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Original Article

PRRX1 drives tamoxifen therapy resistance through induction of epithelial-mesenchymal transition in MCF-7 breast cancer cells

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Abstract: Purpose: Resistance to endocrine therapies is a major cause of disease relapse and mortality in estrogen receptor (ER)-positive breast cancers, which has been associated with tumor epithelial-mesenchymal transition (EMT). In this study, we investigated the contribution of the EMT-inducing factor paired related homeobox 1 (PRRX1) to tamoxifen (TAM) resistance acquired *in vitro* using ER-positive MCF-7 breast cancer cells. Methods: PRRX1 was overexpressed in MCF-7 cells through transfection; cells transfected with a blank vector served as the control. The morphological changes and transfection efficiency were observed by inverted fluorescence microscopy. The expression of ER and EMT-related proteins and genes was evaluated by Western blot and real-time polymerase chain reaction analysis, respectively. Finally, we evaluated the EMT features of the breast cancer cells and their response to TAM treatment. Results: The transfection efficiency was greater than 80%, and the expression level of PRRX1 protein was significantly higher after transfection, whereas the expression of ER protein was significantly lower after transfection. The overexpression of PRRX1 changed the morphology of breast cancer cells from a "paving stone" to a long spindle shape. The mRNA expression levels of *PRRX1* and vimentin were significantly higher, whereas that of E-cadherin was significantly lower after transfection. The proliferative level of the breast cancer cells after transfection was significantly increased at 12, 24 and 48 h after treatment with TAM. At 24 h of TAM treatment, the half-maximal inhibitory concentration of the transfected cells was significantly higher than that before transfection. Moreover, the PRRX1-overexpressing MCF-7 breast cancer cells acquired an EMT phenotype and displayed decreased levels of ER targets to ultimately acquire resistance to TAM. Conclusions: PRRX1 overexpression can induce EMT to promote resistance to TAM in MCF-7 breast cancer cells, partly by reducing ER expression. It is suggested that in clinical practice, PRRX1 gene expression detection can be performed in patients with hormone-receptor-positive breast cancer to guide our medication and prognosis.

Keywords: Breast cancer, PRRX1, EMT, tamoxifen, drug resistance

Introduction

Breast cancer is one of the most common female malignancies worldwide, and approximately 70% of all breast cancers are positive for a hormone receptor [1]. Endocrine therapy is one of the main treatments for hormone receptor-positive breast cancer to reduce the risk of recurrence, and is also the primary treatment for patients with advanced and recurrent metastases [2]; however, the emergence of drug resistance represents the main cause of treatment failure, so endocrine treatment resistance has become an urgent problem to be solved. Epithelial-mesenchymal transition (EMT) refers to the process by which epithelial cells are transformed into interstitial phenotypes, which changes not only the cell morphology but is also accompanied by the loss of epithelial markers, including E-cadherin, and the gain of interstitial phenotype markers such as vimentin [3, 4]. Several EMT-related transcription factors have been identified to date, including cell transcription factors and transforming growth factor 1 [5, 6]. EMT is closely associated with the acquisition of the drug resistance of cells, and can be induced by exposure to antitumor drugs, radiotherapy, or hypoxia condi-

Table 1. PCR primer sequences

Gene	Primer sequences (5'-3')	Product length
PRRX1	F: GCACAGGCGGATGAGAAC	116 bp
	R: TCTTCTGAGTTCAGCTGGTCAT	
Vimentin	F: TTGACAATGCGTCTCTGGCA	256 bp
	R: CGTGAGGTCAGGCTTGGAAA	
E-cadherin	F: ACACCATCCTCAGCCAAGA	116 bp
	R: CGTAGGGAAACTCTCTCGGT	
GAPDH	F: TCATGGGTGTGAACCATGAGAA	146 bp
	R: GGCATGGACTGTGGTCATGAG	

tions. Moreover, the interstitial phenotype of tumor cells is more susceptible to drug resistance than the epithelial phenotype [7]. Paired related homeobox 1 (PRRX1) is a newly discovered EMT-inducing factor, and its abnormal expression is closely related with the occurrence and development of a variety of tumors [8, 9]. Moreover, PRRX1 is highly expressed in tumor cells such as gastric and malignant glioma cells, and can promote the EMT process in tumor cells [10, 11]. Although silencing PRRX1 expression was shown to inhibit EMT in breast cancer cells [12], there has been relatively sparse research conducted on the relationship between EMT and breast cancer endocrine resistance. In particular, a decrease of estrogen receptor (ER) expression is closely related to EMT, and is associated with resistance to endocrine therapy in patients with ER-positive breast cancer [13]; however, the mechanism contributing to the decline in ER expression appears to be complex and is not yet clear. Thus, in this study, we evaluated the potential role of PRRX1 in contributing to endocrine therapy resistance and the role of EMT in this process. To this end, we established PRRX1-overexpressing ER-positive breast cancer cells (MCF-7 cell line), which were treated with tamoxifen (TAM), and evaluated the effects on the cell morphology and expression of ER and EMT-related genes.

Material and methods

Cell culture and transient transfection

The MCF-7 cell line was provided by Affiliated Hospital Laboratory of Qingdao University, and the cells were maintained and cultured in a 1640 medium containing 10% fetal bovine serum (FBS) at 37°C in a saturated humidity incubator with 5% CO_2. At 80-90% confluence, the cells were passaged by trypsinization, and the culture medium was replaced after one or two days.

MCF-7 cells were inoculated in 24-well plates and left until 50-70% of the cells adhered to the plate. In the experimental group, the cells were transfected with a recombinant plasmid carrying the *PRRX1* gene (pEX-3/PRRX1; GenePharma Company, Shanghai, China). The cells in the blank control group were transfected with the blank pEX-3 vector (GenePharma), and the negative control group was untransfected MCF-7 cells. The culture medium was replaced the day before transfection, and then transfection was performed using Lipofectamine 2000 reagent according to the manufacturer's instructions to generate stable PRRX1-overexpressing MCF-7 cells. After two days, the morphology and transfection efficiency were observed under an inverted fluorescence microscope.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using RNAiso Plus (TaKaRa, China Bao Biological Engineering Co., Ltd.) reagent according to the manufacturer's instructions, and reverse transcription was performed using the PrimeScript™ RT reagent kit (TaKaRa, China Bao Biological Engineering Co., Ltd.). qRT-PCR was performed with the SYBR Premix Ex Taq system using primers synthesized and sequenced by Shanghai Bioengineering Technology Service Co., Ltd. (**Table 1**). The levels of E-cadherin, vimentin, and *PRRX1* transcripts were normalized to those of the reference gene *GAPDH* using the DCt method. The reaction conditions were a pre-denaturation step at 95°C for 30 sec, followed by 40 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 30 sec, 95°C for 1 min, and cooling at 40°C cooling. Agarose gel electrophoresis was used to test primer specificity.

Cell proliferation analysis

The cell suspension was diluted to 5×10^4 cells/mL after preparation using a cell counting plate, and 100 mL of the suspension was transferred to a 96-well plate, cultured for 24 h, and

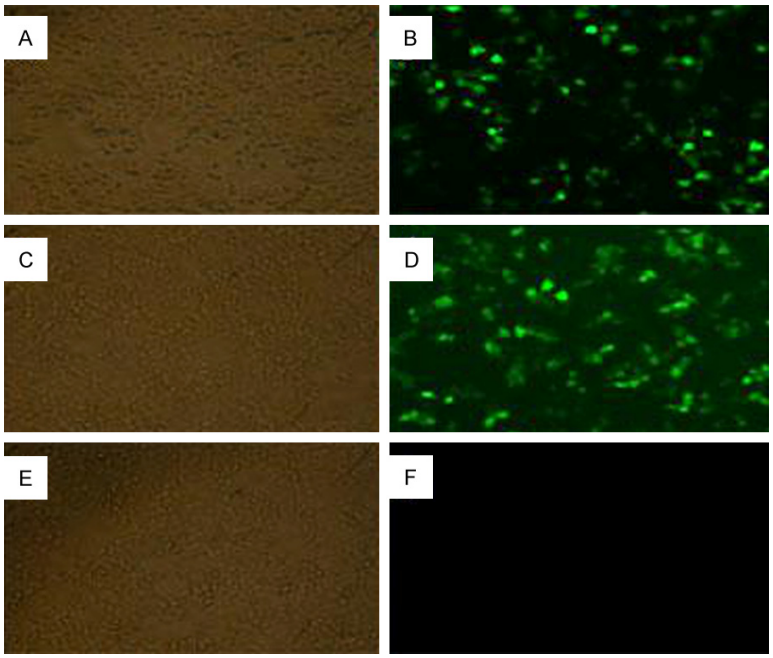

Figure 1. A. MCF-7 cells transfected with a *PRRX1* expression vector (ordinary light, × 200). B. MCF-7 cells transfected with a *PRRX1* expression vector (fluorescence, × 200); C. MCF-7 cells transfected with a blank vector (ordinary light, × 200); D. MCF-7 cells transfected with a blank vector (fluorescence, × 200). E. Untransfected MCF-7 cells (ordinary light, × 200); F. Untransfected MCF-7 cells (fluorescence, × 200).

TAM solution was added at various concentrations: 1×10^{-7} mol/L, 5×10^{-7} mol/L, 1×10^{-6} mol/L, 5×10^{-6} mol/L, 1×10^{-5} mol/L. Three wells were established for each TAM concentration, and the control wells were equilibrated with the cell suspension and an equal volume of 1640 medium containing 10% FBS. The cells were cultured for 24 h, and then 10 mL of Cell Counting Kit-8 (CCK8) (YiYuan Biotechnology limited, Guangzhou, China.) solution was added. After 2 h, the optical density value was measured at 450 nm, which was used to determine the cell proliferation rate and the concentration at which 50% of the cells are inhibited (IC50 value).

Western blot analysis

In brief, the three groups of cells showing good growth were lysed in a RIPA buffer and the protein concentration was measured with a BCA kit (TaKaRa, China Bao Biological Engineering Co., Ltd.). From each group, 20 μg of the protein lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane for 1.5 h. The membrane was blocked in 5% skim milk and subsequently incubated with the rabbit anti-human ERα, rabbit anti-human PRRX1, or mouse anti-human GAPDH primary antibody (Abcam) overnight at 4°C. After washing the membrane three times for 5 min each in Tris-buffered saline containing 1% Tween-20, it was incubated with the corresponding secondary antibody at room temperature for 1 h. The membranes were visualized with the ECL detection reagent and analyzed with a protein gel imaging analysis system (ViberFusion, France).

Statistical analysis

Correlation analysis was performed using SPSS 17.0 software. The least-significant difference t-test was used to compare data between two groups. All experiments were repeated at least three times. Single-factor analysis of variance was used to compare data among groups. $P < 0.05$ was considered statistically significant.

Results

Transfection efficiency and morphological changes due to PRRX1 overexpression

After transfection for 24 h, the cells were observed under an inverted fluorescence microscope using ordinary light and fluorescent light. Overall, the transfection efficiency of cells transfected with PRRX1 and blank vector reached up to 80% (**Figure 1B**, **1D**); the MCF-7 cells did not fluoresce (**Figure 1F**). Cells that grew in good condition were observed under normal light microscopy, which revealed that the morphology of untransfected cells and of cells transfected with the blank vector displayed a “paving stone” shape and were closely positioned to one another (**Figure 1C**, **1E**), whereas the cells transfected with the *PRRX1* expression vector showed a more “spindle” morphology with loose connections between cells (**Figure 1A**).

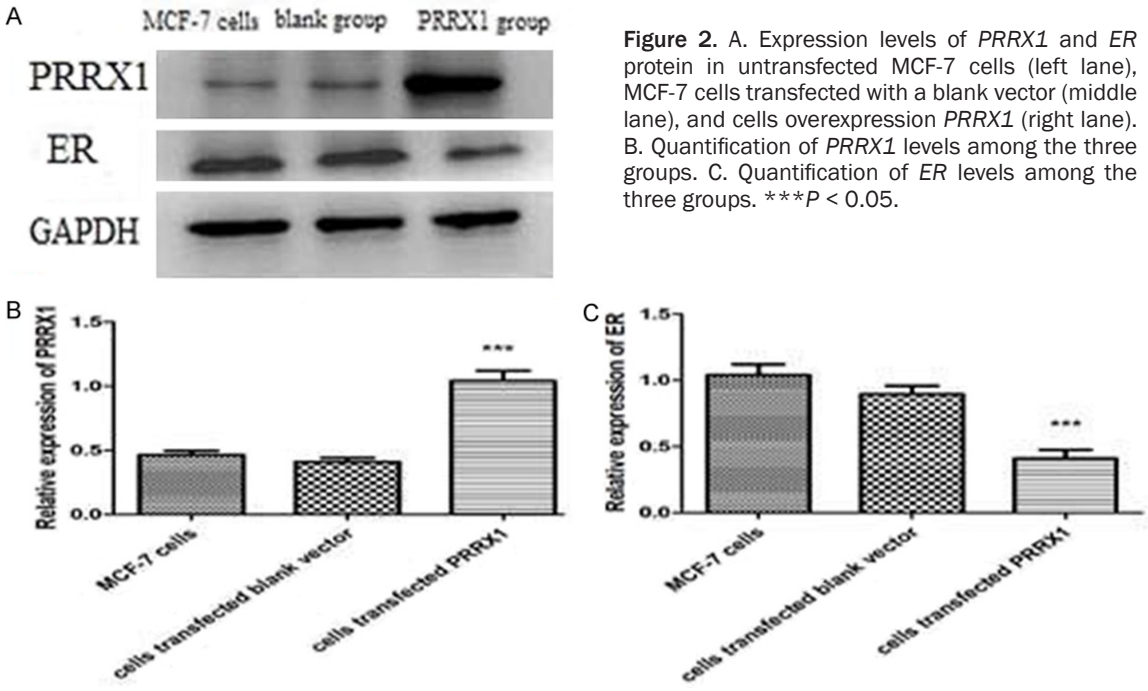

Figure 2. A. Expression levels of *PRRX1* and *ER* protein in untransfected MCF-7 cells (left lane), MCF-7 cells transfected with a blank vector (middle lane), and cells overexpression *PRRX1* (right lane). B. Quantification of *PRRX1* levels among the three groups. C. Quantification of *ER* levels among the three groups. ***$P < 0.05$.

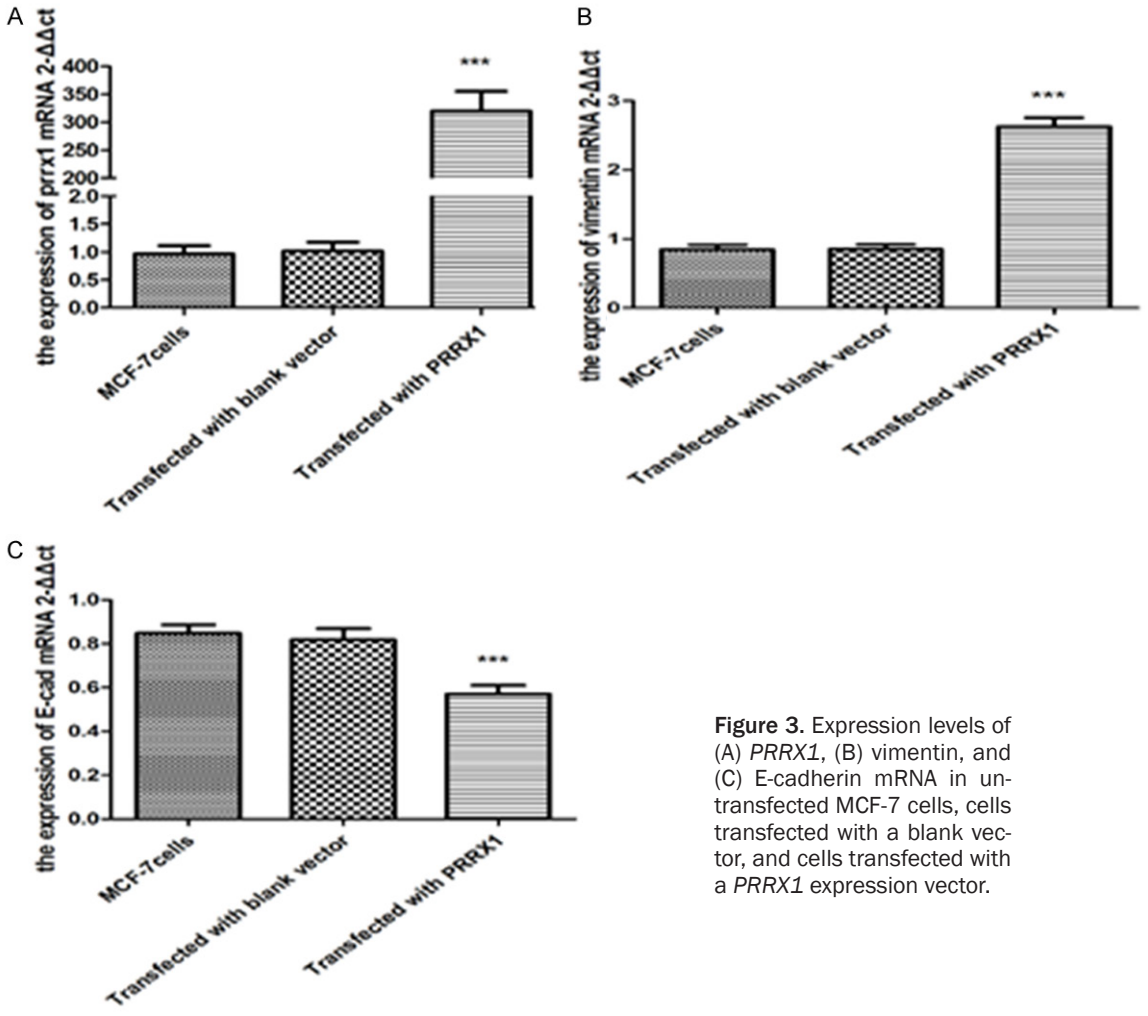

Figure 3. Expression levels of (A) *PRRX1*, (B) vimentin, and (C) E-cadherin mRNA in untransfected MCF-7 cells, cells transfected with a blank vector, and cells transfected with a *PRRX1* expression vector.

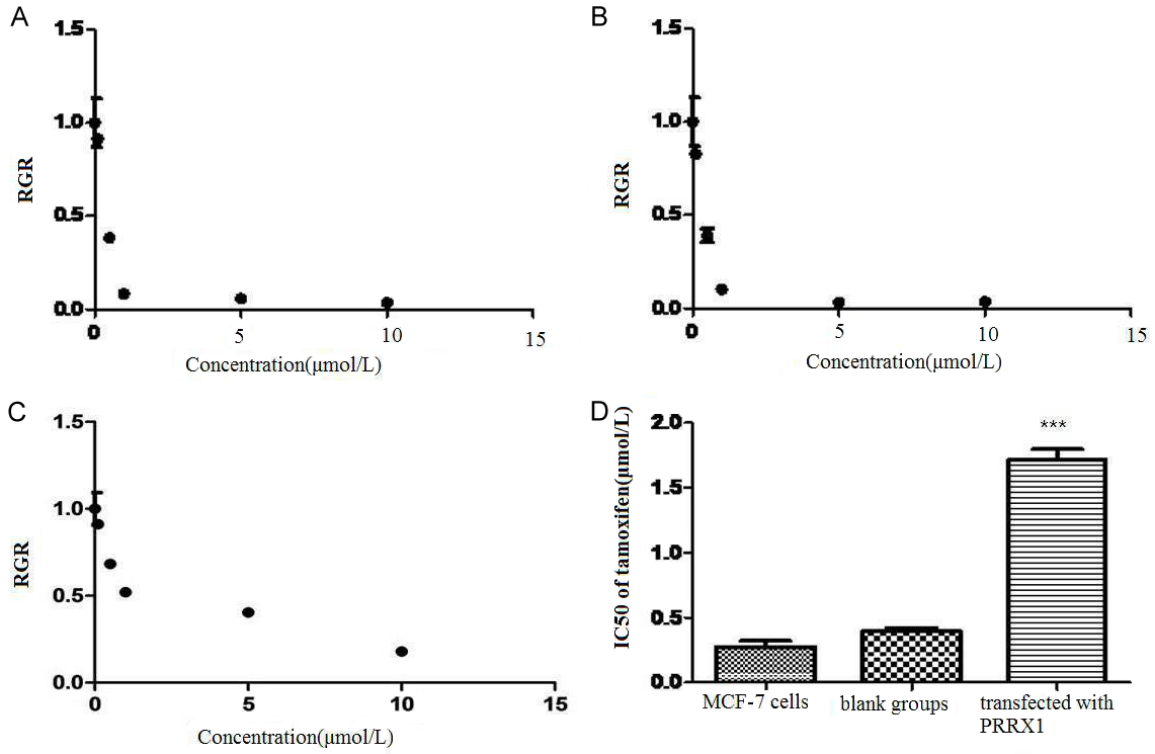

Figure 4. Cell relative growth rate (RGR) of (A) untransfected MCF-7 cells, (B) cells transfected with a blank vector, and (C) cells transfected with a *PRRX1*-overexpression vector exposed to different concentrations of tamoxifen. (D) IC50 values of tamoxifen for the three groups of cells.

Effect of PRRX1 on ER protein expression

After transfection for 48 h, the expression level of the PRRX1 protein was significantly increased ($P < 0.05$), whereas very low and similar expression levels were detected in the two control groups ($P = 0.33$). By contrast, the expression level of ER protein was significantly decreased in the MCF-7 cells overexpressing PRRX1 compared to the pre-transfection level ($P < 0.05$), but was present at high and similar levels in the two control groups ($P = 0.96$) (**Figure 2**).

Effect of PRRX1 overexpression on E-cadherin and vimentin expression

After transfection for 48 h, the expression levels of *PRRX1* and vimentin mRNA increased significantly ($P < 0.05$, **Figure 3A**, **3B**), whereas the expression level of Ecadherin mRNA decreased significantly ($P < 0.05$, **Figure 3C**). There was also a statistically significant difference in the expression levels of *PRRX1*, vimentin, and E-cadherin between the overexpression group and control groups ($P < 0.05$), whereas there were no significant differences detected between the blank control group and untransfected cells ($P > 0.05$).

Effect of PRRX1 overexpression on resistance to TAM

Analysis of the cell proliferation rate with the CCK8 assay showed that the cells overexpressing PRRX1 had a significantly higher proliferation rate than that measured before transfection (**Figure 4A-C**). At 24 h after TAM treatment, the IC50 of cells transfected with PRRX1 was significantly higher ($P < 0.05$, **Figure 4D**) than that of the two control groups, with no difference between the control groups ($P = 0.093$). This result indicated that the growth capacity of PRRX1-overexpressing cells was significantly improved under exposure to the anti-hormone receptor drug TAM.

Discussion

Breast cancer is a hormone receptor-dependent tumor, and approximately 70% of all breast tumors are ER-positive for which endocrine therapy remains the main treatment option [14,

15]. TAM is the most effective and most commonly used drug in endocrine therapy, which is administered to patients with both post- and pre-menopausal breast cancer, representing the cornerstone in endocrine therapy for breast cancer [16, 17]. However, intrinsic or acquired resistance to TAM is the main reason for treatment failure and has emerged as an important clinical challenge [18, 19]. EMT is the process by which epithelial cells are transformed into cells with interstitial phenotypes. The molecular-level changes during EMT include the loss of E-cadherin and keratin expression, which is an epithelial cell adhesion molecule, and the acquisition of mesenchymal marker expression, including vimentin and fibronectin [20-22]. When tumor cells lose the epithelial phenotype to obtain the interstitial phenotype, they also lose the close connections between cells, accompanied by a change in cell morphology [23]. In line with these changes, in our study, after successful transfection of a *PRRX1* expression vector, the morphology of ER-positive MCF-7 breast cancer cells changed from the original paving stone shape to an elongated shape, and the epithelial features were lost toward a shift to mesenchymal characteristics along with looser cell-cell connections. Compared with the control group, the expression level of E-cadherin mRNA significantly declined and the expression level of vimentin mRNA significantly increased. Therefore, PRRX1 clearly influences the characteristic events of cells undergoing EMT.

There are only a few limited reports on the complex EMT-mediated mechanism of TAM resistance in hormone receptor-positive breast cancer cells. Some studies have shown that TAM resistance is related to the expression of ER or an abnormal ER signaling pathway [24, 25], and the ER expression level is generally decreased in TAM-resistant cell lines *in vitro*, accompanied by morphological changes from an epithelial to interstitial phenotype. PRRX1 is an inducing factor for EMT and can regulate the expression of multiple genes [26]; however, its role in regulating ER expression has not been investigated to date. The results of the present study suggest that PRRX1-induced EMT can lead to a reduction in ER expression in MCF-7 breast cancer cells, indicating that PRRX1-induced EMT could result in resistance to TAM by reducing ER expression. Indeed, compared with the control group, the IC50 value of TAM was significantly higher for the PRRX1-overexpressing cells, indicating that PRRX1-induced EMT in MCF-7 cells could be a mechanism of TAM resistance.

These findings provide a new perspective for understanding EMT and endocrine drug resistance mechanisms. However, given the complexity of drug resistance mechanisms, further studies are needed to uncover the complex pathway underlying the regulation between PRRX1 and ER.

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Disclosure of conflict of interest

None.

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