling Technology, Inc.). LCC2 cells were treated with 50 μ M LY294002 for 24 h in DMEM

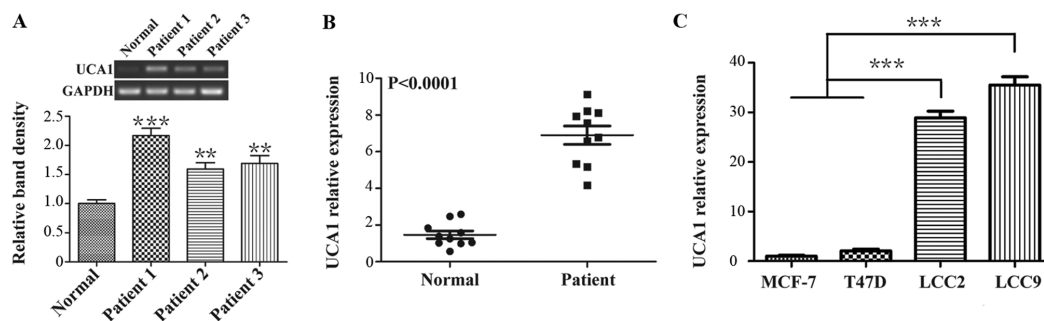


Figure 1. Expression level of UCA1 is higher in breast cancer. (A) Reverse transcription-polymerase chain reaction analysis of UCA1 expression in normal tissues and breast cancer tissues. (B) Quantitative polymerase chain reaction analysis of UCA1 expression in 10 normal and 10 breast cancer tissues. (C) Quantitative polymerase chain reaction analysis of UCA1 expression in tamoxifen-sensitive MCF-7 and T47D cells and in tamoxifen-resistant LCC2 and LCC9 cells. Data were analyzed using independent samples Student's t-test or one-way analysis of variance followed by a LSD or SNK test. ** $P < 0.01$ and *** $P < 0.001$ as indicated or vs. normal group. UCA1, urothelial cancer associated 1.

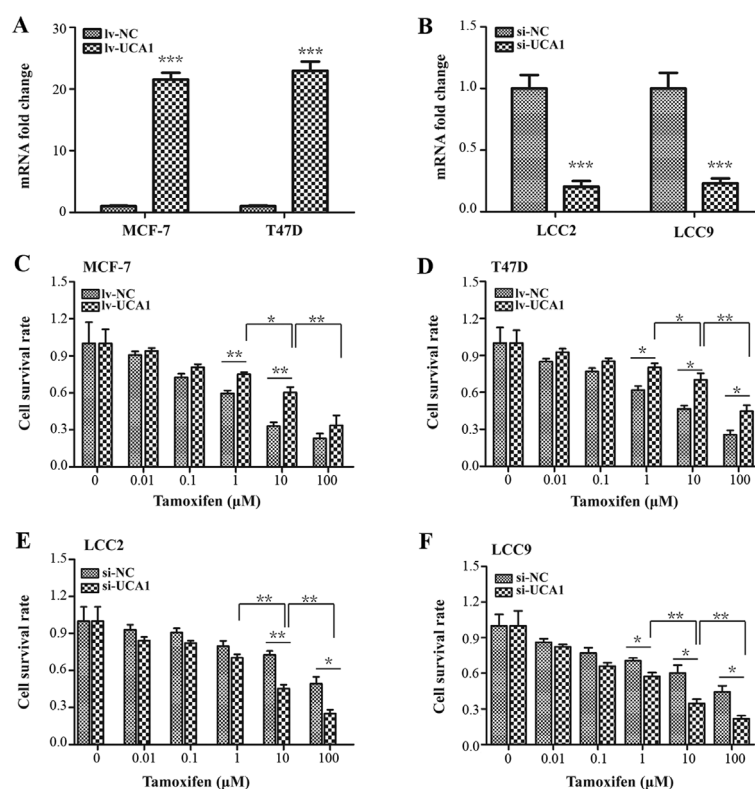


Figure 2. UCA1 contributes to tamoxifen resistance in breast cancer cells. Overexpression of UCA1 enhanced tamoxifen resistance in MCF-7 and T47D cells and knockdown of UCA1 enhanced tamoxifen sensitivity in LCC2 and LCC9 cells. (A and B) Delivery efficiency of the lentiviral carrying UCA1 DNA and the siRNA was assessed using quantitative polymerase chain reaction. (C and D) Overexpression of UCA1 increased the cell survival rate of MCF-7 and T47D cells. (E and F) Knockdown of UCA1 decreased the cell survival rate of LCC2 and LCC9 cells. Data were analyzed using the independent samples Student's t-test or one-way analysis of variance followed by a LSD or SNK test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ as indicated or vs. control groups. UCA1, urothelial cancer associated 1; si, small interfering RNA; NC, negative control; lv, lentivirus.

supplemented with 10% FBS. Subsequent qPCR and western blot analysis were conducted at 24 h post-inhibition.

Statistical analysis. Data were presented as the mean \pm standard error of the mean of at least three independent experiments. Statistical significance between two groups was determined using one-way analysis of variance followed by an LSD or Dunnett's post hoc test or the Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference. All analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

UCA1 expression is upregulated in tamoxifen-resistant breast cancer cells. Firstly, 1 normal breast tissue and 3 breast cancer tissues were randomly selected from the 20 samples, and the level of UCA1 expression was detected using RT-PCR. The PCR results revealed that the level of UCA1 expression was significantly increased in breast cancer tissues compared with normal tissues ($P < 0.001$ and $P < 0.01$; Fig. 1A). Following this, the UCA1 expression levels in all the 10 normal breast tissues and 10 breast cancer tissues were assessed by qPCR. As indicated in

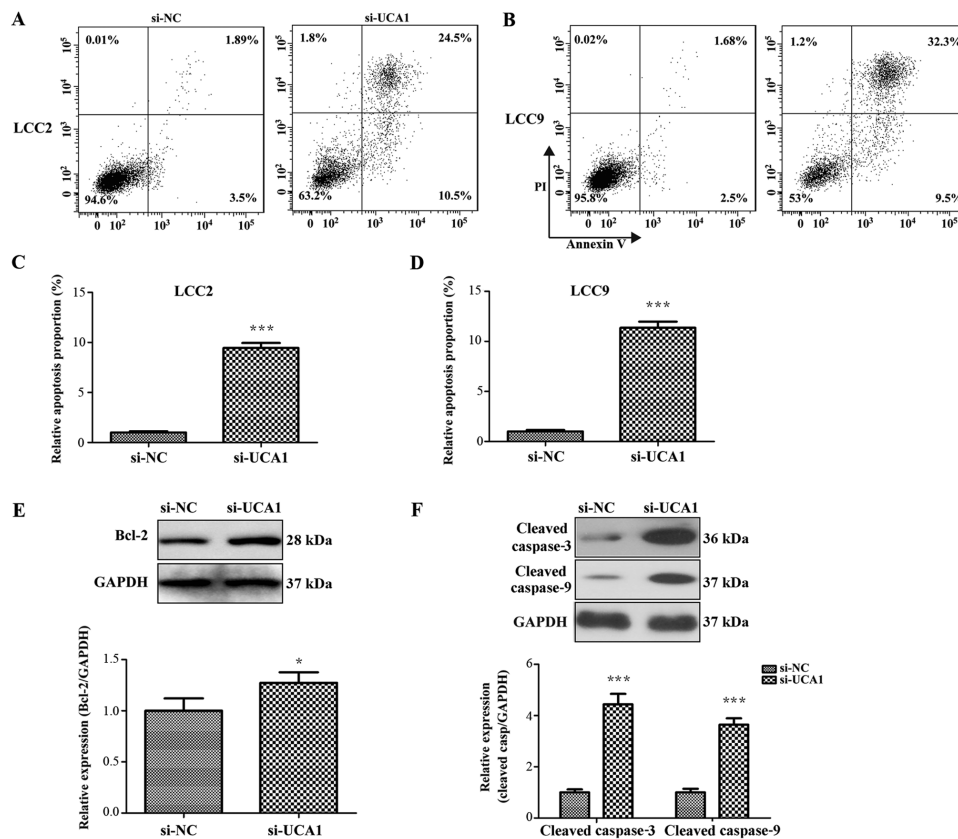


Figure 3. Knockdown of UCA1 increases cell apoptosis in tamoxifen-resistant breast cancer cells. (A and B) Flow cytometry analysis revealed cell apoptosis of LCC2 and LCC9 cells transfected with si-UCA1 increased following tamoxifen treatment. (C and D) Proportion of cell apoptosis in si-NC and si-UCA1 breast cancer cells following tamoxifen treatment. (E and F) Detection of cell apoptosis-associated factors, including Bcl-2, cleaved caspase-3 and -9 by western blot analysis. Upper panel, western blot bands; lower panel, relative band density to GAPDH. Data were analyzed using the independent samples Student's t-test. * $P < 0.05$ and *** $P < 0.001$ vs. control groups. UCA1, urothelial cancer associated 1; si, small interfering RNA; NC, negative control; Bcl-2, B-cell leukemia/lymphoma 2.

Fig. 1B, the mean expression level of UCA1 in the breast cancer group was 4.68-fold greater when compared with that in the normal control group ($P < 0.0001$). These data indicated a positive association between breast cancer and the expression of UCA1.

According to these results, the expression levels of UCA1 in tamoxifen-sensitive cells, MCF-7 and T47D, and in the tamoxifen-resistant cells, LCC2 and LCC9, were assessed using qPCR (Fig. 1C). It was revealed that the level of UCA1 expression in LCC2 and LCC9 cells was >20-fold greater when compared with that in MCF-7 and T47D cells ($P < 0.001$), suggesting a positive association between tamoxifen resistance and UCA1 expression in breast cancer cells.

UCA1 affects the cell viability of breast cancer cells treated with tamoxifen. In order to further confirm the contribution of UCA1 to tamoxifen resistance, the WST-1 assay was performed to detect the cell survival rate following UCA1 knockdown or overexpression in breast cancer cells.

The delivery efficiencies of the lentivirus carrying UCA1 DNA and the siRNA were assessed. As indicated in Fig. 2A, UCA1 expression was significantly elevated by 21.67- and 22.97-fold in lentivirus-transduced MCF-7 and T47D cells compared with the lv-NC group, respectively ($P < 0.001$). Furthermore, UCA1 expression was significantly downregulated to 0.2-fold and 0.23-fold in the UCA1-siRNA transfected LCC2 and LCC9 cells when compared with the si-NC group, respectively (Fig. 2B; $P < 0.001$).

Following treatment with increasing concentrations of tamoxifen (0, 0.01, 0.1, 1, 10 and 100 μM), it was observed that the cell survival rates of UCA1-overexpressed cells were significantly increased compared with the lv-NC group in the presence of 1 or 10 μM tamoxifen in MCF-7 cells and in the presence of 1, 10 or 100 μM tamoxifen in T47D cells (Fig. 2C and D; $P < 0.05$ and $P < 0.01$). Conversely, UCA1 silencing significantly decreased the cell survival rate compared with the si-NC group in the presence of 10 or 100 μM tamoxifen in LCC2 cells or in the presence of 1, 10 or 100 μM tamoxifen in LCC9 cells (Fig. 2E and F; $P < 0.05$ and $P < 0.01$). Specifically, the cell survival rates significantly changed in the 10 μM tamoxifen treatment group compared with the 1 μM tamoxifen treatment group (Fig. 2C-F; $P < 0.05$ or $P < 0.01$). However, in the 100 μM tamoxifen treatment group, the cell survival rates of the control and the experimental group were significantly decreased compared with the 10 μM tamoxifen treatment group, indicating that a high concentration of tamoxifen promoted non-specific cytotoxicity (Fig. 2C-F; $P < 0.01$).

Flow cytometry results indicated that the cell apoptosis rate of si-UCA1 LCC2 cells (35%) and si-UCA1 LCC9 cells (41.8%) was significantly increased following 10 μM tamoxifen treatment when compared with the negative control (si-NC, 5.39% and 4.18%; Fig. 3A-D; $P < 0.001$). Several apoptosis-associated factors were also measured by western blot analysis. Results indicated that the expression levels of

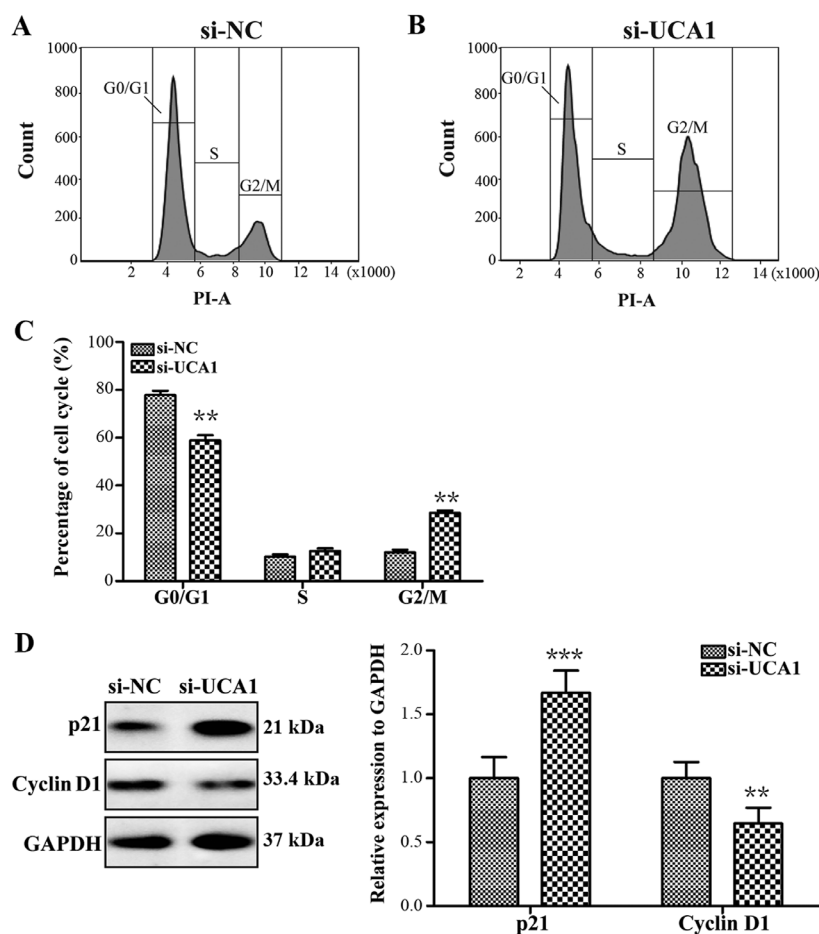


Figure 4. UCA1 silencing promotes G₂/M phase cell cycle arrest following tamoxifen treatment. (A-C) si-NC LCC2 and si-UCA1 LCC2 cells treated with 10 μ M tamoxifen exhibited increased G₂/M cell cycle distribution. (D) Western blot analysis determined the expression levels of p21 and cyclin D1 in si-NC LCC2 and si-UCA1 LCC2 cells. GAPDH was used as a loading control. Data were analyzed using the independent samples Student's t-test. ** $P < 0.01$ and *** $P < 0.001$ vs. control groups. UCA1, urothelial cancer associated 1; si, small interfering RNA; NC, negative control.

Bcl-2, cleaved caspase-3 and cleaved caspase-9 were significantly increased in si-UCA1 LCC2 cells (Fig. 3E and F; $P < 0.05$ and $P < 0.001$). These data suggest that UCA1 contributed to the tamoxifen resistance in breast cancer cells.

UCA1 silencing promotes G₂/M phase cell cycle arrest following tamoxifen treatment. A previous study have demonstrated that UCA1 could promote bladder cancer progression (33). Therefore, the cell cycle distribution in LCC2 cells post-UCA1 knockdown was assessed. si-UCA1 LCC2 cells treated with 10 μ M tamoxifen for 24 h exhibited significant G₂/M phase arrest (Fig. 4A-C; $P < 0.01$), and the expression level of cell cycle-associated factor p21 was significantly upregulated and the expression level of cyclin D1 was significantly downregulated (Fig. 4D; $P < 0.001$ and $P < 0.01$).

UCA1 recruits EZH2 to the p21 promoter and represses p21 expression. It was reported that EZH2 could inhibit the expression of p21 and that p21 is a target of UCA1 (41). It was speculated in the present cell model that p21 may also be suppressed by UCA1 through the recruitment of EZH2 on the p21 promoter. Therefore, RIP analysis was performed. The results indicated that, compared with the IgG control antibody, UCA1 was significantly enriched by EZH2 antibody (Fig. 5A; $P < 0.01$).

ChIP analysis was further performed to demonstrate whether UCA1 inhibited p21 expression by interacting with EZH2. As indicated in Fig. 5B, EZH2 and H3K27me3 could bind to the p21 promoter region directly. However, in the si-UCA1 LCC2 cells, the binding of EZH2 and H3K27me3 to the p21 promoter region was significantly weakened ($P < 0.01$). This finding suggested that UCA1 repressed the expression of p21 via the recruitment of EZH2 and H3K27me3.

UCA1 contributes to tamoxifen resistance in breast cancer cells through the PI3K/AKT signaling pathway. CREB-binding protein, a key nuclear transcription factor in the PI3K/AKT signaling pathway, serves an important role in cell cycle progression (42). A previous study demonstrated that cell cycle progression was greatly arrested in UCA1 knockdown cells, and CREB expression levels were significantly downregulated simultaneously (33). In the present study, it was investigated whether UCA1 could influence the expression of CREB. As indicated in Fig. 6A, CREB and p-CREB expression levels were reduced in si-UCA1 LCC2 cells. Band density analysis revealed that the level of CREB and the p-CREB expression significantly decreased 3.06-fold and 2.1-fold when compared with the control group (Fig. 6B; $P < 0.001$ and $P < 0.01$).

Considering that the PI3K/AKT signaling pathway is pivotal for the maintenance of normal cell cycle progression

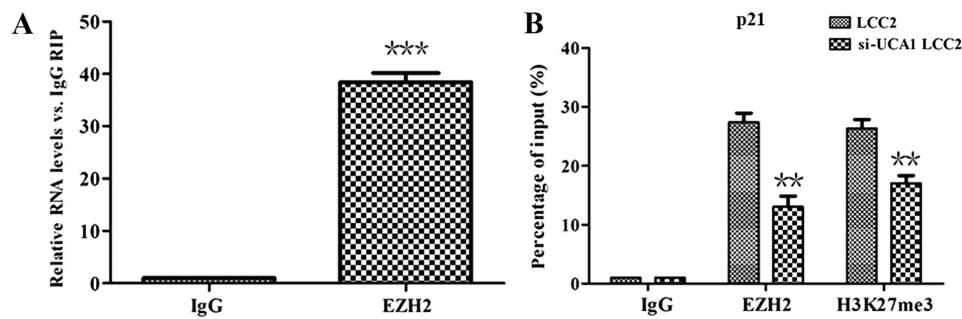


Figure 5. UCA1 represses the expression of p21 via the recruitment of EZH2 in breast cancer cells. (A) UCA1 immunoprecipitated with EZH2 was determined by quantitative polymerase chain reaction and presented as fold enrichment relative to IgG immunoprecipitates in breast cancer cells. (B) Chromatin immunoprecipitation quantitative polymerase chain reaction was used to analyze EZH2 and H3K27me3 binding to the p21 promoter regions in LCC2 and si-UCA1 LCC2 cells. IgG was used as a negative control. Data were analyzed using the independent samples Student's t-test. ** $P < 0.01$ and *** $P < 0.001$ vs. control group. UCA1, urothelial cancer associated 1; si, small interfering RNA; EZH2, enhancer of zeste homolog 2.

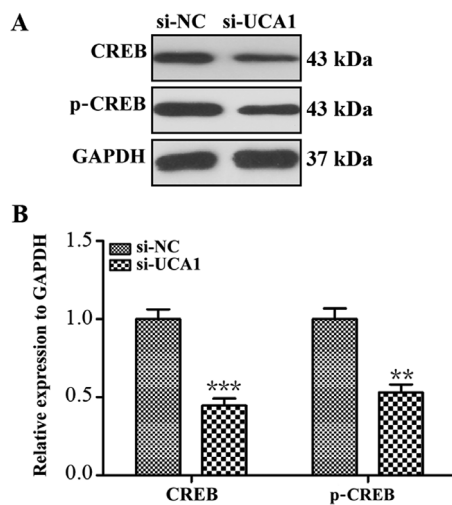


Figure 6. UCA1 silencing reduces CREB and p-CREB expression. (A) Western blot analysis revealed that the expression of CREB and p-CREB was reduced in si-UCA1 LCC2 cells compared with si-NC LCC2 cells. (B) Relative band density of the blots normalized to GAPDH. Data were analyzed using independent samples Student's t-test. ** $P < 0.01$ and *** $P < 0.001$ vs. control group. UCA1, urothelial cancer associated 1; si, small interfering RNA; NC, negative control; p-, phospho; CREB, CAMP responsive element binding protein.

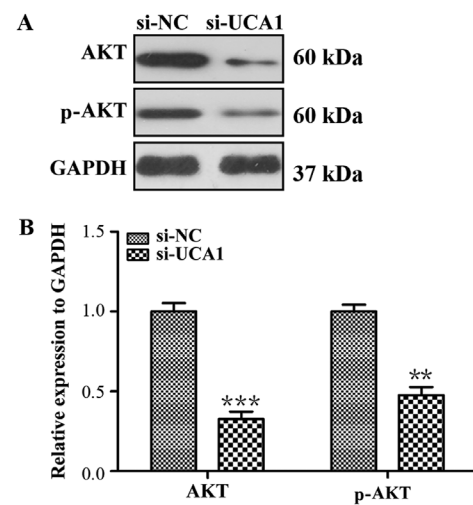


Figure 7. UCA1 silencing represses the activation of AKT. (A) Western blot analysis revealed that the expression of AKT and p-AKT were greatly reduced in si-UCA1 LCC2 cells compared with si-NC LCC2 cells. (B) Relative band density of the blots normalized to GAPDH. Data were analyzed using independent samples Student's t-test. ** $P < 0.01$ and *** $P < 0.001$ vs. control group. UCA1, urothelial cancer associated 1; si, small interfering RNA; NC, negative control; p-, phospho.

and is associated with CREB expression (43,44), it was further assessed whether the PI3K/AKT signal pathway could regulate the expression of CREB in si-UCA1 LCC2 cells in the present study. As indicated in Fig. 7, the expression levels of AKT and p-AKT were significantly reduced in si-UCA1 LCC2 cells ($P < 0.0001$ and $P < 0.01$), suggesting that UCA1 was involved in the activation of AKT.

In order to further verify whether UCA1 could regulate CREB through the PI3K/AKT signaling pathway, LCC2 cells were treated with the PI3K inhibitor LY294002 for 24 h. qPCR analysis revealed that LCC2 cells treated with LY294002 exhibited significantly decreased UCA1 expression levels (Fig. 8A; $P < 0.01$). Furthermore, the phosphorylation of CREB and AKT was also significantly repressed in LCC2 cells were treated with LY294002 (Fig. 8B and C; $P < 0.001$ and $P < 0.05$). Taken together, these results further indicated that UCA1 regulated the activation of CREB and impacted cell cycle progression through PI3K/AKT-dependent signaling.

Discussion

Breast cancer currently remains the most common female malignancy in the world (45). Tamoxifen is the most frequently used endocrinotherapy for ER⁺ breast cancer (46). Despite great treatment advances in improving the survival rate of patients with breast cancer, almost 30% of patients treated with tamoxifen may develop resistance to the drug (47). Numerous studies have focused on the function of lncRNA, and emerging evidence has demonstrated that lncRNAs significantly contribute to various aspects of cancer biology and have been identified as critical players of drug resistance in cancer therapy (44). However, the underlying mechanisms for tamoxifen resistance are largely unknown. In the present study, it was indicated that UCA1 expression was significantly increased in tamoxifen-resistant breast cancer compared with tamoxifen-sensitive breast cancer. Following the knockdown of UCA1, breast cancer cells exhibited a significant increase in G₂/M phase cell cycle arrest.

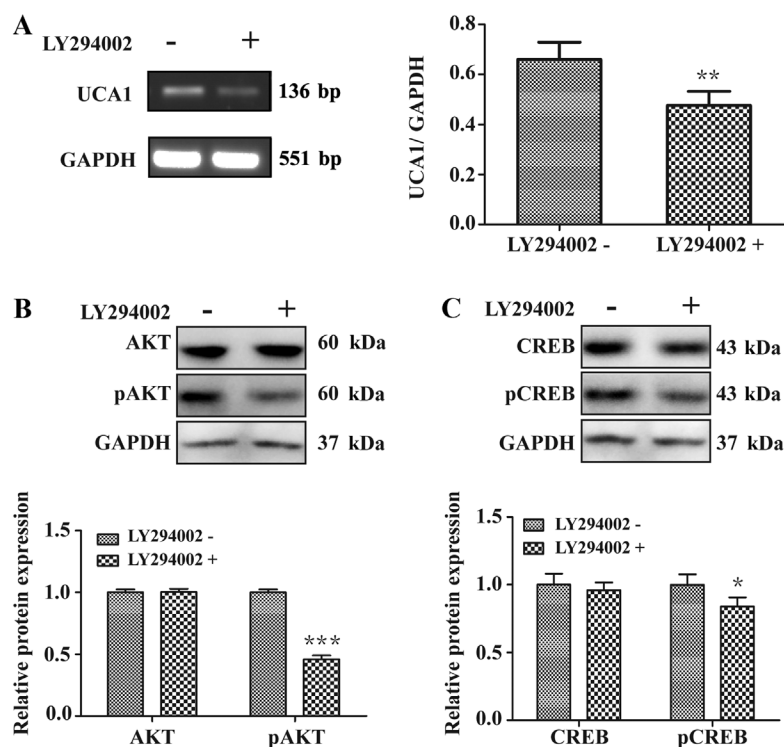


Figure 8. UCA1 regulates the activation of CREB through PI3K/AKT-dependent signaling. (A) Quantitative polymerase chain reaction analysis indicated that the PI3K pathway inhibitor, LY294002, inhibited the expression of UCA1 in LCC2 cells. (B) The expression of AKT/p-AKT was determined by western blot analysis. (C) The expression of CREB/p-CREB was determined by western blot analysis. Data were analyzed using independent samples Student's t-test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control groups. CREB, CAMP responsive element binding protein; p-, phospho; UCA1, urothelial cancer associated 1; PI3K, phosphoinositide 3-kinase.

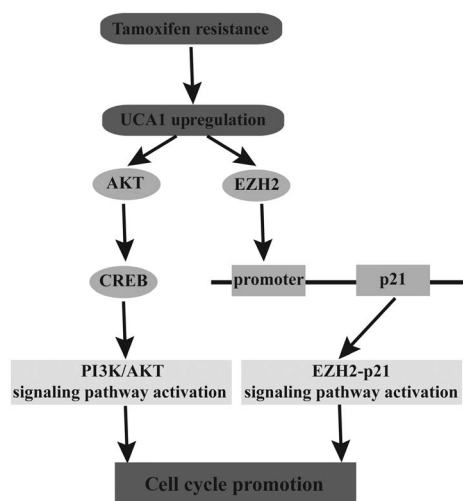


Figure 9. Diagram depicting the role of UCA1 in tamoxifen resistance in breast cancer. UCA1 is physically associated with EZH2, and suppresses the expression of p21 by impacting the binding of H3K27me3 on the p21 promoter. Furthermore, UCA1 impacts the activation of CREB and AKT, and contributes to tamoxifen resistance via PI3K/AKT-dependent signaling in human breast cancer. UCA1, urothelial cancer associated 1; PI3K, phosphoinositide 3-kinase; EZH2, enhancer of zeste homolog 2.

UCA1 has been reported to be upregulated and to exert its oncogenic activity and enhance chemoresistance in several cancer types (23,26,35,48). It has been reported that UCA1 can increase chemosensitivity through a CREB-miR-196a-5p paradigm in bladder cancer (49). Various studies have demonstrated that UCA1 expression is elevated in breast cancer. For

example, Liu *et al* (50) revealed that UCA1 regulates tamoxifen resistance through the Wnt/ β -catenin signaling pathway in breast cancer. Consistent with these reports, in the present study it was demonstrated that UCA1 was significantly increased in tamoxifen-resistant breast cancer. Following treatment with tamoxifen, the expression levels of Bcl-2 and cleaved caspase-3 and -9 were increased in si-UCA1 LCC2 and si-UCA1 LCC9 cells, which demonstrated that UCA1 contributed to tamoxifen drug resistance in breast cancer cells. Bcl-2 protein is a critical component in cell apoptotic signaling. It blocks the increased permeability of the mitochondrial membrane and prevents the release of cytochrome *c* (51). Several studies have reported lncRNA-mediated sequestering of miR expression, whereas some miRs can directly target Bcl-2 and affect the function of Bcl-2 (52-54). It was presumed that UCA1 regulated Bcl-2 through a similar manner. However, the exact reason for this change remains to be further studied.

The PI3K/AKT signaling pathway serves an important role in cell growth, cell cycle distribution, apoptosis and survival of human cancer (55). AKT and CREB are two key molecules in this pathway. lncRNA may regulate the activation of the PI3K/AKT signaling pathway and affect tumorigenesis and drug sensitivity. For example, miR-21 can modulate tamoxifen sensitivity of breast cancer cells through the PI3K/AKT/mTOR signaling pathway (56). In the present study, it was demonstrated that knockdown of UCA1 in LCC2 cells induced an apparent G₂/M phase arrest and altered the expression of p21 and cyclin D1.

A previous study reported that p21 transcription could be repressed through recruitment of EZH2, which was mediated

by UCA1 in renal cell carcinoma cells (40). EZH2 is a histone methyltransferase that catalyzes the trimethylation of H3K27me3 of target genes. The levels of EZH2 are frequently elevated in breast cancer (30). The present study indicated that p21 transcription was repressed by EZH2 through H3K27me3, which was mediated by UCA1 in breast cancer cells. These data demonstrated that UCA1 could modulate the cell cycle through EZH2 and H3K27me3 in breast cancer cells.

CREB, a proto-oncogenic transcription factor, is crucial in cell cycle regulation of breast cancer cells (57). In the present study, the association between the expression of UCA1 with the expression of CREB was assessed by western blot analysis. Results demonstrated that CREB and p-CREB expression levels were significantly decreased when UCA1 was suppressed. CREB is mediated by various protein kinases, including AKT and PI3K (58). Likewise, it was indicated in the present study that AKT expression was positively associated with UCA1 expression. A previous study reported that CREB could be positively regulated by AKT kinase activity (33). Furthermore, the present results confirmed that the expression levels of p-AKT and p-CREB were inhibited by the PI3K inhibitor, LY294002, and this was consistent with a previous report (33). These data demonstrated that UCA1 could regulate CREB through AKT via PI3K/AKT signaling.

In conclusion, to the best of our knowledge the present study demonstrated for the first time that UCA1 regulates tamoxifen resistance through the EZH2/p21 axis and the PI3K/AKT signaling pathway in breast cancer (Fig. 9). Based on the present results, UCA1 may be considered a novel biomarker of poor response to tamoxifen and a potential therapeutic intervention target of breast cancer endocrinotherapy.

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Availability of data and materials

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

Authors' contributions

SL contributed to the design of the study and wrote the manuscript. ZL and DY performed the experiments and analyzed the data. HL and YL helped perform the analysis with constructive discussions. All authors have read and approved this manuscript.

Ethics approval and consent to participate

Permission to use the clinical samples for research purposes was obtained and approved by the Ethics Committee of the First Hospital of Jilin University.

Patient consent for publication

Informed consents were obtained from all patients.

Competing interests

The authors declare no conflict of interest.

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