ERRα confers oncogenesis and cisplatin resistance via transcriptionally activating CCNE2 in breast cancer

Jiahui Liu
    General Hospital of Southern Theater Command of PLA

Jinwen Feng
    Beijing Proteome Research Center

Junfeng Xuan
    General Hospital of Southern Theater Command of PLA

Ling Huang
    General Hospital of Southern Theater Command of PLA

Bin Xiao
    General Hospital of Southern Theater Command of PLA

Yi Zhu
    General Hospital of Southern Theater Command of PLA

Zhenzhan Kuang
    General Hospital of Southern Theater Command of PLA

Weiyun Zhang
    General Hospital of Southern Theater Command of PLA

Lijie Song
    General Hospital of Southern Theater Command of PLA

Ye Zhou
    General Hospital of Southern Theater Command of PLA

Chen Ding
    Beijing Proteome Research Center

Hongbin Zhang
    General Hospital of Southern Theater Command of PLA

Linhai Li
    General Hospital of Southern Theater Command of PLA

Zhaohui Sun
    General Hospital of Southern Theater Command of PLA

Quan Zhou (✉ springzhou@yeah.net)
    General Hospital of Southern Theater Command of PLA
Abstract

Background: Platinum is widely used in the neoadjuvant and metastatic treatment of breast cancer, but increasingly drug resistance is the main cause of cancer recurrence and treatment failure. Our study aimed to investigate the mechanisms by which upregulation of estrogen-related receptor α (ERRα) induced chemoresistance in breast cancer.

Methods: Immunohistochemistry (IHC) was used to determine the expression of ERRα in breast cancer and adjacent tissues. Functional analyses (in vitro and in vivo) were performed to confirm the role of ERRα in cancerogenesis and cisplatin chemoresistance in breast cancer. RNA-sequencing, ChIP and dual luciferase assays were performed to identify the mechanisms by which ERRα promotes chemoresistance in breast cancer.

Results: Analyses of ERRα expression among a case-control cohort of 63 annotated tumor specimens demonstrated that ERRα expression was highly expressed in breast cancer tissues. Overexpression of ERRα promoted cell proliferation and metastasis of breast cancer in vitro and in vivo, increased chemoresistant of cisplatin and enhanced the pluripotency; while ERRα knockdown resulted in the opposite effects. We show that ERRα can directly induce CCNE2 expression through binding its promoter region then enhanced the pluripotency of cancer cells and facilitated chemoresistance. Overexpression of CCNE2 could reverse the sensitivity of breast cancer cells to cisplatin caused by ERRα depletion, thus resulting in accelerated tumor growth.

Conclusions: Our study demonstrates that ERRα acts as oncogene in breast cancer and promotes cisplatin resistance by regulating the transcription of CCNE2 and may serve as a therapeutic target in breast cancer.

Background

Breast cancer is one of the most common malignancies that threatens the life of women worldwide. According to 2020 global cancer statistics, breast cancer mortality accounts for 6.9% of total cancer deaths, and there were an estimated 416,000 new breast cancer cases in China [1]. Currently, chemotherapy is one of the major treatments for breast cancer[2]. However, the increasing chemotherapy resistance is the leading causes of recurrence and death. Identification of novel prognostic markers and understanding their mechanisms may provide a basis for designing future therapeutic strategies for breast cancer patients.

Transcription factors are closely related to the occurrence and development of various diseases[3]. Nuclear receptors form a large family of ligand-inducible transcription factors that regulate all aspects of biology and frequent dysregulation in tumors. The most prominent nuclear receptor in breast cancer biology is estrogen receptor-α (ERα), which is the final effector of the estrogen signaling pathway[4].
The molecular basis and mechanisms of actions of ER have been intensively investigated and elucidated. Studies have found estrogen-related receptors (ERRs), which have similar structure with ERs but not activated by estrogen, are also partly involved in the complex signal transduction system of estrogen, and closely related to estrogen-induced diseases, though established as major regulators of energy metabolism[5].

ERRα belongs to the family of orphan nuclear receptors for which endogenous ligands are not yet determined[6]. Estrogen-related receptors (ERRs), the first discovered orphan nuclear receptors, includes ERRα, ERRβ and ERRγ. In 1988, ERRα (45.5 kDa, 423 amino acid residue) and ERRβ (56.2 kDa, 508 amino acid residue) were discovered by Giguère et al[7]. ERRα is involved in the estrogen signal pathway and also an important transcription factor regulating cellular energy metabolism[8, 9]. In recent years, studies have found that ERRα is closely related to the progression of estrogen-dependent and estrogen-independent tumors, and regulates the occurrence, progression, metastasis and drug resistance of various tumors[10–17]. It serves a critical role in the mediation of acquired resistance, leading to recurrence and metastasis. It has been reported that lapatinib induces the degradation of ERRα. However, reactivated mTOR signal in lapatinib-resistant breast cancer cells restores ERRα expression to trigger metabolic adaptations that confer resistance[18]. Similarly, researchers found ERRα activates SHMT2 transcription by binding to its promoter region, thereby enhancing breast cancer resistance to lapatinib[19]. ERRα also mediates osteosarcoma cell chemoresistance by regulating ABCB1, which encoded an important ABC membrane transporter for drug efflux [20]. Our previous data showed that ERRα expression was elevated by cisplatin stimulation in a dose-dependent manner and we presumed it might contribute to cisplatin resistance in breast cancer cells. Collectively, these findings suggest that ERRα plays a pivotal role in the acquired drug-resistance of tumors, and may act as a potential treatment target. However, the critical role of ERRα and its association with cellular sensitivity to cisplatin in breast cancer are unclear, which therefore are worthy of exploring.

In this study, we identify a function of orphan nuclear receptor ERRα in cancerogenesis and cisplatin chemoresistance in breast cancer. ERRα expression is upregulated in breast cancer and promotes proliferation and metastasis in vitro and in vivo. In addition, it is observed that elevated expression of ERRα is closely related to increased cisplatin resistance in breast cancer. Here, we also demonstrate that ERRα promotes the stem cell-like phenotype and induces cisplatin resistance in breast cancer cells via regulating CCNE2. These findings may contribute to new targets and insights for diagnosis and treatment of breast cancer.

**Materials And Methods**

**Patient samples and immunohistochemistry**

For the analysis of ERRα expression levels, we purchased the human breast cancer tissue microarray from Shanghai outdo biotech company (TMA, HBreD139Su01; Shanghai Outdo Biotech). Tissues were collected from January 2001 to August 2004. All patients were completely informed before the collection
of the tissue samples and written informed consent was provided as well. A total of 84 cancer-adjacent normal tissues and 138 cancer tissues from patients with breast cancer were included.

Tissues were hydrated, antigen repaired and circled. After blocking for 30 min in 10% normal goat serum, anti-ERRα (Santa Cruz Biotechnology, USA) were applied and incubated overnight at 4°C. After incubation in HRP-secondary antibody, sections were washed with phosphate-buffered saline (PBS) and the chromogen reaction was performed with diamino benzidine (DAB). The Image-Pro Plus 6.0 System image analysis system was used for quantitative analysis.

**Cell Culture**

MDA-MB-231 and BT-474 cell lines were purchased from the Cell Bank of Shanghai Academy of Chinese Sciences and the MCF-7 and MCF7/DDP cell lines were purchased from the Meisen Chinese Tissue Culture Collections. MDA-MB-231 and MCF-7 cell lines were cultured in Dulbecco’s Modified Eagle Medium (Gibco, USA) and BT-474 cell line was cultured in Roswell Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C under a 5% CO2 atmosphere.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted using Trizol reagent (Invitrogen, Canada) and reverse transcribed using the Evo M-MLV RT Premix (Accurate Biology, China). Quantitative PCR was conducting using a CFX96 fluorescence quantitative PCR instrument and SYBR Green dye (Accurate Biology, China). Relative expression of genes was calculated by \(2^{-\Delta\Delta Ct}\) values. The primer sequences were shown in the attached Additional file 4: Table S1.

**Western Blotting**

Total cellular proteins were extracted using RIPA lysis buffer, separated by 10% SDS-PAGE electrophoresis, and transferred to PVDF membranes. The membranes were incubated overnight at 4°C in blocking solution containing the following antibodies: ERRα (Santa Cruz Biotechnology, USA), E-cadherin (Cell Signaling Technology, USA), ZO-1 (Cell Signaling Technology, USA), β-Catenin (Cell Signaling Technology, USA), Vimentin (Cell Signaling Technology, USA), Slug (Cell Signaling Technology, USA), Cyclin D1 (Cell Signaling Technology, USA), Cyclin D3 (Cell Signaling Technology, USA), CDK4 (Cell Signaling Technology, USA), CDK6 (Cell Signaling Technology, USA), Cyclin E1 (Cell Signaling Technology, USA), Cyclin E2 (Cell Signaling Technology, USA), Cyclin A2 (Cell Signaling Technology, USA), CDK2 (Cell Signaling Technology, USA), Cyclin B1 (Cell Signaling Technology, USA), cdc2 (Cell Signaling Technology, USA), and GAPDH (Cell Signaling Technology, USA). After incubation with HRP-linked anti-rabbit/mouse IgG (Cell Signaling Technology, USA) for 2 h, immunoreactive bands were visualized using ECL (Millipore, USA) and detected using the OI900 fully automatic chemiluminescence image analysis system. GAPDH was used as an internal reference.

**Plasmid Plasmids, virus constructs, and retroviral infection of target cells**
The PCR products were resolved by 1% agarose gel electrophoresis and inserted into the pLVX-mCMV-ZsGreenpuro vector by NotI/EcoRI co-digestion and ligation using T4 DNA ligase to form overexpression plasmids. These plasmids were then electroporated into Escherichia coli DH5a competent cells and positive transformants selected on plates containing chloramphenicol. Correct insertion was confirmed by sequencing and comparison to the NCBI BLAST program. Silencing of endogenous ERRα was performed by cloning shRNA construct into the PLENT-U6-GFP-PURO. Transfection of plasmids was performed using Lipofectamine 3000 transfection reagent kit (Invitrogen, USA) strictly according to the manufacturer’s instructions. Stable cell lines were selected using 0.5 µg/ml puromycin (WEST GENE, China) for 7 days after infection. After maintaining with 0.25 µg/mL puromycin for one month, ERRα gene overexpressing or silencing was confirmed by western blotting and qRT-PCR.

**Colone Formation Assay**

Cells were seeded in 6-well plates at a density of 1×10^3 and cultured at 37°C under 5% CO2. After 10 days, colonies were washed twice with PBS, fixed with 4% paraformaldehyde for 60 min, and stained with 1% crystal violet (LEAGENE, China) for 30 min. Colonies were photographed and counted.

**Cell Proliferation Analysis**

The cell viability and the inhibitory concentration 50% (IC_{50}) of cisplatin was determined using a cell counting kit 8 (CCK8) assay. Cells were seeded onto 96 well plates at 5×10^3 cells/well in 100 µl medium with 10% FBS. The number of viable cells was estimated using a CCK8 kit according to the manufacturer’s instructions (Dojindo, Japan). After allowing the cells to adhere overnight, complete medium was replaced with medium containing serially diluted cisplatin reagent (0, 1, 2, 4, 8, 16, 32, 64, 128 and 256 µM). After incubating for 48 h, 10 µl of CCK8 solution was added to each well for 2 h at 37°C. The optical density of each well at 450 nm was determined using a microplate reader (Thermo Fisher Scientific, USA).

**Transwell Assay**

Cell migration was also evaluated by transwell migration assays (Coming, USA), and cell invasion was assessed using matrigel invasion chambers (Coming, USA). Briefly, the upper chambers were seeded with 8×10^4 cells in serum-free culture medium, and 800 µl culture medium containing 10% FBS was added to the lower chambers. After 24 h of culture at 37°C under 5% CO2, cells on the reverse side of the insert (migrating/invading cells) were stained with 0.5% crystal violet and three fields were randomly selected and photographed at ×100 magnification.

**Wound Healing Assay**

Cells were seeded in 6-well plates at 8×10^4 /well and cultured for 12 h. When cells reached 80%-90% confluence, a scratch was made through the center of each well using a 200 µl sterile pipette tip. The cells were then washed twice with PBS, incubated in serum-free culture medium, and photographed at 0 h, 24 h and 48 h to assess cell migration into the bare region (wound healing). Images were analyzed by ImageJ software to calculate the % wound closure.
Tumor sphere formation assays

$5 \times 10^3$ cells were incubated in low-attachment six-well plate (Corning, USA) containing serum-free culture medium, 20 ng/ml epidermal growth factor (EGF) (Zhongqiao Xinzhou Biotechnology, China) and 10 ng/ml basal fibroblast growth factor (bFGF) (Zhongqiao Xinzhou Biotechnology, China). After culturing for at least 10 days, cells were collected for secondary spherulation. Another 10 days late, tumor sphere numbers were counted under an inverted microscope using the 50× and 100× magnification lens.

Cell Cycle Assay

At 48 h after transfection, cells were collected and fixed overnight with 75% alcohol at -20°C. After washing with PBS, cells were incubated with propidium iodide (PI)/RNase A solution (Absin, China) for 20 min at 37°C. Samples were analyzed within 1 h of staining using a CytoFLEX flow cytometer (Beckman-Coulter, USA) and ModFit LT software.

RNA-Sequencing analysis

RNA purity was checked using the kaiaoK5500®Spectrophotometer (Kaiao, China). RNA integrity and concentration were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, USA). A total amount of 2 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer’s recommendations andindex codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). First strand cDNA was synthesized using random hexamer primer and RNase H. Second strand cDNA synthesis was subsequently performed using buffer, dNTPs, DNA polymerase I and RNase H. The library fragments were purified, then terminal repair, A-tailing and adapter added were implemented. The aimed products were retrieved and PCR was performed, then the library was completed. The clustering of the index-coded samples was performed on a cBot cluster generation system using HiSeq PE Cluster Kit v4-cBot-HS (Illumina, USA) according to the manufacturer’s instructions. After cluster generation, the libraries were sequenced on an Illumina platform and 150 bp paired-end reads were generated.

Chromatin immunoprecipitation (ChIP)

ChIP-seq and ChIP-qPCR was employed to analyze genomic DNA sequences bound to ERRα using NovoNGS® CUT&Tag 3.0 High-Sensitivity Kit (Novoprotein, China). According to the kit instructions, protein-DNA complexes were crosslinked, immunoprecipitated, purified and subjected to PCR analysis. The primer sequences were shown in the attached Additional file 5: Table S2.

Dual luciferase assays
Cells were seeded into 24-well plates to reach a confluency of 60–70%. The truncated CCNE2 promoter constructs were cloned to pGL3-luciferase reporter plasmids. The pGL3-luciferase reporter plasmids and pRL-TK renilla were transfected into MDA-MB-231 cells using Lipofectamine™ 3000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. After 48 h of transfection, luciferase activity was detected using the dual luciferase reporter assay system (Promega, USA).

**Mice xenograft models**

The animal studies were approved by the Institute of Biological and Medical Engineering, Guangdong Academy of Sciences, and all the experiments conform to the relevant regulatory standards. In the tumor model, BALB/c nude mice were randomly divided into four groups (n = 3 mice/group). 8×10^6 stable cells were resuspended in sterile PBS, then inoculated subcutaneously into the right armpit of the mice. Upon the subcutaneous tumor size reaching a diameter of approximately 5 mm, mice were treated with sterile water (control), or CDDP (5 mg/kg) every 3 days, for up to 26 days. Upon experimental endpoint, the mice were sacrificed, and the tumors were weighed and photographed.

**Statistical analysis**

All statistical analyses were conducted using SPSS version 26 and GraphPad Prism 8. Data are presented as mean ± standard deviation (SD) of three independent experiments. Two group means were compared by Student's t test and more than two group means by one-way ANOVA. A P-value < 0.05 (two-tailed) was considered statistically significant for all tests.

**Results**

**ERRα promotes proliferation and metastasis in breast cancer cells**

Recently, ERRα has been identified to be upregulated in various tumors[10, 12, 15], which we validated in breast cancer. We first performed IHC staining to detect ERRα expression in breast cancer and adjacent tissues. As expected, the result showed ERRα expression was upregulated in breast cancer tissues (Additional file 1: Fig. S1 A-B). In 63 cases of paired clinical samples, a paired sample t test showed that ERRα was expressed in breast cancer tissue at a higher level than in adjacent tissues (Additional file 1: Fig. S1. C). As well known, HER2-positive breast cancers are highly aggressive with poor prognoses. The outcome of HER2-positive breast cancer patients has dramatically improved with the advent of numerous anti-HER2 molecular-targeted therapies[21–23]. The ERRα expression was determined to be significantly correlated with the expression level of HER2. We observed a significantly higher expression level of ERRα among HER2-positive patients (Additional file 6: Table S3). We next explored the role of ERRα in breast cancer cells progression. Overexpression and knockdown efficiency of ERRα were first confirmed by Western blotting and qRT-PCR (Fig. 1A-B). Clone formation and CCK8 assays showed that knockdown of ERRα significantly suppressed cell viability while overexpression significantly increased (Fig. 1C-D).

Meanwhile, overexpression of ERRα also promoted breast cancer cells migration and invasion while ERRα
deficiency repressed in transwell assays (Fig. 1E). Consistently, ERRα could enhance the expression of β-Catenin, Vimentin, and Slug and downregulate the expression of E-cadherin and ZO-1, suggesting its roles in promoting epithelial-mesenchymal transition (EMT) of breast cancer cells (Fig. 1F). These results demonstrate that ERRα contributes to the proliferation, migration and invasion ability of breast cancer cells.

**ERRα promoted cisplatin resistance and the stemness of breast cancer cells**

We next explored the relationship between ERRα and cisplatin chemosensitivity. We compared the ERRα mRNA levels and IC_{50} values in five breast cancer cell lines (Fig. 2A-B). It was identified that ERRα mRNA expression levels were positively correlated with IC_{50} values (Fig. 2C). To investigate the potential role of ERRα in mediating cisplatin resistance, we used cisplatin to stimulate MDA-MB-231 cells at series time (0 h, 12 h, 24 h, 48 h) and ERRα expression was observed to be gradually induced (Fig. 2D). Moreover, the expression of ERRα protein in MDA-MB-231 cells after cisplatin treatment was up-regulated in a dose-dependent manner with increasing concentrations of cisplatin (Fig. 2E). In addition, IC_{50} value for cisplatin increased in ERRα-overexpressing MDA-MB-231 cells, and reduced in ERRα-knockdown BT-474 cells (Fig. 2F). Many studies have reported that cancer stem cells (CSCs) contributed to chemoresistance and unfavorable clinical outcomes[24, 25]. We observed that ERRα promoted tumor sphere formation, which was a typical characteristic of CSCs. (Fig. 2G). Collectively, ERRα might contribute to cisplatin resistance in breast cancer cells.

**ERRα knockdown sensitized the resistant cells to cisplatin.**

To further investigate the role of ERRα in cisplatin resistance of breast cancer cells, we purchased cisplatin-resistant breast cancer cells MCF7/DDP for following experiments (Fig. 3A). Western blotting and qRT-PCR showed that the protein and mRNA expressions of ERRα were upregulated in MCF7/DDP cells compared with their parental MCF-7 cells (Fig. 3B-C). We therefore constructed ERRα knockdown MCF7/DDP cells (Fig. 3D-E). Compared with the control group, the IC_{50} of cisplatin in the ERRα deficiency group was significantly decreased (Fig. 3F). The clone formation, CCK8, transwell and wound healing assays also demonstrated that downregulation of ERRα increased the sensitivity to cisplatin in MCF7/DDP cells (Fig. 3G-J). We also observed that ERRα facilitated tumor sphere formation and the expression of stem cell-related molecules, including SOX2, OCT4, Lin28, CD133 and CD44 in vitro (Fig. 3K-L). Next, flow cytometry revealed that ERRα knockdown reduced the proportion of cells in S phase and increased the proportion in G1 phase (Fig. 3M). It indicates that ERRα was a positive regulator of cell cycle progression, while inhibiting ERRα could induce slightly cell cycle accumulation at the G1-S transition. Furthermore, western blotting indicated that the expression levels of cell cycle regulators CDK2, 4, and 6 as well as CyclinA2, B1, D1, D3, E1, and E2 were reduced by ERRα knockdown compared to
control cells (Fig. 3N). These results proved that ERRα knockdown reduced cisplatin resistance to breast cancer in vitro.

**High expression of ERRα conferred cisplatin resistance to breast cancer in vivo**

We employed a nude mouse xenograft model to further explore the role of ERRα in breast cancer chemoresistance in vivo. First, we injected the MDA-MB-231-Vector and MDA-MB-231-ERRα cells into mice to generate transplanted tumors of BALB/c nude mice. ERRα overexpression resulted in a significant increase in tumor volume and weight, compared to the control group (Fig. 4B-D). Next, to validate whether ERRα contributed to cisplatin resistance in vivo, 5 mg/kg cisplatin was injected intraperitoneally into indicated group every 3 days starting from day 11 post cell injection (Fig. 4A). Significant tumor growth inhibition was observed when mice were treated with cisplatin. However, the treatment effect of cisplatin was poorer in ERRα overexpression group, compared with the control group (Fig. 4B-D). These results proved that ERRα overexpression promoted cisplatin chemoresistance to breast cancer in vivo.

**Genome-wide characterization of ERRα transcriptional binding sites in breast cancer cells**

As a transcription factor, ERRα plays a regulatory role by binding to specific DNA sequences. To further determine the mechanism of ERRα function in cisplatin-resistance, we explored the genomic regulating and binding pattern of ERRα between ERRα-overexpressing MDA-MB-231 cells and control cells by RNA-seq and CUT&Tag. RNA-seq analysis identified 659 mRNA transcripts were up-regulated and 517 mRNA transcripts were down-regulated (Fig. 5A). The significantly upregulated genes were functionally analyzed using Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. According to GO enrichment analysis, upregulated genes involved predominantly in ‘Inflammatory response’, ‘Positive regulation of endothelial cell proliferation’, ‘Positive regulation of gene expression’ and ‘Immune response’. The proteins encoded by these genes were mainly located in ‘Extracellular space’, ‘Plasma membrane’ and ‘Chloride channel complex’ and enriched in the MF annotations ‘Receptor binding’, ‘Transcriptional activator activity’, ‘Sequence-specific DNA binding’, ‘Ion channel activity’ and ‘Growth factor activity’. According to KEGG enrichment analysis, these genes were involved in ‘Signaling pathways regulating pluripotency of stem cells’, ‘PI3K-Akt signaling pathway’, ‘TGF-beta signaling pathway’ and ‘Hematopoietic cell lineage’ (Fig. 5B).

CUT&Tag is an emerging approach for the study of protein-DNA interactions which provides efficient high-resolution sequencing libraries for profiling diverse chromatin components. Heatmaps demonstrate the distribution of reads on both sides of the peak summits (Fig. 5C). Analysis of the distance distribution between the peak summit and the transcription start site (TSS) of genes revealed that the majority of ERRα binding sites were near the TSS (Fig. 5D). We used ChIPseeker to analyze the distribution of peak in each function area and found that most of the binding sites were located in promoter regions (44.65%) (Fig. 5E). The motifs shared between the peaks were scanned and the five motifs with the most significant differences were selected for display (Fig. 5F). We also compared our ERRα binding sites
profile with previous research by Etienne et al[26]. While AMPK activation induced more than 34,000 ERRα binding genomic regions in BT-474 cell line, our data showed increased the total number of binding sites for ERRα by more than 70%, namely about 59,000, in ERRα overexpression MDA-MB-231 cells. In addition, location analysis of binding sites showed dramatic increasing promoter ratio, demonstrating ERRα functions as a transcription factor to regulate gene transcription (Additional file 2: Fig. S2). When coming to regulated genes associated with an ERRα binding event, there was also a remarkable increased number in our dataset, with over 6,000 genes overlap in both two researches (Additional file 2: Fig. S2C).

To determine whether the differentially expressed genes induced by ERRα overexpression were also bound by ERRα, we integrated the CUT&Tag and RNA-seq datasets and compared the expression of genes with a ERRα binding site. Among the 1176 differentially expressed genes, 587 genes were bound by ERRα. Eleven genes which qualified the preliminary screening and reported to be related to drug resistance were further chosen for the qRT-PCR verification (Fig. 5G). We further checked the expression of BCL2A1, PDGFRα, FLT1, CCNE2, WNT4, SPHK1, FOS, KDR, ASS1, FHIT and ASNS in ERRα-overexpressing MDA-MB-231 cells, ERRα-knockdown BT-474 cells and MCF7/DDP cells. The qRT-PCR results showed that after overexpressing ERRα, the mRNA level of CCNE2 increased, whereas after ERRα knockdown, the levels of CCNE2 decreased (Fig. 5H). We also found that cisplatin induced CCNE2 expression in breast cancer cells (Fig. 5H). To investigate whether ERRα can directly regulate CCNE2 gene expression, we used JASPAR software (http://jaspar.genereg.net/) to predicted the binding sites of ERRα in the CCNE2 promoter, identifying five binding sites in CCNE2 promoter region. Furthermore, analysis of CPTAC breast cancer dataset showed significant positive correlation between ERRα and CCNE2 (Fig. 5I). Serially truncated CCNE2 promoter constructs were cloned to pGL3-luciferase reporter plasmids and transfected into MDA-MB-231 cells. The results of the luciferase assay showed that the regulatory region may be located between −2000 and −1691 bp (Fig. 5J). ChIP-qPCR assays further confirmed that ERRα binds to the −2000 to −1691 bp region.

**ERRα mediated the chemoresistance of breast cancer cells via regulating the transcription of CCNE2.**

CCNE2 was significantly upregulated in multiple cancer datasets, especially in breast cancer tissues (Fig. 6A-B). To further verify whether does ERRα exert its biological function through CCNE in breast cancer, we performed the rescue experiment in MDA-MB-231, BT-474 and MCF7/DDP cells (Additional file 3: Fig. S3). Compared with the control group, the IC_{50} vale of cisplatin in CCNE2 deficiency group was significantly decreased, and increased in CCNE2-overexpressing cells (Fig. 6C-E). The clone formation, CCK8, transwell and wound healing assays revealed that CCNE2 was essential for ERRα-mediated proliferative and metastatic effects in breast cancer cells after cisplatin treatment (Fig. 6F-I). Overexpression of CCNE2 reversed G1 accumulation caused by the depletion of ERRα, thus promoting G1-S transition in breast cancer cells (Fig. 6L). CSCs are known to show strong resistance to chemotherapy. Tumor sphere formation in ERRα-silenced MCF7/DDP cells was robustly promoted by the overexpression of CCNE2. Similarly, CCNE2 promotes the expression of stem cell-related molecules, including SOX2, OCT4, Lin28, CD133 and CD44 (Fig. 6J-K). Therefore, ERRα mediates the chemoresistance of breast cancer cells by targeting CCNE2.
Discussion

Breast cancer is one of the most common forms of cancer in females. About 70–80% of patients with nonmetastatic disease are curable, while advanced breast cancer with distant metastasis is considered incurable by current therapies. At present, the treatments for breast cancer include surgery, endocrine therapy, radiation therapy, chemotherapy, and targeted therapy. Since there is no targeted therapy available for TNBC, chemotherapeutic agents (e.g., platinum, paclitaxel) remain the current first-line therapy for the patients with TNBC[27]. Chemotherapy is one of the most efficient therapeutic methods for oncotherapy, but increasingly serious drug resistance is the main cause of cancer recurrence and treatment failure. Adjuvant chemotherapy substantially reduces the risk of recurrence and death in breast cancer. Doxorubicin, cyclophosphamide, 5-fluorouracil, platinum, and other chemotherapy drugs are widely used in breast cancer treatment, but the side effects of toxic and chemoresistance limit the therapeutic effects for patients. Accordingly, discovering novel therapeutic targets to overcome chemoresistance are crucial for establishing effective treatments for breast cancer patients. Cisplatin, which is among the most effective and widely used chemotherapeutic agents employed for treatment of solid tumors, is involved in DNA damage by forming covalent adducts with DNA. Platinum drugs are highly effective at initial treatment, but tumor recurrence and metastasis often result in the failure of treatment due to drug resistance. The mechanisms of cisplatin-induced apoptosis are complex, as it may cause different stresses such as DNA damage, endoplasmic reticulum stress, oxidative stress. Various cisplatin-induced stress signals can activate distinct pathway through specific transcription factors that act as the ultimate drug targets. The mechanism of acquiring resistance to platinum has been under investigation for nearly half a century and researchers have found multiple resistance mechanisms across tumors, including decreased drug uptake, increased drug efflux, increased DNA repair activity and increased resistance to apoptosis et al[28–30].

As the final signal executors of cell signal transduction, transcription factors play very important roles in tumor drug resistance associated pathway such as DNA damage repair and apoptosis. For instance, p53 has been established as a central mediator of the DNA damage and other cellular stress responses. Platinum-based drug therapy can stabilize p53/p73 through ATR and MAPK-mediated phosphorylation and induce the expression of its downstream target genes, resulting in drug resistance[31]. Many chemotherapeutics promote the activation of the NF-kB pathway, which plays a role in cancer progression and chemoresistance by activating multiple genes including anti-apoptotic genes [32]. Several transcription factors involved in cisplatin resistance have been identified, including Y-box binding protein-1 (YB-1), CCAAT-binding transcription factor 2 (CTF2), activating transcription factor 4 (ATF4), zinc-finger factor 143 (ZNF143) and mitochondrial transcription factor A (mtTFA)[33]. Therefore, we consider that transcription factors can be used as a potential target for tumor chemosensitivity.

In recent years, transcription factor ERRα has received more and more attention from researchers, as it is not only an important transcription factor regulating cellular energy metabolism but also closely related
to the progression of estrogen-dependent tumors and estrogen-independent tumors, regulating the occurrence, development, metastasis and drug resistance of various tumors. However, there was relatively few studies on the role of ERRα in the chemoresistance of cancers. It has been reported that ERRα triggered resistance to lapatinib by altering the glutamine metabolic pathway in tumor cells[18]. Li demonstrated that ERRα activated SHMT2 transcription to enhance the resistance of breast cancer cells to lapatinib via modulating the mitochondrial metabolic adaption[19]. ERRα also mediated chemoresistance of osteosarcoma cells by regulating ABCB1[20]. Similarly, Huang found that ERRα-induced increased expression of ABCC4 could promote docetaxel efflux and lead to the development of drug resistance in prostate cancer[34]. Collectively, these studies indicate that ERRα plays a critical role in tumor-acquired drug resistance and may serve as a potential therapeutic target.

Our IHC data showed that ERRα was highly expressed in breast cancer tissues. Consistent with our results, many studies have found that the expression level of ERRα was significantly upregulated in various tumors, such as breast cancer[12], ovarian cancer[15] and colorectal cancer[10], which predicted shorter survival. Thus, ERRα may be an important biomarker for evaluating the prognosis of breast cancer patients. Interestingly, the ERRα expression was determined to be significantly correlated with the expression level of HER2. We observed a significantly higher expression level of ERRα among HER2-positive patients. Meanwhile, HER2-positive BT-474, UACC-812 and SK-BR-3 breast cancer cells exhibited higher ERRα level. It can be attributed to the activation of HER2 initiating a signaling cascade that leads ultimately to the phosphorylation of several serine residues located at the N-terminus of ERRα and these modifications have been shown to increase receptor transcriptional activity[35–37]. Besides, HER2 and insulin-like growth factor (IGF)-I receptor signaling pathways increase the expression of PGC-1β through the induction of c-MYC[38]. On the other hand, the activated phosphoinositide 3-kinase (PI3K)/Akt pathway induces the expression of PGC-1α by activating the mTOR/YY-1 pathway. Therefore, the activity of ERRα may be regulated by HER2 signaling pathways.

In this study, we reported a new molecular mechanism that might potentially explain the emergence of chemoresistance in breast cancer patients. We observed that overexpression of ERRα in breast cancer cells associated with cisplatin resistance. Mechanically, ERRα perhaps enhanced the pluripotency of cancer cells and promoted the development of chemoresistance via targeting CCNE2. Studies have identified CCNE1 as the potential target gene for ERRα based on genome-wide identification[39, 40]. Although ERRα was reported to repress cyclin E2, it may be due to PGC-1α cofactor competition[41]. CPTAC database showing a high correlation between ERRα level and CCNE2 expression level[42]. Importantly, the luciferase and ChIP-qPCR assay showed that the regulatory region may be located between −2000 and −1691 bp. In this interval, our predicted CCNE2 promoter binding site contains a TTAAGGTCA sequence, which is consistent with the identified canonical ERRE sequence recognized by ERRα[43]. Our study also illustrated that overexpression of CCNE2 could reverse the sensitivity of breast cancer cells to cisplatin caused by the depletion of ERRα, thus resulting in accelerated tumor growth. These findings suggest that ERRα may act as a potential therapeutic target to enhance the cisplatin response for patients with chemoresistant breast cancer.
Cyclin E represents a component of the core cell cycle machinery. In mammalian cells, cyclinE1 and cyclinE2 play important roles in the transition of the cell cycle from G1 to S phase by interacting with cyclin-dependent kinases (CDKs) and regulating multiple downstream molecules such as RB1 and transcription factor E2F family members. Cyclin E2 was discovered according to amino acid sequence similarity to CyclinE1[44]. Cyclin E2 mRNA has an open reading frame encoding a protein of 404 amino acids and a predicted molecular weight of 47 kDa. The encoded protein shows 47% similarity to human Cyclin E1 and contains a cyclin box motif unique to all cyclins. Aberrantly elevated cyclin E-CDK2 activity has been demonstrated in a number of different types of tumors, and overexpression of Cyclin E mediated the resistance of tumor cells to various therapeutic drugs. Genome-wide analyses of ovarian cancers, in which CCNE1 is frequently amplified, revealed that such amplification is associated with intrinsic resistance to standard platinum-taxane chemotherapy[45]. Amplification of CCNE1 and overexpression of cyclin E1 underlie resistance to trastuzumab therapy in HER2 + breast cancer[46]. Zhang also found that downregulation of CCNE1 expression inhibited cell proliferation and enhanced the sensitivity of gastric cancer cells to cisplatin[47].

CSCs are a small subset of tumor cells that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor. CSCs were first reported in 1994 during a study on human acute myeloid leukaemia (AML)[48]. A decade later, CSCs were also identified in solid tumors such as breast, glioblastoma, prostate, and colorectal tumors[49–52]. There are three main hypotheses regarding the origin of CSCs. The first is a belief that CSCs are derived from normal tissue stem cells. This is based on the fact that normal tissue cells tend to undergo apoptosis already when the genetic mutation has not accumulated enough to initiate carcinogenesis, whereas stem cells eventually undergo carcinogenesis because of their unlimited proliferative capacity and the tendency for the genetic mutation to efficiently accumulate in adult stem cells. Another view holds that CSCs may develop from progenitor cells. The number of progenitor cells is more abundant in adult tissue than the stem cells, which makes oncogenic transformation more likely. There is also an argument that cancer cells may arise from differentiated cells, that somehow dedifferentiate to become more stem cell like[53]. One of the most important features of CSCs is their ability to resist traditional therapies. CSCs evade conservative therapy approaches by becoming dormant, increasing DNA repair, decreasing apoptosis, and interacting with their supporting microenvironment. Therefore, increasing knowledge about the biological functionality of CSCs and conducting research on targeted drugs and gene therapy can provide new insight into cancer biology and deliver new treatment options.

Cyclin E has been shown involved in the maintenance of stemness. Cyclin E-CDK2 (along with D-cyclins) maintained the undifferentiated, pluripotent state of ESCs by phosphorylating the key pluripotency regulators, NANOG, OCT4, and SOX2[54]. Cyclin E also promoted stemness and inhibited the differentiation of neural stem cells. Overexpression of cyclin E1 in embryonic mouse brains shortened G1 phase, increased self-renewal of neural stem cells, and inhibited neurogenic differentiation[55]. However, the role of cyclin E in CSCs has been poorly studied. High expression of CSCs marker CD133 in invasive breast cancer was positively correlated with cyclin E expression[56]. More importantly, the heightened oncogenicity of low-molecular weight cyclin E (LMW-E) related to its ability to promote CSC
properties, supporting the design of therapeutic strategies to target this unique function[57]. However, LMW-E protein overexpression in mammary epithelial cells resulted in increased expression of full-length cyclin E[58]. In addition, Cyclin E forms a complex with and activates CDK2, which further hyperphosphorylates pRb, resulting in releasing free E2F transcription factors. In turn, E2F factors promote transcription of Cyclin E, leading to activation of CDK2/Cyclin E complexes, thus providing a positive feedback loop[59]. As the downstream signaling molecules of the cyclin E–CDK2 pathway, E2Fs are widely involved in the regulation of the biological characteristics of CSCs[60]. Therefore, we speculate that Cyclin E is related to the maintenance of CSCs. We observed that low expression of ERRα restricted the sphere formation of breast cancer cells, and the expression levels of stem cell-related molecules were decreased along with the reduction of ERRα expression levels. Consistent with our hypothesis, overexpression of CCNE2 in ERRα-silenced breast cancer cells could reverse the inhibition of sphere formation ability caused by knockdown of ERRα. Therefore, ERRα promotes the stemness of breast cancer cells by targeting CCNE2 and leads to cisplatin resistance, which may be the formation of CSCs to facilitate the repair of cisplatin-induced DNA damage in breast cancer cells. The high DNA repair and anti-apoptotic activities of CSCs correspond to the anti-tumor mechanism of cisplatin, which inhibits the division and proliferation of tumor cells by causing DNA replication disorders.

Furthermore, ERRα belongs to the orphan nuclear receptor family member. As a transcription factor, its lipid-soluble hormone receptor properties enable it to directly enter the nucleus to regulate the transcriptional expression of target genes. In the central position of transcriptional regulation, it plays an important role in regulating various physiological activities of the body. Evidence indicates that transcription factors bind directly to DNA sites and are hyperactive in most human cancer cells, which makes them become promising targets for anticancer drug development. In our study, we demonstrated that ERRα binds to promoter elements of CCNE2 and strongly activates its transcription in breast cancer cells. Although further in vivo and clinical studies are needed to confirm our conclusion, it provides a rational mechanism for cisplatin resistance in breast cancer.

There are, however, limitations of our study. First, whether ERRα regulate CCNE2 expression through recruitment of its co-activator in breast cancer needs further improvement. Second, we did not explore the expression levels of ERRα in cisplatin resistant breast cancer patient tissues. Further clinical collection is needed to assess it. Finally, cisplatin in combination with specific antagonists may provide new therapeutic avenues for cisplatin resistant breast cancer patients, which also requires further experiments.

Conclusions

Collectively, our study demonstrates that ERRα plays an important role in the acquisition of chemoresistance by promoting the transcription of CCNE2 thereby enhancing the pluripotent stemness of breast cancer cells. Therefore, ERRα may be used as a prognostic biomarker in breast cancer patients, as well as a predictive biomarker of cisplatin sensitivity or acquired cisplatin resistance. The combination of
cisplatin and ERRα-specific antagonists may be beneficial in the treatment of cisplatin-resistant breast cancer patients.

**Abbreviations**

ERRα
estrogen-related receptor α
HER2
human epidermal growth factor receptor 2
ERRs
estrogen-related receptors
IHC
immunohistochemistry
DMEM
Dulbecco's modified Eagle Medium
qRT-PCR
quantitative RT-PCR
CCK-8
cell counting kit 8
ChIP
Chromatin immunoprecipitation
EMT
epithelial-mesenchymal transition
CSCs
cancer stem cells
GO
gene ontology
KEGG
kyoto encyclopedia of genes and genomes
TSS
transcription start site.

**Declarations**

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**Author’s contributions**
QZ, ZH S and LH L designed and supervised the research. JH L and QZ carried out the research. JW F, JF X, LH, and BX analyzed the data. YZ, ZZ K, WY Z, LJ S and YZ assisted some experiments, contributed to and figure production; JH L and QZ wrote the paper. HB Z and CD assisted in manuscript revision. All authors contributed to the article and approved the submitted version.

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**Availability of data and materials**

Any non-commercially available reagents or data from the studies are available upon reasonable request.

**Ethics approval and consent to participate**

This study is compliant with all relevant ethical regulations regarding animal research. All animal experiments were reviewed and approved by the Institute of Biological and Medical Engineering, Guangdong Academy of Sciences.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing interests.

**Author details**

1 Laboratory of Basic Medical Science, General Hospital of Southern Theater Command of PLA, Guangdong, Guangzhou, 510000, China. 2 Department of Laboratory Medicine, General Hospital of Southern Theater Command of PLA, Guangdong, Guangzhou, 510000, China. 3 Department of Clinical Laboratory, Zhongshan City People's Hospital, The Affiliated Zhongshan Hospital of Sun Yat-Sen University, Zhongshan, China. 4 State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine; National Center for Protein Sciences (The PHOENIX Center, Beijing), Beijing 102206, China. 5 Human Phenome Institute, Fudan University, Shanghai, 201203, China. 6 The First College of Clinical Medicine, Southern Medical University, Guangdong, Guangzhou, China. 7 Qingyuan People's Hospital, The Sixth Affiliated Hospital of Guangzhou Medical University, Guangdong, Qingyuan, 511500, China.
References


Figures
Figure 2

**ERRα promoted cisplatin resistance and the stemness of breast cancer cells.** (A) qRT-PCR analysis for ERRα mRNA level in normal breast epithelial cell line (MCF-10A) and five breast cancer cell lines. Each bar represents the mean ± SD of three independent experiments. (B) Five breast cancer cells cisplatin IC_{50} values were measured. Each bar represents the mean ± SD of three independent experiments. (C) Correlation between ERRα expression and cisplatin IC_{50} values in five breast cancer cells. Effects of...
cisplatin on ERRα protein expression with 4 μM at different time points (D) or different concentrations treatment at 48 h(E) in MDA-MB-231cells. (F) The effect of ERRα overexpression and knockdown on IC_{50} value of cisplatin in MDA-MB-231 and BT-474 cells. Each bar represents the mean ± SD of three independent experiments. (G) Representative micrographs and quantification of the tumor spheres formed by ERRα-overexpressing MDA-MB-231 cells and ERRα-silenced BT-474 cells subjected to the indicated treatments. Each bar represents the mean ± SD of three independent experiments. *P<0.05.

**Figure 4**

**High expression of ERRα confers cisplatin resistance to breast cancer in vivo.** Cisplatin regime (A), tumor images (B) tumor volume (mean ± SD, n = 3) (C) and tumor weight (mean ± SD, n = 3) (D) of each group show ERRα overexpression promote cisplatin chemosensitivity in vivo. The cisplatin was given 5 mg /kg through intraperitoneal injection. The image of tumors harvested at the end time point is shown in the insets. *P<0.05, ***P<0.001.
Figure 5

Genome-wide characterization of ERRα transcriptional binding sites in breast cancer cells. (A) Volcano plot of RNASeq results. (B) GO functional annotation and KEGG pathway enrichment analysis of significantly upregulated genes were conducted proteins using DAVID. (C) The distribution of reads on both sides of the peak summits. (D) Plot representing the density of ERRα binding sites distribution around the TSS in ChIP-seq. (E) Pie diagram showed the ratios of ERRα binding sites located relative to a
transcription unit. (F) Five common motifs with the most significant differences among peaks. (G) Venn diagram analysis shows intersection genes from ERRα targeted ChIP-seq data and ERRα overexpressed RNA-seq data. (H) The effect of ERRα overexpression and knockdown on candidate genes mRNA expressions were detected by qRT-PCR. Each bar represents the mean ± SD of three independent experiments. (I) Correlation between ERRα protein expression and CCNE2 mRNA expression in CPTAC breast cancer dataset. (J) Serially truncated CCNE2 promoter constructs were cloned to pGL3-luciferase reporter plasmids and transfected into MDA-MB-231 cells. Each bar represents the mean ± SD of three independent experiments. (K) A ChIP assay demonstrated the direct binding of ERRα to the CCNE2 promoter in MDA-MB-231 cells. M: Marker. (L) qRT-PCR of the ChIP products validated the binding capacity of ERRα to the CCNE2 promoter. Each bar represents the mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001. ns, no significance.

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