MOLECULAR MECHANISMS OF DRUG RESISTANCE AND STRATEGIES OF SENSITIZATION IN BREAST CANCER, 2nd Edition

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Obg-Like ATPase 1 Enhances Chemoresistance of Breast Cancer *via* Activation of TGF-β/Smad Axis Cascades

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Understanding the molecular mechanism of drug resistance helps to identify an effective target for breast cancer therapy. In this study we investigated the regulatory role of Obg-like ATPase 1 which is involved in multiple uses of drug resistance against breast cancer. Paclitaxel resistant cell line (MCF-7-PTR) was developed by a continuous increasing paclitaxel concentration. MTT assay was used to validate either acquired resistant or OLA1 modified cell lines. qRT-PCR, western blotting, apoptosis, and cell cycle assays were executed to evaluate gene and protein expression in cell lines. A series of *in vitro* assays was performed in the cells with RNAi-mediated knockdown to expound the regulatory function of OLA1 in breast cancer. We demonstrated that OLA1 was highly correlated with either acquired or intrinsic resistance of breast cancer. Further study showed that escalated expression of OLA1 promoted the EMT process in tumor cells through TGF-β/Smad signaling cascades, resulting in the enhanced expression of anti-apoptosis-related proteins (cleaved caspase3, Bax, Bcl-2) and the strengthening depolymerization of microtubules in tumor cells. Our findings revealed that OLA1 enhanced the anti-apoptotic ability and elucidated a regulatory role of OLA1 in promoting chemotherapy resistance of breast cancer. Chemo-sensitivity of the disease can be thus enhanced significantly by knocked down OLA1, which led to the inactivation of the TGF-β/Smad signaling cascades, polymerized microtubules, and promoted cell apoptosis. Our data suggest that OLA1 may be developed as a potential target to improve chemotherapy of patients with breast cancer.

Keywords: Obg-like ATPase 1, breast cancer, chemosensitivity, γ-tubulin, multidrug resistance, paclitaxel, 5-FU

INTRODUCTION

Breast cancer is one of the leading fatal cancers among women worldwide. According to statistics, there were an estimated 2.1 million breast cancer patients globally in 2018, and one out of every four female cancer patients is a breast cancer patient (Bray et al., 2018). Although breast cancer is a relatively easily diagnosed cancer in most countries, this does not prevent it from being a most lethal disease. The resistance of tumor cells to chemotherapy is still a critical issue breast cancer therapy.

Epithelial-to-mesenchymal transition (EMT), which is associated with the acquisition of stem cell properties, metastasis, and resistance to therapy (Mani et al., 2008; Pastushenko et al., 2018), is a process in which the characteristics of epithelial cells change to the mesenchymal phenotype. Reasonably, breast cancer that remains after chemotherapy shows mesenchymal phenotypes and tumor initiation characteristics (Creighton et al., 2009). In fact, the evidence that EMT promotes tumor metastasis is insufficient, but EMT can indeed promote tumor chemoresistance in various cancers (Fischer et al., 2015; Zheng et al., 2015; Diepenbruck and Christofori, 2016; Ye et al., 2017). Emerging evidence has proven that EMT makes tumor cells more chemoresistance when cells are transfected with specific hallmark genes of EMT, including Wnt and the transforming growth factor-β (TGF-β) (Cufi et al., 2010; Li et al., 2013; Mallini et al., 2014). Actually, the TGF-β pathway has a crucial role in EMT induction in a variety of tissue types (Xu et al., 2009; Lamouille et al., 2014). Adding TGF-β to culture mediums of epithelial cells is a convenient method to induce EMT (Xu et al., 2009). Exposure of tumor cells to TGF-β and TNF-α induces the EMT process and generates cells with a cancer stem cell-like phenotype, which is shown by the increased self-renewal capacity resulting in greatly improved tumorigenicity, and enhanced resistance to oxaliplatin, etoposide, and paclitaxel (Asiedu et al., 2011).TGF-β signaling possesses both Smad and non-Smad pathways, and crosstalk with numerous signal transduction pathways to advance EMT processes at multiple levels, including PI3K-AKT-mTOR, Wnt, Notch, and ERK, p38, and JUN N-terminal kinase (JNK) MAPK pathways (Xu et al., 2009; Lamouille et al., 2014). A study showed that breast cancer MDA-231 cells treated with cisplatin increased TGF-β mRNA expression. When TGF-β neutralizing antibodies were used to block the activity of TGF-β in tumor cells, breast cancer cells resumed sensitivity to the drug (Ohmori et al., 1998). Moreover, MCF-7/tamoxifen-resistant cells experienced the EMT process driven by an intensive endogenous TGF-β/Smad signaling pathway. Ectopic supplements of TGF-β promoted a mesenchymal transition of MCF-7 cells showing a resistant phenotype (Shi et al., 2013).

Paclitaxel (PTX) is a microtubule-stabilizing agent which is approved by the Food and Drug Administration(FDA) for the therapy of ovarian, breast (Gampenrieder et al., 2013; Zardavas and Piccart, 2015), and lung cancer, as well as leukaemias and lymphomas (Wertz et al., 2011). Paclitaxel in general induces mitotic arrest to mediate arrested cell death. Microtubules play pivotal roles in basic cellular processes and are targets used for anti-tubulin chemotherapeutics (Wertz et al., 2011). Microtubules are composed of tubulin monomers joined together by non-covalent bonds. There are two subunits of tubulin: α-tubulin and β-tubulin. Either assembly or disassembly of tubulin is highly relied on in cellular GTP and GDP (Muller-Reichert et al., 1998; Wang and Nogales, 2005; Alushin et al., 2014). γ-tubulin, not a component of microtubules, is involved in the assembly of microtubules (Oegema et al., 1999). It is important in the nucleation and polar orientation of microtubules (Joshi et al., 1992). It is mainly found in centrosomes and spindle poles because they are the most abundant microtubule nucleation areas (Wolf and Joshi, 1996).

Obg-like ATPase 1(OLA1) is a p-loop GTPase belonging to the TRAFAC (translation factor related) class, the Obg family and the YchF subgroup. The main functions of TRAFAC GTPase include: translation factors and ribosomal connexin, signal transduction, intracellular transport, and stress response proteins (Leipe et al., 2002; Verstraeten et al., 2011). OLA1 is highly conserved from bacteria to humans, and unlike other Obg family members, exercises both GTPase and ATPase activities (Koller-Eichhorn et al., 2007; Gradia et al., 2009). OLA1 was a DNA damage related and cell growth regulated gene, and decreased cellular sensitivity to doxorubicin in colon cancer cell (Sun et al., 2010). Currently, literature reports that OLA1 was involved in EMT transformation in different tumor cells (Zhang et al., 2009b; Bai et al., 2016). EMT is not a prerequisite for metastasis but contributes to chemoresistance (Fischer et al., 2015). However, whether OLA1 also mediates the EMT process in drug-resistant cells is not known yet. Thus far, there is no report showing whether OLA1 is associated with breast cancer drug resistance. Recent studies showed that OLA1 can also interact with γ-tubulin and form a complex with breast cancer 1 (BRCA1) and BRCA1-associated RING domain protein (BARD1), leading to the recruitment of receptors for activated C kinase 1 (RACK1) to regulate the centrosome (Matsuzawa et al., 2014; Yoshino et al., 2018; Yoshino et al., 2019). We hypothesize that OLA1 may regulate paclitaxel resistance of breast cancer by interfering tubulin expression. In this study, we show that OLA1 is positively correlated with the development of drug resistance by inducing the EMT process through activation of TGF-β/Smad signaling pathway in breast cancer. Our results indicate OLA1 can be developed as a novel valuable target for an improvement of breast cancer chemotherapy.

Abbreviations: BARD1, BRCA1-associated RING domain protein; BRCA1, breast cancer 1; CCND1, Cyclin D1; DMEM, Dulbecco's modified Eagle's medium; ECM, Extracellular matrix; EMT, epithelial-mesenchymal transition; EMT-TFs, EMT-transcription factors; ER, Estrogen receptor; FBS, fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GTEx, Genotype-Tissue Expression; GSEA, Gene set enrichment analysis; Her2, Human epidermal growth factor receptor 2; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; mRNA, Messenger RNA; OLA1, Obg-like ATPase 1; PBS, phosphate-buffered saline; PCR, Polymerase chain reaction; PR, Progesterone receptor; RACK1, receptor for activated C kinase 1; RT-qPCR, reverse transcription quantitative PCR; SD, standard deviation; ShRNA, Short hairpin RNA; siRNA, small interfering RNA; TCGA, The Cancer Genome Atlas.

MATERIALS AND METHODS

Materials

PTX (#S1150) and Fluorouracil (5-FU, #S1209) were purchased from Selleck Chemicals LLC (Shanghai, China). Antibodies against OLA1 (also as GTPBP9, #PA5-31227) and Snail (#PA5-11923) were purchased from Invitrogen (San Diego, CA, USA). Antibodies against GAPDH (#5174), Slug (#9585P), Vimentin (#3932S), Smad3 (#9523P), p-Smad3 (#9520P), Smad4 (#9515P), Cleaved caspase3 (#9661), Bax (#2772), and Bcl2 (#2876S) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against gamma tubulin (#ab11316), Zeb1 (#ab180905) and E-cadherin (#ab76055) were purchased from Abcam (Cambridge, MA, USA). GAPDH was purchased from ProteinTech Inc (Wuhan, China). The second antibodies were purchased from Abbkine Inc (Wuhan, China). SuperEnhanced chemiluminescence (ECL) detection reagents and RIPA lysis buffer were purchased from Applygen Technologies Inc. (Beijing, China). Apoptosis Kit was purchased from KeyGEN BioTECH Inc (Nanjing, China). (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Solarbio Inc (Beijing, China). All other reagents were from Beyotime (Haimen, China) and Sangon Biotech (Shanghai, China).

Cell Culture

Human MCF-7 breast carcinoma cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences(Shanghai, China), and routinely grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Cat#12430054) containing 10% fetal bovine serum (PAN, Germany) and a mixture of 100 IU/ml penicillin and 100 μg/ml streptomycin (Solarbio, Beijing, China) in an incubator at 37 °C, 5% CO_2. The paclitaxel-resistant sublines (MCF-7-PTR) were derived from MCF-7 by continuous exposure to PTX. MCF-7-PTR cells were cultured continuously in a medium containing 10% FBS supplemented with 100 nM PTX. MDA-MB-231 were kindly provided by Dr. Zhengzheng Shi, and the culture conditions were similar with MCF-7 as described above.

Cell Viability Assay

MTT assay was used to determine the viability of the treated cells. Digest, collect, and count cells and 6000 cells were seeded onto 96-well plates and incubated overnight at 37°C. Paclitaxel at different concentrations (0, 1.56, 3.13, 6.25, 12.50, 25.0, 50.0, and 100.0 μM) were added to each well with different incubation times. Thereafter, 20 μL MTT solution (5 mg/mL) was added to each individual well. After incubating for 4 h at 37°C, the media was aspirated and 150 μL DMSO was added to dissolve the formazan crystals. The absorbance was measured at 490nm by the microplate reader. Six replicate wells were included in each analysis and at least three independent experiments were conducted. The cell inhibition rate and IC50 were calculated respectively by SPSS. The same method was used for the measurement of 5-Fu.

Cell Proliferation

Cell proliferation ability was assessed using an MTT assay. The silence group was transfected with either siOLA1 or silence negative control (siNC) for 12h before use. Cells were seeded at a density of 2×10^3 cells/well in 96-well plates. In the experimental group transfected with interfering RNA, the culture medium containing the transfection reagent and the interfering RNA was replaced once on the fourth day of the experiment. Cells, on day 1, 2, 3, 4, 5, 6, and 7 were incubated with 20 μL MTT solution at 37°C for 4 h. With the incubation medium removed, 150 μL DMSO was added to each well. The absorbance was measured at 490 nm.

Construction of Paclitaxel Resistant Cell Lines

Low-concentration induction method was used to construct drug-resistant cell lines. Briefly, the IC50 of paclitaxel on MCF-7 was detected, and the appropriate initial concentration (100 nM) was selected and added into the culture flask for incubation. After 24 h, the medicated medium was aspirated, washed with PBS, and added to the normal medium until the cells were over 80% and passaged. The medium was incubated n with equal concentration of the drug depending on cell growth, or a four-fold dose of the drug was added. This process was cycled back and forth until the resistance met the experimental needs. The establishment cycle of acquired drug-resistant cells was about six months.

Small Interfering RNA Transfections

Small interfering RNA (siRNA) for OLA1 (Cat#EHU113781) and siRNA Universal Negative Control #1 (Cat#SIC001) were purchased from Sigma-Aldrich. Cells seeded in a 6-well plate were transiently transfected with 100 pM siRNA with Lipo2000 Transfection Reagent (Thermo Scientific) according to manufacturer's instructions.

Establishment of the Stable OLA1 Knockdown MDA-MB-231 Cell Lines.

Small hairpin RNA (shRNA) lentiviral used for stable silencing of OLA1 (shOLA1) and the control non-targeting plasmid (shNC) were purchased from GenePharma (Nanjing, China) by inserting the following short-hairpin sequences into the pGLV3/H1/GFP/Puro vector:

5′-CCGGGAGGAAATGATTGGGCCCATTCTCGAG AATGGGCCCAAT CATTTCCTCTTTTTTG-3′ for sh-OLA1 and 5′-CCGGCAACAAGATGAAGAG CACCAACTCG AGTTGGTGCTCTTCATCTTGTTGTTTTTG-3′ for Small hairpin control (shNC). shRNA transfections and protocol were followed the recommendations by GenePharma (China). The shNC and shOLA1 vectors were transfected into MDA-MB-231 cells. The knockdown efficiency of the target gene was verified by qRT-PCR and western blot analysis.

qRT-PCR Analysis

Total RNA was extracted from the cells using TRIzol (Cat#15596026, Invitrogen, CA, USA), and the concentration and quality were determined by a microplate reader (DU730, Beckman, CA, USA). The nucleotides were reverse-transcribed into cDNA according to the instructions of the PrimeScript™ RT Reagent Kit (Cat#RR037A, Takara, Japan). After amplification

and dilution, the assay was performed on the *LightCycler480 II* (Roche, USA). The gene primer was as follows: GAPDH (glyceraldehyde 3-phosphate dehydrogenase): Forward primer: 5-CATGAGAAGTATGACAACAGCCT Reverse primer: 5-AGTCCTTCCACGATACCAAAGT; OLA1: Forward primer: 5-TGGACAAGTATGACCCAGGT Reverse primer: 5-GCTGCAAACCCAGCCTTAATG. The other primer sequences are provided in the supplemental material (**Supplementary Table 1**).

Western Blotting Analysis

Protein was extracted from the cells using RIPA buffer, added with PMSF to avoid degrading, and stored at -80°C. The BCA protein concentration detection kit was used for quantification, and the loading buffer was added in proportion to boil at 95 °C and stored in a refrigerator at -20 °C. SDS-PAGE gel was prepared and 30 μg of protein sample was added to each lane. The target protein band was cut and transferred to the PVDF membrane, and the milk was blocked for 2 h. The membrane was washed three times with TBST (10 min/time), added with a primary antibody at 4°C overnight, then washed three times with TBST (10 min/time), and the secondary antibody was incubated for 2 h. After TBST washing, the membrane was incubated with ECL high-sensitivity developer and then developed in ChemiDoc Imaging Systems (BIO-RAD, USA).

Apoptosis Analysis

Cells (2×10^5) were seeded onto 6-well plates for each group overnight then treated with paclitaxel (20 μM) for the indicated time. After incubation, the medium was collected, and the cells were digested with trypsin without EDTA and incorporated into the previously collected medium, where total cells were collected by centrifugation. Following the steps of Annexin V-FITC/PI double staining kit, staining reagents were added twice in turn, incubated at room temperature for 10 min in the dark, and then apoptosis analysis was performed by flow cytometry.

Cell Cycle Analysis

Cells were stained with propidium iodide (PI) using the cell cycle kit (#KGA511, KeyGEN BioTECH, Nanjing, China) according to the provided protocol. Briefly, cells were harvested, washed in ice-cold phosphate-buffered saline (PBS), and fixed in 70% cold ethanol for 2h at 4°C. After two PBS washes, cells were treated with RNase A/PI staining buffer and assayed with an FACS Calibur (BD Biosciences, San Jose, CA, USA) flow cytometer using Cell Quest software. The cell cycle distribution was analyzed using BD CellQuest™ Pro Analysis software (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Data were presented as mean ± standard deviation (SD). IC50 (mean ± 95% confidence interval) of chemotherapeutics in breast cancer was calculated by SPSS23.0, and other statistic results were carried by GraphPad Prism 8. A two-sided tail non-paired Student's t test was used to compare the differences of two groups. Kaplan-Meier analysis and logrank test was used to assess statistical significance of survival rate. P <0.05 was considered statistically significant.

RESULTS

OLA1 Was Upregulated in Breast Cancer

We first analyzed OLA1 expression profile across all tumor samples and paired normal tissues in the RNA sequencing data from Gene Expression Profiling Interactive Analysis (GEPIA) (Tang et al., 2017). We found that OLA1 has a ubiquitous expression in the brain, thyroid, and 25 other tissues from the body map (**Supplementary Figure 1A**), and is highly expressed in breast cancer, pancreatic cancer, colorectal cancer, and other cancer tissues (**Supplementary Figure 1B**). Through matching with The Cancer Genome Atlas (TCGA) normal and Genotype-Tissue Expression (GTEx) data, we found that OLA1 is significantly upregulated in breast cancer (N=1085) compared to their paired normal tissue (N=291) (**Figure 1A**). Notably, the Kaplan-Meier (KM) Plotter analysis (Nagy et al., 2018) showed that OLA1 expression may negatively correlate with the overall survival and relapse free survival of breast invasive carcinoma (BRCA) patients (**Figures 1B, C**). Although the *P*-value is more than 0.05, the survival time with OLA1 highly expressed cohort is much less than the lower, suggesting OLA1 may play a regulatory role in drug resistance.

Upregulated OLA1 Was Also Observed in Acquired Drug-Resistant Cell Line MCF-7-PTR

To understand the regulatory role of OLA1 in breast cancer resistance to PTX, MCF-7 resistant cell line to PTX was developed (named as MCF-7-PTR) through continuous induction of MCF-7 with PTX at a low concentration (100 nM). To understand whether multiple drug resistance of MCF-7-PTR was also conferred, the resistance of MCF-7-PTR to 5-Fu was also examined and showed that multiple drug resistance of breast cancer cells were formed, as shown in **Figure 2**. MTT assays showed that the IC50 of MCF-7-PTR to paclitaxel was 50.87 ± 31.85 μM compared to the parental cells MCF-7 (IC50 6.17 ± 2.93 μM), and the drug resistance index was 8.24 (**Figure 2B**). The IC50 of MCF-7-PTR to 5-Fu was 1173.19 ± 688.62 μM as compared to the parental cells MCF-7 (i.e. 91.84 ± 42.38 μM), and the drug resistance index was 12.77 (**Figure 2C**). During development of the drug antagonistic to breast cancer, cell morphology was changed significantly, observed to be more dispersed and irregular in resistant cells than that of the parental cells (**Figure 2A**). To find out whether endogenous OLA1 is related to the development of drug resistance in breast cancer, the endogenic level of OLA1 in both mRNA (**Figure 2D**) and protein levels (**Figure 2E**) in MCF-7-PTR was analyzed, and showed that both endogenous levels of mRNA and protein of OLA1 were indeed significantly higher than that of the parental cells (**P*< 0.05, ***P* < 0.01), indicating that OLA1 plays a regulatory role in the development of tumor drug resistance.

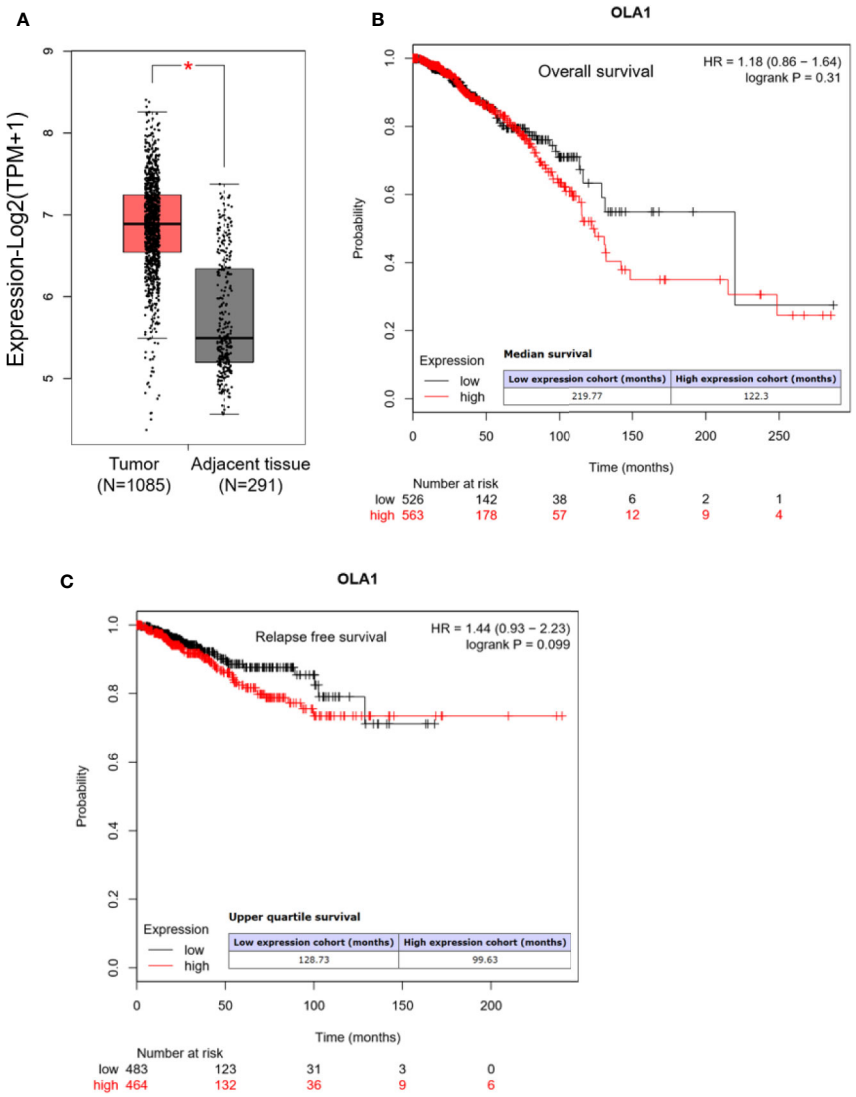

FIGURE 1 | OLA1 is upregulated in breast cancer. **(A)** The average expression level of OLA1 in patient's breast cancer tissue was higher than adjacent tissues in TCGA and GTEx breast cancer dataset. **(B, C)** Kaplan-Meier overall survival and relapse free survival curves for patients with breast cancer stratified by high and low expression of OLA1.

Knockdown of OLA1 Enhanced Chemo-Sensitivity of the Acquired Drug Resistance of Breast Cancer

To further determine the regulatory role of OLA1 in drug resistance, small interfering RNA of OLA1 was successfully used to knockdown the endogenic level of OLA1 in MCF-7-PTR, as shown in both mRNA and protein levels (**Figures 3A, B**). Acquired resistant cell MCF-7-PTR regained its sensitivity to paclitaxel after knocking down of the endogenous OLA1 ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) (**Figures 3C, D**), as shown in the flow cytometry analysis of MCF-7-PTR (**Figure 3E**). To further confirm apoptosis induced by siRNA-OLA1 treatment, the expression level of Bcl-2 and the proapoptotic protein Bax and apoptosis terminal factor Caspase-3 were examined and showed that Bcl-2 was significantly decreased while Bax and Caspase-3 were increased remarkably (**Figure 3F**). Intriguingly, regardless of whether OLA1 was silenced or not, no significant effect on cell proliferation of MCF-7-PTR was observed (**Figure 3G**).

Knockdown of OLA1 Enhanced Chemo-Sensitivity of Intrinsic Drug Resistance of Breast Cancer

To understand whether endogenous OLA1 is also associated with intrinsic drug resistance of breast cancer cells, a triple-negative breast cancer cell line MDA-MB-231 was utilized. The endogenic level of OLA1 in MDA-MB-231 cells was significantly

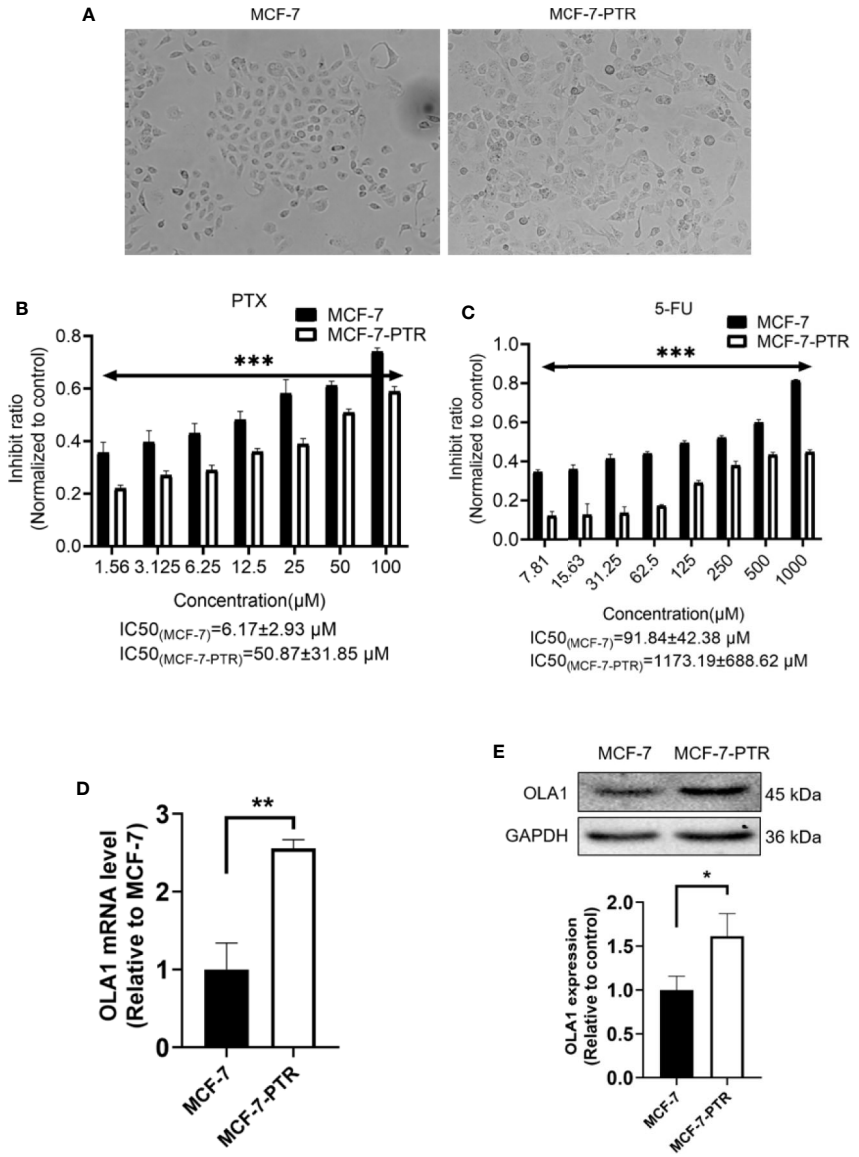

FIGURE 2 | Upregulated of OLA1 in acquired drug-resistant cell line MCF-7-PTR. **(A)** Morphology of paclitaxel-induced MCF-7-PTR cells and the parent MCF-7 cells (100X). **(B)** Drug resistance assay for enhanced expression of OLA1 promotes MCF-7-PTR cell resistance to PTX. **(C)** Drug resistance assay for enhanced expression of OLA1 promotes MCF-7-PTR cell resistance to 5-Fu. MCF-7 cells and MCF-7-PTR cells were analyzed for the presence of OLA1 by RT-PCR **(D)**, Western blotting **(E)**. The relative fold-change was compared with MCF-7 cells (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's t-test).

higher than MCF-7 (**Supplementary Figure 1C**), while it can be remarkably suppressed if it was silenced (**Figures 4A, B**). To understand whether knocked down OLA1 in MDA-MB-231 can enhance the chemosensitivity of the triple negative breast cancer, Knocked down of OLA1 stably in MDA-MB-231 was performed (**Figures 4A, B**), and it was found that the expression of OLA1 was meaningfully decreased in the transfected group compared with the control group (**$P < 0.01$) (**Figures 4C, D**). The sensitivity to paclitaxel of MDA-MB-231 cells in the knockdown OLA1 group was not changed at low doses of the drug, but significantly enhanced at high doses of the drug (**Figures 4E–H**). The enhanced sensitivity to paclitaxel of MDA-MB-231 was further confirmed by analysis of flow cytometry when OLA1 was knocked down as shown in **Figure 4I**. Decreased level of Bcl-2 and the increased levels of Bax and Caspase-3 were also observed (**Figure 4J**), suggesting that knock down of OLA1 also promoted the sensitivity of breast cancer cells with intrinsic resistance to chemotherapy. This notion was further confirmed by retarded cell proliferation (**Figure 4K**), which was supported by an arrested activity in the cell cycle indicated by a blockage at the G1/S phase (**Figure 4L**) and the reduced CCND1 (**Figure 6D**). However, there was no change

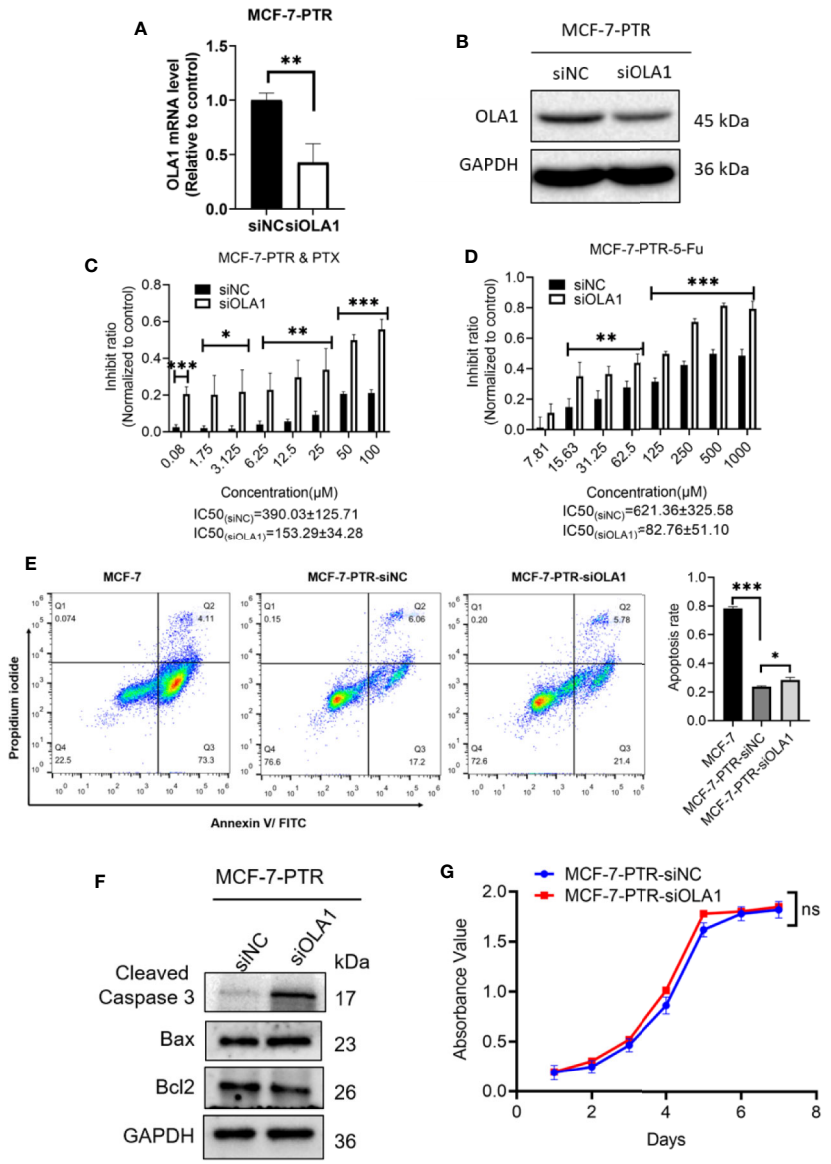

FIGURE 3 | Knockdown of OLA1 suppress the chemoresistance and enhanced chemo-sensitivity of acquired drug resistance cell in breast cancer. **(A, B)** were mRNA and protein levels of OLA1 with or without siOLA1 in MCF-7-PTR. **(C, D)** Depletion of OLA1 enhances the PTX or 5-Fu sensitivity of MCF-7-PTR cells by specific siRNA respectively. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Unpaired Student's t-test.) **(E)** Annexin V-FITC and PI staining of the indicated cells treated with PTX (20 μM) for 24 h (apoptosis rate is the sum of early and late apoptosis rates). (*$P < 0.05$, ***$P < 0.001$, Unpaired Student's t-test.) **(F)** Western blotting analysis of cleaved caspase3, Bax, and Bcl2 in the indicated cells; GAPDH was used as a loading control. **(G)** Cell proliferation curve was drawn in the indicated cells. ns, no significance.

observed in the expression of CCND1 in MCF-7-PTR (**Figure 6C**), consistent with the cell growth, suggesting that there may be other interactive factors involved in OLA1 signaling cascade.

Downregulation of OLA1 Inhibited EMT Progression in Drug-Resistant Breast Cancer Cell Lines

EMT is a biological process that allows polarized epithelial cells to undergo a variety of biochemical changes, making them exhibit a mesenchymal cell phenotype, including increased migratory capacity, invasiveness, and enhanced anti-apoptotic capabilities. To understand the molecular mechanism underlying OLA1-mediated drug resistance to breast cancer cells, biomarkers in the EMT process were analyzed. The results showed that knocked down OLA1 in the MDA-MB-231-shOLA1 decreased the expression of Snail, MMP9, Vimentin, Slug, zeb-1 ($P < 0.05$) (**Figures 5A, C**), and incremental E-cadherin (**Figure 5C**). We also found that the expression of Snail, MMP9, Vimentin, Slug, and Zeb-1 was significantly increased in MCF-7-PTR cells ($P < 0.05$) (**Figures 5B, D**) and decreased E-

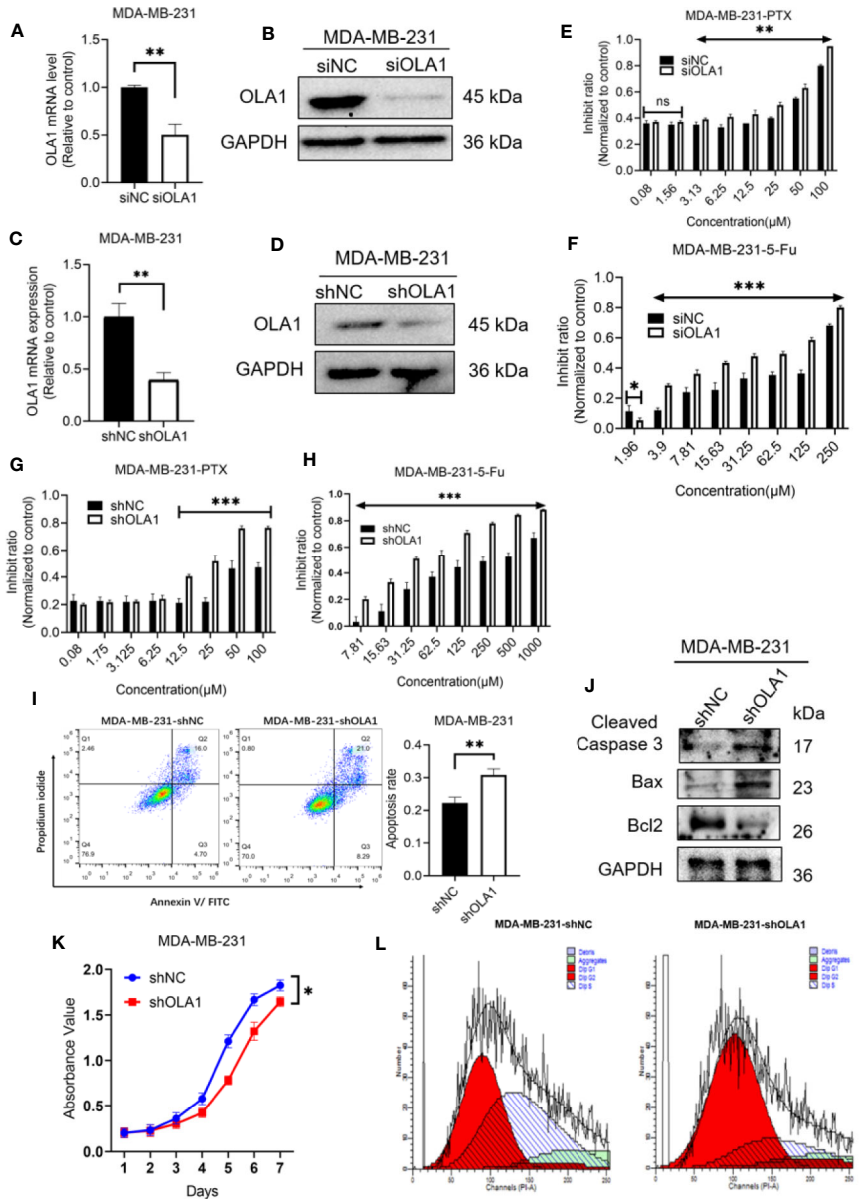

FIGURE 4 | Knockdown of OLA1 suppress chemoresistance and enhanced chemo-sensitivity of endogenous drug resistance cell in breast cancer. **(A, B)** were mRNA and protein level of OLA1 with or without siOLA1 in MDA-MB-231. **(C, D)** were mRNA and protein level of OLA1 with or without shOLA1 in MDA-MB-231. **(E, F)** Depletion of OLA1 enhances the PTX or 5-Fu sensitivity of MDA-MB-231 cells by specific siRNA respectively. **(G, H)** Knockdown of OLA1 enhances the PTX or 5-Fu sensitivity of MDA-MB-231 cells by short hairpin RNA. **(I)** Annexin V-FITC and PI staining of the indicated cells treated with PTX (20 μM) for 24 h. **(J)** Western blotting analysis of cleaved caspase3, Bax and Bcl2 in the indicated cells; GAPDH was used as a loading control. **(K)** Cell proliferation curve was drawn in indicated cells. **(L)** The cell-cycle distribution was assessed following transduction by flow cytometry. Data represent the mean ± SD from three independent experiments. (*$P < 0.05$ **$P < 0.01$, ***$P < 0.001$, Student's t-test).

cadherin, suggesting that OLA1 may regulate EMT process positively. To understand the effects of chemoresistance of MCF-7 cells with the effects of the extinction of OLA1 in MDA-MB-231 cells, SNAI1, VIM, ZEB1, and CDH1 in MCF-7 PTR cells transfected with siOLA1 were detected (**Figure 5E**). The results showed that knocked down OLA1 in the MCF-7-PTR decreased the EMT process.

OLA1 Induced EMT Phenotype *via* TGF-β/Smad Pathway in Breast Cancer

To further investigate the molecular mechanisms of OLA1 regulated EMT resulting in drug resistance to breast cancer, biomarkers in TGF-β/Smad pathway including TGFβ1, TGFβ2, SMAD3, and SMAD4 were characterized in the following cell lines: MCF-7 and MCF-7-PTR, and MDA-MB-231 cells with or

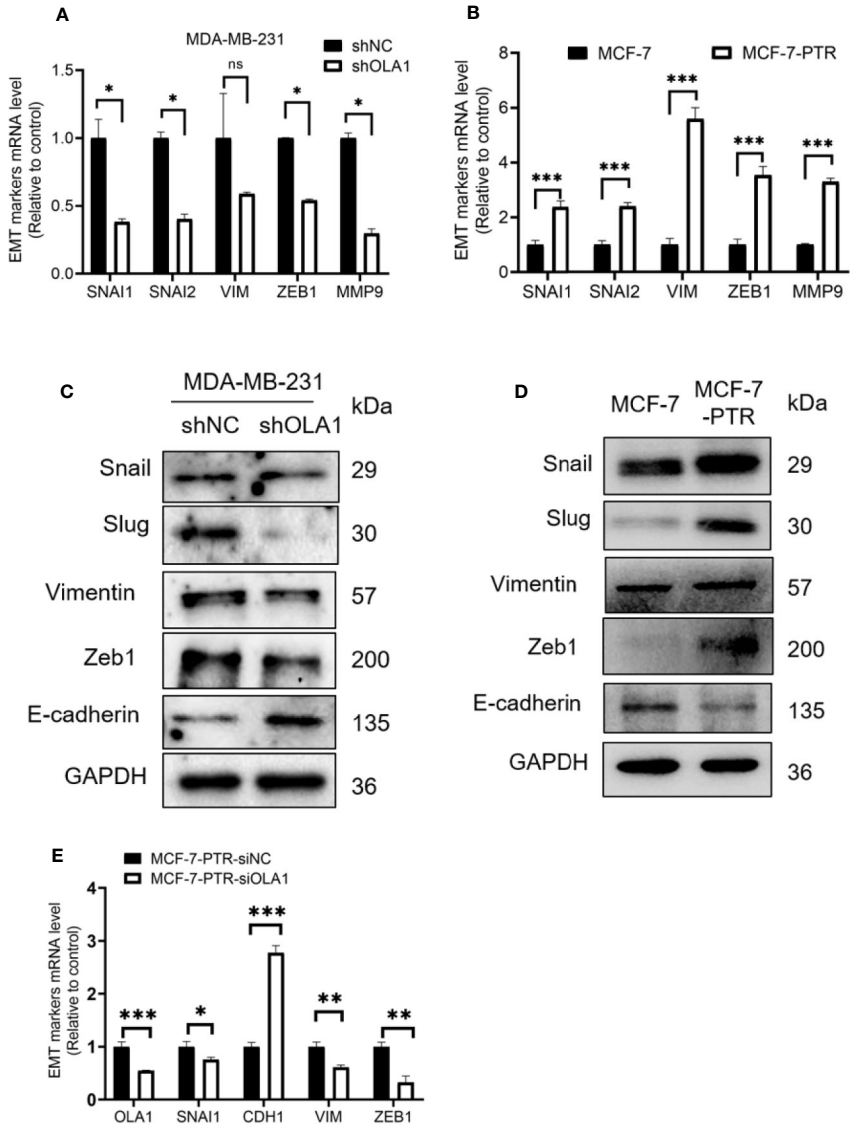

FIGURE 5 | Down regulated OLA1 inhibits EMT progress in drug-resistant cell lines. **(A)** The effect of OLA1 knockdown on expression of Snail, Slug, Vimentin, Zeb1, and MMP9 were evaluated by qRT-PCR. **(B)** mRNA levels of Snail, Slug, Vimentin, Zeb1, and MMP9 in MCF-7-PTR were calculated comparing with MCF-7. **(C)** The effect of OLA1-KD on E-cadherin, Vimentin, and Snail 1, Slug, and Zeb1 protein levels were confirmed by western blotting. **(D)** Protein levels of E-cadherin, Vimentin, and Snail 1, Slug, and Zeb1 in MCF-7-PTR were calculated comparing with MCF-7. **(E)** mRNA levels of OLA1, Snail, E-cadherin, Vimentin, and Zeb1 in MCF-7-PTR-siOLA1 were calculated comparing with siNC. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's t-test). ns, no significance.

without OLA1 knockdown. The results showed that TGF-β/SMAD was activated in MCF-7-PTR cells in contrast to MCF-7 (**Figures 6A, E**). Knockdown of OLA1 decreased the expression of TGF-β1, TGF-β2, SMAD4, and SMAD3 significantly ($P < 0.05$) (**Figures 6B, F**), indicating that TGF-β/Smad but not Wnt signaling was inhibited in MDA-MB-231-shOLA1 cells (**Figures 6C, D**). The relationship OLA1 with TGF β 1, SMAD3, and SMAD4 was also validated with Pearson Correlation analysis by GEPIA software. The results showed that the correlation between OLA1 and TGF β 1, SMAD3, and SMAD4 was small, or negatively correlated (**Figures 6G–I**, left) in the breast mammary tissue of the GTEx database and the normal breast tissue of TCGA. However, OLA1 and TGF β 1, SMAD3 SMAD4 has a strong positive correlation in the breast cancer tissue of the TCGA database (**Figures 6G–I**, right). These results indicate that OLA1 deficiency weakens the EMT phenotype through the inhibition of the TGF-β/Smad pathway in either the acquired or intrinsic drug resistant cell lines.

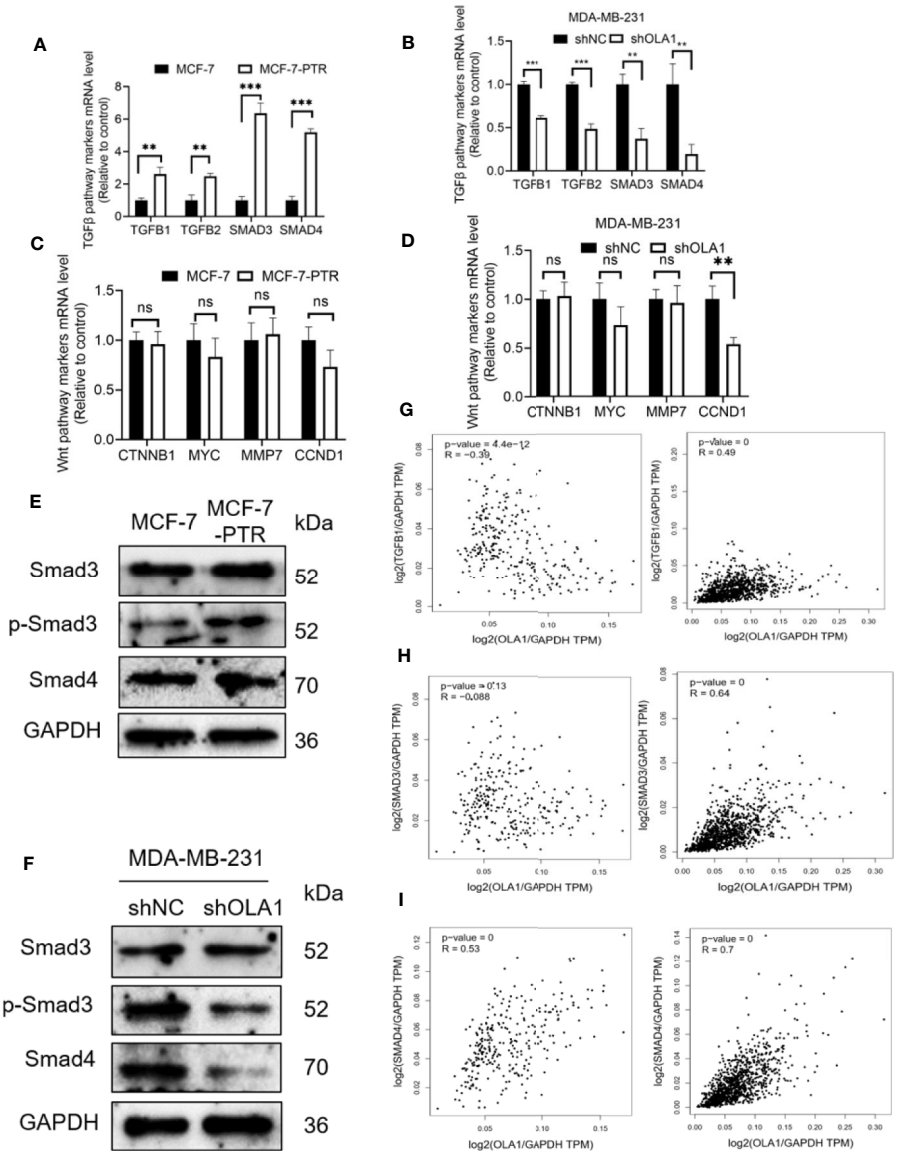

FIGURE 6 | OLA1 induces EMT phenotype by TGFβ/Smad pathway in breast cancer. TGFβ/Smad signaling marker mRNA level was confirmed in MCF-7-PTR **(A)**, and MDA-MB-231-shOLA1 **(B)** compared with their Control. Wnt signaling marker mRNA level was confirmed in MCF-7-PTR **(C)**, and MDA-MB-231-shOLA1 **(D)** compared with their Control. **(E)** Protein level of Smad3, p-Smad3 and Smad 4 in MCF-7-PTR was calculated comparing with MCF-7. **(F)** Protein level of Smad3, p-Smad3 and Smad 4 in MDA-MB-231-shOLA1 was calculated comparing with MDA-MB-231. **(G)** mRNA level Pearson Correlation analysis between OLA1 and TGFB1. Left: Relationship in GTEx Breast mammary tissue and TCGA BRCA normal data. Right: Relationship TCGA BRCA tumor data. **(H)** mRNA level Pearson Correlation analysis between OLA1 and Smad3. Left: Relationship in GTEx Breast mammary tissue and TCGA BRCA normal data. Right: Relationship TCGA BRCA tumor data. **(I)** mRNA level Pearson Correlation analysis between OLA1 and Smad4. Left: Relationship in GTEx Breast mammary tissue and TCGA BRCA normal data. Right: Relationship TCGA BRCA tumor data. (**$P < 0.01$, ***$P < 0.001$, Student's t-test). ns, no significance.

Mechanism of OLA1 Increases the Resistance of Paclitaxel in Breast Cancer

Ingenuity Pathway Analysis (IPA) was used to clarify the underling the mechanism of paclitaxel resistance regulated by OLA1. As the picture shows (**Figure 7A**), γ-tubulin acts as a bridge linking OLA1 to paclitaxel. We also tested the expression of γ-tubulin in drug-resistant cell lines by knocking down the expression of OLA1. The results showed that the expression of γ-tubulin decreased significantly with the weakening of OLA1 (**Figure 7B**). Our study provides a novel insight that reveals the role of OLA1 in tamoxifen and paclitaxel resistant breast cancer. The mechanism of OLA1 participation in the resistance of paclitaxel in breast cancer was drawn (**Figure 7C**).

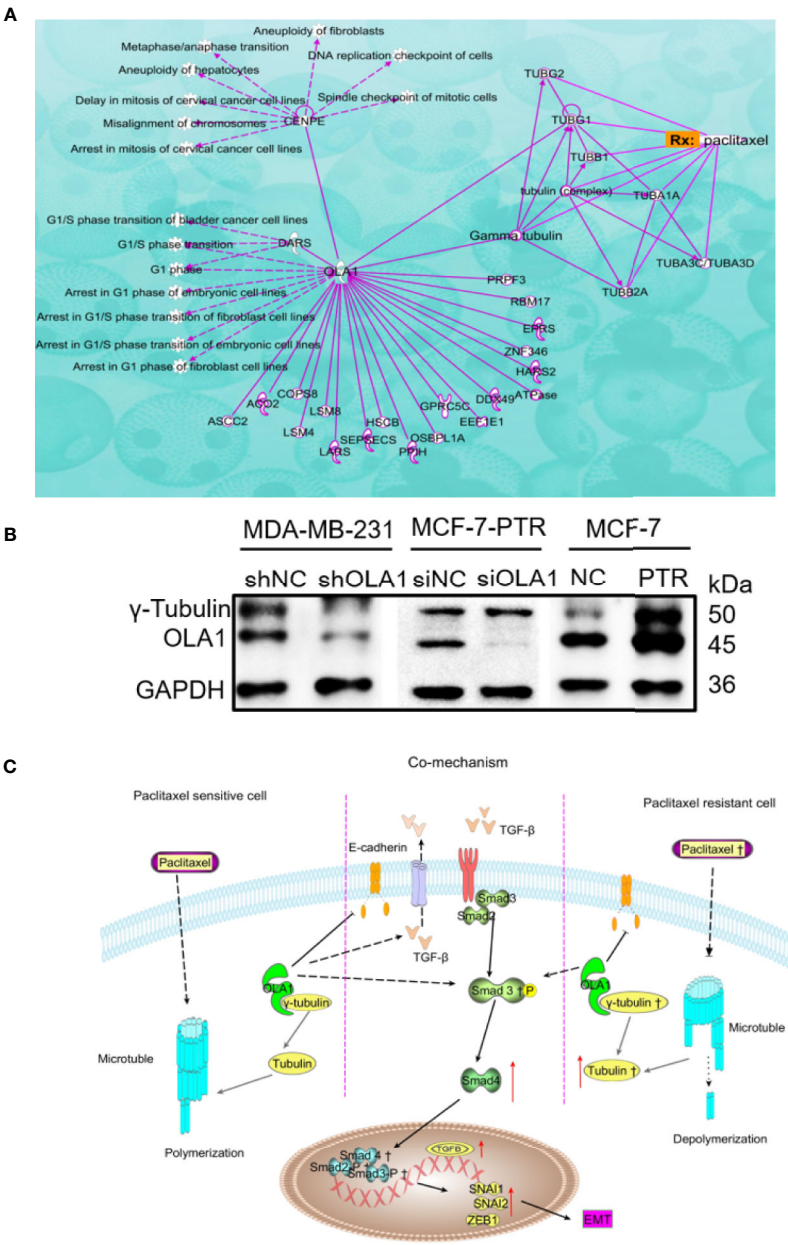

FIGURE 7 | Mechanism of OLA1 increases the resistance of paclitaxel in breast cancer. **(A)** IPA analysis between OLA1 and γ-tubulin in cell cycle regulation. **(B)** OLA1 and γ-tubulin levels in indicated cells. **(C)** Mechanism of OLA1 regulates the resistance in different breast cancer cells.

DISCUSSION

OLA1 belongs to the YchF subfamily p-Loop GTPase. The YchF family protein structure mainly includes three domains: N-terminus G domain, the coiled-coil domains on both sides, and the C-terminus TGS domain (ThrRS, GTPase, Spot). OLA1 is highly conserved compared with other Obg family members, but the NKxD consensus sequence in the G4 domain is replaced by NxxE, so it lacks the specificity of binding nucleotides, so it can hydrolyze both ATP and GTP. OLA1 also plays a vital role in cell signal transduction, intracellular transport, cell stress and embryonic development, and protein translation. As an enzyme that also has transport functions and hydrolyzes ATP activity, we can easily associate the influential role played by members of the ABC protein superfamily in tumor multidrug resistance research, including ABCB1 (MDR1), ABCC1, ABCG, etc. Through bioinformatics analysis, we found that overexpression of OLA1 has been found for various types of cancer, including breast cancer, and may be connected with poor survival, (Zhang et al., 2009a; Zhang et al., 2009b; Sun et al., 2010;

Bai et al., 2016; Ding et al., 2016; Huang et al., 2020). Therefore, let us justify the hypothesis that OLA1 may be associated with breast cancer resistance.

Tumor metastasis is an important reason for cancer multidrug resistance. EMT plays a consequential role in the process of tumor invasion, and propels cancer cells increased tumor-initiating and metastatic potency with a stubborn resistance to elimination by multiple therapies (Dongre and Weinberg, 2019). Conversely, some chemotherapeutic drugs, while killing cancer cells, also promote the metastasis of cancer cells (Chang et al., 2017; Karagiannis et al., 2017; Keklikoglou et al., 2019). For example, George Karagiannis et al. found that paclitaxel can change the tumor metastasis microenvironment and promote breast cancer metastasis (Karagiannis et al., 2017). Recently, Ioanna Keklikoglou found that the commonly used chemotherapy drugs paclitaxel and doxorubicin can promote the release of exosome by tumors, change the microenvironment in the lung, and promote lung metastasis in breast cancer (Keklikoglou et al., 2019). The loss of the epithelial phenotype and the acquisition of interstitial characteristics are the main features of EMT occurrence.

According to reports, decreased OLA1 expression weakens breast cancer cell motility and invasion (Zhang et al., 2009b). We constructed a paclitaxel-resistant cell line MCF-7-PTR, and found that the drug-resistant cell line promotes the EMT process and OLA1 was highly expressed. The resistance cell MCF-7-PTR with morphology change is associated with the morphology of MCF-7-ADR which is induced by continuous concentration of Adriamycin, and indicated that the resistant cell line may have much heterogeneity. EMT-transcription factors (EMT-TFs) such as TWIST1, SNAIL1, SLUG, and ZEB1, can trigger the EMT process by either directly or indirectly restraining E-cadherin expression (Graham et al., 2008; Taube et al., 2010). Moreover, OLA1 contributes to EMT in lung cancer by modulating the GSK3β/Snail/E-cadherin signaling. OLA1-knockdown cells are more resistant to TGFβ1-induced EMT in A549 cell line. The TGF-β signaling has a proven role in expediting EMT by down-regulating E-cadherin *via* certified EMT-TFs such as TWIST1, SNAIL1, and SLUG (Mallini et al., 2014). Knockdown of OLA1 in lung adenocarcinoma cells can attenuate the TGF-β-induced EMT process and restore E-cadherin expression (Bai et al., 2016). Knockdown of OLA1 caused Egr1, a regulator of oxidative stress, to be down-regulated as well as Smad, and could reverse the process of mouse embryo fibroblast transformation induced by a metal mixture (Martinez-Baeza et al., 2016). Meanwhile, EGR1 is a TGF-β/Smad target that up-regulates the expression of collagen genes and undertakes a crucial role in regulating TGF-β stimulation (Chen et al., 2006). Therefore, this evidence leads us to believe that the chemoresistance of breast cancer cells caused by OLA1 may be achieved through the TGF-β/Smad pathway. In our results, we demonstrated that OLA1 is a positive regulator of the EMT process regardless in the paclitaxel acquired resistance (MCF-7-PTR) cell or endogenous drug resistance cell (MDA-MB-231) in breast cancer. Knockdown of OLA1, TGFB1, TGFB2, SMAD3, and SMAD4 were down regulated accordingly, and p-Smad3 and Smad4 were decreased as well. While in the MCF-7-PTR, in which OLA1 was highly expressed, it had converse results. That is to say, OLA1 can activate the TGF-β/Smad pathway to induce the EMT process in breast drug resistance cells. Bcl-2 has been proven to be an anti-apoptotic protein and is overexpressed in multiple malignant tumors. Bcl-2 inhibits apoptosis by binding to Bax and blocking Bax oligomerization. In our study, in either acquired drug-resistant or intrinsic resistant cell lines, knockdown of OLA1 can cause a decrease in Bcl-2 expression and increase of Bax and cleaved caspase3, indicating improvement of the chemosensitivity of breast cancer.

MCF-7 is an ER-positive breast cancer cell line that is relatively sensitive to tamoxifen in clinical treatment. However, as the process toward the treatment progresses, it is easier to produce tolerance to chemotherapeutics. MDA-MB-231 is a type of triple-negative breast cancer cell line with negative expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2), and usually as endogenous resistant breast cancer cells. This type of breast cancer is prone to greatly metastasize and to endogenous resistance, which undoubtedly brings great difficulties to the treatment of breast cancer. Tamoxifen-resistant breast cancer cells highly overexpress BARD1 and BRCA1, resulting in chemoresistance to DNA-damaging therapies including cisplatin and doxorubicin, but not to paclitaxel (Zhu et al., 2018). This suggests that microtubule-targeting drugs may be given more priority to DNA-damaging agents for treating tamoxifen-resistant breast cancer patients (Zhu et al., 2018). In addition, microtubule-targeting drugs such as paclitaxel, despite their side effects, are still considered the standard therapy against triple negative breast cancer (Anderhub et al., 2019). However, it must not be ignored that some cancer cells will "seek away" slyly and cause patients to become resistant to treatment. Therefore, we want to further study the mechanism of paclitaxel resistance by constructing a paclitaxel-resistant cell line for breast cancer and accumulate therapeutic evidence in the treatment of tamoxifen-resistant breast cancer. Interestingly, in knock down OLA1 expression, the proliferation of MDA-MB-231-shOLA1 was decreased, and cell cycle G1/S marker CCND1 was reduced significantly. From the cell cycle analysis carried by flow cytometry, the MDA-MB-231-shOLA1 in the experimental group was blocked at the G1/S phase. While acquired resistance cell line MCF-7-PTR had no significant changes both in proliferation and cell cycle.

MCF-7-PTR is a drug-resistant cell line induced by paclitaxel. Paclitaxel resistance may be a key factor that causes the difference between the two phenotypes of cells. The breast and ovarian cancer-specific tumor suppressor BRCA1, along with its heterodimer partner BARD1, plays a critical role in DNA repair, drug resistance, centrosome regulation, and transcription (Matsuzawa et al., 2014). OLA1 directly interacted with BARD1, BRCA1, and γ-tubulin in centrosomal regulation (Matsuzawa et al., 2014; Yoshino et al., 2018). Further, tamoxifen-resistant breast cancer cells express observably more BARD1 and BRCA1, lending chemoresistance to DNA-

damaging therapy especially in ER-positive breast cancer patients (Zhu et al., 2018). Therefore, we believe that OLA1 may be involved in paclitaxel resistance due to the interaction with γ-tubulin in breast cancer. IPA was used to clarify the underling mechanism of paclitaxel resistance regulated by OLA1. As the picture shows (**Figure 7A**), γ-tubulin acts as a bridge linking OLA1 to paclitaxel, which further shows that our guess is reasonable. OLA1 participates in normal spindle assembly and in the cell cycle regulation process (Xie et al., 2020). The typical centrosome is considered to be the center of microtubule organization and is necessary for spindle assembly (So et al., 2019). Centrosome-associated protein E (CENPE) accumulates in the G2 phase of the cell and is involved in microtubule depolymerization activity near the centromere region. OLA1 can directly interact with CENPE and participate in the G1/S cycle process of various cells, which fully illustrates that OLA1 does participate throughout the process of paclitaxel-mediated resistance to Anti-tubulin. Simultaneously, we tested the expression of γ-tubulin in drug-resistant cell lines by knocking down the expression of OLA1. The results showed that the expression of γ-tubulin decreased significantly with the weakening of OLA1 (**Figure 7B**). OLA1 deficiency attenuates tubulin formation and thus regains the sensitivity of paclitaxel in breast cancer.

CONCLUSIONS

Our study provides a novel insight into revealing the distinct role of OLA1 in tamoxifen and paclitaxel resistant breast cancer. Long-term chemotherapeutic agent exposure facilitates translocation of OLA1 to cell membranes, leading to active TGF-β/Smad signaling pathway and accelerating the EMT process. OLA1 target γ-tubulin to depolymerization microtubules and avoid cell cycle block in paclitaxel-resistant cancer cells instead of tamoxifen resistant breast cancer cells. Blockage of OLA1 may be a potential method to improve the survivability of chemoresistant breast cancer patients. However, doubtless further investigations, including *in vivo* animal model studies and prospective clinical observations, are needed.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

GGu, JL, and XM participated in the study design, performed experiments, analyzed and interpreted data, and wrote the manuscript. YP, BX, and GGa participated in the study design and data analysis. JH and XT contributed to the acquisition of data. JZ, HZ, and HW revised the manuscript critically for important intellectual content. GGu totally revised and edited the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00666/full#supplementary-material

REFERENCES

Alushin, G. M., Lander, G. C., Kellogg, E. H., Zhang, R., Baker, D., and Nogales, E. (2014). High-Resolution Microtubule Structures Reveal the Structural Transitions in alpha beta-Tubulin upon GTP Hydrolysis. *Cell* 157, 1117–1129. doi: 10.1016/j.cell.2014.03.053

Anderhub, S. J., Mak, G. W.-Y., Gurden, M. D., Faisal, A., Drosopoulos, K., Walsh, K., et al. (2019). High Proliferation Rate and a Compromised Spindle Assembly Checkpoint Confers Sensitivity to the MPS1 Inhibitor BOS172722 in Triple-Negative Breast Cancers. *Mol. Cancer. Ther.* 18, 1696–1707. doi: 10.1158/1535-7163.MCT-18-1203

Asiedu, M. K., Ingle, J. N., Behrens, M. D., Radisky, D. C., and Knutson, K. L. (2011). TGF beta/TNF alpha-Mediated Epithelial-Mesenchymal Transition Generates Breast Cancer Stem Cells with a Claudin-Low Phenotype. *Cancer Res.* 71, 4707–4719. doi: 10.1158/0008-5472.CAN-10-4554

Bai, L., Yu, Z., Rubio, V., Chen, H., Li, Y., Zhang, J., et al. (2016). OLA1 contributes to epithelial-mesenchymal transition in lung cancer by modulating the GSK3β/snail/E-cadherin signaling. *Oncotarget* 7, 10402–10413. doi: 10.18632/oncotarget.7224

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68, 394–424. doi: 10.3322/caac.21492

Chang, Y. S., Jalgaonkar, S. P., Middleton, J. D., and Hai, T. (2017). Stress-inducible gene Atf3 in the noncancer host cells contributes to chemotherapy-exacerbated breast cancer metastasis. *Proc. Natl. Acad. Sci. U. S. A.* 114, E7159–E7168. doi: 10.1073/pnas.1700455114

Chen, S. J., Ning, H., Ishida, W., Sodin-Semrl, S., Takagawa, S., Mori, Y., et al. (2006). The early-immediate gene EGR-1 is induced by transforming growth factor-beta and mediates stimulation of collagen gene expression. *J. Biol. Chem.* 281, 21183–21197. doi: 10.1074/jbc.M603270200

Creighton, C. J., Li, X., Landis, M., Dixon, J. M., Neumeister, V. M., Sjolund, A., et al. (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc. Natl. Acad. Sci. U. S. A.* 106, 13820–13825. doi: 10.1073/pnas.0905718106

Cufi, S., Vazquez-Martin, A., Oliveras-Ferraros, C., Martin-Castillo, B., Joven, J., and Menendez, J. A. (2010). Metformin against TGFbeta-induced epithelial-to-mesenchymal transition (EMT): from cancer stem cells to aging-associated fibrosis. *Cell Cycle* 9, 4461–4468. doi: 10.4161/cc.9.22.14048

Diepenbruck, M., and Christofori, G. (2016). Epithelial-mesenchymal transition (EMT) and metastasis: yes, no, maybe? *Curr. Opin. Cell Biol.* 43, 7–13. doi: 10.1016/j.ceb.2016.06.002

Ding, Z., Liu, Y., Rubio, V., He, J., Minze, L. J., and Shi, Z. Z. (2016). OLA1, a Translational Regulator of p21, Maintains Optimal Cell Proliferation Necessary for Developmental Progression. *Mol. Cell Biol.* 36, 2568–2582. doi: 10.1128/MCB.00137-16

Dongre, A., and Weinberg, R. A. (2019). New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. *Nat. Rev. Mol. Cell Biol.* 20, 69–84. doi: 10.1038/s41580-018-0080-4

Fischer, K. R., Durrans, A., Lee, S., Sheng, J., Li, F., Wong, S. T., et al. (2015). Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. *Nature* 527, 472–476. doi: 10.1038/nature15748

Gampenrieder, S. P., Rinnerthaler, G., and Greil, R. (2013). Neoadjuvant chemotherapy and targeted therapy in breast cancer: past, present, and future. *J. Oncol.* 2013, 732047. doi: 10.1155/2013/732047

Gradia, D. F., Rau, K., Umaki, A. C. S., De Souza, F. S. P., Probst, C. M., Correa, A., et al. (2009). Characterization of a novel Obg-like ATPase in the protozoan Trypanosoma cruzi. *Int. J. Parasitol.* 39, 49–58. doi: 10.1016/j.ijpara.2008.05.019

Graham, T. R., Zhau, H. E., Odero-Marah, V. A., Osunkoya, A. O., Kimbro, K. S., Tighiouart, M., et al. (2008). Insulin-like growth factor-I-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells. *Cancer Res.* 68, 2479–2488. doi: 10.1158/0008-5472.CAN-07-2559

Huang, S., Zhang, C., Sun, C., Hou, Y., Zhang, Y., Tam, N. L., et al. (2020). Obg-like ATPase 1 (OLA1) overexpression predicts poor prognosis and promotes tumor progression by regulating P21/CDK2 in hepatocellular carcinoma. *Aging (Albany NY)* 12, 3025–3041. doi: 10.18632/aging.102797

Joshi, H. C., Palacios, M. J., Mcnamara, L., and Cleveland, D. W. (1992). Gamma-tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature* 356, 80–83. doi: 10.1038/356080a0

Karagiannis, G. S., Pastoriza, J. M., Wang, Y., Harney, A. S., Entenberg, D., Pignatelli, J., et al. (2017). Neoadjuvant chemotherapy induces breast cancer metastasis through a TMEM-mediated mechanism. *Sci. Transl. Med.* 9, 397. doi: 10.1126/scitranslmed.aan0026

Keklikoglou, I., Cianciaruso, C., Guc, E., Squadrito, M. L., Spring, L. M., Tazzyman, S., et al. (2019). Chemotherapy elicits pro-metastatic extracellular vesicles in breast cancer models. *Nat. Cell Biol.* 21, 190–202. doi: 10.1038/s41556-018-0256-3

Koller-Eichhorn, R., Marquardt, T., Gail, R., Wittinghofer, A., Kostrewa, D., Kutay, U., et al. (2007). Human OLA1 Defines an ATPase Subfamily in the Obg Family of GTP-binding Proteins. *J. Biol. Chem.* 282, 19928–19937. doi: 10.1074/jbc.M700541200

Lamouille, S., Xu, J., and Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. *Nat. Rev. Mol. Cell Biol.* 15, 178–196. doi: 10.1038/nrm3758

Leipe, D. D., Wolf, Y. I., Koonin, E. V., and Aravind, L. (2002). Classification and evolution of P-loop GTPases and related ATPases11Edited by J. Thornton. *J. Mol. Biol.* 317, 41–72. doi: 10.1006/jmbi.2001.5378

Li, Y., Ma, J., Qian, X., Wu, Q., Xia, J., Miele, L., et al. (2013). Regulation of EMT by Notch signaling pathway in tumor progression. *Curr. Cancer Drug Targets* 13, 957–962. doi: 10.2174/15680096113136660101

Mallini, P., Lennard, T., Kirby, J., and Meeson, A. (2014). Epithelial-to-mesenchymal transition: what is the impact on breast cancer stem cells and drug resistance. *Cancer Treat Rev.* 40, 341–348. doi: 10.1016/j.ctrv.2013.09.008

Mani, S. A., Guo, W., Liao, M.-J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., et al. (2008). The Epithelial-Mesenchymal Transition Generates Cells with Properties of Stem Cells. *Cell* 133, 704–715. doi: 10.1016/j.cell.2008.03.027

Martinez-Baeza, E., Rojas, E., and Valverde, M. (2016). Metal mixture (As-Cd-Pb)-induced cell transformation is modulated by OLA1. *Mutagenesis* 31, 463–473. doi: 10.1093/mutage/gew010

Matsuzawa, A., Kanno, S., Nakayama, M., Mochiduki, H., Wei, L., Shimaoka, T., et al. (2014). The BRCA1/BARD1-interacting protein OLA1 functions in centrosome regulation. *Mol. Cell* 53, 101–114. doi: 10.1016/j.molcel.2013.10.028

Muller-Reichert, T., Chretien, D., Severin, F., and Hyman, A. A. (1998). Structural changes at microtubule ends accompanying GTP hydrolysis: Information from a slowly hydrolyzable analogue of GTP, guanylyl (alpha,beta) methylenediphosphonate. *Proc. Natl. Acad. Sci. United States America* 95, 3661–3666. doi: 10.1073/pnas.95.7.3661

Nagy, A., Lanczky, A., Menyhart, O., and Gyorffy, B. (2018). Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. *Sci. Rep.* 8, 9227. doi: 10.1038/s41598-018-27521-y

Oegema, K., Wiese, C., Martin, O. C., Milligan, R. A., Iwamatsu, A., Mitchison, T. J., et al. (1999). Characterization of two related Drosophila gamma-tubulin complexes that differ in their ability to nucleate microtubdes. *J. Cell Biol.* 144, 721–733. doi: 10.1083/jcb.144.4.721

Ohmori, T., Yang, J. L., Price, J. O., and Arteaga, C. L. (1998). Blockade of tumor cell transforming growth factor-betas enhances cell cycle progression and sensitizes human breast carcinoma cells to cytotoxic chemotherapy. *Exp. Cell Res.* 245, 350–359. doi: 10.1006/excr.1998.4261

Pastushenko, I., Brisebarre, A., Sifrim, A., Fioramonti, M., Revenco, T., Boumahdi, S., et al. (2018). Identification of the tumour transition states occurring during EMT. *Nature* 556, 463–468. doi: 10.1038/s41586-018-0040-3

Shi, X. P., Miao, S., Wu, Y., Zhang, W., Zhang, X. F., Ma, H. Z., et al. (2013). Resveratrol Sensitizes Tamoxifen in Antiestrogen-Resistant Breast Cancer Cells with Epithelial-Mesenchymal Transition Features. *Int. J. Mol. Sci.* 14, 15655–15668. doi: 10.3390/ijms140815655

So, C., Seres, K. B., Steyer, A. M., Monnich, E., Clift, D., Pejkovska, A., et al. (2019). A liquid-like spindle domain promotes acentrosomal spindle assembly in mammalian oocytes. *Science* 364, 6447. doi: 10.1126/science.aat9557

Sun, H., Luo, X., Montalbano, J., Jin, W., Shi, J., Sheikh, M. S., et al. (2010). DOC45, a novel DNA damage-regulated nucleocytoplasmic ATPase that is overexpressed in multiple human malignancies. *Mol. Cancer Res.* 8, 57–66. doi: 10.1158/1541-7786.MCR-09-0278

Tang, Z., Li, C., Kang, B., Gao, G., Li, C., and Zhang, Z. (2017). GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res.* 45, W98–W102. doi: 10.1093/nar/gkx247

Taube, J. H., Herschkowitz, J. I., Komurov, K., Zhou, A. Y., Gupta, S., Yang, J., et al. (2010). Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc. Natl. Acad. Sci. U. S. A* 107, 15449–15454. doi: 10.1073/pnas.1004900107

Verstraeten, N., Fauvart, M., Versées, W., and Michiels, J. (2011). The Universally Conserved Prokaryotic GTPases. *Microbiol. Mol. Biol. Rev.* 75, 507–542. doi: 10.1128/MMBR.00009-11

Wang, H. W., and Nogales, E. (2005). Nucleotide-dependent bending flexibility of tubulin regulates microtubule assembly. *Nature* 435, 911–915. doi: 10.1038/nature03606

Wertz, I. E., Kusam, S., Lam, C., Okamoto, T., Sandoval, W., Anderson, D. J., et al. (2011). Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. *Nature* 471, 110–114. doi: 10.1038/nature09779

Wolf, K. W., and Joshi, H. C. (1996). Microtubule organization and distribution of gamma-tubulin in male meiosis of lepidoptera. *Mol. Reprod. Dev.* 45, 547–559. doi: 10.1002/(SICI)1098-2795(199612)45:4<547::AID-MRD17>3.0.CO;2-T

Xie, D., Zhang, J., Ding, J., Yang, J., and Zhang, Y. (2020). OLA1 is responsible for normal spindle assembly and SAC activation in mouse oocytes. *PeerJ* 8, e8180. doi: 10.7717/peerj.8180

Xu, J., Lamouille, S., and Derynck, R. (2009). TGF-β-induced epithelial to mesenchymal transition. *Cell Res.* 19, 156–172. doi: 10.1038/cr.2009.5

Ye, X., Brabletz, T., Kang, Y., Longmore, G. D., Nieto, M. A., Stanger, B. Z., et al. (2017). Upholding a role for EMT in breast cancer metastasis. *Nature* 547, E1–E3. doi: 10.1038/nature22816

Yoshino, Y., Qi, H., Fujita, H., Shirota, M., Abe, S., Komiyama, Y., et al. (2018). BRCA1-interacting Protein OLA1 Requires Interaction with BARD1 to

Regulate Centrosome Number. *Mol. Cancer Res.* 16, 1499–1511. doi: 10.1158/1541-7786.MCR-18-0269

Yoshino, Y., Qi, H., Kanazawa, R., Sugamata, M., Suzuki, K., Kobayashi, A., et al. (2019). RACK1 regulates centriole duplication by controlling localization of BRCA1 to the centrosome in mammary tissue-derived cells. *Oncogene* 38, 3077–3092. doi: 10.1038/s41388-018-0647-8

Zardavas, D., and Piccart, M. (2015). Neoadjuvant therapy for breast cancer. *Annu. Rev. Med.* 66, 31–48. doi: 10.1146/annurev-med-051413-024741

Zhang, J., Rubio, V., Lieberman, M. W., and Shi, Z. Z. (2009a). OLA1, an Obg-like ATPase, suppresses antioxidant response via nontranscriptional mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* 106, 15356–15361. doi: 10.1073/pnas.0907213106

Zhang, J. W., Rubio, V., Zheng, S., and Shi, Z. Z. (2009b). Knockdown of OLA1, a regulator of oxidative stress response, inhibits motility and invasion of breast cancer cells. *J. Zhejiang Univ. Sci. B.* 10, 796–804. doi: 10.1631/jzus.B0910009

Zheng, X., Carstens, J. L., Kim, J., Scheible, M., Kaye, J., Sugimoto, H., et al. (2015). Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. *Nature* 527, 525–530. doi: 10.1038/nature16064

Zhu, Y., Liu, Y., Zhang, C., Chu, J., Wu, Y., Li, Y., et al. (2018). Tamoxifen-resistant breast cancer cells are resistant to DNA-damaging chemotherapy because of upregulated BARD1 and BRCA1. *Nat. Commun.* 9, 1595. doi: 10.1038/s41467-018-03951-0

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Notch Signaling Pathway and Endocrine Resistance in Breast Cancer

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Nearly 70% of breast cancers express the estrogen receptor (ER) and are hormone-dependent for cell proliferation and survival. Anti-estrogen therapies with aromatase inhibitors (AIs), selective estrogen receptor modulators (SERMs) or selective estrogen receptor down regulators (SERDs) are the standard endocrine therapy approach for ER positive breast cancer patients. However, about 30% of patients receiving endocrine therapy will progress during the therapy or become endocrine resistance eventually. The intrinsic or acquired endocrine resistance has become a major obstacle for endocrine therapy. The mechanism of endocrine resistance is very complicated and recently emerging evidence indicates dysregulation of Notch signaling pathway contributes to endocrine resistance in breast cancer patients. The potential mechanisms include regulation of ER, promotion of cancer stem cell (CSC) phenotype and mesenchymal cell ratio, alteration of the local tumor microenvironment and cell cycle. This review will summarize the latest progress on the investigation of Notch signaling pathway in breast cancer endocrine resistance.

Keywords: breast cancer, Notch signaling pathway, endocrine resistance, estrogen receptor, anti-estrogen therapy

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INTRODUCTION

Breast cancer has become the leading common cancer and the second largest cause of death among women worldwide (Siegel et al., 2020). A majority of breast cancer shows ER expression (Yip and Rhodes, 2014) and anti-estrogen therapy is considered as the most effective treatment for them. Current anti-estrogen drugs include SERMs (i.e., tamoxifen), AIs (i.e., letrozole and anastrozole), and SERDs (i.e., Faslodex/Fulvestrant). It has been considered a revolutionized progress in endocrine therapies which significantly decreases cancer-related mortality and improves the survival rate (Tremont et al., 2017). But about 30% patients treated with endocrine therapy will develop recurrence even though initially respond well (D'Souza et al., 2018). Thus the resistance has been believed as a pivotal obstacle leading to breast cancer treatment failure.

The endocrine mechanisms in breast cancer are complex and multiple with diverse molecules and pathways involved. 1) Direct or indirect ER related signaling pathway: As ERα has been proven to be the main target in endocrine therapy, changes in ERα expression or function including ERα

loss or ESR1 mutations or epigenetic modification all contribute to endocrine independence (Toy et al., 2013; Gelsomino et al., 2016; Tecalco-Cruz and Ramirez-Jarquin, 2018; Fontes-Sousa et al., 2019). 2) Non-ER related signaling pathways, such as promotion of stemness of cancer cells and EMT, dysregulation of cell cycle, crosstalk with cells tyrosine kinase growth factor signaling pathways, influence of tumor microenvironment and drug metabolism also act crucial parts in endocrine resistance (Rani et al., 2019).

Both experimental studies and clinical observations suggested that the aberrant activation of Notch signaling pathway was very common in breast cancer and it was depicted in most of regulating pathway related to endocrine resistance (Acar et al., 2016).

In this review, we will sum up the latest development aiming at the role of Notch signaling pathway and discuss the complicated evidence underlie its impact on endocrine resistance. The potential for Notch correlated cancer therapy is also highlighted.

THE NOTCH SIGNALING PATHWAY

Notch signaling pathway is highly conserved in eukaryotes which involves two kinds of adjacent cells, signal sending and receiving cells (Wilson and Radtke, 2006; Gazave et al., 2009). In mammals, the key components of Notch signaling pathway are four Notch receptors, five Notch ligands and DNA-binding protein CSL [CBF-1/RBP-Jκ, Su(H), Lag-1]. Compared with other cell signaling pathways, Notch is relatively simple in structure and there is no second messenger involved in the activation process, so it cannot produce cascade amplification like others. The activation process of Notch signal pathway is as follows.

In the canonical Notch pathway, Notch receptors undergo two successive proteolytic cleavages (Bray, 2006; Kopan and Ilagan, 2009). After reaching the membrane and activated by the ligand on the neighboring cell, the Notch receptors can be cleaved by a disintegrin and metalloprotease (ADAM) family at Site 2 (S2) and then by γ-secretase at Site 3 (S3). Afterwards, Notch intracellular domain (NICD) was released to nucleus (Brou et al., 2000; Sprinzak et al., 2010). NICD translocates to the nucleus and forms a complex with the DNA-binding protein CSL and the coactivator Master-mind-like (MAML) family to regulate transcription of downstream target genes (Wu et al., 2000). In this way, the travelling NICD transduces the signal not only from cell to cell but also from extracellular to intracellular.

In the non-canonical Notch signaling mechanisms, the discovered interactions mainly focused on interplay between NICD and downstream effectors. For instance, NICD can directly interact with β-catenin (Jin et al., 2009), Smad proteins (Blokzijl et al., 2003), and HIF-1α (Gustafsson et al., 2005), thereby providing a crosstalk between Notch and the Wnt, TGFβ and hypoxia-dependent signaling pathways. However, it is worth mentioning that most Notch-correlated cancer phenotypes can be perturbed by the canonical rather than non-canonical Notch signaling.

Notch signaling regulates numerous cellular processes including cancer stem cell renewal, angiogenesis, proliferation, apoptosis, and EMT (Miele et al., 2006). More recently, it was reported that dysregulation of Notch signaling pathway was involved in endocrine resistance and combined Notch with estrogen signaling inhibition had showed synergistic effect in ERα positive breast cancer (Acar et al., 2016). In next section, we'll describe the potential mechanisms whereby Notch promotes endocrine resistance in depth.

NOTCH SIGNALING AND ENDOCRINE RESISTANCE IN BREAST CANCER

More freshly, Notch signaling was found to be an important pathway mediating endocrine resistance in breast cancer cells (Magnani et al., 2013). As an illustration, Paola Rizzo and his colleagues reported that Notch inhibition potentiated the effects of tamoxifen in ERα positive cells, T47D:A18. When they combined γ-Secretase inhibitors (GSI) and 4-OH-tamoxifen (Tam) together, combination treatment reduced the growth significantly more than either drug alone (Rizzo et al., 2008). These data suggested that GSIs may be a promising therapeutic target to overcome resistance for antiestrogen treatment. Besides Notch1, Yun et al. revealed that Notch4 also played an essential role in endocrine resistance as measurements of DNA content verified that Notch4-ICD in T47D:A18 increased DNA synthesis in the absence of estrogen, indicating that overexpression of Notch4-ICD could stimulate proliferation through estrogen-independent and Tam-resistant mechanisms (Yun et al., 2013).

To design new therapeutic strategies based on Notch signaling, Notch regulation and the context-dependent interactions between Notch and other relevant pathways needs to be taken into well consideration.

The Regulation of ER by Notch Signaling

As we know, ERα dysregulation performs a central role in the acquisition of resistance to endocrine therapy in breast cancer. Previous studies demonstrated that Notch signaling pathway could directly or in-directly regulate ER expression or activity (**Figure 1**).

Regulation of ER Expression and Its Downstream Genes by Notch Family Members

There was interaction between the estrogen receptor and Notch in breast cancer (Rizzo et al., 2008). Breast cancer cells expressed Notch1 and Notch4 proteins at variable steady-state levels regardless of the ER status. But when examined basal centromere binding factor (CBF-1)–dependent reporter activity, researchers found an inverse correlation between Notch activity and ERα expression in breast cancer cells. In another word, activity of Notch was higher in MDA-MB231 cell line than in MCF-7 or T47D cell line. Notch activity was inhibited by estradiol (E2) significantly

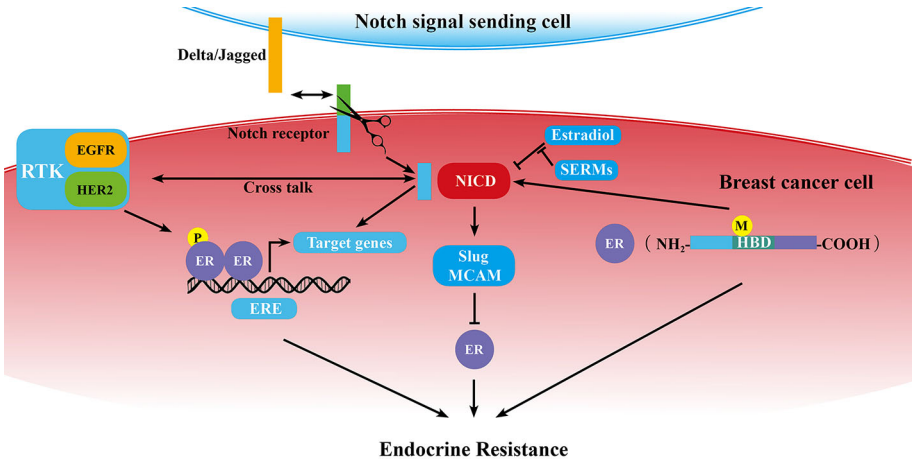

FIGURE 1 | Schematic diagram of the correlation of Notch signaling pathway and ER in breast cancer endocrine resistance. ER is at the center of endocrine resistance observed in breast cancer cells. Notch signaling modulates endocrine therapy through cooperating with ER in a complex network as mentioned in some sections of this review. P, phosphorylation; M, mutation; →, promotion; ⊥, inhibition; ↔, receptor ligand-binding.

(P = 0.0025) *via* changing the cellular distribution of Notch1 in ERα positive (ERα+) cell lines and SERMs blocked its effect. In T47D:A18 cells (ERα + cell lines), GSI with an IC50 of 0.84 μmol/L exhibited strong inhibition effect on the growth *in vitro*. Moreover, combination of GSI and 4-OH-Tam had significantly more growth inhibition than either drug alone, even at very low concentrations. *In vivo*, treatment with GSI alone or tamoxifen alone blocked the growth of T47D:A18 xenografts with similar efficacy, but combination showed stronger effect. Another research (Hao et al., 2010) reported that E2 target genes, such as pS2, vascular endothelial growth factor-α (VEGFα), cyclin-D1, CD44, and c-Myc were upregulated by overexpression Notch1-ICD in the absence of E2. They further sought to identify the molecular mechanisms whereby Notch activated ER-dependent transcription without E2 using pS2 transcriptional model. They found Jagged-1 stimulated the recruitment of Notch1, IKKα, MAML1, p300/CBP, and ERα to the pS2 promoter by Chromatin immunoprecipitation assay (CHIP). The formation of above supramolecular complex contributed to activate a subset of ERα-responsive genes transcription in the absence of E2. In general, E2 inhibits Notch activation and SERMs reactivates Notch in breast cancer cells. Meanwhile Notch activates ERα-dependent transcription, demonstrating there is a feedback mechanism regulating the Notch1-ERα crosstalk. Altogether, Notch may promote endocrine resistance by affecting ERα activity.

Beyond that, some transcriptional factors, such as Snail (Scherbakov et al., 2012), Slug (Li et al., 2015; Bai et al., 2017), Twist (Vesuna et al., 2012), ZEB1 (Zhang et al., 2017), MCAM (Liang et al., 2017), were shown to mediate endocrine resistance through directly repressing ERα expression. In breast cancer cells, Notch1 (Shao et al., 2015) or Notch4 (Zhou et al., 2020) can promote the expression of Slug by activating the Slug promoter. We recently published an article which showed Notch1 could also transcriptionally activate MCAM in breast cancer cells (Zeng et al., 2020). So Notch1-MCAM signaling pathway is possibly another method leading to endocrine resistance in breast cancer. In a word, apart from directly activation of ERα downstream gene expression, Notch1/4 may indirectly influence ERα expression contributing to endocrine resistance.

In contrast to Notch1 and 4, our previous study (Dou et al., 2017) showed that Notch3 was mainly expressed in luminal breast cancer cells but not in either basal-like or HER2 (human epidermal growth factor receptor 2)-positive breast cancer cell line. Notch3 expression displayed strong positive correlation with ERα both in protein and mRNA level. When Notch3 was silenced *via* siRNA, ERα was decreased. Conversely, overexpression of Notch3 resulted in upregulation of ERα. We also found that Notch3 specifically bound to the CSL binding element of the ERα promoter and transcriptionally activated ERα expression by CHIP and Electrophoretic mobility shift assay (EMSA). In addition to such direct regulation, it was also found Notch3 could indirectly increase ERα expression by GATA3. We found that protein and mRNA level of Notch3 and GATA-3 was positively correlated especially in luminal breast cancer cells. There were two putative CSL-binding sites located upstream of GATA-3 promoter (-829-834 bp and -665-670 bp). CHIP, EMSA, and dual reporter assay certificated Notch3 activated GATA-3 transcription by binding to CSL-binding elements in the GATA-3 promoter in MCF-7 and MDA-MB231 cells (Lin et al., 2018). GATA3 helped to maintain a luminal phenotype by activating ERα (Eeckhoute et al., 2007). Unlike Notch1 and Notch4, the role of Notch3 in anti-estrogen therapy need to further research, perhaps Notch3 expression can increase sensitivity to endocrine therapy.

ER Mutations, ER Modification, and Notch Signaling

A recent study confirmed that hot spot mutations in hormone binding domain (HBD) of ERα/ESR1 like Y537N, Y537S, D538G

alterations, promoted transcription in an ER-dependent manner and proliferation even in the absence of estrogen, leading to endocrine resistance (Toy et al., 2013). Compared with wild-type ESR1, mutant cells displayed an increase in $CD44^+/CD24^-$ ratio, mammosphere formation, migratory capabilities, and self-renewal through highly expressed Notch signaling components, like receptors, ligands and target genes (Gelsomino et al., 2018). It also demonstrated that ERα-Y537S could not enhance BCSCs once Notch signaling was inhibited, which reaffirming the importance of the correlation between ER and Notch in ERα-Y537S-mediated BCSC enrichment. Therefore, the development of Notch inhibitors will be new strategies to prevent or delaying disease progress and relapsing-onset in ER mutant positive patients.

Apart from the above mutations, modification of ERα such as phosphorylation, contributing to ligand-independent transcription of ERα-dependent genes, also promoted resistance to anti-estrogen therapy (Korobeynikov et al., 2019). HBD-ERα mutants exhibited an overexpressed S118 phosphorylation located within the AF-1 domain. When transfecting S118A-ERα (a plasmid where a serine was changed to an alanine to eliminate phosphorylation at S118) in MCF-7 cell, it was detected a lower expression of Notch4-ICD, Notch4 and HES1, and reduction in mammosphere forming efficiency (MFE) only in Y537S-ERα mutant cells. These results indicated that Notch4 activation was required for phosphorylation of S118 to increase BCSC activity in ERα mutant cells (Gelsomino et al., 2018). In addition, it was reported that receptor tyrosine kinases (RTK) and several other pathways, including the CDK2 complex and CDK7/TFIIH complex enhanced the phosphorylation of ERα (Rani et al., 2019). RTK includes epidermal growth factor receptors (EGFR), vascular endothelial growth factor receptor (VEGFR), insulin-like growth factor-I receptor, et al. RTKs promoted ER phosphorylation through at least two pathways: RAS–RAF–ERK and PI3K-AKT pathway (Ali and Coombes, 2002), which enabled ERα positive breast cancer cells to escape from anti-estrogen therapies. Furthermore, a meaningful crosstalk existed between Notch and the RTK. When overexpressing active Notch1, EGFR expression was increased. On the other hand, Notch1 overexpression could reverse EGFR inhibitor-induced cell toxicity, suggesting mutual positive regulation existed between Notch1 and EGFR (Dai et al., 2009). In addition, a study which analyzed the statistical data of histological and immunophenotypic parameters from 98 invasive breast cancer patients found that Notch2 and HER2, also known as human epidermal growth factor receptor 2, had positive correlation (Florena et al., 2007). In this way, Notch may indirectly promote endocrine resistance by ER phosphorylation through RTK pathways.

In brief, HBD-ESR1 mutations and ERα phosphorylation result in endocrine resistance and subsequent progression or relapse by means of increased BCSC activity induced by activating Notch signaling. Though the early detection of ER mutations is an immense difficulty for breast cancer, developing approaches targeting Notch pathway to prevent disease development and metastatic will be a valuable clinical decision.

The Crosstalk of Notch and Other Signaling Pathway Which Involves in Endocrine Resistance

In addition to the direct regulation of ER expression, Notch could cooperate with other pathways and cause endocrine resistance.

There is an abundance of evidence that the number of breast cancer stem cells (BCSCs) rose during antiestrogen treatment for ERα positive tumors (Creighton et al., 2009; O'Brien et al., 2011). Notch1 and Notch4 have been validated to regulate breast cancer stem cells by recent studies (Harrison et al., 2010; Gonzalez et al., 2014). Harrison H and his colleagues demonstrated that in stem cell-enriched cell populations, Notch4 signaling activity was elevated to 8-fold than differentiated cells; however, Notch1 signaling was only 4-fold higher. Their finding verified that Notch4 may produce more robust effect in maintaining breast cancer stemness (Harrison et al., 2010). Simões BM declared that short-term treatment with antiestrogens agents impaired cell proliferation yet improved breast CSCs activity through Jagged-1/Notch4 receptor activation in tumor tissue derived from breast cancer patients and xenograft (PDX) tumors (Simoes et al., 2015). Another study also showed that in ER positive breast cancer treatment with FKBPL-based therapeutics inhibited endocrine therapy resistant stem cells *via* downregulating DLL4 and Notch4 (McClements et al., 2019). In breast cancer, from non-CSCs to CSCs, CSC activity could be stimulated following exposure to estrogen *via* paracrine signaling. *In vitro* and *in vivo*, Gefitinib (EGFR inhibitor) and GSI were proven to barricade CSC activity induced by estrogen and GSIs showed more effective than Gefitinib (Harrison et al., 2013). In sum, these evidences demonstrated that detected Notch-sensitive CSCs might predict endocrine sensitivity and using Notch blockade may be an effective therapeutics for breast cancer.

It has been claimed that Notch signaling plays critical roles in acceleration of EMT in breast cancer cells which are drug-resistant. Results from Bui QT and his colleagues revealed that mesenchymal marker proteins in Tam-resistant human breast cancer (TamR-MCF-7) cells were highly expressed compared to MCF-7 cells. They proved Notch4 was instrumental in regulating EMT signaling in TamR-MCF-7 cells, but not Notch1. These results might hit upon a potential strategy to prevent metastasis in TAM-resistant breast cancer (Bui et al., 2017). Lombardo Y also found that endocrine therapies resistant cells overexpressed Nicastrin and Notch4 with mesenchymal phenotype (Lombardo et al., 2014). In another paper, it was declared that DMXL2 was increased in some endocrine therapy resistant breast cancer cells where DMXL2 promoted EMT *via* activating Notch signaling through V-type ATPase dependent acidification (Faronato et al., 2015).

Recent evidence displayed that Notch signaling pathway was engaged in the differentiation of tumor-associated macrophages (TAMs) in breast cancer (Palaga et al., 2018). Liu H detected that increased upregulation levels of Jagged-1 led to macrophage

differentiation toward M2-TAMs (Liu et al., 2017). TAM secreted CC-chemokine ligand 2 (CCL2), which resulted in breast cancer endocrine resistance by activation of the PI3K/Akt/mTOR pathway (Li et al., 2020). Adipocytes and breast cancer cells could secrete Interleukin (IL) 6. IL-6 was found to trigger a potential Notch3/Jagged-1 loop in autocrine/paracrine mode to boost BSCS self-renewal in the mammary gland (Sansone et al., 2007). Sansone P revealed that the inhibition of IL6R/IL6-Notch3 pathways combined with hormone therapy restored ERα expression and switched $CD133^{hi}$ self-renewal from IL6/Notch3-dependent to an ER-dependent one (Sansone et al., 2016).

Moreover, Rizzo and his colleagues demonstrated that no matter in MDA-MB231 or T47D cell, Notch1 knockdown or GSI treatments led to cyclins A and B1 downregulation, thus G2 arrest. In T47D cells, Notch inhibition strengthened the effects of tamoxifen. And *in vivo*, GSI, and tamoxifen treatment caused regression of T47D tumors (Rizzo et al., 2008). To this extent, Notch is capable of promoting endocrine resistance by regulate cell cycle.

CONCLUSIONS

Hormone receptor positive breast cancer accounts for 70% of all breast cancer patients. For this type of patients, despite advances in therapy, antiestrogen drugs to block ERα function is still the most meaningful approach. Unfortunately, a considerable proportion of tumors eventually develop resistance during the course of the treatment. Therefore, there is an urgent requirement to study the underlying resistance mechanism and identify novel targets in hormone receptor positive breast cancers for therapeutic intervention. Lately, increasing preclinical and clinical evidence had shown that Notch signaling pathway led to antiestrogen resistance which was related with the regulation of ER expression/activity, maintenance of CSCs and mesenchymal phenotype, crosstalk with other tyrosine kinase growth factor signaling pathways and impact on tumor microenvironment. Takebe et al. demonstrated that in Phase II clinical trials, therapy of GSI MK-0752 combined with docetaxel effectively improved the health of patients who had advanced breast cancer, indicating that chemotherapy resistance might be reversed by targeted inhibition of Notch pathway (Takebe et al., 2014). But no clinical results are launched about combination of GSI with antiestrogen. Treatment targeting both ER and Notch may hold a promising future in overcoming endocrine resistance.

AUTHOR CONTRIBUTIONS

J-WB and MW designed the project and wrote the manuscript (they contributed equally to this work). J-WL searched related articles from PubMed, Medline, and Google Scholar. The whole project was arranged and supervised by G-JZ. All authors contributed to the article and approved the submitted version.

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REFERENCES

Acar, A., Simoes, B. M., Clarke, R. B., and Brennan, K. (2016). A Role for Notch Signalling in Breast Cancer and Endocrine Resistance. *Stem Cells Int.* 2016, 2498764. doi: 10.1155/2016/2498764

Ali, S., and Coombes, R. C. (2002). Endocrine-responsive breast cancer and strategies for combating resistance. *Nat. Rev. Cancer* 2 (2), 101–112. doi: 10.1038/nrc721

Bai, J. W., Chen, M. N., Wei, X. L., Li, Y. C., Lin, H. Y., Chen, M., et al. (2017). The zinc-finger transcriptional factor Slug transcriptionally downregulates ERalpha by recruiting lysine-specific demethylase 1 in human breast cancer. *Oncogenesis* 6 (5), e330. doi: 10.1038/oncsis.2017.38

Blokzijl, A., Dahlqvist, C., Reissmann, E., Falk, A., Moliner, A., Lendahl, U., et al. (2003). Cross-talk between the Notch and TGF-beta signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. *J. Cell Biol.* 163 (4), 723–728. doi: 10.1083/jcb.200305112

Bray, S. J. (2006). Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* 7 (9), 678–689. doi: 10.1038/nrm2009

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., et al. (2000). A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol. Cell* 5 (2), 207–216. doi: 10.1016/s1097-2765(00)80417-7

Bui, Q. T., Im, J. H., Jeong, S. B., Kim, Y. M., Lim, S. C., Kim, B., et al. (2017). Essential role of Notch4/STAT3 signaling in epithelial-mesenchymal transition of tamoxifen-resistant human breast cancer. *Cancer Lett.* 390, 115–125. doi: 10.1016/j.canlet.2017.01.014

Creighton, C. J., Li, X., Landis, M., Dixon, J. M., Neumeister, V. M., Sjolund, A., et al. (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc. Natl. Acad. Sci. U. S. A.* 106 (33), 13820–13825. doi: 10.1073/pnas.0905718106

D'Souza, A., Spicer, D., and Lu, J. (2018). Overcoming endocrine resistance in metastatic hormone receptor-positive breast cancer. *J. Hematol. Oncol.* 11 (1), 80. doi: 10.1186/s13045-018-0620-6

Dai, J., Ma, D., Zang, S., Guo, D., Qu, X., Ye, J., et al. (2009). Cross-talk between Notch and EGFR signaling in human breast cancer cells. *Cancer Invest.* 27 (5), 533–540. doi: 10.1080/07357900802563036

Dou, X. W., Liang, Y. K., Lin, H. Y., Wei, X. L., Zhang, Y. Q., Bai, J. W., et al. (2017). Notch3 Maintains Luminal Phenotype and Suppresses Tumorigenesis and Metastasis of Breast Cancer via Trans-Activating Estrogen Receptor-α. *Theranostics* 7 (16), 4041. doi: 10.7150/thno.19989

Eeckhoute, J., Keeton, E. K., Lupien, M., Krum, S. A., Carroll, J. S., and Brown, M. (2007). Positive cross-regulatory loop ties GATA-3 to estrogen receptor alpha expression in breast cancer. *Cancer Res.* 67 (13), 6477–6483. doi: 10.1158/0008-5472.CAN-07-0746

Faronato, M., Nguyen, V. T., Patten, D. K., Lombardo, Y., Steel, J. H., Patel, N., et al. (2015). DMXL2 drives epithelial to mesenchymal transition in hormonal therapy resistant breast cancer through Notch hyper-activation. *Oncotarget* 6 (26), 22467–22479. doi: 10.18632/oncotarget.4164

Florena, A. M., Tripodo, C., Guarnotta, C., Ingrao, S., Porcasi, R., Martorana, A., et al. (2007). Associations between Notch-2, Akt-1 and HER2/neu expression in invasive human breast cancer: a tissue microarray immunophenotypic analysis on 98 patients. *Pathobiology* 74 (6), 317–322. doi: 10.1159/000110024

Fontes-Sousa, M., Amorim, M., Salta, S., Palma De Sousa, S., Henrique, R., and Jeronimo, C. (2019). Predicting resistance to endocrine therapy in breast cancer: It's time for epigenetic biomarkers (Review). *Oncol. Rep.* 41 (3), 1431–1438. doi: 10.3892/or.2019.6967

Gazave, E., Lapebie, P., Richards, G. S., Brunet, F., Ereskovsky, A. V., Degnan, B. M., et al. (2009). Origin and evolution of the Notch signalling pathway: an overview from eukaryotic genomes. *BMC Evol. Biol.* 9, 249. doi: 10.1186/1471-2148-9-249

Gelsomino, L., Gu, G., Rechoum, Y., Beyer, A. R., Pejerrey, S. M., Tsimelzon, A., et al. (2016). ESR1 mutations affect anti-proliferative responses to tamoxifen through enhanced cross-talk with IGF signaling. *Breast Cancer Res. Treat* 157 (2), 253–265. doi: 10.1007/s10549-016-3829-5

Gelsomino, L., Panza, S., Giordano, C., Barone, I., Gu, G., Spina, E., et al. (2018). Mutations in the estrogen receptor alpha hormone binding domain promote stem cell phenotype through notch activation in breast cancer cell lines. *Cancer Lett.* 428, 12–20. doi: 10.1016/j.canlet.2018.04.023

Gonzalez, M. E., Moore, H. M., Li, X., Toy, K. A., Huang, W., Sabel, M. S., et al. (2014). EZH2 expands breast stem cells through activation of NOTCH1 signaling. *Proc. Natl. Acad. Sci. U. S. A* 111 (8), 3098–3103. doi: 10.1073/pnas.1308953111

Gustafsson, M. V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., et al. (2005). Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev. Cell* 9 (5), 617–628. doi: 10.1016/j.devcel.2005.09.010

Hao, L., Rizzo, P., Osipo, C., Pannuti, A., Wyatt, D., Cheung, L. W., et al. (2010). Notch-1 activates estrogen receptor-alpha-dependent transcription via IKKalpha in breast cancer cells. *Oncogene* 29 (2), 201–213. doi: 10.1038/onc.2009.323

Harrison, H., Farnie, G., Howell, S. J., Rock, R. E., Stylianou, S., Brennan, K. R., et al. (2010). Regulation of breast cancer stem cell activity by signaling through the Notch4 receptor. *Cancer Res.* 70 (2), 709–718. doi: 10.1158/0008-5472.CAN-09-1681

Harrison, H., Simoes, B. M., Rogerson, L., Howell, S. J., Landberg, G., and Clarke, R. B. (2013). Oestrogen increases the activity of oestrogen receptor negative breast cancer stem cells through paracrine EGFR and Notch signalling. *Breast Cancer Res.* 15 (2), R21. doi: 10.1186/bcr3396

Jin, Y. H., Kim, H., Ki, H., Yang, I., Yang, N., Lee, K. Y., et al. (2009). Beta-catenin modulates the level and transcriptional activity of Notch1/NICD through its direct interaction. *Biochim. Biophys. Acta* 1793 (2), 290–299. doi: 10.1016/j.bbamcr.2008.10.002

Kopan, R., and Ilagan, M. X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* 137 (2), 216–233. doi: 10.1016/j.cell.2009.03.045

Korobeynikov, V., Borakove, M., Feng, Y., Wuest, W. M., Koval, A. B., Nikonova, A. S., et al. (2019). Combined inhibition of Aurora A and p21-activated kinase 1 as a new treatment strategy in breast cancer. *Breast Cancer Res. Treat* 177 (2), 369–382. doi: 10.1007/s10549-019-05329-2

Li, Y., Wu, Y., Abbatiello, T. C., Wu, W. L., Kim, J. R., Sarkissyan, M., et al. (2015). Slug contributes to cancer progression by direct regulation of ERalpha signaling pathway. *Int. J. Oncol.* 46 (4), 1461–1472. doi: 10.3892/ijo.2015.2878

Li, D., Ji, H., Niu, X., Yin, L., Wang, Y., Gu, Y., et al. (2020). Tumor-associated macrophages secrete CC-chemokine ligand 2 and induce tamoxifen resistance by activating PI3K/Akt/mTOR in breast cancer. *Cancer Sci.* 111 (1), 47–58. doi: 10.1111/cas.14230

Liang, Y. K., Zeng, D., Xiao, Y. S., Wu, Y., Ouyang, Y. X., Chen, M., et al. (2017). MCAM/CD146 promotes tamoxifen resistance in breast cancer cells through induction of epithelial-mesenchymal transition, decreased ERalpha expression and AKT activation. *Cancer Lett.* 386, 65–76. doi: 10.1016/j.canlet.2016.11.004

Lin, H. Y., Liang, Y. K., Dou, X. W., Chen, C. F., Wei, X. L., Zeng, D., et al. (2018). Notch3 inhibits epithelial-mesenchymal transition in breast cancer via a novel mechanism, upregulation of GATA-3 expression. *Oncogenesis* 7 (8), 59. doi: 10.1038/s41389-018-0069-z

Liu, H., Wang, J., Liu, Z., Wang, L., Liu, S., and Zhang, Q. (2017). Jagged1 modulated tumor-associated macrophage differentiation predicts poor prognosis in patients with invasive micropapillary carcinoma of the breast. *Med. (Baltimore)* 96 (16), e6663. doi: 10.1097/MD.0000000000006663

Lombardo, Y., Faronato, M., Filipovic, A., Vircillo, V., Magnani, L., and Coombes, R. C. (2014). Nicastrin and Notch4 drive endocrine therapy resistance and epithelial to mesenchymal transition in MCF7 breast cancer cells. *Breast Cancer Res.* 16 (3), R62. doi: 10.1186/bcr3675

Magnani, L., Stoeck, A., Zhang, X., Lánczky, A., Mirabella, A. C., Wang, T. L., et al. (2013). Genome-wide reprogramming of the chromatin landscape underlies endocrine therapy resistance in breast cancer. *Proc. Natl. Acad. Sci. U. S. A.* 110 (16), E1490–E1499. doi: 10.1073/pnas.1219992110

McClements, L., Annett, S., Yakkundi, A., O'Rourke, M., Valentine, A., Moustafa, N., et al. (2019). FKBPL and its peptide derivatives inhibit endocrine therapy resistant cancer stem cells and breast cancer metastasis by downregulating DLL4 and Notch4. *BMC Cancer* 19 (1), 351. doi: 10.1186/s12885-019-5500-0

Miele, L., Golde, T., and Osborne, B. (2006). Notch signaling in cancer. *Curr. Mol. Med.* 6 (8), 905–918. doi: 10.2174/156652406779010830

O'Brien, C. S., Farnie, G., Howell, S. J., and Clarke, R. B. (2011). Breast Cancer Stem Cells and Their Role in Resistance to Endocrine Therapy. *Hormones Cancer* 2 (2), 91–103. doi: 10.1007/s12672-011-0066-6

Palaga, T., Wongchana, W., and Kueanjinda, P. (2018). Notch Signaling in Macrophages in the Context of Cancer Immunity. *Front. Immunol.* 9, 652. doi: 10.3389/fimmu.2018.00652

Rani, A., Stebbing, J., Giamas, G., and Murphy, J. (2019). Endocrine Resistance in Hormone Receptor Positive Breast Cancer-From Mechanism to Therapy. *Front. Endocrinol. (Lausanne)* 10, 245. doi: 10.3389/fendo.2019.00245

Rizzo, P., Miao, H., D'Souza, G., Osipo, C., Song, L. L., Yun, J., et al. (2008). Cross-talk between notch and the estrogen receptor in breast cancer suggests novel therapeutic approaches. *Cancer Res.* 68 (13), 5226–5235. doi: 10.1158/0008-5472.Can-07-5744

Sansone, P., Storci, G., Tavolari, S., Guarnieri, T., Giovannini, C., Taffurelli, M., et al. (2007). IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J. Clin. Invest.* 117 (12), 3988–4002. doi: 10.1172/JCI32533

Sansone, P., Ceccarelli, C., Berishaj, M., Chang, Q., Rajasekhar, V. K., Perna, F., et al. (2016). Self-renewal of CD133(hi) cells by IL6/Notch3 signalling regulates endocrine resistance in metastatic breast cancer. *Nat. Commun.* 7, 10442. doi: 10.1038/ncomms10442

Scherbakov, A. M., Andreeva, O. E., Shatskaya, V. A., and Krasil'nikov, M. A. (2012). The relationships between snail1 and estrogen receptor signaling in breast cancer cells. *J. Cell Biochem.* 113 (6), 2147–2155. doi: 10.1002/jcb.24087

Shao, S., Zhao, X., Zhang, X., Luo, M., Zuo, X., Huang, S., et al. (2015). Notch1 signaling regulates the epithelial-mesenchymal transition and invasion of breast cancer in a Slug-dependent manner. *Mol. Cancer* 14, 28. doi: 10.1186/s12943-015-0295-3

Siegel, R. L., Miller, K. D., and Jemal, A. (2020). Cancer statistics 2020. *CA Cancer J. Clin.* 70 (1), 7–30. doi: 10.3322/caac.21590

Simoes, B. M., O'Brien, C. S., Eyre, R., Silva, A., Yu, L., Sarmiento-Castro, A., et al. (2015). Anti-estrogen Resistance in Human Breast Tumors Is Driven by JAG1-NOTCH4-Dependent Cancer Stem Cell Activity. *Cell Rep.* 12 (12), 1968–1977. doi: 10.1016/j.celrep.2015.08.050

Sprinzak, D., Lakhanpal, A., Lebon, L., Santat, L. A., Fontes, M. E., Anderson, G. A., et al. (2010). Cis-interactions between Notch and Delta generate mutually exclusive signalling states. *Nature* 465 (7294), 86–90. doi: 10.1038/nature08959

Takebe, N., Nguyen, D., and Yang, S. X. (2014). Targeting notch signaling pathway in cancer: clinical development advances and challenges. *Pharmacol. Ther.* 141 (2), 140–149. doi: 10.1016/j.pharmthera.2013.09.005

Tecalco-Cruz, A. C., and Ramirez-Jarquin, J. O. (2018). Polyubiquitination inhibition of estrogen receptor alpha and its implications in breast cancer. *World J. Clin. Oncol.* 9 (4), 60–70. doi: 10.5306/wjco.v9.i4.60

Toy, W., Shen, Y., Won, H., Green, B., Sakr, R. A., Will, M., et al. (2013). ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat. Genet.* 45 (12), 1439–1445. doi: 10.1038/ng.2822

Tremont, A., Lu, J., and Cole, J. T. (2017). Endocrine Therapy for Early Breast Cancer: Updated Review. *Ochsner J.* 17 (4), 405–411.

Vesuna, F., Lisok, A., Kimble, B., Domek, J., Kato, Y., van der Groep, P., et al. (2012). Twist contributes to hormone resistance in breast cancer by downregulating estrogen receptor-alpha. *Oncogene* 31 (27), 3223–3234. doi: 10.1038/onc.2011.483

Wilson, A., and Radtke, F. (2006). Multiple functions of Notch signaling in self-renewing organs and cancer. *FEBS Lett.* 580 (12), 2860–2868. doi: 10.1016/j.febslet.2006.03.024

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., and Griffin, J. D. (2000). MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat. Genet.* 26 (4), 484–489. doi: 10.1038/82644

Yip, C. H., and Rhodes, A. (2014). Estrogen and progesterone receptors in breast cancer. *Future Oncol.* 10 (14), 2293–2301. doi: 10.2217/fon.14.110

Yun, J., Pannuti, A., Espinoza, I., Zhu, H., Hicks, C., Zhu, X., et al. (2013). Crosstalk between PKCalpha and Notch-4 in endocrine-resistant breast cancer cells. *Oncogenesis* 2, e60. doi: 10.1038/oncsis.2013.26

Zeng, D., Liang, Y. K., Xiao, Y. S., Wei, X. L., Lin, H. Y., Wu, Y., et al. (2020). Inhibition of Notch1 reverses EMT and chemoresistance to cisplatin via direct downregulation of MCAM in triple-negative breast cancer cells. *Int. J. Cancer*. 147(2), 490–504. doi: 10.1002/ijc.32911

Zhang, J., Zhou, C., Jiang, H., Liang, L., Shi, W., Zhang, Q., et al. (2017). ZEB1 induces ER-alpha promoter hypermethylation and confers antiestrogen resistance in breast cancer. *Cell Death Dis.* 8 (4), e2732. doi: 10.1038/cddis.2017.154

Zhou, L., Wang, D., Sheng, D., Xu, J., Chen, W., Qin, Y., et al. (2020). NOTCH4 maintains quiescent mesenchymal-like breast cancer stem cells via transcriptionally activating SLUG and GAS1 in triple-negative breast cancer. *Theranostics* 10 (5), 2405–2421. doi: 10.7150/thno.38875

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JNK and Autophagy Independently Contributed to Cytotoxicity of Arsenite combined With Tetrandrine *via* Modulating Cell Cycle Progression in Human Breast Cancer Cells

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Novel therapeutic strategies for breast cancer are urgently needed due to the sustained development of drug resistance and tumor recurrence. Trivalent arsenic derivative (arsenite, As^{III}) has been reported to induce cytotoxicity in breast cancer cells. We recently demonstrated that As^{III} plus tetrandrine (Tetra), a Chinese plant-derived alkaloid, exerted potent antitumor activity against human breast cancer cells, however, the underlying mechanisms for their action have not been well defined. In order to provide fundamental insights for understanding the action of As^{III} plus Tetra, the effects of the combined regimen on two breast cancer cell lines T47D and MDA-MB-231 were evaluated. Compared to T47D cells, MDA-MB-231 cells were much more susceptible to the synergistic cytotoxic effects of As^{III} and Tetra. Besides the induction of apoptotic/necrotic cell death, S-phase arrest and autophagic cell death were also observed in MDA-MB-231 cells. Exposure of MDA-MB-231 cells to As^{III} and Tetra caused the activation of MAPKs. Cytotoxicity of the combined regimen in MDA-MB-231 cell was significantly abrogated by SP600125, a potent c-Jun N-terminal kinase (JNK) inhibitor. However, similar abrogation was not caused by p38 and ERK inhibitors. The addition of either autophagy inhibitors (3-methyladenine or wortmannin) or SP600125 corrected the combined regimen-triggered S-phase arrest, whereas had little effect on the apoptosis/necrosis induction in the cells. Surprisingly, SP600125NC, a negative control for SP600125, significantly strengthened S-phase arrest and the cytotoxicity induced by

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the combined regimen. The addition of SP600125 did not alter autophagy induction. In conclusion, the cytotoxicity of As^{III} combined with Tetra was attributed to the induction of S-phase arrest, apoptotic/necrotic and autophagic cell death. The enhanced cytotoxicity of the two drugs by SP600125NC might be explained by its capability to strengthen S-phase arrest. Our results suggested that JNK and autophagy independently contributed to the cytotoxicity *via* modulating cell cycle progression. The study further provides fundamental insights for the development of As^{III} in combination with Tetra for patients with different types of breast cancer.

Keywords: arsenite, tetrandrine, breast cancer cells, JNK, combination therapy, cell cycle arrest, autophagy

INTRODUCTION

In spite of recent progress in early detection, diagnosis, and targeted treatment options, breast cancer is still the most frequently diagnosed cancer among women worldwide and one of the leading causes of cancer-related deaths for women (Taylor et al., 2015; Fitzmaurice et al., 2017). Novel therapeutic strategies are urgently needed due to the sustained development of drug resistance, tumor recurrence, and metastasis (Taylor et al., 2015; Fitzmaurice et al., 2017).

It has been demonstrated that arsenic trioxide (As_2O_3, a trivalent arsenic derivative) exhibits high therapeutic efficacy against relapsed and refractory acute promyelocytic leukemia patients. The great therapeutic achievements thus encouraged more researchers to explore its potential future application for other malignant neoplasms (Dilda and Hogg, 2007; Yuan et al., 2010). We have demonstrated that arsenic compounds such as arsenic disulfide (As_2S_2) exhibits inhibitory effects against various types of cancer cells including breast cancer cell lines (Hu et al., 2014a; Hu et al., 2014b; Zhao et al., 2018a; Zhao et al., 2018b; Zhao et al., 2019). We also demonstrated the differentiation-inducing activity of clinically achievable concentrations of arsenite (As^{III}, a trivalent arsenic compound) combined with tetrandrine (Tetra), a traditional Chinese herbal medicine, in breast cancer cell lines (Yu et al., 2019). We thus suggested that the combined regimen of As^{III} and Tetra should be valuable in the development of differentiated therapeutic approach to combat breast cancer. In addition, we demonstrated cytocidal effect of As^{III} against estrogen receptor (ER)-positive MCF-7 breast cancer cell line, and indicated that Tetra synergistically strengthened the cytotoxicity of As^{III} (Yao et al., 2017). Our recent *in vitro* and *in vivo* study also demonstrated antitumor activity of As^{III} combined with Tetra against human triple-negative breast cancer (TNBC) cell line MDA−MB−231 (Yuan et al., 2018).

Anti-cancer therapy involves many novel therapeutic interventions, such as modification of tumor microenvironment, innate immune gene response, the induction of apoptotic and/or autophagic cell death in premalignant and malignant cells (Yao et al., 2017; Yoshino et al., 2018; Khare et al., 2019). Additionally, the role of necrotic cell death in chemotherapeutic treatment has been increasing appreciated since tumor cells evolve diverse strategies to evade apoptosis during tumor development (Cui et al., 2011; Xu et al., 2014). In this regard, we have demonstrated that autophagic and necrotic cell death contributed to the cytocidal effects of As^{III} in combination with Tetra in breast cancer cells (Yuan et al., 2018). In addition, S-phase arrest associated with the alterations of cell cycle regulators such as p21, p27 and cyclin D1 was also observed (Yuan et al., 2018). Despite this, the correlation between S-phase arrest and autophagic/necrotic cell death has not yet been clarified.

Mitogen-activated protein kinases (MAPKs) are known to be involved in a variety of cellular responses including cell division, proliferation, differentiation and cell death. The MAPKs include c-Jun NH2-terminal protein kinase (JNK), p38 kinase and extracellular signal-regulated kinase (ERK) (Cargnello and Roux, 2011). ERK usually serves as a survival mediator implicated in cytoprotection (Kikuchi et al., 2013; Kawiak et al., 2019). On the other hand, JNK and p38 MAPK are generally considered to be involved in cell death induction by diverse stimuli (Hu et al., 2014b; Kikuchi et al., 2014; Deng et al., 2018; Qiao et al., 2019). Of note, recent emerging evidence has demonstrated a strong association between the activation of JNK and antitumor agent-mediated cytotoxicity such as cell cycle arrest as well as autophagic cell death in breast cancer cells (Wang et al., 2016; Xie et al., 2017; Kong et al., 2020). Our previous report has demonstrated the contribution of S-phase arrest, autophagic and necrotic cell death to the cytotoxicity of As^{III} combined with Tetra in breast cancer cell line MDA-MB-231 (Yuan et al., 2018). However, whether the activation of MAPKs occurs and links to the combined regimen-triggered cellular responses have not yet been investigated.

A previous study (Yu et al., 2017) has demonstrated a clear difference between MCF-7 and T47D cells in the response to progesterone, although both MCF-7 and T47D are ER-positive breast cancer cell lines and share the similarities in phenotypic and molecular characteristics (Aka and Lin, 2012). In this study, in order to provide fundamental insights for understanding the action of As^{III} combined with Tetra in breast cancer cells, the cytotoxicity of the combined regimen was first evaluated in both T47D and MDA-MB-231 cells. The relation between autophagic cell death and apoptotic/necrotic cell death as well as cell cycle arrest was also explored in MDA-MB-231 cells, which showed a relatively high susceptibility to the combined regimen. Given critical roles of MAPKs in a variety of cellular responses, the relation between its activation and the combined regimen-mediated cytotoxicity was also evaluated. The association of activation of JNK, which was found to be closely related to the cytotoxicity, with various cellular responses such as cell cycle

progression and autophagic cell death was further clarified. Tamoxifen (TAM) is known as a selective ER modulator and has been widely used in chemotherapy of breast cancer. Since previous reports have demonstrated that TAM induced cytotoxicity including apoptosis in different types of breast cancer cells regardless of ER status (Liu et al., 2014; Yeh et al., 2014), TAM was used as a positive control in the current study.

MATERIALS AND METHODS

Materials

Sodium arsenite ($NaAsO_2$, As^{III}) (>99% purity) and tetrandrine (99.2% purity) were purchased from Tri Chemical Laboratories (Yamanashi, Japan) and National Institutes for Food and Drug Control (Beijing, China), respectively. Fetal bovine serum (FBS) was purchased from Nichirei Biosciences (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, phenazine methosulfate (PMS), and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Propidium iodide (PI), proteinase K, ribonuclease A (RNaseA), 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)3.2-5-[(phenylamino)carbony]-2*H*-tetrazolium hydroxide (XTT), and tamoxifen were purchased from Sigma-Aldrich (St. Louis, MO, USA). MAPK inhibitors and their negative controls (JNK inhibitor SP600125 and its negative control SP600125NC; p38 MAPK inhibitor SB203580 and its negative control SB202474; ERK inhibitor PD98059) were purchased from Calbiochem (La Jolla, CA, USA). Autophagy inhibitors, 3-methyladenine (3-MA) and wortmannin, were purchased from Wako Pure Chemical Industries and Calbiochem, respectively. Can Get Signal® Immunoreaction Enhancer Solution was purchased from Toyobo Co., Ltd. (Osaka, Japan).

Cell Culture and Treatment

Breast cancer cell lines, MDA-MB-231 and T47D, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Health Science Research Resources Bank (HSRRB, Osaka, Japan), respectively. MDA-MB-231 cells were cultured in DMEM medium, and T47D cells were cultured in RPMI-1640 medium, both of which were supplemented with 10% heat-inactivated FBS and 100 U/ml of penicillin and 100 μg/ml of streptomycin, in a humidified 5% CO_2 atmosphere at 37°C. Based on our recent work (Yuan et al., 2018), both cancer cells were treated with various concentrations of As^{III} (5, 10, and 15 μM) and Tetra (5.6, 6.4, and 7.2 μM), alone or in combination, for 48 h. Tetra was dissolved in DMSO, and no cytotoxicity of the final concentrations of DMSO was observed in the current experimental system.

Cell Viability Assay

The cell viability was measured by XTT dye-reduction assay as described previously (Yoshino et al., 2018; Yuan et al., 2019). Relative cell viability was expressed as the ratio of the absorbance of each treatment group against that of the corresponding untreated control group. Data are shown as mean ± standard deviation (SD) from more than three independent experiments. In order to evaluate whether the two drugs, As^{III} and Tetra, generated synergistic, antagonistic, or additive effects, a combination index (CI) was determined as reported previously, using the computer software ComboSyn (Combosyn Inc. NJ, USA) for drug combinations and for general dose–effect analysis, which was developed by Chou (2006; 2010). The effect of the combination treatment was defined as a synergistic effect if CI < 1, an additive effect if CI = 1 or an antagonistic effect if CI > 1 (Chen et al., 2014; Yao et al., 2017). In order to evaluate whether the activation of JNK, p38 and ERK is implicated in the cytotoxicity of As^{III} and Tetra against MDA-MB-231 cells, which possessed a relatively high susceptibility to the combinational treatment in the current study, the cells were treated with respective potent inhibitor at the indicated concentrations for 30 min prior to treatment with 10 μM As^{III}+6.4 μM Tetra in the presence or absence of each inhibitor for an additional 48 h, followed by the XTT assay as described above.

Annexin V/PI Analysis

The TACS™ Annexin V-FITC apoptosis detection Kits (Trevigen, MD, USA) was used for the detection of apoptotic and necrotic cells according to the method described previously (Yuan et al., 2015; Yoshino et al., 2018). Briefly, after treatment for 48 h with various concentrations of As^{III} (5, 10, and 15 μM) and Tetra (5.6, 6.4, and 7.2 μM), alone or in combination, cells were washed with PBS. Cells were then incubated for 15 min in 100 μl of reaction buffer, which containing annexin V-FITC and PI, followed by addition of 400 μl of binding buffer. Fluorescence intensities of FITC and PI were measured by a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA, USA). A total of 30,000 events were acquired and data were analyzed by Diva software. Annexin V(−)PI(−), annexin V(+)PI(−), annexin V(+)PI(+), and annexin V(−)PI(+) cells were defined as viable, early apoptotic, late apoptotic, and necrotic cells, respectively. In order to clarify whether autophagy induction or the activation of JNK is associated with the induction of apoptosis and necrosis, MDA-MB-231 cells were treated with either autophagy inhibitors (3-MA or wortmannin) or JNK inhibitor (SP600125), at the indicated concentrations for 30 min prior to treatment with 10 μM As^{III}+6.4 μM Tetra in the presence or absence of each inhibitor for an additional 48 h, followed by the annexin V/PI as described above.

Cell Cycle Analysis

After treatment with 10 μM As^{III}+6.4 μM Tetra for 48 h, cell cycle analysis was performed using a FACSCanto flow cytometer (Becton–Dickinson) according to a method reported previously (Kikuchi et al., 2013; Yao et al., 2017). Briefly, cells were washed twice with PBS, fixed with 1% paraformaldehyde/PBS for 30 min, washed twice again with PBS, permeabilized in 70% (v/v) cold ethanol and kept at −20°C for at least 4 h. Cell pellets were then washed twice with PBS after centrifugation and incubated with 0.25% Triton-X 100 for 5 min on ice. After centrifugation and washing with PBS, cells were resuspended in 500 μl of PI/RNase A/PBS (5 μg/ml of PI and 0.1% RNase A in PBS) and incubated

for 30 min in the dark at room temperature. A total of 10,000 events were acquired and Diva software and Mod-Fit LT™ Ver.3.0 (Verity Software House, ME, USA) were used to calculate the number of cells at each G_0/G_1, S and G_2/M phase fraction. In order to explore whether JNK or autophagy contributes to the cytotoxicity of the combined regimen by modulating cell cycle progression, MDA-MB-231 cells were treated with JNK inhibitor or autophagy inhibitors at the indicated concentrations for 30 min prior to treatment with 10 μM As^{III}+6.4 μM Tetra in the presence or absence of each inhibitor for an additional 48 h, followed by the cell cycle analysis as described above.

Western Blot Analysis

For protein samples preparation, cell pellets (approximately 1-2×10^6 cells per 110 μl Laemmli buffer) were suspended in lysis buffer (Laemmli buffer containing 100 mM DTT, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepestain, 1 mM PMSF). The suspensions of cells were sonicated using a sonicator (Qsonica, LLC, CT, USA) with 10 short bursts of 2 s followed by intervals of 2 s for cooling. The suspensions were kept at all times in an ice bath. Sonicated cells were heated in 95°C for 5 min, and then centrifuged at 13,000 g for 15 min at 4°C. Protein concentrations of the supernatant were determined according to Bradford's method using the protein assay dye reagent (Bio-Rad, CA, USA) according to the manufacturer's instructions, and using BSA as the standard. Western blot analysis was carried out according to the methods previously described (Yuan et al., 2009). Briefly, after separation of proteins on a sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, followed by transferring to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp, MA, USA), protein bands were detected using the following primary antibodies and dilution ratios: mouse anti-human β-actin (1:5,000 dilution; cat. no. A-5441; Sigma–Aldrich, MO, USA), rabbit anti-human LC3 (1:1,000 dilution; cat. no. 12741), rabbit anti-human phospho-SAPK/JNK (Thr183/Tyr185, 1:1,000 dilution; cat. no. 9251) and SAPK/JNK (1:1,000 dilution; cat. no. 9252), rabbit anti-human phospho-p38 (Thr180/Tyr182, 1:1,000 dilution; cat. no. 9211) and p38 (1:1,000 dilution; cat. no. 9212), rabbit anti-human phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:2000 dilution; cat. no. 4370) and p44/42 MAPK (Erk1/2) (137F5) (1:1,000 dilution; cat. no. 4695) (Cell Signaling Technology, MA, USA). Blotted protein bands were detected with respective horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence (ECL) Western blot analysis system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Relative amounts of the immunoreactive proteins were calculated from the density of the gray level on a digitized image using a program, NIH Image 1.60.

Statistical Analysis

Experiments were independently repeated three times, and the results are presented as the means ± SD of the three assays. Statistical analysis was conducted using one-way ANOVA followed by Dunnett's post-test. A probability level of $p<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Synergistic Cytotoxicity of As^{III} Combined With Tetra in Human Breast Cancer Cell Lines

Cell viability of MDA-MB-231 and T47D cells was determined by XTT assay following treatment for 48 h with As^{III} and Tetra, alone or in combination, at the indicated concentrations. As shown in **Figure 1**, treatment with As^{III} alone (5, 10, and 15 μM) resulted in a similar growth inhibition in both cancer cells. Tetra alone (5.6, 6.4, and 7.2 μM) induced a clear dose-dependent decrease in cell viability of MDA-MB-231, but not in T47D, indicating that MDA-MB-231 cells were more sensitive to Tetra compared to T47D. Intriguingly, synergistic cytocidal effect of the two drugs was observed in MDA-MB-231 cells when treated

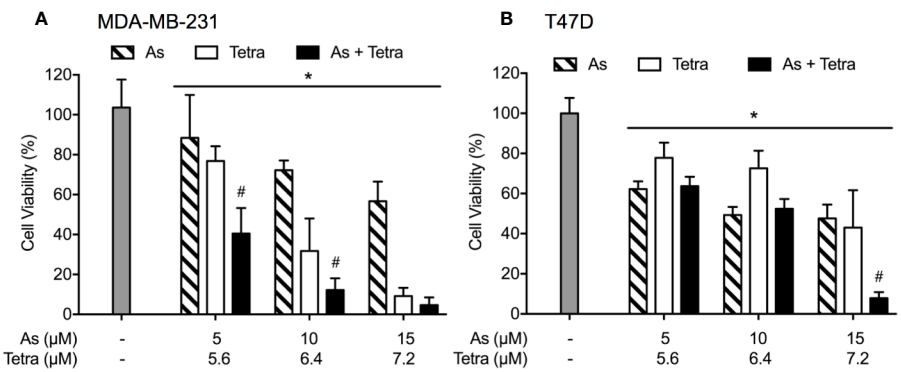

FIGURE 1 | Synergistic cytotoxicity of As^{III} combined with Tetra in human breast cancer cell lines. Following treatment with various concentrations of As^{III} alone (5, 10, and 15 μM), Tetra alone (5.6, 6.4, and 7.2 μM), or their combination for 48 h, the cell viability of MDA-MB-231 **(A)** and T47D **(B)** was determined by XTT assay. Relative cell viability was calculated as the ratio of the absorbance at 450 nm of each treatment group against those of the corresponding untreated control group. Data are shown as the means and SD from more than three independent experiments. *$p<0.05$ vs. control; #$p<0.001$ vs. each alone. As, As^{III}; Tetra, tetrandrine.

with a combination of a relatively low concentration of As^{III} and Tetra (CI values were 0.9951 and 0.9967 for the treatment of 5 μM As^{III}+5.6 μM Tetra and 10 μM As^{III}+6.4 μM Tetra, respectively). However, similar synergistic effect was only observed in T47D cells following the treatment with a combination of a relatively high concentration of the two drugs (CI value was 0.6921 for the treatment of 15 μM As^{III} +7.2 μM Tetra). These results thus indicated that the sensitivity of MDA-MB-231 to the combinatorial treatment was substantially higher than that of T47D.

Contribution of Apoptosis and Necrosis to the Cytotoxicity of As^{III} Combined With Tetra in Breast Cancer Cells

After exposure of both breast cancer cells for 48 h to the indicated concentrations of As^{III} and Tetra, alone or in combination, annexin V/PI analysis was conducted to explore whether apoptosis and/or necrosis contribute to the cytotoxicity of As^{III} combined with Tetra. TAM, a widely used in chemotherapy of breast cancer, has been shown to induce apoptosis of breast cancer cells including MDA-MB-231 and T47D (Liu et al., 2014; Yeh et al., 2014). In line with these previous reports, both cancer cells treated with 20 μM TAM underwent early and late stage apoptosis in comparison with control group, as evidenced by an increase in the number of annexin V(+)PI (-) and annexin V(+)PI(+) cells (**Figures 2A, B** and **3A, B**). A higher percentage of annexin V(-)PI(+) cells was further detected in MDA-MB-231 compared to T47D cells, indicating the induction of necrotic cell death in both cells by TAM (**Figures 2A, C** and **3A, C**).

In comparison to TAM, treatment with either As^{III} or Tetra alone hardly induced apoptosis, except that the highest concentrations of 15 μM As^{III} slightly but significantly induced apoptosis in both cells (**Figures 2A, B** and **3A, B**). Intriguingly, synergistic apoptosis-inducing activities of As^{III} combined with Tetra, regardless of their concentrations, were observed in MDA-MB-231 cells (CI values were 0.3873, 0.3245, and 0.1204 for the treatment of 5 μM As^{III}+5.6 μM Tetra, 10 μM As^{III}+6.4 μM Tetra, and 15 μM As^{III}+7.2 μM Tetra, respectively) (**Figures 2A, B**). Of note, the combined regimen of 15 μM As^{III}+7.2 μM Tetra, and TAM exhibited very similar apoptosis-inducing activity in the cells (**Figures 2A, B**). On the other hand, treatment with the combination of As^{III} and Tetra showed only a modest but significant increase in the apoptosis-inducing activity in T47D cells in comparison with control group (**Figures 3A, B**). Besides apoptosis-inducing activity, necrosis-inducing activity of 15 μM As^{III} was also observed in MDA-MB-231 cells, and further enhanced by the addition of 7.2 μM Tetra (**Figure 2C**). In addition, 10 μM As^{III} combined with 6.4 μM Tetra also exhibited necrosis-inducing activity in the cells (**Figure 2C**). Despite this, no necrosis-inducing activity of the two drugs, either alone or in combination, was recognized in T47D cells (**Figure 3C**). Since a relatively high susceptibility of MDA-MB-231 cells to As^{III} combined with Tetra was observed, a detailed analysis of the cytotoxicity of the combined regimen was carried out using the cells in the following study.

Autophagy Contributed to the Cytotoxicity of As^{III} Combined With Tetra in MDA-MB-231 Cells by Modulating Cell Cycle Progression

We have recently reported the involvement of activation of autophagic cell death in the combined regimen-mediated cytotoxicity of breast cancer cells (Yao et al., 2017; Yuan et al., 2018). We also demonstrated that As^{III} in combination with Tetra induced S-phase arrest in MDA-MB-231 cells (Yuan et al., 2018). Herein, both the induction of autophagy and S-phase arrest were first confirmed in MDA-MB-231 cells following the exposure to the combined regimen of 10 μM As^{III}+6.4 μM Tetra for 48 h (**Supplementary Figures 1** and **2**). Previous studies have demonstrated a close association between autophagy and apoptosis as well as cell cycle arrest induction in different types of cancer cells including MDA-MB-231 (Cheng et al., 2015; Gao et al., 2015; Lee et al., 2016; Lin et al., 2016; Chen et al., 2019). In order to clarify whether there was a link between autophagy and apoptosis/necrosis as well as S-phase arrest, two autophagy inhibitors, 3-MA and wortmannin, were used in the current study. After treatment for 48 h with 10 μM As^{III}+6.4 μM Tetra in the presence or absence of 3-MA (0.25 and 1.0 mM) or wortmannin (0.25 and 1.0 μM), the effects of inhibitors on the alteration of apoptosis/necrosis induction and cell cycle profiling in MDA-MB-231 cells were investigated. As shown in **Figure 4**, in comparison to the combined regimen-treatment group, the addition of 3-MA or wortmannin, regardless of the concentrations of each respective inhibitor, had little effect on the apoptosis/necrosis induction, indicating almost no association between autophagy and apoptosis/necrosis induction. It is worthy of note that the combined regimen-triggered S-phase arrest was successfully corrected by the addition of a relatively high concentration of 3-MA (1 mM) and wortmannin (1 μM), respectively (**Figures 5A, C**). Interestingly, a clear increase in the cell populations in the G_2/M phase was concomitantly observed (**Figures 5A, B, D**).

Involvement of JNK Activation in the Cytotoxicity of As^{III} Combined With Tetra in MDA-MB-231 Cells

To explore whether MAPK signaling pathways are involved in the cytocidal effect of As^{III} combined with Tetra, the activation of JNK, p38 and ERK was determined in MDA-MB-231 cells following the treatment with the indicated concentrations of As^{III} and Tetra, alone or in combination, for 48 h. In comparison to control groups, exposure to As^{III} or Tetra exhibited little effect on the ratio of phospho-JNK/JNK, except for the highest concentrations of As^{III} (15 μM) with the capability to increase the ratio (**Figures 6A, B**). Of note, a substantial increase in the ratio of phospho-JNK/JNK was detected following the treatment with 10 μM As^{III} combined with 6.4 μM Tetra (**Figures 6A, B**). Similar alterations in the ratio of phospho-ERK/ERK were also detected (**Figures 6A, C**). As shown in **Figures 6A, D**, a significant increase in the ratio of phospho-p38/p38 was detected following the exposure to various concentrations

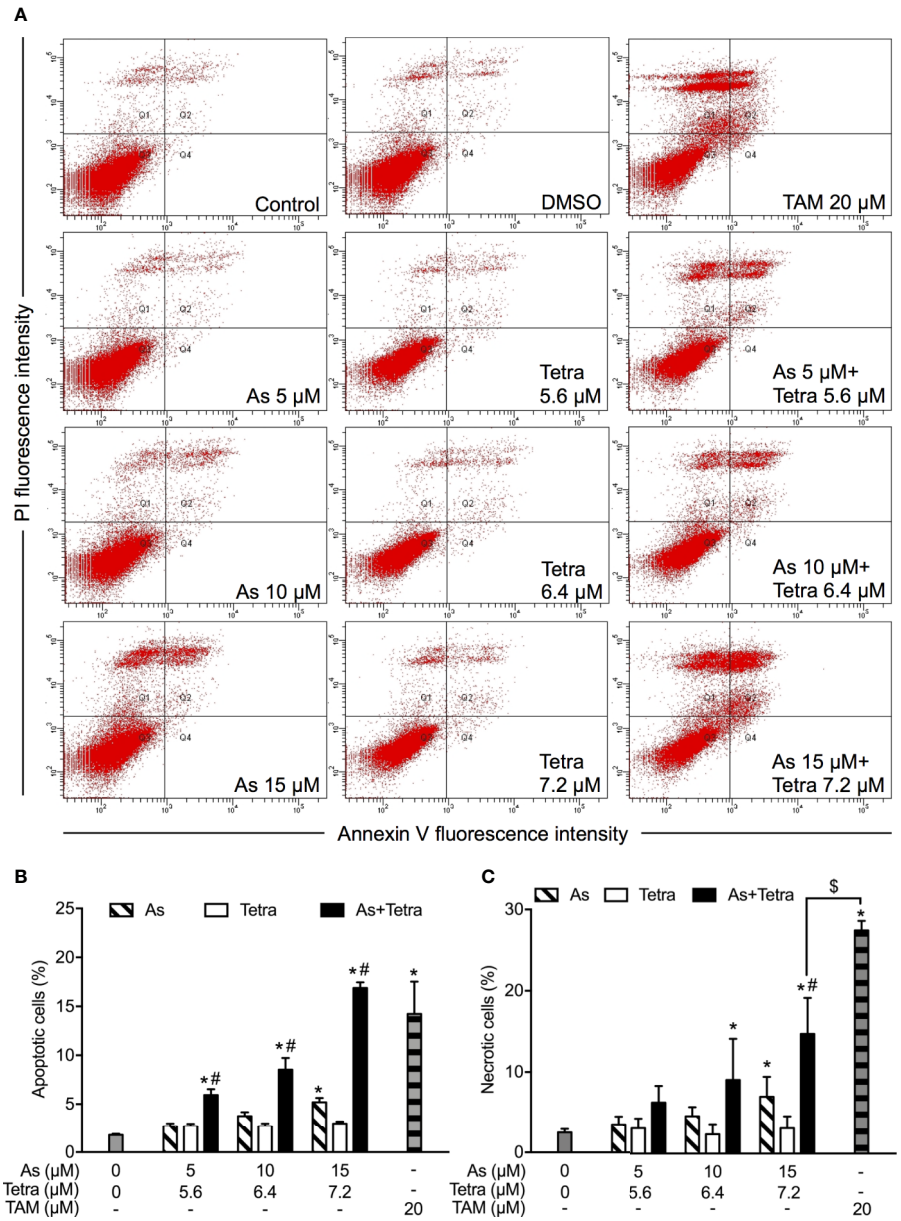

FIGURE 2 | Induction of apoptotic and necrotic cell death in MDA-MB-231 cells by As^{III} combined with Tetra. **(A)** After treatment with indicated concentrations of As^{III} and Tetra, alone or in combination, for 48 h, cells were stained with annexin V-FITC and PI, and analyzed by flow cytometry. Annexin V(–)PI(–) cells, annexin V(+)PI(–) cells, annexin V(+)PI(+) cells, and annexin V(–)PI(+) cell represent viable cells, early apoptotic cells, late apoptotic/necrotic cells, and necrotic cells, respectively. **(A)** Representative dot plots from three independent experiments are shown. Quantifications in the percentages of apoptotic cells **(B)** and necrotic cells **(C)** are shown, respectively. *p<0.05 vs. control; $^{\#}$p<0.05 vs. each alone; $^{\$}$p<0.01 vs. TAM. TAM (20 μM) used as an inducer (positive control) for the induction of apoptosis and necrosis. As, As^{III}; Tetra, tetrandrine; TAM, tamoxifen.

of Tetra alone (5.6, 6.4, and 7.2 μM), and the increase was not influenced by the addition of As^{III}. In addition, only a modest increase in the ratio of phospho-p38/p38 was observed following the exposure of As^{III} alone (5, 10, and 15 μM). These results indicated the activation of each MAPK in the cells treated with the combined regimen, although the degree of their activation was different to some extent.

Next, in order to clarify whether the respective activation of JNK, p38 and ERK is implicated in the cytotoxicity, cell viability of MDA-MB-231 cells was investigated following the exposure for 48 h to 10 μM As^{III} and 6.4 μM Tetra, alone or in combination, in the presence or absence of potent inhibitors of JNK, p38, and ERK, respectively. Consistent with the results in **Figure 1**, a significant decrease in cell viability was induced by 10

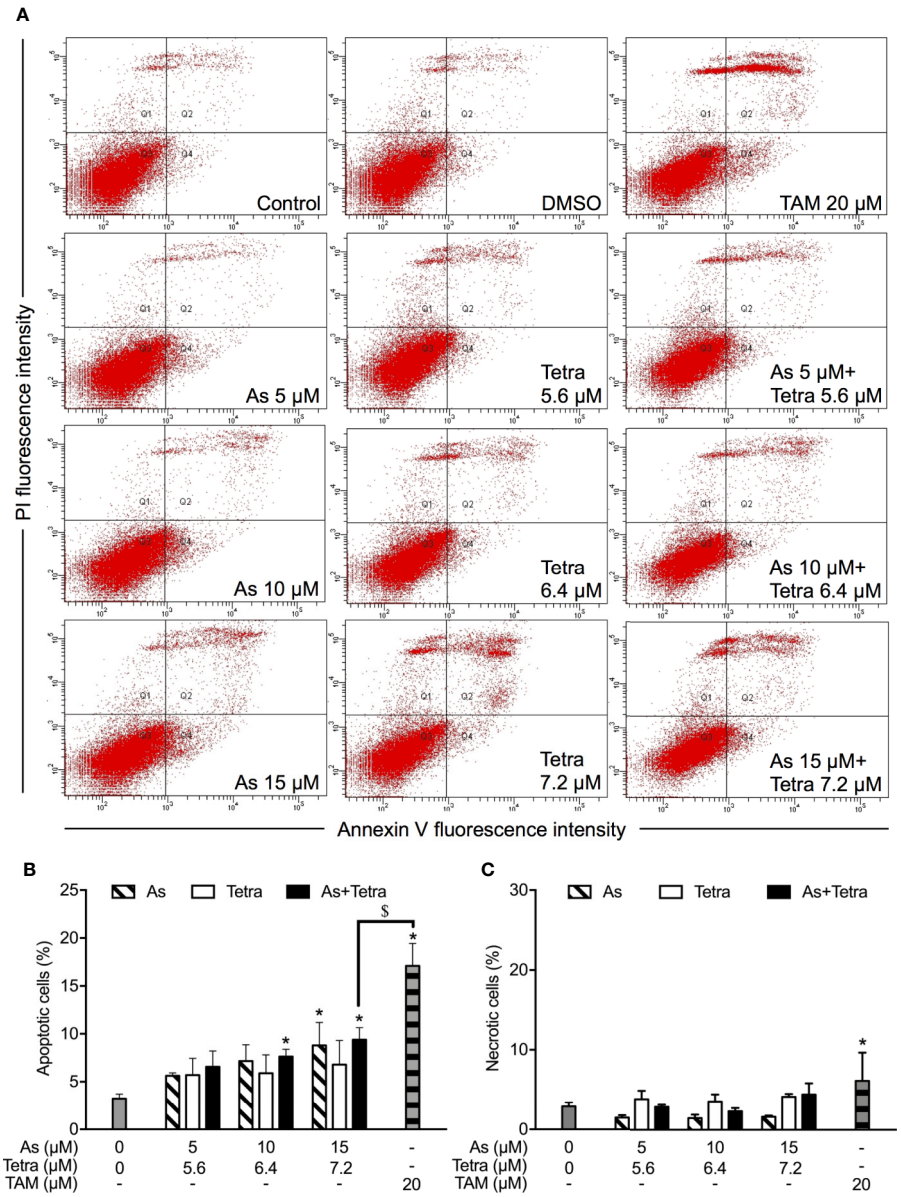

FIGURE 3 | Induction of apoptotic and necrotic cell death in T47D cells by As^{III} combined with Tetra. **(A)** After treatment with indicated concentrations of As^{III} and Tetra, alone or in combination, for 48 h, cells were stained with annexin V-FITC and PI, and analyzed by flow cytometry. Annexin V(–)PI(–) cells, annexin V(+)PI(–) cells, annexin V(+)PI(+) cells, and annexin V(–)PI(+) cell represent viable cells, early apoptotic cells, late apoptotic/necrotic cells, and necrotic cells, respectively. **(A)** Representative dot plots from three independent experiments are shown. Quantifications in the percentages of apoptotic cells **(B)** and necrotic cells **(C)** are shown, respectively. *p<0.05 vs. control; $^{\$}$p<0.05 vs. TAM. TAM (20 μM) used as an inducer (positive control) for the induction of apoptosis and necrosis. As, As^{III}; Tetra, tetrandrine; TAM, tamoxifen.

μM As^{III} and 6.4 μM Tetra, each alone, and further strengthened by their combination (**Figure 7**). Notably, the combined regimen-triggered cytotoxicity was partially but significantly abrogated by the addition of 10 μM SP600125, an inhibitor for JNK, but not SP600125NC, a negative control for SP600125 (**Figure 7A**). Conversely, the addition of SP600125NC intensified the cytotoxicity of As^{III} and Tetra, each alone (**Figure 7A**). As shown in **Figure 7B**, the cytotoxicity of the combined regimen was hardly altered by 1 μM of PD98059, an inhibitor for ERK, however, was significantly augmented by 10 μM of PD98059. In addition, no alteration in the cytotoxicity of the combined regimen was observed regardless of the presence of 10 μM of SB203580, a specific inhibitor of p38 MAPK, indicating almost no involvement of p38 MAPK in the cytotoxicity (**Figure 7C**). The addition of 10 μM of SB202474, a negative control for SB203580, interestingly enhanced the cytotoxicity of 6.4 μM Tetra alone as well as the combined regimen, although there was no obvious plausible explanation for the enhancement right now

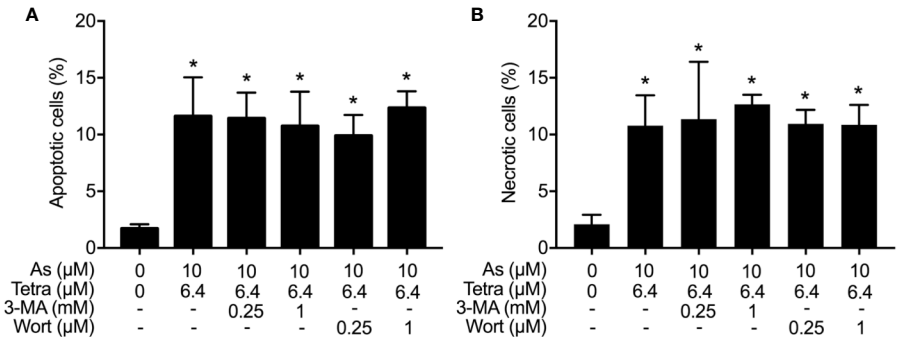

FIGURE 4 | Effects of autophagy inhibitors on the induction of apoptosis and necrosis in MDA-MB-231 cells treated with the combination of As^{III} and Tetra. After treatment with 10 μM As^{III}+6.4 μM Tetra in the presence or absence of 3-MA (0.25, 1.0 mM) or wortmannin (0.25, 1.0 μM) for 48 h, cells were stained with annexin V-FITC and PI, and analyzed by flow cytometry. The percentages of apoptotic cells **(A)** and necrotic cells **(B)** were quantified by the same manner as described in the legend of **Figures 2** and **3**. *p<0.0001 vs. control. As, As^{III}; Tetra, tetrandrine; Wort, wortmannin.

(**Figure 7C**). All of MAPK inhibitors and their respective negative control itself had no effect on the cell viability of MDA-MB-231 (**Figure 7**).

Implication of JNK Activation in the Cytotoxicity of MDA-MB-231 Cells Treated With As^{III} in Combination With Tetra Through Modulating Cell Cycle Progression

In order to provide detailed evidence for the implication of JNK activation in the combined regimen-triggered cytotoxicity, alterations of the induction of apoptosis and necrosis were first investigated following the exposure of MDA-MB-231 cells to 10 μM As^{III} combined with 6.4 μM Tetra in the presence or absence of SP600125 or its negative control for 48 h. As shown in **Figures 8A, B**, a significant increase in the number of apoptotic cells was not altered by the addition of SP600125, whereas the increase was slightly but significantly enhanced by the addition of SP600125NC. The combined regimen-triggered necrosis was also not affected by both SP600125 and its negative control, SP600125NC (**Figures 8A, C**), indicating little involvement of JNK activation in the induction of apoptosis and necrosis. In addition, substantial upregulation of the expression of LC3 was not affected by either SP600125 or SP600125NC (**Figure 9**), indicating that JNK activation and the induction of autophagy independently occurred in the cells.

Next, the effect of SP600125 on the alteration of cell cycle profiling was further investigated. Consistent with results in **Figure 5** and **Supplementary Figure 2**, S-phase arrest along with a significant decrease in the cell populations in the G_0/G_1 phase was confirmed in MDA-MB-231 cells after treatment with 10 μM As^{III} combined with 6.4 μM Tetra for 48 h (**Figures 10A–C**). In comparison, S-phase arrest was modestly but significantly reversed by the addition of 10 μM SP600125 (**Figures 10A, C**). Of note, a remarkable increase in the cell populations in the G_2/M phase along with a further decrease in the cell populations in the G_0/G_1 phase was concomitantly observed (**Figures 10A, B, D**). On the other hand, S-phase arrest was further enhanced by the addition of 10 μM SP600125NC (**Figures 10A, C**). Intriguingly, no alteration in the cell populations in the G_2/M phase was observed when combining 10 μM SP600125NC to the combinatorial treatment, although a further decrease in the cell populations in the G_0/G_1 phase was recognized (**Figures 10A, B, D**). The addition of SP600125NC itself, but not SP600125, slightly but significantly induced S-phase arrest of the cells, although both of them induced measurable decrease and increase in the cell populations in the G_0/G_1 and G_2/M phase, respectively (**Figures 10A, B, D**).

DISCUSSION

In this study, we demonstrated that the combined regimen of As^{III} and Tetra exerted a synergistic cytotoxic effect against T47D (**Figure 1**), which was in good agreement with our previous study on MCF-7 cells (Yao et al., 2017). In line with our recent work (Yuan et al., 2018), we also confirmed the synergistic cytocidal effect of the two drugs in MDA-MB-231 cells, and further indicated that MDA-MB-231 cells were markedly more susceptible to the combinatorial treatment than T47D cells (**Figure 1**). Collectively, As^{III} and Tetra, which have been used as medicinal agents, should be valuable in the development of novel therapeutic approaches to combat different types of breast cancers in spite of their estrogen dependency.

Despite the fact that the aim of anticancer therapy has been commonly focused on the induction of apoptosis in premalignant and malignant cells, the role of necrotic cell death in chemotherapeutic treatment has been increasing appreciated since tumor cells evolve diverse strategies to evade apoptosis during tumor development (Cui et al., 2011; Xu et al., 2014). In this regard, the combined regimen of a relatively low concentration of each drug (10 μM As^{III}+6.4 μM Tetra) induced both apoptosis and necrosis in MDA-MB-231 cells (**Figure 2**). Intriguingly, the combined regimen of 15 μM As^{III}+7.2 μM Tetra and TMA exhibited very similar apoptosis-inducing activity in MDA-MB-231 cells, providing very meaningful *in vitro* experimental data for

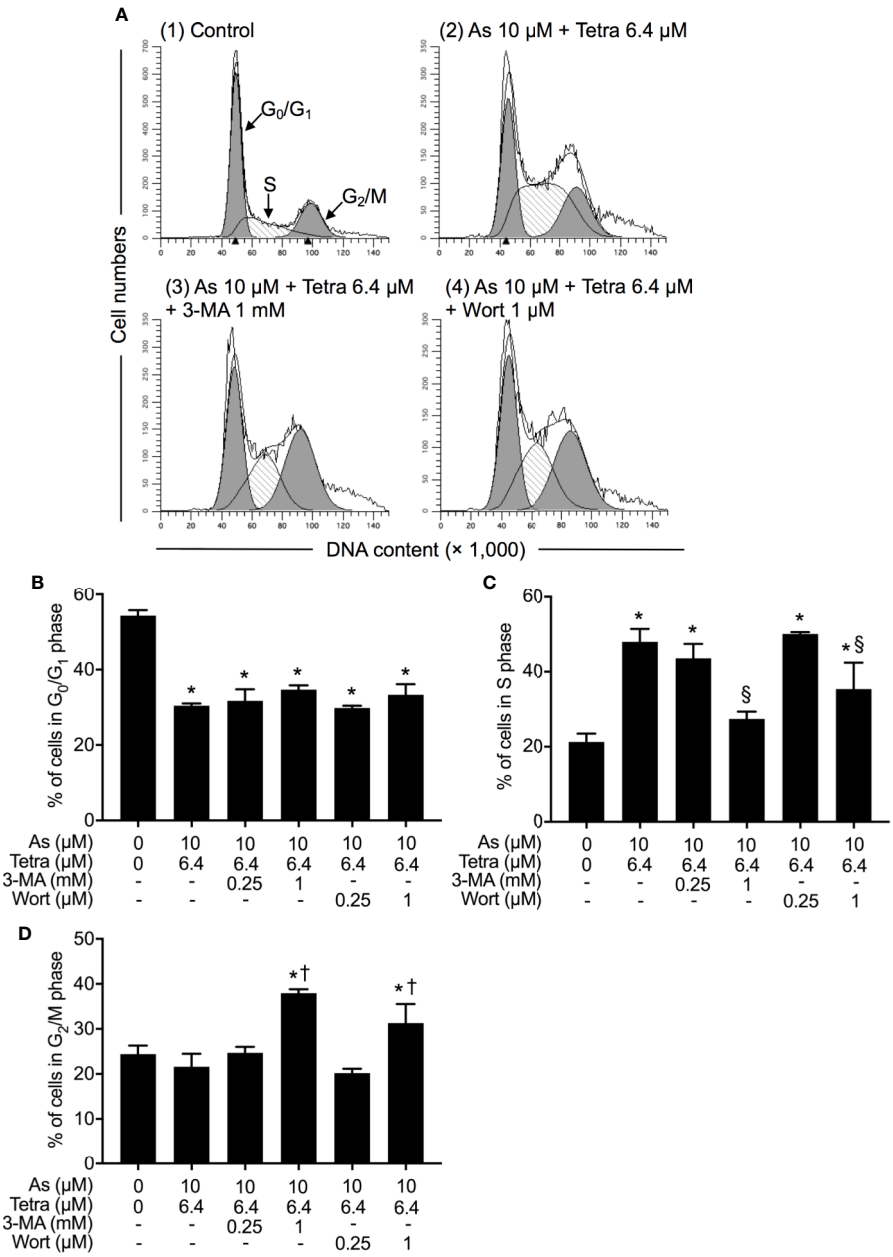

FIGURE 5 | Contribution of autophagy to the cytotoxicity of As^{III} combined with Tetra in MDA-MB-231 cells by modulating cell cycle progression. **(A–D)** After treatment with 10 μM As^{III}+6.4 μM Tetra in the presence or absence of 3-MA (0.25, 1.0 mM) or wortmannin (0.25, 1.0 μM) for 48 h, cell cycle profiling was performed by FACSCanto flow cytometer. Analyzed data and profiles for each G_0/G_1 and G_2/M phase using Diva software and ModFit LT™ ver.3.0. are shown in the gray area. Cells at S phase are shown as shaded area. A representative FACS histogram from three separate experiments is shown **(A)**. *p<0.05, vs. control; §p<0.05, †p<0.01, vs. 10 μM As^{III} +Tetra 6.4 μM. As, As^{III}; Tetra, tetrandrine; Wort, wortmannin.

breast cancer drug development, although more detailed analyses including *in vivo* experiments are obviously needed.

In addition to the induction of apoptotic/necrotic cell death, our results also demonstrated that autophagic cell death and S-phase arrest (**Supplementary Figures 1** and **2**) contributed to the cytocidal effects of the combined regimen of 10 μM As^{III}+6.4 μM Tetra in MDA-MB-231 cells. Although autophagy has been linked to apoptotic/necrotic cell death in many cases (Nikoletopoulou et al., 2013; Yoshida, 2017; Chen et al., 2019), our experimental results demonstrated that the addition of either 3-MA or wortmannin, two autophagy inhibitors, successfully corrected the combined regimen-triggered S-phase arrest, however, had little effect on the apoptosis/necrosis induction (**Figures 4** and **5**). Autophagy has been demonstrated to act either as a cytoprotective process or a pro-death factor in different cellular contexts (White and DiPaola, 2009;

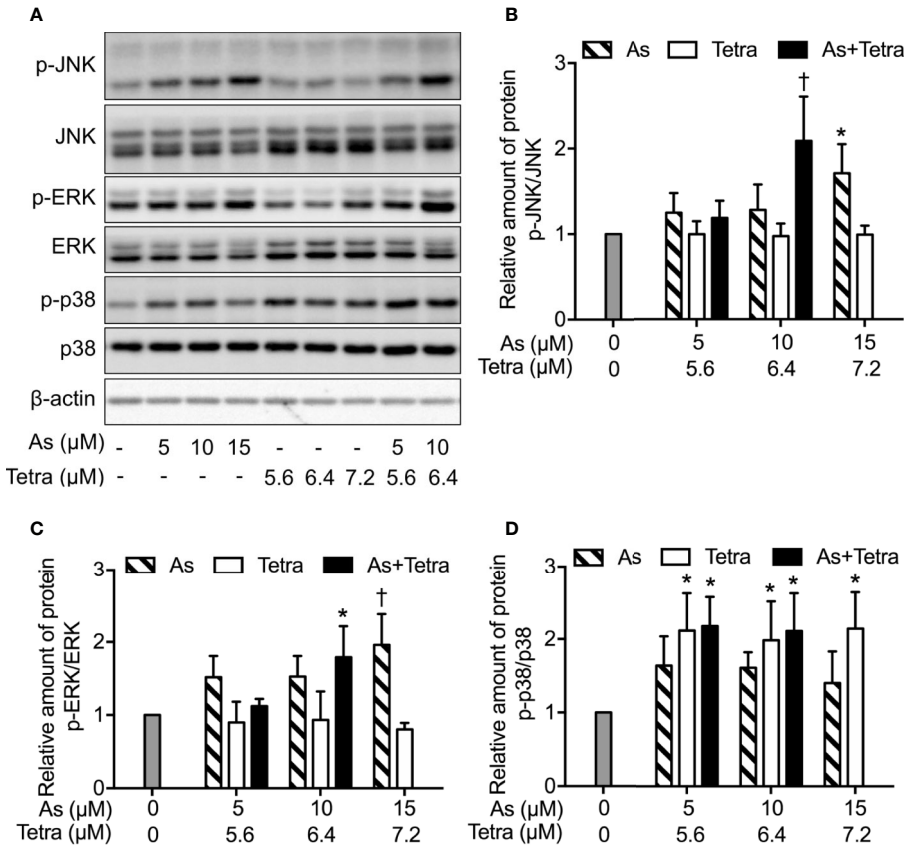

FIGURE 6 | MAPK activation in MDA-MB-231 cells treated with As^{III} and Tetra, alone or in combination. **(A–D)** Following treatment for 48 h with the indicated concentrations of As^{III} and Tetra, alone or in combination, the expression profiles of both phosphorylated and total forms of JNK, ERK, and p38 were analyzed using western blotting. Representative images of the expression profile of each protein are shown from three independent experiments. The relative expression levels were expressed as the ratios between the phosphorylated active form and total form of each target protein expression levels, and compared with those of untreated control group, respectively. Results are shown as the means ± SD from three independent experiments. p-JNK, p-ERK, and p-p38 represent phospho-JNK, phospho-ERK, and phospho-p38, the respective phosphorylated active form of each MAPK. *$p<0.05$, †$p<0.01$, vs. control. As, As^{III}; Tetra, tetrandrine. Since enough cells cannot be collected in the group treated with 15 μM As^{III} in combination with 7.2 μM Tetra due to its strong cytotoxicity, western blot analyses were not conducted.

Mathiassen et al., 2017). A recent review article has demonstrated that in response to different exogenous cellular stress stimuli, adequate autophagy can be activated to induce degradation of cell cycle arrest-related proteins such as p27, consequently contributes to tumorigenesis and/or drug resistance (Zheng et al., 2019). On the other hand, anticancer agents cause overactivated autophagy to breakdown cell cycle regulators including cyclin-dependent kinases or cyclin to induce permanent cell cycle arrest and autophagy-related cell death (Maes et al., 2013; Zheng et al., 2019). While the correlative induction of cell-cycle arrest and autophagy has been discussed, the molecular mechanisms linking them together remain poorly characterized (Maes et al., 2013; Mathiassen et al., 2017; Zheng et al., 2019). Based on the previous observations and our findings, we suggest that the abrogation of the overactivated autophagy might promote cell cycle progression, and consequently inhibit the cytotoxic effects of As^{III} combined with Tetra in MDA-MB-231 cells, although more detailed mechanisms underlying the crosstalk between cell cycle progression and autophagy are obviously needed to clarify.

We also demonstrated that the cytocidal effect of As^{III} combined with Tetra was significantly abolished by SP600125, a potent inhibitor of JNK, but not by SB203580, a specific inhibitor for p38, suggesting the contribution of JNK, instead of p38, to the cytotoxicity (**Figure 7**). In line with previous reports showing that ERK usually served as a survival mediator implicated in cytoprotection (Yao et al., 2012; Kikuchi et al., 2013), the combined regimen-triggered cytotoxicity was clearly augmented by PD98059, an inhibitor of ERK, suggesting that activation of ERK might compensate for the cytocidal stimuli. Although the activation of JNK has been deeply implicated in apoptosis/necrosis induction in different types of cancer cells (Deng et al., 2018; Qiao et al., 2019), inhibition of JNK by SP600125 did not alter the induction of apoptosis/necrosis (**Figure 8**), suggesting little involvement of JNK activation. Similarly, substantial upregulation of the expression of LC3

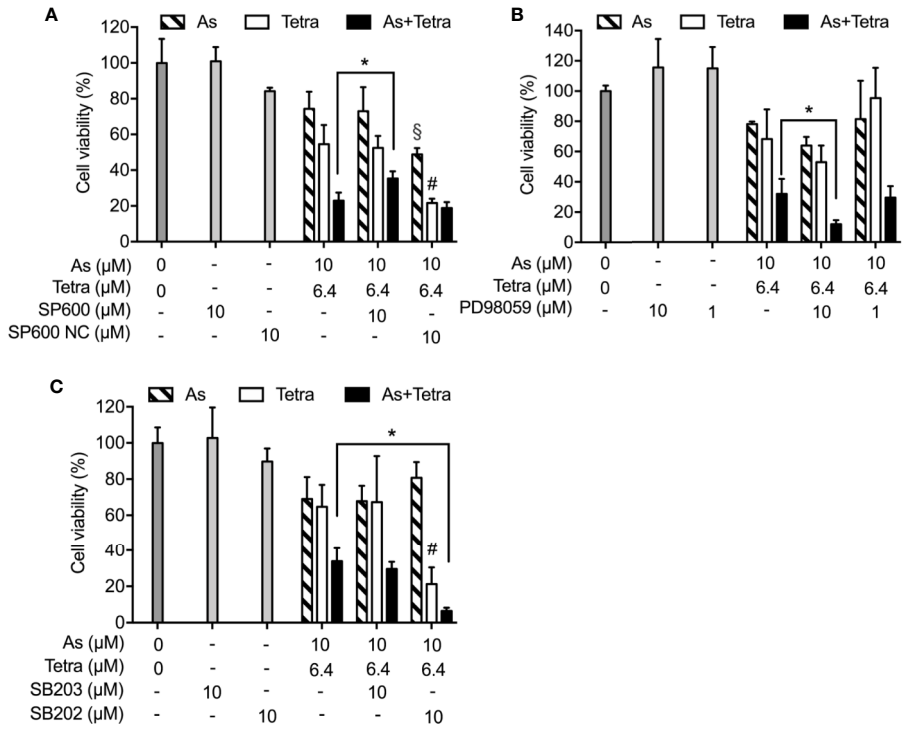

FIGURE 7 | Abrogation of the cytotoxicity of As^{III} combined with Tetra by JNK inhibition in MDA-MB-231 cells. Following the treatment for 48 h with 10 μM As^{III} and 6.4 μM Tetra, alone or in combination, in the presence or absence of MAPK inhibitors and their negative controls [JNK inhibitor SP600125 and its negative control SP600125NC **(A)**; ERK inhibitor PD98059 **(B)**; p38 MAPK inhibitor SB203580 and its negative control SB202474 **(C)**], the cell viability of MDA-MB-231 was determined by XTT assay. Relative cell viability was calculated as the ratio of the absorbance at 450 nm of each treatment group against those of the corresponding untreated control group. Data are shown as the means and SD from more than three independent experiments. *$p<0.01$ vs. the combination; $^{§}p<0.01$, vs. 10 μM As^{III} alone; $^{\#}p<0.01$, vs. 6.4 μM Tetra alone. As, As^{III}; Tetra, tetrandrine; SP600, SP600125; SP600 NC, SP600125NC; SB203, SB203580; SB202, SB202474.

was not affected by SP600125 (**Figure 9**), suggesting that JNK activation and autophagy independently contributed to the cytotoxicity of As^{III} combined with Tetra in MDA-MB-231 cells.

In comparison, SP600125 modestly but significantly corrected S-phase arrest, which was accompanied by a significant increase and decrease in the cell populations in the G_2/M and G_0/G_1 phase, respectively (**Figure 10**). These results thus suggested that the combined regimen-triggered cytotoxicity was attributed to JNK activation-associated with S-phase arrest. In agreement with this opinion, we interestingly observed that SP600125NC, a negative control for SP600125, significantly enhanced the cytotoxicity of As^{III} and Tetra, alone or in combination (**Figures 7** and **8**), which might be explained by the capability of SP600125NC to strengthen S-phase arrest (**Figure 10**) although the mechanisms underlying the strengthening remain to be clarified. Recently, Xie et al. demonstrated that Pu-erh tea water extract induced growth inhibition of MDA-MB-231 cells through S-phase arrest associated with upregulation of p21 and downregulation of cyclin D1, cyclin E, all of which was mediated *via* JNK activation (Xie et al., 2017). They further showed that co-treatment with SP600125 restored the water extract-induced alterations of p21, cyclin D1, and cyclin E, suggesting that S-phase arrest occurred through the activation of JNK-related cell death pathway (Xie et al., 2017). Most recently, Kong et al. have demonstrated that cardamonin, a naturally occurring chalcone isolated from large black cardamom, induces G_2/M arrest and apoptosis in breast cancer cells including MDA-MB-231 (Kong et al., 2020). They further clarified that SP600125 blocked FOXO3a expression and nuclear translocation, and significantly diminished the expression of FOXO3a and the upregulation of p21 and p27, two target genes of FOXO3a (Kong et al., 2020). Similar to these previous reports, we recently also demonstrated that S-phase arrest associated with the upregulation of FOXO3a, p21, p27 along with decreased cyclin D1 expression contributed to the anticancer activity of As^{III} and Tetra against MDA-MB-231 cells (Yuan et al., 2018). Collectively, we suggest that the activation of JNK-FOXO3a pathway probably plays a critical role in the combined regimen-triggered cytotoxicity in MDA-MB-231 cells, although more detailed analyses are needed to clarify this opinion.

CONCLUSION

Our results demonstrated that besides apoptosis/necrosis, autophagic cell death and cell cycle arrest were also involved in the cytotoxicity of As^{III} combined with Tetra in breast cancer

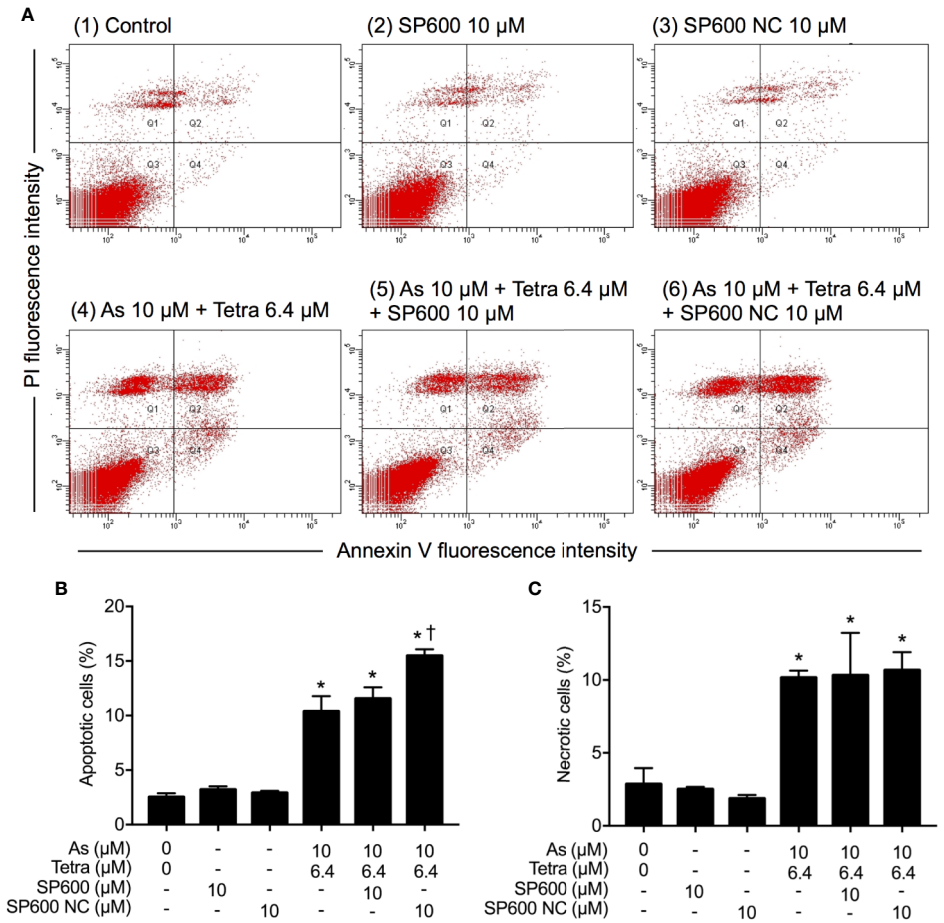

FIGURE 8 | Effect of SP600125 on the induction of apoptosis and necrosis in MDA-MB-231 cells treated with As^{III} combined with Tetra. After treatment with 10 μM As^{III}+6.4 μM Tetra in the presence or absence of 10 μM of SP600125 and its negative control SP600125NC for 48 h, cells were stained with annexin V-FITC and PI, and analyzed by flow cytometry. **(A)** Representative dot plots from three independent experiments are shown. The percentages of apoptotic cells **(B)** and necrotic cells **(C)** were quantified by the same manner as described in the legend of Figures 2 and 3. *p<0.0001 vs. control, †p<0.0001, vs. 10 μM As^{III}+6.4 μM Tetra. As, As^{III}; Tetra, tetrandrine; SP600, SP600125; SP600 NC, SP600125NC.

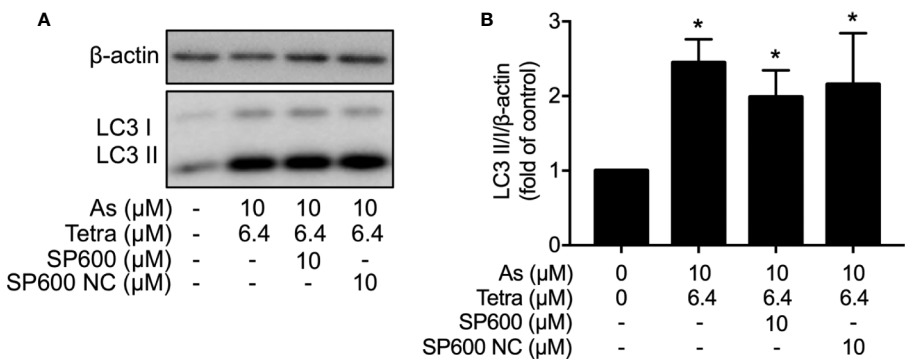

FIGURE 9 | Effect of SP600125 on the induction of autophagy in MDA-MB-231 cells treated with As^{III} combined with Tetra. After treatment with 10 μM As^{III}+6.4 μM Tetra in the presence or absence of 10 μM of SP600125 and its negative control SP600125NC for 48 h, the expression of LC3 protein was analyzed using western blot. **(A)** Representative images of the expression profile of LC3 are shown from three independent experiments. **(B)** The relative expression level was expressed as the ratio between LC3 protein and β-actin protein expression levels, and compared with those of untreated control group. Results are shown as the means ± SD from three independent experiments. *p<0.05 vs. control. As, As^{III}; Tetra, tetrandrine; SP600, SP600125; SP600 NC, SP600125NC.

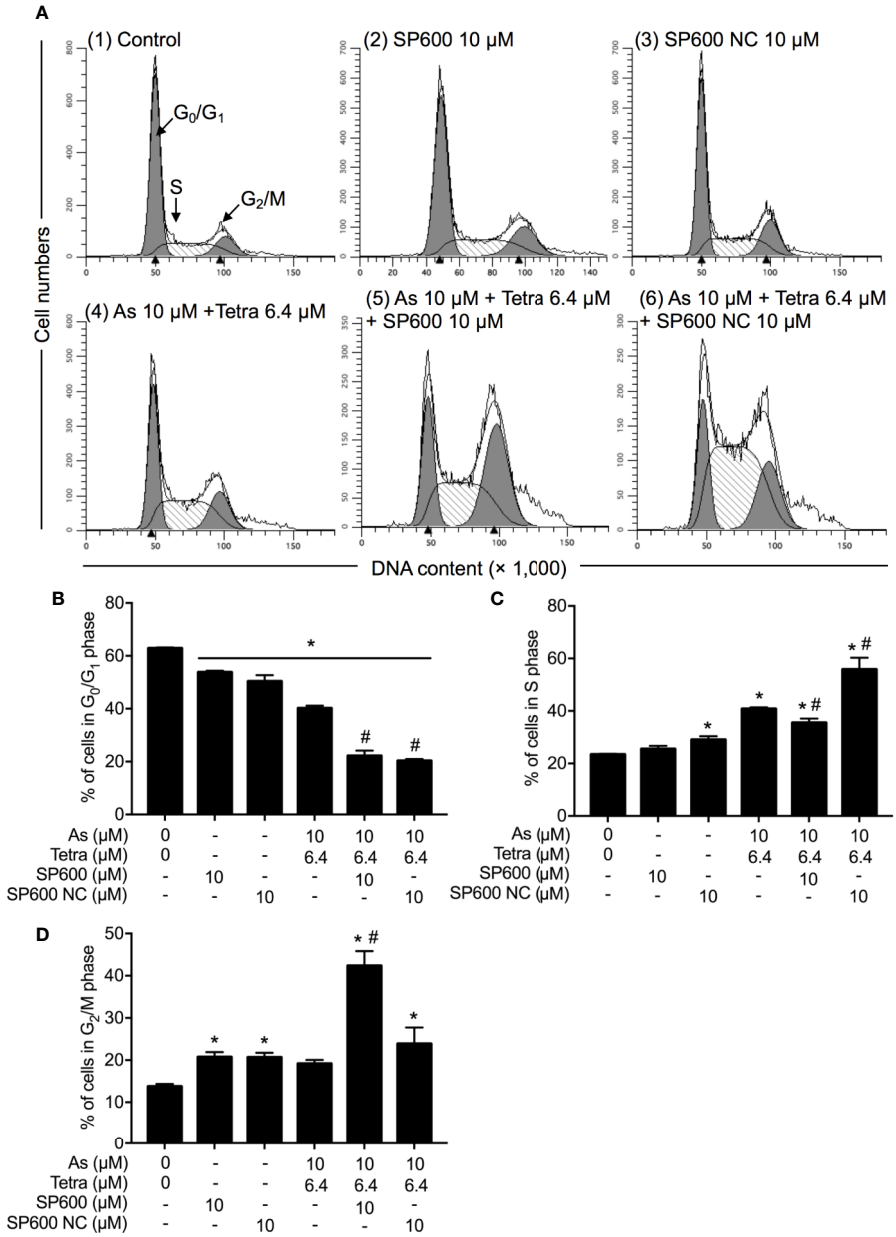

FIGURE 10 | Association of JNK activation with cell cycle progression of MDA-MB-231 cells treated with As^{III} in combination with Tetra. **(A–D)** After treatment with 10 μM As^{III}+6.4 μM Tetra in the presence or absence of 10 μM of SP600125 or its negative control SP600125NC for 48 h, cell cycle profiling was performed by FACSCanto flow cytometer. Analyzed data and profiles for each G_0/G_1 and G_2/M phase using Diva software and ModFit LT™ ver.3.0. are shown in the gray area. Cells at S phase are shown as shaded area. A representative FACS histogram from three separate experiments is shown **(A)**. *p<0.05, vs. control; #p<0.05, vs. 10 μM As^{III}+Tetra 6.4 μM. As, As^{III}; Tetra, tetrandrine; SP600, SP600125; SP600 NC, SP600125NC.

cells, and that MDA-MB-231 cells were markedly more susceptible to the combinatorial treatment than T47D cells. Therefore, we suggest that the combined regimen could be a broadly applicable approach to combat different types of breast cancer cells. We further demonstrated that the activation of JNK and autophagy independently contributed to the cytotoxicity of As^{III} combined with Tetra *via* modulating cell cycle progression in MDA-MB-231 cells. TNBC has been characterized by highly aggressive metastatic behavior and represents one of the most difficult subtypes of breast cancer (Carey et al., 2010). In view of this, the combination of As^{III} and Tetra, both of which have been used in the clinic, should be valuable in developing a novel therapeutic strategy to cease the uncontrolled proliferation of cancer cells in patients with TNBC. Our findings provide

valuable insights into the development of the novel therapeutic for different types of breast cancer, especially TNBC.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

BYu and BYua contributed equally to this study. BYua conceived and designed the study and drafted the manuscript. BYu and BYua performed the experiments. JL, AK, HK, HH, XH, MO, MS, TH, YF, XP, and NT assisted interpretation of the results with BYua. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.01087/full#supplementary-material

REFERENCES

Aka, J. A., and Lin, S. X. (2012). Comparison of functional proteomic analyses of human breast cancer cell lines T47D and MCF7. *PLoS One* 7 (2), e31532. doi: 10.1371/journal.pone.0031532

Carey, L., Winer, E., Viale, G., Cameron, D., and Gianni, L. (2010). Triple-negative breast cancer: disease entity or title of convenience? *Nat. Rev. Clin. Oncol.* 7 (12), 683–692. doi: 10.1038/nrclinonc.2010.154

Cargnello, M., and Roux, P. P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* 75 (1), 50–83. doi: 10.1128/mmbr.00031-10

Chen, Y., Li, P., Yang, S., Tong, N., Zhang, J., and Zhao, X. (2014). Tetrandrine enhances the anticancer effects of arsenic trioxide in vitro. *Int. J. Clin. Pharmacol. Ther.* 52 (5), 416–424. doi: 10.5414/cp201939

Chen, Z., Tian, D., Liao, X., Zhang, Y., Xiao, J., Chen, W., et al. (2019). Apigenin Combined With Gefitinib Blocks Autophagy Flux and Induces Apoptotic Cell Death Through Inhibition of HIF-1alpha, c-Myc, p-EGFR, and Glucose Metabolism in EGFR L858R+T790M-Mutated H1975 Cells. *Front. Pharmacol.* 10, 260. doi: 10.3389/fphar.2019.00260

Cheng, S. M., Chang, Y. C., Liu, C. Y., Lee, J. Y., Chan, H. H., Kuo, C. W., et al. (2015). YM155 down-regulates survivin and XIAP, modulates autophagy and induces autophagy-dependent DNA damage in breast cancer cells. *Br. J. Pharmacol.* 172 (1), 214–234. doi: 10.1111/bph.12935

Chou, T. C. (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol. Rev.* 58 (3), 621–681. doi: 10.1124/pr.58.3.10

Chou, T. C. (2010). Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* 70 (2), 440–446. doi: 10.1158/0008-5472.can-09-1947

Cui, Q., Jiang, W., Guo, J., Liu, C., Li, D., Wang, X., et al. (2011). Relationship between hypermethylated MGMT gene and osteosarcoma necrosis rate after chemotherapy. *Pathol. Oncol. Res.* 17 (3), 587–591. doi: 10.1007/s12253-010-9354-7

Deng, L. J., Qi, M., Peng, Q. L., Chen, M. F., Qi, Q., Zhang, J. Y., et al. (2018). Arenobufagin induces MCF-7 cell apoptosis by promoting JNK-mediated multisite phosphorylation of Yes-associated protein. *Cancer Cell Int.* 18, 209. doi: 10.1186/s12935-018-0706-9

Dilda, P. J., and Hogg, P. J. (2007). Arsenical-based cancer drugs. *Cancer Treat Rev.* 33 (6), 542–564. doi: 10.1016/j.ctrv.2007.05.001

Fitzmaurice, C., Allen, C., Barber, R. M., Barregard, L., Bhutta, Z. A., Brenner, H., et al. (2017). Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups 1990 to 2015: A Systematic Analysis for the Global Burden of Disease Study. *JAMA Oncol.* 3 (4), 524–548. doi: 10.1001/jamaoncol.2016.5688

Gao, L., Wang, Y., Xu, Z., Li, X., Wu, J., Liu, S., et al. (2015). SZC017, a novel oleanolic acid derivative, induces apoptosis and autophagy in human breast cancer cells. *Apoptosis* 20 (12), 1636–1650. doi: 10.1007/s10495-015-1179-0

Hu, X. M., Yuan, B., Tanaka, S., Song, M. M., Onda, K., Tohyama, K., et al. (2014a). Arsenic disulfide-triggered apoptosis and erythroid differentiation in myelodysplastic syndrome and acute myeloid leukemia cell lines. *Hematology* 19 (6), 352–360. doi: 10.1179/1607845413y.0000000138

Hu, X. M., Yuan, B., Tanaka, S., Zhou, Q., Onda, K., Toyoda, H., et al. (2014b). Involvement of oxidative stress associated with glutathione depletion and p38 mitogen-activated protein kinase activation in arsenic disulfide-induced differentiation in HL-60 cells. *Leuk. Lymphoma* 55 (2), 392–404. doi: 10.3109/10428194.2013.802779

Kawiak, A., Domachowska, A., and Lojkowska, E. (2019). Plumbagin Increases Paclitaxel-Induced Cell Death and Overcomes Paclitaxel Resistance in Breast Cancer Cells through ERK-Mediated Apoptosis Induction. *J. Nat. Prod.* 82 (4), 878–885. doi: 10.1021/acs.jnatprod.8b00964

Khare, V. M., Saxena, V. K., Tomar, A., Nyinawabera, A., Singh, K. B., Ashby, C. R.Jr., et al. (2019). Cytokine gene expression following RSV-A infection. *Front. Biosci. (Landmark Ed)* 24, 463–481. doi: 10.2741/4729

Kikuchi, H., Yuan, B., Yuhara, E., Takagi, N., and Toyoda, H. (2013). Involvement of histone H3 phosphorylation through p38 MAPK pathway activation in casticin-induced cytocidal effects against the human promyelocytic cell line HL-60. *Int. J. Oncol.* 43 (6), 2046–2056. doi: 10.3892/ijo.2013.2106

Kikuchi, H., Yuan, B., Yuhara, E., Imai, M., Furutani, R., Fukushima, S., et al. (2014). Involvement of histone H3 phosphorylation via the activation of p38 MAPK pathway and intracellular redox status in cytotoxicity of HL-60 cells induced by Vitex agnus-castus fruit extract. *Int. J. Oncol.* 45 (2), 843–852. doi: 10.3892/ijo.2014.2454

Kong, W., Li, C., Qi, Q., Shen, J., and Chang, K. (2020). Cardamonin induces G2/M arrest and apoptosis via activation of the JNK-FOXO3a pathway in breast cancer cells. *Cell Biol. Int.* 44, 177–188. doi: 10.1002/cbin.11217

Lee, E., Lee, C. G., Yim, J. H., Lee, H. K., and Pyo, S. (2016). Ramalin-Mediated Apoptosis Is Enhanced by Autophagy Inhibition in Human Breast Cancer Cells. *Phytother. Res.* 30 (3), 426–438. doi: 10.1002/ptr.5544

Lin, Y., Wang, Y., Liu, X., Yan, J., Su, L., and Liu, X. (2016). A novel derivative of tetrandrine (H1) induces endoplasmic reticulum stress-mediated apoptosis and prosurvival autophagy in human non-small cell lung cancer cells. *Tumour Biol.* 37 (8), 10403–10413. doi: 10.1007/s13277-016-4950-0

Liu, C. Y., Hung, M. H., Wang, D. S., Chu, P. Y., Su, J. C., Teng, T. H., et al. (2014). Tamoxifen induces apoptosis through cancerous inhibitor of protein phosphatase 2A-dependent phospho-Akt inactivation in estrogen receptor-negative human breast cancer cells. *Breast Cancer Res.* 16 (5), 431. doi: 10.1186/s13058-014-0431-9

Maes, H., Rubio, N., Garg, A. D., and Agostinis, P. (2013). Autophagy: shaping the tumor microenvironment and therapeutic response. *Trends Mol. Med.* 19 (7), 428–446. doi: 10.1016/j.molmed.2013.04.005

Mathiassen, S. G., De Zio, D., and Cecconi, F. (2017). Autophagy and the Cell Cycle: A Complex Landscape. *Front. Oncol.* 7:51. doi: 10.3389/fonc.2017.00051

Nikoletopoulou, V., Markaki, M., Palikaras, K., and Tavernarakis, N. (2013). Crosstalk between apoptosis, necrosis and autophagy. *Biochim. Biophys. Acta* 1833 (12), 3448–3459. doi: 10.1016/j.bbamcr.2013.06.001

Qiao, Z., Cheng, Y., Liu, S., Ma, Z., Li, S., and Zhang, W. (2019). Casticin inhibits esophageal cancer cell proliferation and promotes apoptosis by regulating mitochondrial apoptotic and JNK signaling pathways. *Naunyn Schmiedebergs Arch. Pharmacol.* 392 (2), 177–187. doi: 10.1007/s00210-018-1574-5

Taylor, S., Lam, M., Pararasa, C., Brown, J. E., Carmichael, A. R., and Griffiths, H. R. (2015). Evaluating the evidence for targeting FOXO3a in breast cancer: a systematic review. *Cancer Cell Int.* 15 (1), 1. doi: 10.1186/s12935-015-0156-6

Wang, K., Zhang, C., Bao, J., Jia, X., Liang, Y., Wang, X., et al. (2016). Synergistic chemopreventive effects of curcumin and berberine on human breast cancer cells through induction of apoptosis and autophagic cell death. *Sci. Rep.* 6:26064. doi: 10.1038/srep26064

White, E., and DiPaola, R. S. (2009). The double-edged sword of autophagy modulation in cancer. *Clin. Cancer Res.* 15 (17), 5308–5316. doi: 10.1158/1078-0432.ccr-07-5023

Xie, J., Yu, H., Song, S., Fang, C., Wang, X., Bai, Z., et al. (2017). Pu-erh Tea Water Extract Mediates Cell Cycle Arrest and Apoptosis in MDA-MB-231 Human Breast Cancer Cells. *Front. Pharmacol.* 8:190. doi: 10.3389/fphar.2017.00190

Xu, Y., Lin, Z., Zhao, N., Zhou, L., Liu, F., Cichacz, Z., et al. (2014). Receptor interactive protein kinase 3 promotes Cisplatin-triggered necrosis in apoptosis-resistant esophageal squamous cell carcinoma cells. *PLoS One* 9 (6), e100127. doi: 10.1371/journal.pone.0100127

Yao, J., Qian, C. J., Ye, B., Zhang, X., and Liang, Y. (2012). ERK inhibition enhances TSA-induced gastric cancer cell apoptosis via NF-kappaB-dependent and Notch-independent mechanism. *Life Sci.* 91 (5-6), 186–193. doi: 10.1016/j.lfs.2012.06.034

Yao, M., Yuan, B., Wang, X., Sato, A., Sakuma, K., Kaneko, K., et al. (2017). Synergistic cytotoxic effects of arsenite and tetrandrine in human breast cancer cell line MCF-7. *Int. J. Oncol.* 51 (2), 587–598. doi: 10.3892/ijo.2017.4052

Yeh, W. L., Lin, H. Y., Wu, H. M., and Chen, D. R. (2014). Combination treatment of tamoxifen with risperidone in breast cancer. *PLoS One* 9 (6), e98805. doi: 10.1371/journal.pone.0098805

Yoshida, G. J. (2017). Therapeutic strategies of drug repositioning targeting autophagy to induce cancer cell death: from pathophysiology to treatment. *J. Hematol. Oncol.* 10 (1), 67. doi: 10.1186/s13045-017-0436-9

Yoshino, Y., Yuan, B., Okusumi, S., Aoyama, R., Murota, R., Kikuchi, H., et al. (2018). Enhanced cytotoxic effects of arsenite in combination with anthocyanidin compound, delphinidin, against a human leukemia cell line, HL-60. *Chemico-Biol. Interact.* 294, 9–17. doi: 10.1016/j.cbi.2018.08.008

Yu, S., Kim, T., Yoo, K. H., and Kang, K. (2017). The T47D cell line is an ideal experimental model to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer. *Biochem. Biophys. Res. Commun.* 486 (3), 752–758. doi: 10.1016/j.bbrc.2017.03.114

Yu, B., Yuan, B., Kiyomi, A., Kikuchi, H., Hayashi, H., Hu, X., et al. (2019). Differentiation induction of human breast cancer cells by arsenite in combination with tetrandrine. *Am. J. Transl. Res.* 11 (12), 7310–7323.

Yuan, B., Ohyama, K., Takeichi, M., and Toyoda, H. (2009). Direct contribution of inducible nitric oxide synthase expression to apoptosis induction in primary smooth chorion trophoblast cells of human fetal membrane tissues. *Int. J. Biochem. Cell Biol.* 41 (5), 1062–1069. doi: 10.1016/j.biocel.2008.09.031

Yuan, B., Yoshino, Y., Kaise, T., and Toyoda, H. (2010). "Application of Arsenic Trioxide Therapy for Patients with Leukaemia," in *Biological Chemistry of Arsenic, Antimony and Bismuth*. Ed. H. Sun (Chichester: John Wiley Sons, Ltd.), 263–292.

Yuan, B., Okusumi, S., Yoshino, Y., Moriyama, C., Tanaka, S., Hirano, T., et al. (2015). Delphinidin induces cytotoxicity and potentiates cytocidal effect in combination with arsenite in an acute promyelocytic leukemia NB4 cell line. *Oncol. Rep.* 34 (1), 431–438. doi: 10.3892/or.2015.3963

Yuan, B., Yao, M., Wang, X., Sato, A., Okazaki, A., Komuro, H., et al. (2018). Antitumor activity of arsenite in combination with tetrandrine against human breast cancer cell line MDA-MB-231 in vitro and in vivo. *Cancer Cell Int.* 18, 113. doi: 10.1186/s12935-018-0613-0

Yuan, B., Shimada, R., Xu, K., Han, L., Si, N., Zhao, H., et al. (2019). Multiple cytotoxic effects of gamabufotalin against human glioblastoma cell line U-87. *Chem. Biol. Interact.* 314, 108849. doi: 10.1016/j.cbi.2019.108849

Zhao, Y., Onda, K., Yuan, B., Tanaka, S., Kiyomi, A., Sugiyama, K., et al. (2018a). Arsenic disulfideinduced apoptosis and its potential mechanism in two and threedimensionally cultured human breast cancer MCF7 cells. *Int. J. Oncol.* 52 (6), 1959–1971. doi: 10.3892/ijo.2018.4357

Zhao, Y., Yuan, B., Onda, K., Sugiyama, K., Tanaka, S., Takagi, N., et al. (2018b). Anticancer efficacies of arsenic disulfide through apoptosis induction, cell cycle arrest, and pro-survival signal inhibition in human breast cancer cells. *Am. J. Cancer Res.* 8 (3), 366–386.

Zhao, Y., Onda, K., Sugiyama, K., Yuan, B., Tanaka, S., Takagi, N., et al. (2019). Antitumor effects of arsenic disulfide on the viability, migratory ability, apoptosis and autophagy of breast cancer cells. *Oncol. Rep.* 41 (1), 27–42. doi: 10.3892/or.2018.6780

Zheng, K., He, Z., Kitazato, K., and Wang, Y. (2019). Selective Autophagy Regulates Cell Cycle in Cancer Therapy. *Theranostics* 9 (1), 104–125. doi: 10.7150/thno.30308

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frontiers in Pharmacology

ORIGINAL RESEARCH
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The Hippo Transducer YAP/TAZ as a Biomarker of Therapeutic Response and Prognosis in Trastuzumab-Based Neoadjuvant Therapy Treated HER2-Positive Breast Cancer Patients

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Background: We explored the therapeutic and prognostic effect of YAP/TAZ intensityinHER2-positive breast cancer patients. We also investigated the relationship between YAP/TAZ expression and Trastuzumab-resistance.

Methods: We collected clinicopathological information from 397 cases. We evaluated therapeutic and prognostic effect of YAP/TAZ and other variables. We also cultivated Trastuzumab-resistance cell lines and explored relationship between YAP/TAZ and Trastuzumab-resistance.

Results: Over-expression of YAP/TAZ was remarkable in Trastuzumab-resistant cells, and so did HER3 and HER2/HER3 heterodimer. Inhibition of YAP/TAZ expression reversed Trastuzumab-resistance.YAP/TAZ deficiency contributed to favorable therapeutic response, and so did hormone receptor insufficiency and chemotherapy dosage inferiority. Deficient YAP/TAZ intensity and abundant hormone receptor intensity contributed to better survival. Over-expression of YAP/TAZ was obvious in recurrent cases in comparison with their matching primary lesions. Prognostic superiority of insufficient YAP/TAZ intensity was more outstanding in hormone receptor negative cases. Over-expression of YAP/TAZ and HER3 was generally synchronous. Absence of HER3 expression in residual lesions might correlate with better breast cancer-free survival.

Conclusions: Over-expression of YAP/TAZ as well as HER-3 and HER2/HER3 heterodimer was synchronously remarkable in Trastuzumab-resistant cell lines. Inhibition of YAP/TAZ expression reversed Trastuzumab resistance. Deficient YAP/TAZ intensity as well as insufficient hormone receptor intensity and high chemotherapy dosage contributed to favorable therapeutic response. Deficient YAP/TAZ intensity and abundant hormone receptor intensity contributed to better survival, and so did absence of HER3expression in residual lesions. Prognostic superiority of YAP/TAZ expression

depended on hormone receptor status. Cases with synchronous over-expression of YAP/TAZ and HER3 suffered poor survival, which revealed the potential effect of YAP/TAZ-HER2/HER3 crosstalk in prognosis of HER2-positive patients.

Keywords: Trastuzumab-based neoadjuvant therapy, YAP/TAZ, HER2/HER3 heterodimer, breast cancer-free interval, SKBR-3 cell lines

INTRODUCTION

Gene amplification resulted in over-expression of the human epidermal growth factor receptor 2 (HER2), which induced shorter disease-free survival and decreased overall survival. Fortunately, routine use of Trastuzumab altered the natural history of HER2 positive breast cancer (Mittendorf et al., 2009). Trastuzumab was a monoclonal antibody that targeted the extracellular domain of HER2 protein and interfered with HER2-mediated signaling cascade, preventing proliferation and eventually leading to cell death (Hudis, 2007). Adjuvant use of Trastuzumab reduced relapse in HER2 positive cases (Perez et al., 2011). Neoadjuvant use of Trastuzumab improved pathological complete response rates (Gianni et al., 2010).

Despite these successes, we noticed that some cases did not respond well to Trastuzumab. For HER2 positive breast cancer patients, disease relapse might occur even after standard anti-HER2 therapy (He et al., 2011; Zhou et al., 2014; Yuan et al., 2015; Zhu et al., 2017; Zhu et al., 2018). Previous literatures indicated that Hippo pathway was an evolutionarily conserved regulator for tissue development (Harvey et al., 2013). Mutations of pathway components caused uncontrolled tissue overgrowth (Tapon et al., 2002), revealing some kind of tumorigenicity (Camargo et al., 2007). Crosstalk of Hippo signaling with other perturbed molecular networks might result in the happening of tumor invasion (Johnson and Halder, 2014). The central role of Hippo pathway focused on degrading of two homologous oncoproteins: the transcriptional co-activator with PDZ-binding motif (TAZ) and Yes-associated protein (YAP). Preliminary clinical studies from a consecutive series of breast cancer patients found that YAP/TAZ over-expression related to shorter disease-free survival, and a statistically obvious correlation between YAP/TAZ and HER2 positivity had also been proved (Bartucci et al., 2015).

In this study, we explored the therapeutic and prognostic effect of YAP/TAZ expression. We also investigated relationship between YAP/TAZ expression and Trastuzumab resistance. We hypothesized that YAP/TAZ-HER2/HER3 crosstalk affected the prognosis of HER2-positive cases. We estimated prognostic effect of YAP/TAZ and HER2/HER3 heterodimer according to our preclinical and clinical findings.

MATERIALS AND METHODS

Study Population

This study enrolled 397 pathology confirmed HER2 positive breast cancer patients from the Breast Cancer Center, Xiangya Hospital, Central South University, between 2012.3 and 2018.3. We excluded patients who suffered inflammatory breast cancer, distant metastasis disease, or bilateral breast tumors. The median follow-up time was 48 months (22–69 months). Xiangya Hospital Ethics Committee reviewed and approved all involved cases. The patients provided their written informed consent to engage in this study.

Study Design and Procedures

In this retrospective study, we gained pathological diagnose *via* core needle biopsy. Cytotoxic therapy was anthacycline and taxane intravenously every 21 days for 8 cycles. Trastuzumab treatment was 8 mg/kg as a loading dose, and then 6mg/kg every 3 weeks for 1 year. All involved cases underwent the above neoadjuvant therapy (NAT). All patients received proper surgical procedure (breast-conserving surgery or modified radical mastectomy) within 1 month after NAT finished. Considering the false negative results of sentinel lymph nodes biopsy after NAT, all patients underwent axillary lymph nodes dissection. Local advanced cases and breast-conserving cases received radiation therapy. Hormone receptor (HR) positive cases underwent proper adjuvant endocrine therapy (**Table 1**).

Formalin-fixed and paraffin-embedded specimens were finally manufactured into 4μm-thick slices and then stained by Hematoxylin and eosin (H&E). All pathological data was available, such as estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki-67 indication. We evaluated signals in both core needle biopsy specimens and residual tumors. The positive status of HR was immunohistochemistry (IHC) staining ER≥1% and/or PR≥1%.The positive status ofHER2 was IHC staining HER2 3+ or FISH +.The positive status of FISH was single-probe average HER2 copy number ≥6.0 signals/cell; or dual-probe HER2/CEP17 ratio of ≥2.0with an average HER2 copy number ≥4.0 signals/cell; or dual-probe HER2/CEP17 ratio of <2.0 with an average HER2 copy number ≥6.0 signals/cell (Wolff et al., 2018).

Our group employed anti-YAP/TAZ to evaluate YAP/TAZ status in diagnostic biopsy specimens (primary lesions and recurrent lesions). We confirmed the positive status of YAP/TAZ when more than 10% tumor cells were nuclear and/or cytoplasmic staining. We graded the IHC staining intensity of YAP/TAZ as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). The calculation method of YAP/TAZ score was multiplying IHC intensity by 1.5 in nuclear stained cases and by0.5 in cytoplasmic stained cases (summing them together in both nuclear and cytoplasmic stained cases). Median score ≤0.5 was YAP/TAZ low expression, whereas median score >0.5 was YAP/TAZ high expression. Two investigators assessed the pathological data independently (Vici et al., 2014).

This study defined pathological complete response (pCR) as no residual invasive breast or lymph node lesions after NAT. We

TABLE 1 | Demographic information of subjects in this study.

Characteristic	No. (n=397)	%
Age (years)		
Median (range)	45 (21-74)	–
≤50	254	63.98
>50	143	36.02
cTNM stage		
II	191	48.11
III	206	51.89
Menstrual status		
Menopause	188	47.36
Non menopause	209	52.64
Histological grade		
I-II	216	54.41
III	181	45.59
Ki67 score (%)		
>14%	223	56.17
≤14%	174	43.83
HR status		
HR+	240	60.45
HR-	157	39.55
YAP/TAZ score		
≤0.5	226	56.93
>0.5	171	43.07
Local therapy[1]		
Mastectomy+ALND+RT	87	21.91
Mastectomy+ALND	236	59.45
BCS+ALND+RT	74	18.64
Pathological response		
pCR	104	26.20
Non-pCR	293	73.80
Lymph nodes after NAC		
>3	281	70.78
≤3	116	29.22
RTDI		
>85%	273	68.77
≤85%	124	31.23

[1]*ALND, axillary lymph node dissection; BCS, breast conserving surgery; RT, radiation therapy.*

employed ultrasound and MRI every 21 days to assess the residual tumor size. We evaluated the clinical response according to the criteria described in solid tumor (RECIST) guideline version 1.1. We calculated objective response rate (ORR) by comparing complete response (CR) and partial response (PR) cases to the total number of involved cases.

Calculation of Dose Intensity

Relative total dose intensity (RTDI): ratio of actual total dose intensity (ATDI) and planned total dose intensity (PTDI) (Loibl et al., 2011).

$$\mathrm{RTDI}(\%) = \frac{\mathrm{ATDi}}{\mathrm{PTDI}} \times 100$$

Planned total dose intensity (PTDI): the planned total dose and the planned treatment duration, average across the chemotherapy agents used.

$$\mathrm{PTDI(mg/week)} = \frac{\text{Planned Total Dose (mg)}}{\text{planned duration of therapy (weeks)}}$$

Actual total dose intensity (ATDI): the ratio of actual total dose and the real treatment duration.

$$\mathrm{ATDI(mg/week)} = \frac{\text{Actual Total Dose (mg)}}{\text{duration of therapy (weeks)}}$$

After calculated separately for each component of the regimen, an average was taken to obtain the final RTDI of the combination.

$$\mathrm{RTDI_{TAC}} = \frac{\mathrm{RTDI_T} + \mathrm{RTDI_A} + \mathrm{RTDI_C}}{3}$$

In this study, we employed SKBR-3 cell line to cultivate Trastuzumab-resistance cell model. We calculated the growth rate of cells *via* colorimetric method. We employed YAP/TAZ inhibitor-1 (Medchemexpress LLC, New Jersy, USA) to evaluate the relationship of YAP/TAZ expression and Trastuzumab resistance. We assessed YAP/TAZ expression according to their localization by western blot analysis. We also used immunodetection assay to estimate the expression of HER3 and HER2/HER3 heterodimer.

Statistical Analysis

We used one-way analysis of variance to clarify relationship between variables and clinical response. We employed logistical regression to explore the impact of variables on pathological remission rate. Univariate analysis and Pearson $\chi 2$ test were both qualified to evaluate the effect of relevant variables on clinical and pathological response in different subgroups. Breast cancer-free interval (BCFI) was proper for the survival analyses, which was the time between surgery and first invasive relapse (local or distant).Cox proportional hazards model was useful to evaluate the prognostic effect of variables, and the results were expressed as hazard ratios (HRs) and 95% confidence intervals (CIs). We employed Kaplan-Meier survival analysis to estimate the relationship between variables and prognosis. We also performed sub-population treatment effect pattern plot (STEPP) methodology and standard method for competing risk analysis to evaluate the disease-specific cumulative incidence and composite recurrence risk. All statistical tests were two-sides and p values less than 0.05 were considered statistically significant. We carried out STEPP analysis using the R software package (R Foundation for Statistical Computing, Vienna, Austria; https://sites.google.com/site/stepprpackage). We carried out other statistical analysis by SPSS version 19.0 for Windows (SPSS Inc, Chicago, USA). Xiangya Clinical Institutional Review Board approved this study. We obtained approvals from the institutional review board before the study procedures began.

RESULTS

Cell Culture and Pharmacological Treatment

Our group noted down cell proliferation at different time intervals. Trastuzumab for present research was residual part

from the clinical practice. The dosage ascended every 7 days (1 to 10 μg/ml). Prior studies indicated that 10 μg/ml was the saturation dose (SD) for SKBR-3 cells (Mittendorf et al., 2006). The cells keeping alive for 7 days during the dosage ascending were cultured and submitted to the subsequent treatment with SD. As the growth rate became synchronous with the parental wild type (WT-SKBR-3) cells, we finally obtained the Trastuzumab-resistant cells (TR-SKBR-3).

Relationship Between YAP/TAZ Expression and Trastuzumab Resistance

As shown in **Figure 1A**, cell vitality of WT-SKBR-3 and TR-SKBR-3 was exactly similar at the starting dose. Subsequently the gap of survival rate became obvious as dose ascending. Furthermore, we synchronously employed YAP/TAZ inhibitor-1 in WT-SKBR-3 and TR-SKBR-3 cells while SD of Trastuzumab was performed. As shown in **Figure 1B**, we observed outstanding difference of vitality between TR cells and WT cells. YAP/TAZ inhibitor reversed Trastuzumab resistance in TR cells, thereby inducing obvious inhibition of their growth rate. By contrast, TR cells always retained outstanding advantage in vitality when YAP/TAZ inhibitor was not performed. These results indicated that over-expression of YAP/TAZ contributed to the resistance of tumor cells to Trastuzumab.

To explore the relationship between YAP/TAZ expression and Trastuzumab resistance, we further estimated YAP/TAZ expression as well as HER3 and HER2/HER3 heterodimer in TR-SKBR-3 cells and WT-SKBR-3 cells. As shown in **Figures 2A, B**, YAP/TAZ expression was generally remarkable in TR-SKBR-3 cells, and nuclear expression of YAP was more outstanding than in cytoplasm. These results correlated over-expression of YAP/TAZ with Trastuzumab resistance and indicated potential localization-dependent expression of YAP/TAZ in TR-SKBR-3 cells. Correspondingly, we also observed remarkable expression of YAP/TAZ in recurrence cases in comparison with their matching primary lesions, which supported the above preclinical findings (**Figures 2C, D**).

To estimate the relationship between HER2/HER3 heterodimer and Trastuzumab resistance, immunodetection assay was performed in the protein obtained from TR cells and WT cells. As shown in **Figures 3** and **4**, over-expression of HER-3 and HER2/HER3 heterodimer was obvious in TR cells in comparison with WT cells. Our findings revealed that HER-3 and HER2/HER3 heterodimer intensity was outstanding in Trastuzumab resistance cells, which were also the YAP/TAZ dominant cells. The synchronous over-expression of YAP/TAZ and HER2/HER3 heterodimer in TR cells correlated the YAP/TAZ- HER2/HER3 crosstalk with Trastuzumab resistance.

Therapeutic Significance of YAP/TAZ Expression

We evaluated the therapeutic effect of variables (e.g., age, menopause status, cTNM stage, histological grade, Ki67, axillary lymph nodes status, RTDI, HR status, and YAP/TAZ status) and showed the results in **Table 2**. Tumor remission was outstanding in YAP/TAZ deficiency cases (p=0.035 for clinical remission and p=0.024 for pathological remission), suggesting that deficient YAP/TAZ expression contributed to better therapeutic response. Insufficient HR intensity and high chemotherapy dosage also contributed to favorable tumor remission (**Table 2**). The therapeutic effect of YAP/TAZ inferiority and HR insufficiency was outstanding when RTDI>85% (**Table 3**). Therapeutic superiority of YAP/TAZ deficiency was also amplified in HR negative patients (**Table 4**). These results indicated that contribution of YAP/TAZ expression to therapeutic response depended on chemotherapy dosage and HR status.

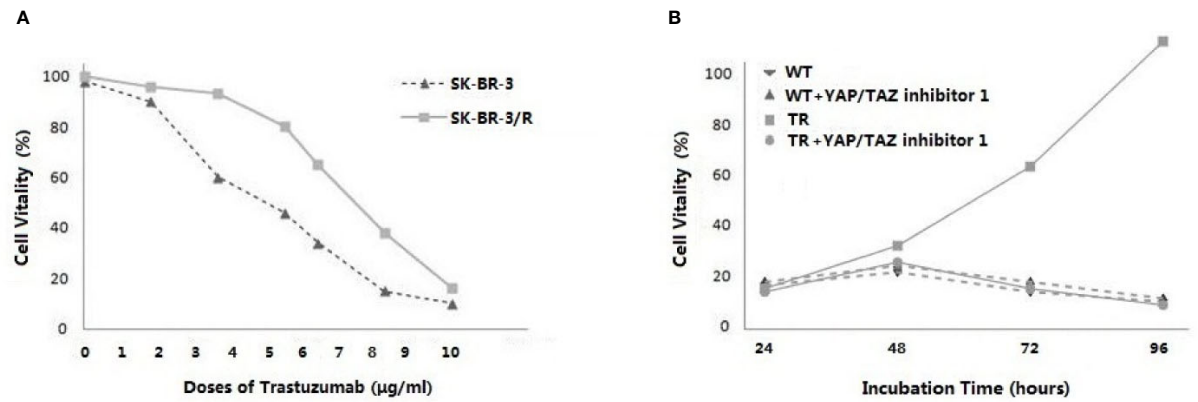

FIGURE 1 | Dose-dependent response was evaluated at different concentrations of Trastuzumab (1 to 10 μg/ml), ascending every 7 days. Cells keeping alive for 7 days during the dosage ascending were collected and cultured with 10μg/ml Trastuzumab. As the growth rate became synchronous with the parental wild type (WT-SKBR-3) cells, the Trastuzumab-resistant cells (TR-SKBR-3) were obtained. Cell vitality of WT-SKBR-3 and TR-SKBR-3 was exactly similar at the starting dose. Subsequently the gap of survival rate became obvious as dose ascending **(A)**. Furthermore, YAP/TAZ inhibitor-1 (Medchemexpress LLC, New Jersy, USA) was synchronously employed in WT-SKBR-3 and TR-SKBR-3 cells while SD of Trastuzumab was performed, in order to evaluate the relationship of YAP/TAZ expression and therapeutic efficacy of Trastuzumab.YAP/TAZ inhibitor reversed the drug resistance, thereby inducing inhibition of vitality in TR cells. By contrast, TR cells without YAP/TAZ inhibitor always retained the outstanding advantage in vitality **(B)**.

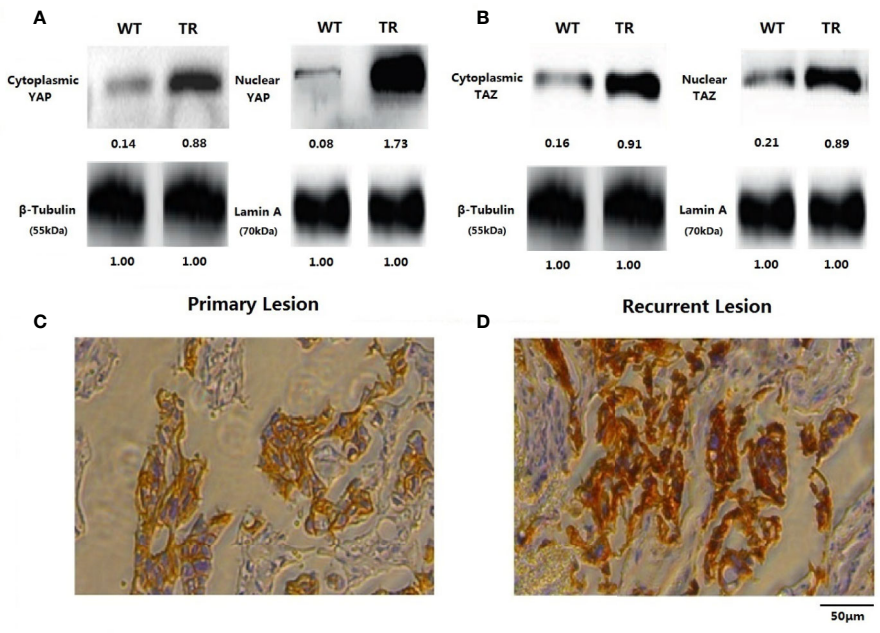

FIGURE 2 | Results of western blot was shown according to the localization of YAP and TAZ. As the weight of reference protein noted, we affixed relative grey level under each band. Nuclear expression of YAP was more outstanding than in cytoplasm **(A)**. Expression of YAP and TAZ were both remarkable in TR-SKBR-3 cells **(A, B)**. Staining intensity of YAP/TAZ in recurrent lesions after first-line Trastuzumab treatment was also significantly stronger than their primary lesions **(C, D)**.

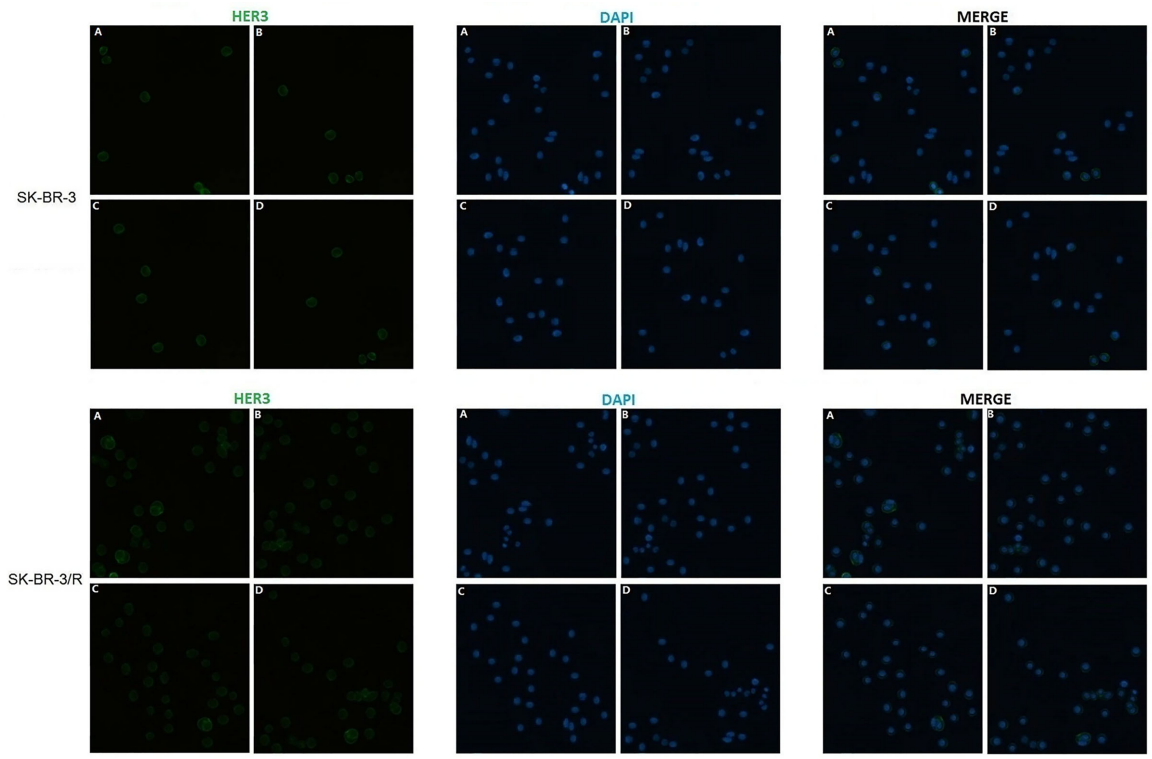

FIGURE 3 | Outstanding increase of HER-3 expression was revealed in TR cells in comparison with WT cells.

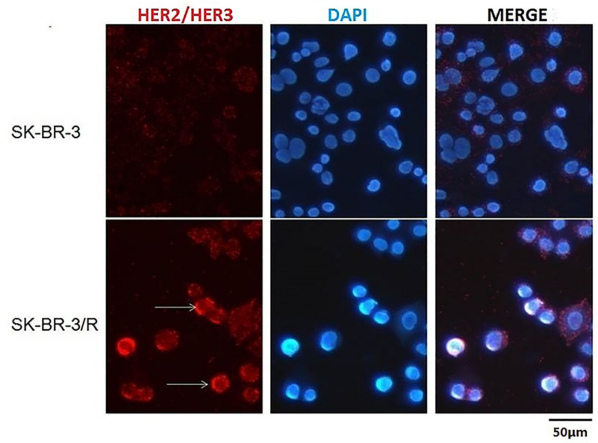

FIGURE 4 | Outstanding increase of HER2/HER3 heterodimer intensity was revealed in TR cells in comparison with WT cells.

Prognostic Significance of YAP/TAZ Expression

As shown in **Figure 5**, inferior YAP/TAZ intensity (p=0.028, OR=0.261, 95%CI 0.081–0.927) and superior HR intensity (p=0.036, OR=0.751, 95%CI 0.279–0.938) correlated with lower recurrence risk. As shown in results of Kaplan-Meier survival analysis, superior HR intensity (p=0.031, **Figure 6A**) and inferior YAP/TAZ intensity (p=0.019, **Figure 6B**) both contributed to improvement of breast cancer-free survival. Compared with HR abundant cases (p=0.057, **Figure 6C**), deficient YAP/TAZ intensity tended to play a more important role in improving the prognosis of HR insufficient patients (p=0.007, **Figure 6D**). These results suggested mutual and interactive prognostic effect of HR intensity and YAP/TAZ expression.

Prognostic Significance of HER3 in Residual Tumors After NAT

According to preclinical study and preliminary clinical research, expression of YAP/TAZ correlated Trastuzumab-resistance and obviously influenced prognostic outcome. In addition, we also noted significant difference of HER3 expression between TR cells and WT cells. Considering the synchronous over-expression of YAP/TAZ and HER2/HER3 heterodimer in TR cells, we hypothesized a potential relationship between HER3 expression and survival in YAP/TAZ sufficient subpopulation. We further evaluated the continuous and composite measure of recurrence risk *via* Cox model including HR, HER2, HER3, and YAP/TAZ in residual tumors, to assess their prognosis effect. Subpopulations with sufficient YAP/TAZ expression generally suffered high composite risk.

Overall, breast cancer-free survival was 90.4% (265/293), ranging from 93.6% in lowest composite risk quartile to 38.9% in highest

TABLE 2 | Association between variables and therapeutic response.

Variables	No.	Clinical response		Pathological response			
		ORR (%)[1]	*P*	pCR (%)	OR	95%CI	*P*
Age							
≤50	254	213 (83.86)	0.526	65 (25.59)	0.836	0.545–1.231	0.189
>50	143	127 (88.81)		39 (27.27)			
Menopause							
Yes	188	163 (86.70)	0.379	49 (26.06)	1.192	0.522–1.340	0.483
No	209	177 (84.69)		55 (26.32)			
cTNM stage							
II	191	168 (87.96)	0.247	50 (26.18)	0.982	0.836–1.407	0.874
III	206	172 (83.50)		54 (26.21)			
Histo-grade							
I-II	216	190 (87.96)	0.883	59 (27.31)	0.985	0.679–1.218	0.260
III	181	150 (82.87)		45 (24.86)			
LN after NAT							
>3	281	238 (84.70)	0.567	74 (26.33)	1.145	0.768–1.374	0.597
≤3	116	102 (87.93)		30 (25.86)			
HR status							
HR+	240	195 (81.25)	0.028	55 (22.92)	0.742	0.508–0.933	0.045
HR-	157	145 (92.36)		49 (31.21)			
Ki67							
>14%	223	191 (85.65)	0.285	58 (26.01)	1.143	0.752–1.393	0.534
≤14%	174	149 (85.63)		46 (26.44)			
YAP/TAZ score							
≤0.5	226	209 (92.48)	0.035	75 (33.19)	0.570	0.482–0.894	0.024
>0.5	171	131 (76.61)		29 (16.96)			
RTDI							
>85%	273	250 (91.58)	0.041	78 (28.57)	0.776	0.517–0.904	0.039
≤85%	124	90 (72.58)		26 (20.97)			

[1]*In this study, 340 patients were observed clinical response (complete or partial remission).*

TABLE 3 | Dose dependent therapeutic response of YAP/TAZ and hormone receptor (HR) status.

Variables	Therapy response							
	RTDI ≤ 85%				RTDI >85%			
	ORR (%)	*P*	pCR	*P*	ORR (%)	*P*	pCR	*P*
YAP/TAZ status								
≤0.5	79.52	0.314	10/57	0.096	98.36	0.035	65/169	0.012
>0.5	71.13		16/67		78.07		13/104	
HR status								
HR+	70.35	0.078	15/75	0.102	84.63	0.041	40/165	0.005
HR-	78.80		11/49		97.98		38/108	

TABLE 4 | Dose dependent therapeutic efficacy of YAP/TAZ in different hormone receptor (HR) status.

HR status	YAP/TAZ status	Therapy response							
		RTDI ≤ 85%				RTDI >85%			
		ORR (%)	*P*	pCR	*P*	ORR (%)	*P*	pCR	*P*
Positive	≤0.5	78.47	0.691	6/35	0.154	96.34	0.047	29/99	0.038
	>0.5	66.10		9/40		80.67		11/66	
Negative	≤0.5	82.66	0.352	4/22	0.098	99.01	0.011	36/70	0.005
	>0.5	74.34		7/27		77.06		2/38	

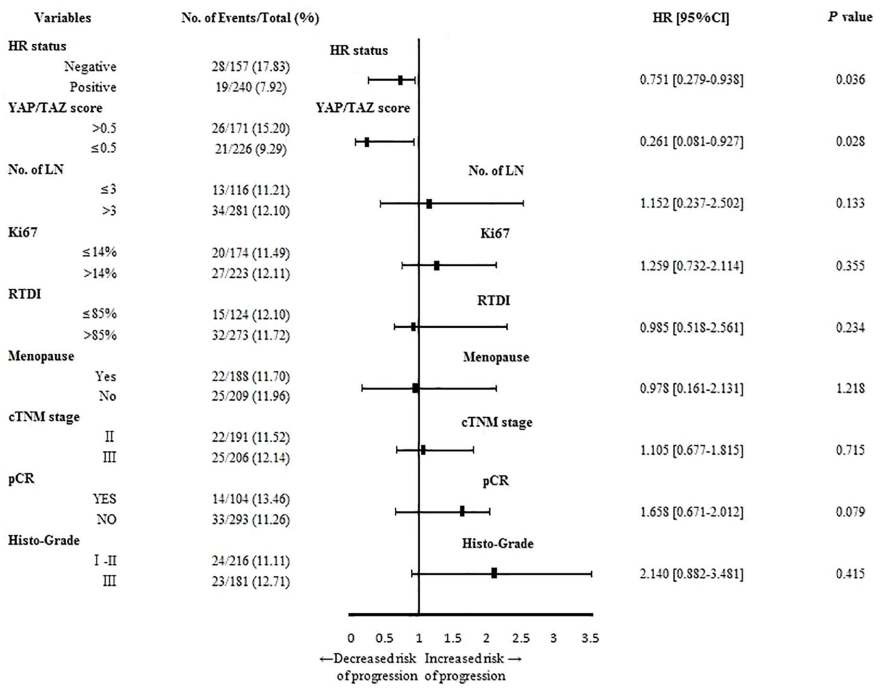

FIGURE 5 | YAP/TAZ insufficiency (p=0.028, OR=0.261, 95%CI 0.081–0.927) and positive HR status (p=0.036, OR=0.751, 95%CI 0.279–0.938) contributed to reduce relapse risk of breast cancer.

composite risk quartile. The continuum of composite risk was also illustrated, ranging from 0.12 in lowest composite risk subpopulation to 3.24 in highest composite risk subpopulation.

As shown in **Figure 7**, prognostic benefit of residual HER3 negative populations was consistently significant when composite risk>1. The discrepancy of survival between subpopulations absolutely rose synchronously with the continuous increasing of composite risk. As shown in **Figure 8**, survival benefit of subpopulations was similar when composite risk was low. In contrast, prognostic superiority of residual HER3 negative

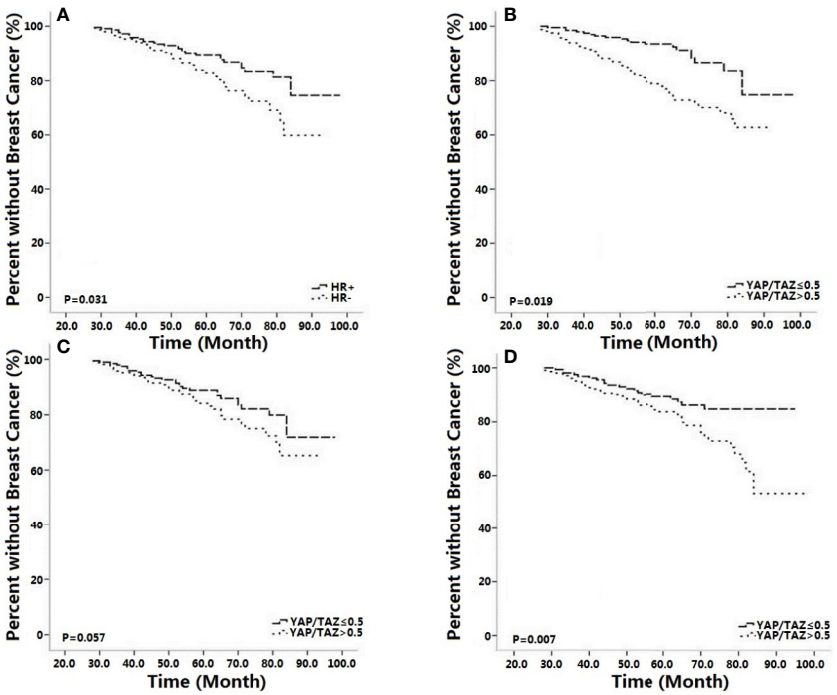

FIGURE 6 | Positive hormone receptor (HR) status [(p=0.031, **(A)]** and inferior YAP/TAZ intensity [(p=0.019, **(B)**] both improved breast cancer free survival. Compared with HR positive cases [(p=0.057, **(C)**], YAP/TAZ insufficiency was more likely to improve outcomes of HR negative patients [(p=0.007, **(D)**].

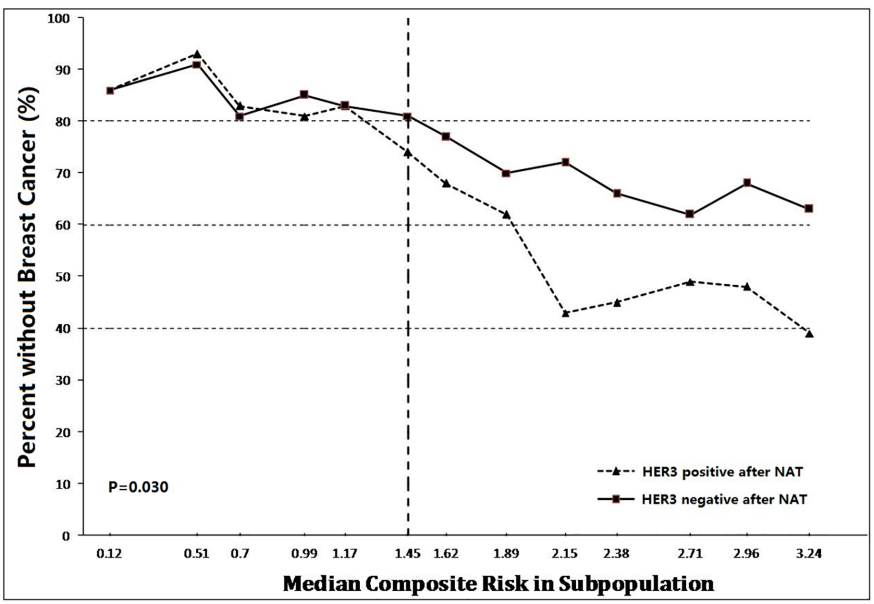

FIGURE 7 | Prognostic benefit of residual HER3 negative populations was consistently significant when composite risk>1. Thereafter, the discrepancy of survival between subpopulations was absolutely raised synchronously with the continuous increasing of composite risk (p=0.03).

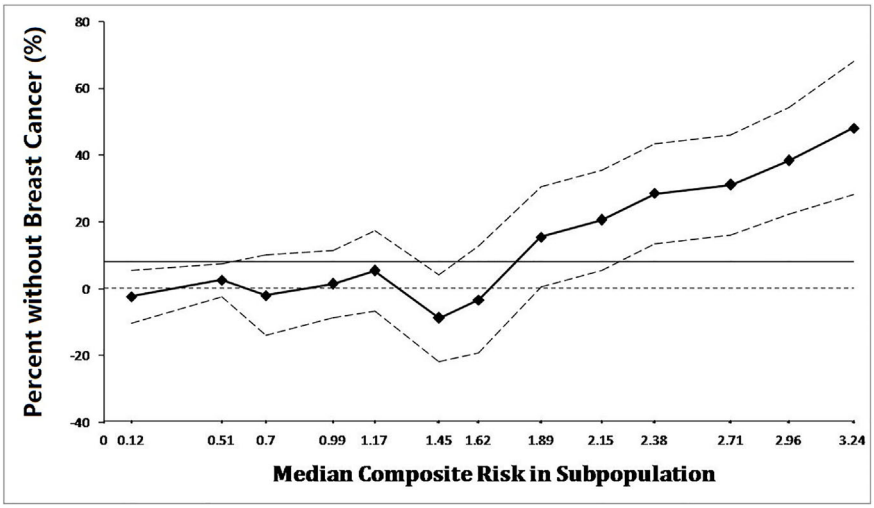

FIGURE 8 | Survival of subpopulations was extremely similar when composite risk was low. In contrast, prognostic superiority of residual HER3 negative subpopulation was outstanding when composite risk rise, as their relapse free survival (>60%) was 40% more than HER3 positive cases (nearly 20%) when composite risk reached the highest point of 3.24.

subpopulation was outstanding when composite risk rise, as their relapse free survival (>60%) was 40% more than HER3 positive cases (nearly 20%) when composite risk reached the highest point of 3.24(p=0.03). These findings revealed that cases with synchronous over-expression of YAP/TAZ and HER3 tended to suffer poor survival, which generally accorded with the results of the above *in vitro* experiments.

DISCUSSION

YAP and TAZ were transcriptional co-activators ubiquitously related with tissue development, and involved in the invasion of breast cancer (Bartucci et al., 2015). They obtained phosphorylation through the Hippo pathway and gained activation *via* cellular density (Zhao et al., 2007; Ota and Sasaki, 2008; Kim et al., 2011). TAZ played important role in the occurrence of breast cancer drug resistance (Cordenonsi et al., 2011). In this study, we revealed the synchronous over-expression of YAP/TAZ as well as HER-3 and HER2/HER3 heterodimer in Trastuzumab-resistant cell lines. We found that inhibition of YAP/TAZ expression reversed Trastuzumab resistance. We clarified the outstanding effect of YAP/TAZ expression in therapeutic response and survival of HER2 positive patients. We also revealed that synchronous over-expression of YAP/TAZ and HER3 contributed to poor survival, which supported the potential prognostic effect of YAP/TAZ-HER2/HER3 crosstalk.

Relationship Between YAP/TAZ and Multiple Cancer-Associated Features

YAP/TAZ expression widely involved in migration and invasion of breast cancer cells (Mi et al., 2015). Knockdown of YAP/TAZ reduced the above migration and invasion (Chan et al., 2008). TAZ promoted a luminal to basal lineage switch, which was confirmed by its depletion in basal and epithelial cells promoted luminal differentiation (Skibinski et al., 2014). Prior studies also reported the similar transforming potential of YAP. Over-expression of YAP caused inhibition of apoptosis and anchorage-independent growth, which induced tumorigenic transformation (Overholtzer et al., 2006). Inhibition of YAP suppressed tumor development and tumor metastasis in a mouse model of breast cancer, suggested that cooperating genetic events were necessary for generating a neoplastic phenotype (Chen et al., 2014). At the preclinical research level, prior literatures prompted that YAP/TAZ played an important role in resistance to anti-cancer drugs and other cancer-associated features such as tumor cell migration and metastasis. At clinical study level, YAP/TAZ expression also related to tumor metastasis and prognostic outcomes.

Role of YAP/TAZ in Breast Cancer Outcomes

Hippo pathway was essential in various pathological processes of breast cancer development. Disturbance Hippo pathway promoted breast cancer metastasis through multiple mechanisms. As the crucial component of Hippo pathway, YAP/TAZ expression played a critical role in tumor cell migration and colonization in tissues (Bos et al., 2009). Prior studies evaluated relationship between YAP/TAZ expression and survival of breast cancer patients, and explored the potential function of YAP/TAZ as a predictive clinical biomarker.

Prior studies had proved that over-expression of YAP related to tumorigenicity (Wang et al., 2012). YAP dysfunction relieved lung metastasis in a genetically engineered mouse model of

breast cancer. Phosphorylated HER3 caused activation of YAP/TAZ in tumor cells, which finally induced bone metastasis (Li et al., 2017). Nuclear expression of TAZ in bone metastasis lesion was significantly higher than its expression in primary tumors (Bartucci et al., 2015).

Mutation in the Hippo signaling pathway also contributed to chemo-resistance of cancer cells, while the absence of TAZ obviously defused the chemo-resistance (Bartucci et al., 2015). Prior studies indicated that over-expression of TAZ promoted chemo-resistance in MCF10 breast cell line and depressed the chemo-sensitivity of MDA-MB-231 breast cell line (Lai et al., 2011). Cultured MCF-10A cell line was competent for YAP activation in invasive breast cancer (Lee et al., 2019). The MDA-MB-231 breast cancer cell line and MCF-7 cell lines were both qualified in research of YAP related breast cancer progression (Hua et al., 2015). Prior study based on MDA-MB-468 and human breast cancer cell line ZR-75-30 had also indicated that YAP/TAZ promoted breast cancer metastasis (Wang et al., 2018).

Patients with superior TAZ expression suffered high risk of tumor relapse and poor outcomes. Over-expression of TAZ caused obvious decline of recurrence-free survival (51.7% in over-expression group versus 78% in negative group; p=0.014) (Bartucci et al., 2015). Activation of TAZ during the metastatic procedure was also observed by comparing primary and metastases lesions (Matteucci et al., 2013). Staining intensity and cellular localization of TAZ brought out a TAZ-based score, which predicted the pathological response of NAT-treated HER2 positive breast cancer (Vici et al., 2014). Over-expression of TAZ might induce residues of HER2-positive tumors after NAT, and high nuclear intensity of TAZ induced poor clinical outcomes (Di Benedetto et al., 2017).

As shown in this study, tumor remission was significant in patients with inferior YAP/TAZ intensity, suggesting that YAP/TAZ expression might relate to the therapeutic efficacy of NAT. Tumor remission of YAP/TAZ insufficient cases was outstanding when RTDI>85%, suggested that therapeutic superiority of insufficient YAP/TAZ was dose-depended (**Tables 2**–**4**). Inferior YAP/TAZ intensity also contributed to reduce the risk of relapse and induce encouraging survival (**Figures 5** and **6**), and these results were similar with the previous findings. According to the results of *in vitro* experiments, over-expression of YAP/TAZ was obvious in Trastuzumab-resistance cells (**Figure 2**), suggested that superior intensity of YAP/TAZ might contribute to the occurrence of drug resistance. Inhibition of YAP/TAZ reversed the above resistance, thereby resuming the therapeutic efficacy of Trastuzumab (**Figure 1**). According to the clinical study of this program, insufficient expression of YAP/TAZ contributed to better survival, while over-expression of YAP/TAZ raised the relapse risk. Correspondingly, we indeed observed remarkable expression of YAP/TAZ in recurrence lesions. These encouraging preclinical and clinical findings provided ideas in the treatment of Trastuzumab resistant cases. Moreover, with the further research of Trastuzumab resistance, doctors should pay attention to individualized treatment. We needed adjusted therapy according to drug sensitivity, without monotony or repetitious tasks. Early intervention might reduce disease relapse in potential drug resistance cases.

Different Trait of YAP/TAZ According to Distinct Subtypes

Prior studies clarified that YAP/TAZ affected the biological behavior of tumor cells according to molecular subtypes of breast cancer (Diaz-Martin et al., 2015). Analysis of TAZ expression in 640 distinct phenotypes of breast cancer patients suggested that over-expression of TAZ was obviously associated with negative HR status, while other literatures reported over-expression of TAZ in HR positive breast cancer (Bartucci et al., 2015; Wang et al., 2016).

Over-expression of YAP/TAZ appeared to be a shared trait according to the intrinsic subtypes, which was associated with prognostic outcomes (Vici et al., 2014; Skibinski et al., 2014; Kim et al., 2014; Bartucci et al., 2015). Superior expression of TAZ was confirmed in basal-like cases in comparison with HR positive patients (Skibinski et al., 2014). Over-expression of TAZ also caused the descent of survival in basal-like cases (Skibinski et al., 2014), and tended to appear synchronously with the existence of HER2 positive subtype (Bartucci et al., 2015). Preclinical studies also claimed that over-expression TAZ was present in HER2-driven mammary tumors (Serrano et al., 2013).

As shown in this study, negative status of HR contributed to tumor remission, and therapeutic superiority of inferior YAP/TAZ intensity was upward in HR negative patients (**Tables 2**–**4**). Although inferior intensity of YAP/TAZ and positive HR status contributed to reduce the risk of relapse and improve survival, superiority of YAP/TAZ insufficiency was more likely to be amplified in HR negative cases (**Figures 5** and **6**). These results suggested that contributions of YAP/TAZ expression to therapeutic efficacy and prognostic outcomes obviously depended on HR status. According to our findings, therapeutic and prognostic effect of HR and YAP/TAZ was mutual and interactive. Inferior HR intensity amplified the therapeutic and prognostic advantage of YAP/TAZ insufficient cases.

Crosstalk Between Hippo Pathway and Other Signaling Pathways

Wide crosstalk between Hippo pathway and other signaling pathways formed complex cellular signaling networks, which obviously affected the development and metastasis of tumors. The activation of AKT increased the probability of YAP to boost the proliferation of MCF10A cells (Overholtzer et al., 2006). Knockdown of YAP inhibited a series of cytokines and vascular invasion of breast cancer cells (Sharif et al., 2015). These prior studies suggested that breast cancer cells might regulate vascular invasiveness *via* YAP and Hippo pathway.

YAP directly activated Pik3cb expression, while YAP required Pik3cb to promote cells proliferation and activate the AKT pathway (Lin et al., 2015). Pik3cb served as a crucial association between Hippo-YAP and PI3K-AKT pathways (Lin

et al., 2015). Mutually stimulatory cross-talk between YAP and PI3K established a feed forward regulatory circuit. YAP increased expression of the PI3K subunit Pik3cb, and PI3K stimulated YAP activity, thereby promoting tumor cells proliferation and survival (Lin et al., 2015). Prior studies also reported that YAP regulated cell metabolism, which was the well-described function of PI3K-AKT signaling (Lin et al., 2015).

ERβ obviously influenced the activation of HER2/HER3/Akt pathways, while the activated HER2/HER3 heterodimer indicated notable activation of PI3K/Akt pathway (Lindberg et al., 2011). Existing of ERβ obviously inhibited the phosphorylated procedure of HER2/HER3 (Lindberg et al., 2011). Previous literatures also observed the up-regulation of HER2 and down-regulation of HER3 in ERβ over-expression cells (Lindberg et al., 2011).

Based on these findings, we attempted to explore the relationship between YAP/TAZ expression and Trastuzumab resistance. As we observed in our preclinical study, HER-3 and HER2/HER3 heterodimer intensity was outstanding in Trastuzumab resistance cells (**Figures 3** and **4**), which was also the YAP/TAZ dominant cells. The synchronous over-expression of YAP/TAZ and HER2/HER3 heterodimer suggested the crosstalk between Hippo-YAP and PI3K-AKT signaling pathways. According to the activation effect of ERβ to HER2/HER3 and Akt pathways, the above crosstalk was more obvious in ER insufficiency cases. Correspondingly, we observed outstanding prognostic inferiority in cases who suffered synchronous over-expression of YAP/TAZ and HER3 (**Figures 7** and **8**). Considering the similar synchronous over-expression in Trastuzumab resistance cells, we believed that YAP/TAZ-HER2/HER3 crosstalk played crucial role in prognosis of HER2 positive patients.

As a single center retrospective study, we were aware the following limitations of our results. Considering the potential localization-dependent expression, we should focus on the nuclear intensity of YAP/TAZ in our future work. Further study should pay more attention to the comparison of primary and residual lesions, which might carry out more meaningful ideas to research about Trastuzumab resistance. Both the primary and recurrence expression of YAP/TAZ should be considered in calculation of composite risk. Besides the assessment of YAP/TAZ in the tumor cells, further studies should also focus on the stromal tissues, thereby identifying the appropriate micro-environment for YAP/TAZ activation. As the above improvement carried out, YAP/TAZ-based biomarker would be more effective for the therapeutic and prognostic evaluation.

CONCLUSIONS

Over-expression of YAP/TAZ as well as HER-3 and HER2/HER3 heterodimer was synchronously remarkable in Trastuzumab-resistant cell lines. Inhibition of YAP/TAZ expression reversed Trastuzumab resistance. Deficient YAP/TAZ intensity as well as insufficient hormone receptor intensity and high chemotherapy dosage contributed to favorable therapeutic response. Deficient YAP/TAZ intensity and abundant hormone receptor intensity contributed to better survival, and so did absence of HER3 expression in residual lesions. Prognostic superiority of YAP/TAZ expression depended on hormone receptor status. Cases with synchronous over-expression of YAP/TAZ and HER3 suffered poor survival, which revealed the potential effect of YAP/TAZ-HER2/HER3 crosstalk in prognosis of HER2-positive patients.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Xiangya Hospital Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-QY and ZX: concept, design, definition of intellectual content, manuscript review. JY: literature search, clinical studies, experimental studies. JY and N-HD: data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing. All authors contributed to the article and approved the submitted version.

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REFERENCES

Bartucci, M., Dattilo, R., Moriconi, C., Pagliuca, A., Mottolese, M., Federici, G., et al. (2015). TAZ is required for metastatic activity and chemoresistance of breast cancer stem cells. *Oncogene* 34, 681–690. doi: 10.1038/onc.2014.5

Bos, P. D., Zhang, X. H., Nadal, C., Shu, W., Gomis, R. R., Nguyen, D. X., et al. (2009). Genes that mediate breast cancer metastasis to the brain. *Nature* 459, 1005–1009. doi: 10.1038/nature08021

Camargo, F. D., Gokhale, S., Johnnidis, J. B., Fu, D., Bell, G. W., Jaenisch, R., et al. (2007). YAP1 increases organ size and expands undifferentiated progenitor cells. *Curr. Biol.* 17, 2054–2060. doi: 10.1016/j.cub.2007.10.039

Chan, S. W., Lim, C. J., Guo, K., Ng, C. P., Lee, I., Hunziker, W., et al. (2008). A role for TAZ in migration, invasion, and tumorigenesis of breast cancer cells. *Cancer Res.* 68, 2592–2598. doi: 10.1158/0008-5472.CAN-07-2696

Chen, Q., Zhang, N., Gray, R. S., Li, H., Ewald, A. J., Zahnow, C. A., et al. (2014). A temporal requirement for Hippo signaling in mammary gland differentiation, growth, and tumorigenesis. *Genes Dev.* 28, 432–437. doi: 10.1101/gad.233676.113

Cordenonsi, M., Zanconato, F., Azzolin, L., Forcato, M., Rosato, A., Frasson, C., et al. (2011). The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. *Cell* 147, 759–772. doi: 10.1016/j.cell.2011.09.048

Di Benedetto, A., Mottolese, M., Sperati, F., Ercolani, C., Di Lauro, L., Pizzuti, L., et al. (2017). Association between AXL, Hippo Transducers, and Survival

Outcomes in Male Breast Cancer. *J. Cell Physiol.* 232, 2246–2252. doi: 10.1002/jcp.25745

Diaz-Martin, J., Lopez-Garcia, M. A., Romero-Perez, L., Atienza-Amores, M. R., Pecero, M. L., Castilla, M. A., et al. (2015). Nuclear TAZ expression associates with the triple-negative phenotype in breast cancer. *Endocr. Relat. Cancer* 22, 443–454. doi: 10.1530/ERC-14-0456

Gianni, L., Eiermann, W., Semiglazov, V., Manikhas, A., Lluch, A., Tjulandin, S., et al. (2010). Neoadjuvant chemotherapy with trastuzumab followed by adjuvant trastuzumab versus neoadjuvant chemotherapy alone, in patients with HER2-positive locally advanced breast cancer (the NOAH trial): a randomised controlled superiority trial with a parallel HER2-negative cohort. *Lancet* 375, 377–384. doi: 10.1016/S0140-6736(09)61964-4

Harvey, K. F., Zhang, X., and Thomas, D. M. (2013). The Hippo pathway and human cancer. *Nat. Rev. Cancer* 13, 246–257. doi: 10.1038/nrc3458

He, M., Guo, Q., and Hu, G. (2011). Reversed urban-rural differences in breast cancer mortality (China, 2002-2008). *Breast Cancer Res. Treat.* 126, 231–234. doi: 10.1007/s10549-010-1276-2

Hua, K., Yang, W., Song, H., Song, J., Wei, C., Li, D., et al. (2015). Up-regulation of miR-506 inhibits cell growth and disrupt the cell cycle by targeting YAP in breast cancer cells. *Int. J. Clin. Exp. Med.* 8, 12018–12027.

Hudis, C. A. (2007). Trastuzumab–mechanism of action and use in clinical practice. *N Engl. J. Med.* 357, 39–51. doi: 10.1056/NEJMra043186

Johnson, R., and Halder, G. (2014). The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment. *Nat. Rev. Drug Discov.* 13, 63–79. doi: 10.1038/nrd4161

Kim, N. G., Koh, E., Chen, X., and Gumbiner, B. M. (2011). E-cadherin mediates contact inhibition of proliferation through Hippo signaling-pathway components. *Proc. Natl. Acad. Sci. U. S. A.* 108, 11930–11935. doi: 10.1073/pnas.1103345108

Kim, S. K., Jung, W. H., and Koo, J. S. (2014). Yes-associated protein (YAP) is differentially expressed in tumor and stroma according to the molecular subtype of breast cancer. *Int. J. Clin. Exp. Pathol.* 7, 3224–3234. doi: 10.1186/bcr3632

Lai, D., Ho, K. C., Hao, Y., and Yang, X. (2011). Taxol resistance in breast cancer cells is mediated by the hippo pathway component TAZ and its downstream transcriptional targets Cyr61 and CTGF. *Cancer Res.* 71, 2728–2738. doi: 10.1158/0008-5472.CAN-10-2711

Lee, J. Y., Chang, J. K., Dominguez, A. A., Lee, H. P., Nam, S., Chang, J., et al. (2019). YAP-independent mechanotransduction drives breast cancer progression. *Nat. Commun.* 10, 1848. doi: 10.1038/s41467-019-09755-0

Li, C., Wang, S., Xing, Z., Lin, A., Liang, K., Song, J., et al. (2017). A ROR1-HER3-lncRNA signalling axis modulates the Hippo-YAP pathway to regulate bone metastasis. *Nat. Cell Biol.* 19, 106–119. doi: 10.1038/ncb3464

Lin, Z., Zhou, P., von Gise, A., Gu, F., Ma, Q., Chen, J., et al. (2015). Pi3kcb links Hippo-YAP and PI3K-AKT signaling pathways to promote cardiomyocyte proliferation and survival. *Circ. Res.* 116, 35–45. doi: 10.1161/CIRCRESAHA.115.304457

Lindberg, K., Helguero, L. A., Omoto, Y., Gustafsson, J. A., and Haldosen, L. A. (2011). Estrogen receptor beta represses Akt signaling in breast cancer cells via downregulation of HER2/HER3 and upregulation of PTEN: implications for tamoxifen sensitivity. *Breast Cancer Res.* 13, R43. doi: 10.1186/bcr2865

Loibl, S., Skacel, T., Nekljudova, V., Luck, H. J., Schwenkglenks, M., Brodowicz, T., et al. (2011). Evaluating the impact of Relative Total Dose Intensity (RTDI) on patients' short and long-term outcome in taxane- and anthracycline-based chemotherapy of metastatic breast cancer- a pooled analysis. *BMC Cancer* 11, 131. doi: 10.1186/1471-2407-11-131

Matteucci, E., Maroni, P., Luzzati, A., Perrucchini, G., Bendinelli, P., and Desiderio, M. A. (2013). Bone metastatic process of breast cancer involves methylation state affecting E-cadherin expression through TAZ and WWOX nuclear effectors. *Eur. J. Cancer* 49, 231–244. doi: 10.1016/j.ejca.2012.05.006

Mi, W., Lin, Q., Childress, C., Sudol, M., Robishaw, J., Berlot, C. H., et al. (2015). Geranylgeranylation signals to the Hippo pathway for breast cancer cell proliferation and migration. *Oncogene* 34, 3095–3106. doi: 10.1038/onc.2014.251

Mittendorf, E. A., Storrer, C. E., Shriver, C. D., Ponniah, S., and Peoples, G. E. (2006). Investigating the combination of trastuzumab and HER2/neu peptide vaccines for the treatment of breast cancer. *Ann. Surg. Oncol.* 13, 1085–1098. doi: 10.1245/ASO.2006.03.069

Mittendorf, E. A., Wu, Y., Scaltriti, M., Meric-Bernstam, F., Hunt, K. K., Dawood, S., et al. (2009). Loss of HER2 amplification following trastuzumab-based neoadjuvant systemic therapy and survival outcomes. *Clin. Cancer Res.* 15, 7381–7388. doi: 10.1158/1078-0432.CCR-09-1735

Ota, M., and Sasaki, H. (2008). Mammalian Tead proteins regulate cell proliferation and contact inhibition as transcriptional mediators of Hippo signaling. *Development* 135, 4059–4069. doi: 10.1242/dev.027151

Overholtzer, M., Zhang, J., Smolen, G. A., Muir, B., Li, W., Sgroi, D. C., et al. (2006). Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proc. Natl. Acad. Sci. U. S. A.* 103, 12405–12410. doi: 10.1073/pnas.0605579103

Perez, E. A., Romond, E. H., Suman, V. J., Jeong, J. H., Davidson, N. E., Geyer, C. E. Jr., et al. (2011). Four-year follow-up of trastuzumab plus adjuvant chemotherapy for operable human epidermal growth factor receptor 2-positive breast cancer: joint analysis of data from NCCTG N9831 and NSABP B-31. *J. Clin. Oncol.* 29, 3366–3373. doi: 10.1200/JCO.2011.35.0868

Serrano, I., McDonald, P. C., Lock, F., Muller, W. J., and Dedhar, S. (2013). Inactivation of the Hippo tumour suppressor pathway by integrin-linked kinase. *Nat. Commun.* 4, 2976. doi: 10.1038/ncomms3976

Sharif, G. M., Schmidt, M. O., Yi, C., Hu, Z., Haddad, B. R., Glasgow, E., et al. (2015). Cell growth density modulates cancer cell vascular invasion via Hippo pathway activity and CXCR2 signaling. *Oncogene* 34, 5879–5889. doi: 10.1038/onc.2015.44

Skibinski, A., Breindel, J. L., Prat, A., Galvan, P., Smith, E., Rolfs, A., et al. (2014). The Hippo transducer TAZ interacts with the SWI/SNF complex to regulate breast epithelial lineage commitment. *Cell Rep.* 6, 1059–1072. doi: 10.1016/j.celrep.2014.02.038

Tapon, N., Harvey, K. F., Bell, D. W., Wahrer, D. C., Schiripo, T. A., Haber, D., et al. (2002). salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* 110, 467–478. doi: 10.1016/S0092-8674(02)00824-3

Vici, P., Mottolese, M., Pizzuti, L., Barba, M., Sperati, F., Terrenato, I., et al. (2014). The Hippo transducer TAZ as a biomarker of pathological complete response in HER2-positive breast cancer patients treated with trastuzumab-based neoadjuvant therapy. *Oncotarget* 5, 9619–9625. doi: 10.18632/oncotarget.2449

Wang, X., Su, L., and Ou, Q. (2012). Yes-associated protein promotes tumour development in luminal epithelial derived breast cancer. *Eur. J. Cancer* 48, 1227–1234. doi: 10.1016/j.ejca.2011.10.001

Wang, J., Rouse, C., Jasper, J. S., and Pendergast, A. M. (2016). ABL kinases promote breast cancer osteolytic metastasis by modulating tumor-bone interactions through TAZ and STAT5 signaling. *Sci. Signal* 9, ra12. doi: 10.1126/scisignal.aad3210

Wang, T., Mao, B., Cheng, C., Zou, Z., Gao, J., Yang, Y., et al. (2018). YAP promotes breast cancer metastasis by repressing growth differentiation factor-15. *Biochim. Biophys. Acta Mol. Basis Dis.* 1864, 1744–1753. doi: 10.1016/j.bbadis.2018.02.020

Wolff, A. C., Hammond, M. E. H., Allison, K. H., Harvey, B. E., Mangu, P. B., Bartlett, J. M. S., et al. (2018). Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. *J. Clin. Oncol.* 142 (11),1364–1382. doi: 10.1200/JCO.2018.77.8738

Yuan, J. Q., Wang, S. M., Tang, L. L., Mao, J., Wu, Y. H., Hai, J., et al. (2015). Relative dose intensity and therapy efficacy in different breast cancer molecular subtypes: a retrospective study of early stage breast cancer patients treated with neoadjuvant chemotherapy. *Breast Cancer Res. Treat.* 151, 405–413. doi: 10.1007/s10549-015-3418-z

Zhao, B., Wei, X., Li, W., Udan, R. S., Yang, Q., Kim, J., et al. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev.* 21, 2747–2761. doi: 10.1101/gad.1602907

Zhou, J., Yan, Y., Guo, L., Ou, H., Hai, J., Zhang, C., et al. (2014). Distinct outcomes in patients with different molecular subtypes of inflammatory breast cancer. *Saudi Med. J.* 35, 1324–1330.

Zhu, Q. N., Wang, G., Guo, Y., Peng, Y., Zhang, R., Deng, J. L., et al. (2017). LncRNA H19 is a major mediator of doxorubicin chemoresistance in breast cancer cells through a cullin4A-MDR1 pathway. *Oncotarget* 8, 91990–92003. doi: 10.18632/oncotarget.21121

Zhu, Q. N., Renaud, H., and Guo, Y. (2018). Bioinformatics-based identification of miR-542-5p as a predictive biomarker in breast cancer therapy. *Hereditas* 155, 17.

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Screening and Identification of Key Biomarkers in Acquired Lapatinib-Resistant Breast Cancer

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Lapatinib, targeting the human epidermal growth factor receptor family members HER1 and HER2, has been approved by the US Food and Drug Administration for use in metastatic HER2-positive breast cancer. However, resistance to lapatinib remains a common challenge to HER2-positive metastatic breast cancer. Until now, the molecular mechanisms of acquired resistance to lapatinib (ALR) have remained unclear. With no definite biomarkers currently known, we aimed to screen for key biomarkers in ALR. In this research, we identified 55 differentially expressed genes (DEGs, 20 upregulated, 35 downregulated) through bioinformatic analysis using microarray datasets GSE16179, GSE38376, and GSE51889 from the Gene Expression Omnibus (GEO) database. The related gene function was explored using the Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The protein-protein interaction (PPI) network was constructed with the Search Tool for the Retrieval of Interacting Genes (STRING) and Cytoscape. The functional enrichment of the DEGs was analyzed, including negative regulation of the B cell apoptotic process, DNA replication, solute:proton symporter activity, synthesis, and degradation of ketone bodies, and metal sequestration by antimicrobial proteins. Analysis of seven hub genes revealed their concentration mainly in DNA replication and cell cycle. Survival analysis revealed that *MCM10* and *SPC24* may be related with poor prognosis in patients with ALR. Meanwhile, the prediction model of lapatinib sensitivity was constructed, and emerging role of the model was further analyzed using several webtools. In conclusion, hub genes are involved in the complex mechanisms underlying ALR in breast cancer and provide favorable support for treatment of ALR in future.

Keywords: HER2-positive breast cancer, acquired lapatinib resistance, biological markers, hub genes, prognostic analysis

INTRODUCTION

Based on molecular markers, breast cancer is divided into four subgroups: luminal A, luminal B, basal-like, and human epidermal growth factor receptor 2 (HER2)-enriched (Perou et al., 2000). Receptor tyrosine-protein kinase HER2, also known as erbB-2, is included in the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (Oh and Bang, 2020). HER2 is overexpressed in 20%–25% of breast cancer patients. HER2 over-expression is known as an aggressive tumor phenotype and is associated with worse survival (Parakh et al., 2017). Lapatinib, a reversible tyrosine kinase inhibitor with specificity for both EGFR and HER2, is approved for treating HER2-positive metastatic breast cancer after disease progression with trastuzumab therapy (Gradishar, 2013; Moasser and Krop, 2015). Compared with capecitabine monotherapy, lapatinib in combination with capecitabine improved objective response rate and progression-free survival (Geyer et al., 2006). Despite the effectiveness of lapatinib in HER2-positive breast cancer, acquired resistance remains a major clinical obstacle. D'Amato et al. have pointed out multiple mechanisms of ALR in breast cancers, including activation of compensatory pathways, mutation of the HER2 kinase domain, and gene amplification (D'Amato et al., 2015). Critically, there are currently no definite biomarkers to predict patients' responses to lapatinib.

With the development of gene sequencing and bioinformatics, increasing number of genetic studies have revealed the mechanism of tumorigenesis and drug resistance. By introducing microarray data and bioinformatic analysis that have been widely applied to investigate whole expression of genes in cancer, researchers have deepened their understanding of the differentially expressed genes (DEGs) and functional enrichment analysis among the complex diseases (Zhu et al., 2017). Although there are some bioinformatic studies corresponding to resistance to anti-HER2 therapies, scarce data and different laboratory conditions make it difficult to acquire reliable results. To overcome the limitation of insufficient data, we identified DEGs through bioinformatic analysis with three Gene Expression Omnibus (GEO) microarray datasets. Additionally, the related gene function was explored with Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, and the protein-protein interaction (PPI) network was determined to express functions to establish a solid theoretical framework for potential molecular mechanisms. In the present study, we aimed to screen for biomarkers in ALR and found that there were 55 DEGs and 7 hub genes, which may be potential biological markers and provide a theoretical support for further treatment of ALR. The specific prediction model was established to evaluate the relationship between clinicopathologic characteristics of breast cancer patients and sensitivity of lapatinib.

MATERIALS AND METHODS

Selection of Microarray Datasets

The GEO (http://www.ncbi.nlm.nih.gov/geo) provides a public platform to obtain different datasets from high-throughput gene expression and genomic hybridization experiments, including platforms, samples, and series (Edgar et al., 2002). We chose the key words "lapatinib resistant" and the organism 'Homo sapiens' (**Figure 1**). Three gene expression datasets [GSE16179 (Liu et al., 2009), GSE38376 (Komurov et al., 2012), GSE51889 (Chen et al., 2013)] were selected from GEO. GSE16179 contained 3 lapatinib-sensitive cell samples and 3 lapatinib-resistant cell samples. GSE38376 contained 3 lapatinib-sensitive cell samples and 3 lapatinib-resistant cell samples. GSE51889 contained 2 lapatinib-sensitive cell samples and 2 lapatinib-resistant cell samples. To establish lapatinib-resistant cells, BT474 and SKBR3 were treated with 1 μM of lapatinib (**Table 1**). The series matrix file and platform were downloaded to convert the probes into the corresponding gene symbol, using Practical Extraction and Report Language (Perl) (https://www.perl.org/) scripts.

Definition of DEGs

Three datasets were merged using Perl scripts to obtain more genes fully. To reduce deviation in data processing, we normalized batch effect using "sva" package in R software and screened the DEGs between lapatinib-sensitive cell samples and lapatinib-resistant cell samples using the "limma" package in R software (http://www.r-project.org/) (Davis and Meltzer, 2007). The difference was considered significant when |logFC| (fold change) was ≥ 1 and P value was < 0.05.

Enrichment Analysis of DEGs

The web-based Gene SeTAnaLysis Toolkit (WebGestalt) (http://www.webgestalt.org/) is one of the most widely used online databases that helps researchers extract biological information from genes of interest (Liao et al., 2019). The goal of GO is to provide a vocabulary that can be applied to the shared genes and proteins, annotating genes, and analyzing biological process (Ashburner et al., 2000). KEGG is a database that sheds light on higher-order functional behaviors from molecular information generated by genome sequencing and other high-throughput experimental techniques (Kanehisa, 2002). Reactome functions as an extended version of a classic metabolic map (Jassal et al., 2020). To confirm characteristic biological functions of DEGs, analyses were performed using WebGestalt. The difference was considered significant when P value was < 0.05 and top 10 would be selected.

Construction of PPI Network

The PPI network was utilized to reveal many functional relationships and interactions among predicted target proteins using the Search Tool for the Retrieval of Interacting Genes (STRING) (version 11.0) database (http://string-db.org) (Szklarczyk et al., 2015). An interaction score > 0.4 was considered statistically significant, and disconnected nodes in the network were hidden. Cytoscape (version 3.7.2) is a bioinformatics software platform for visualizing modules of the PPI network (Smoot et al., 2011). The cytoHubba of Cytoscape is an application for exploring important hubs and clustering an interactome network with topological algorithms (Chin et al., 2014). For each module, the GO, KEGG, and reactome analysis were performed using WebGestalt.

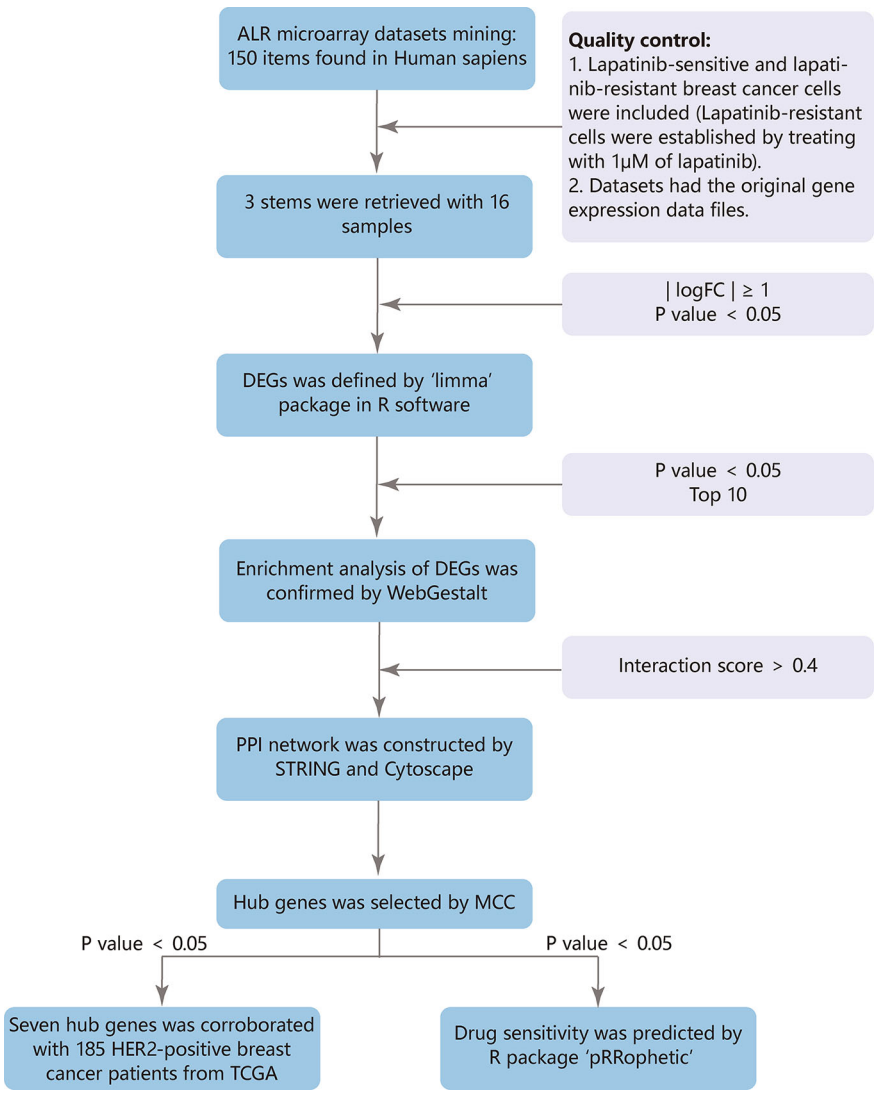


FIGURE 1 | Flow chart.

TABLE 1 | Characteristics of individual studies selected from GEO.

Dataset	Platform	Samples (Cancer cell)		Drug
		Lapatinib-sensitive	Lapatinib-resistant	
GSE16179	GPL570	3 (BT474)	3 (BT474-J4)	Lapatinib
GSE38376	GPL6947	3 (SKBR3)	3 (SKBR3-R)	Lapatinib
GSE51889	GPL6480	2 (SKBR3、BT474)	2 (SKBR3,lapatinib-resistant、BT474,lapatinib-resistant)	Lapatinib

GEO, Gene Expression Omnibus. BT474、SKBR3: an HER2-positive and lapatinib-sensitive cell line. BT474-J4、SKBR3-R: an acquired lapatinib resistant cell line.

Selection of Hub Genes

The hub genes were selected with the Maximal Clique Centrality algorithm (MCC). A network of their co-expressed genes was appraised by GeneMANIA (http://genemania.org/), which can provide gene function and extend the list with similar genes (Warde-Farley et al., 2010). Efficient hierarchical cluster analysis of hub genes was performed using UCSC Cancer Genomics Browser (http://xena.ucsc.edu/) (Kent et al., 2002). To perform survival analysis in a larger number of patients, gene expression data and clinicopathologic data of breast cancer patients were downloaded from The Cancer Genome Atlas (TCGA) at the UCSC Cancer Genomics Browser (http://xena.ucsc.edu/). The

survival analysis of hub genes was constructed by the R packages "survival" and "survminer". The difference was considered significant when P value was < 0.05.

Prediction of Drug Sensitivity

Based on the data from TCGA of patients diagnosed with breast cancer, and drug sensitivity data from the Cancer Genome Project (CGP), the prediction model was performed using R package "pRRophetic" (Garnett et al., 2012; Geeleher et al., 2014a). The half-maximal inhibitory concentration (IC50) of lapatinib in each treated patient was obtained by ridge regression, and the prediction accuracy was measured through 10-fold cross-validation. The default parameters were chosen, including "combat" for removing the batch effect, "allSolidTumors" for tissue type, and mean value for identifying the duplicate gene expression (Geeleher et al., 2014b). The median IC50 was selected for risk stratification to develop a specific model: lower IC50 was more sensitive to lapatinib. GraphPad Prism 7.0 was used to analyze data, and categorical data was compared by chi-square test. The difference was considered significant when P value was < 0.05.

RESULTS

Definition of DEGs in ALR

After normalization of the microarray data, 14,653 genes were merged among three datasets. Fifty-five DEGs are shown in the volcano plot (**Figure 2A**), consisting of 20 upregulated genes and 35 downregulated genes between lapatinib-sensitive cells and lapatinib-resistant cells.

Enrichment Analysis of DEGs

To obtain a comprehensive understanding of the biological effect of 55 DEGs, WebGestalt was applied to describe functional enrichment (**Supplementary Table 1**). GO analysis indicated that negative regulation of B cell apoptotic process, glomerular mesangium development, sequestering of metal ion, DNA-dependent DNA replication, and protein autoubiquitination were the top 5 relevant biological process (**Figure 3A**). For the cellular component, replication terms were mainly enriched (**Figure 3A**). Molecular functions were concentrated mainly on solute:proton symporter activity, chromatin binding, and carbohydrate:proton symporter activity (**Figure 3A**). KEGG pathway analysis revealed that synthesis and degradation of ketone bodies, terpenoid backbone biosynthesis, and butanoate metabolism were three of the most enriched pathways (**Figure 3B**). Reactome pathway analysis demonstrated that DEGs were mainly enriched in metal sequestration by antimicrobial proteins, DNA replication, and cellular hexose transport (**Figure 3B**).

Construction of PPI Network

To understand the biological activity at the protein level, an integrated PPI network of these DEGs was performed (**Figure 2B**), and the most important module was constructed using Cytoscape (**Figure 2C**, **Supplementary Table 2**). Genes in the module were concentrated mainly on cell cycle and DNA replication (**Figures 3C, D**, **Supplementary Table 3**).

Selection of Hub Genes

The hub genes were calculated by Matthews Correlation Coefficient (MCC), and the top seven genes were selected. A network of their co-expressed genes was interpreted by GeneMANIA (**Figure 4**). The hub genes could distinguish breast cancer samples from normal tissue samples through hierarchical clustering (**Figures 5A–C**). When the hub genes were expressed highly, an increasing number of samples presented estrogen receptor status (**Figure 5D**). The survival analysis of hub genes from TCGA was verified in 185 patients diagnosed with HER2-positive breast cancer. Patients with high *MCM10* and *SPC24* expression showed worse overall survival (**Figure 6**).

Prediction of Drug Sensitivity

The specific prediction model could evaluate the relationship between breast cancer patients and sensitivity of lapatinib (**Figure 7**, **Table 2**). A cohort involving 664 breast cancer patients with clinicopathologic data from TCGA was analyzed. The distributions of patients' age and gender were not significantly different between the low-IC50 and high-IC50 groups. The high-IC50 group tended to include more non-white patients (chi-square test, P = 0.03) and more patients with stage II cancer (chi-square test, P = 0.0003). More patients with infiltrating lobular carcinoma and mixed histology were included in low-IC50 group (chi-square test, P = 0.0004). Interestingly, there were more patients with both HER2-positive and hormone receptor-positive and luminal subtype in low-IC50 group (chi-square test, P < 0.0001) indicating that the status of hormone receptor could influence lapatinib sensitivity.

DISCUSSION

Although lapatinib has been approved for treating HER2-positive metastatic breast cancer after trastuzumab failure, acquired resistance to lapatinib (ALR) remains a major clinical challenge. However, the molecular mechanisms of ALR remain unclear. Thus, in-depth study of the mechanism of ALR and discovery of biomarkers with high sensitivity and specificity are of great value to improve the prognosis of patients with HER2-positive breast cancer. Microarray data and bioinformatic analysis have enabled us to explore the whole expression of genes in ALR and improved our understanding of DEGs and functional pathways in complex diseases.

In the current study, DEGs were analyzed using three mRNA microarray datasets between lapatinib-sensitive cell samples and lapatinib-resistant cell samples. Fifty-five DEGs were analyzed, consisting of 20 upregulated genes and 35 downregulated genes. Functional enrichment analysis of GO and KEGG revealed that the majority of DEGs were associated with the following cellular processes: cell cycle, development, apoptosis, and signal transduction. In particular, several terms, including GO terms

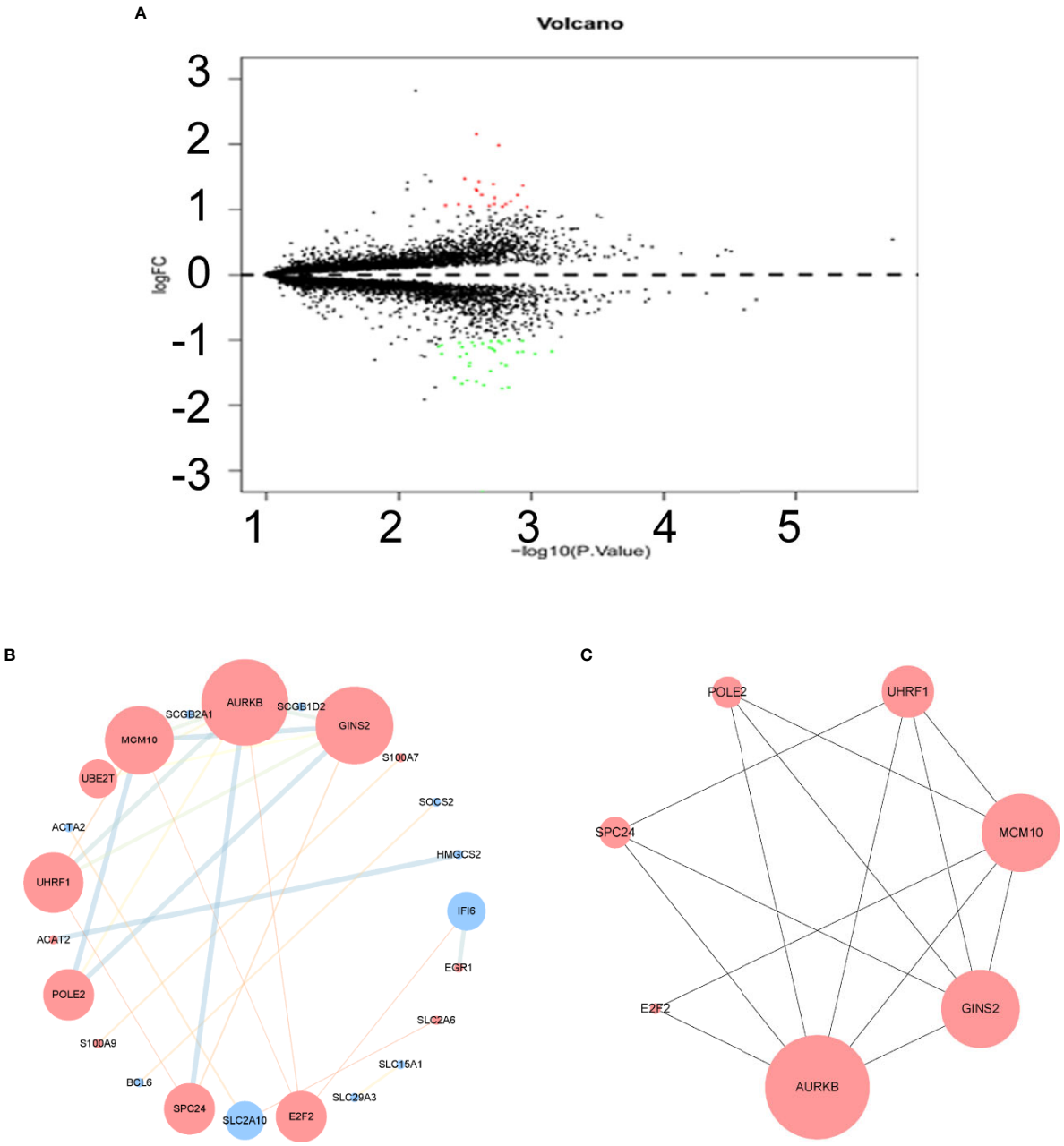


FIGURE 2 | Volcano plot, protein-protein interaction (PPI) network and the most significant module of differentially expressed genes (DEGs). **(A)** DEGs were selected with |logFC|(fold change) ≥ 1 and P value < 0.05 among GSE16179, GSE38376, GSE51889. Upregulated genes are marked in red; downregulated genes are marked in green. **(B)** The PPI network of DEGs was constructed using Cytoscape. Map Node Size to degree, low values to small sizes; upregulated genes are marked in red and downregulated genes are marked in blue. Map Edge Size (Color) to combined score, low values to small sizes (bright colors). **(C)** The most significant module was obtained from PPI network with 7 nodes and 14 edges. Top 7 nodes are ranked by Maximal Clique Centrality algorithm (MCC).

"negative regulation of B cell apoptotic process", "replication fork", "solute:proton symporter activity", KEGG terms "synthesis and degradation of ketone bodies", "terpenoid backbone biosynthesis", and reactome terms "metal sequestration by antimicrobial proteins", "DNA replication", were closely related with classic mechanisms of ALR in breast cancers. The hub genes were calculated by MCC, and the top seven genes including *AURKB*, *GINS2*, *MCM10*, *UHRF1*, *POLE2*, *SPC24*, and *E2F2* were presumed to be associated with drug resistance. Seven hub genes were later corroborated with TCGA data from 185 HER2-positive breast cancer patients. Survival analysis showed that *MCM10* and *SPC24* may be related with poor prognosis in patients with

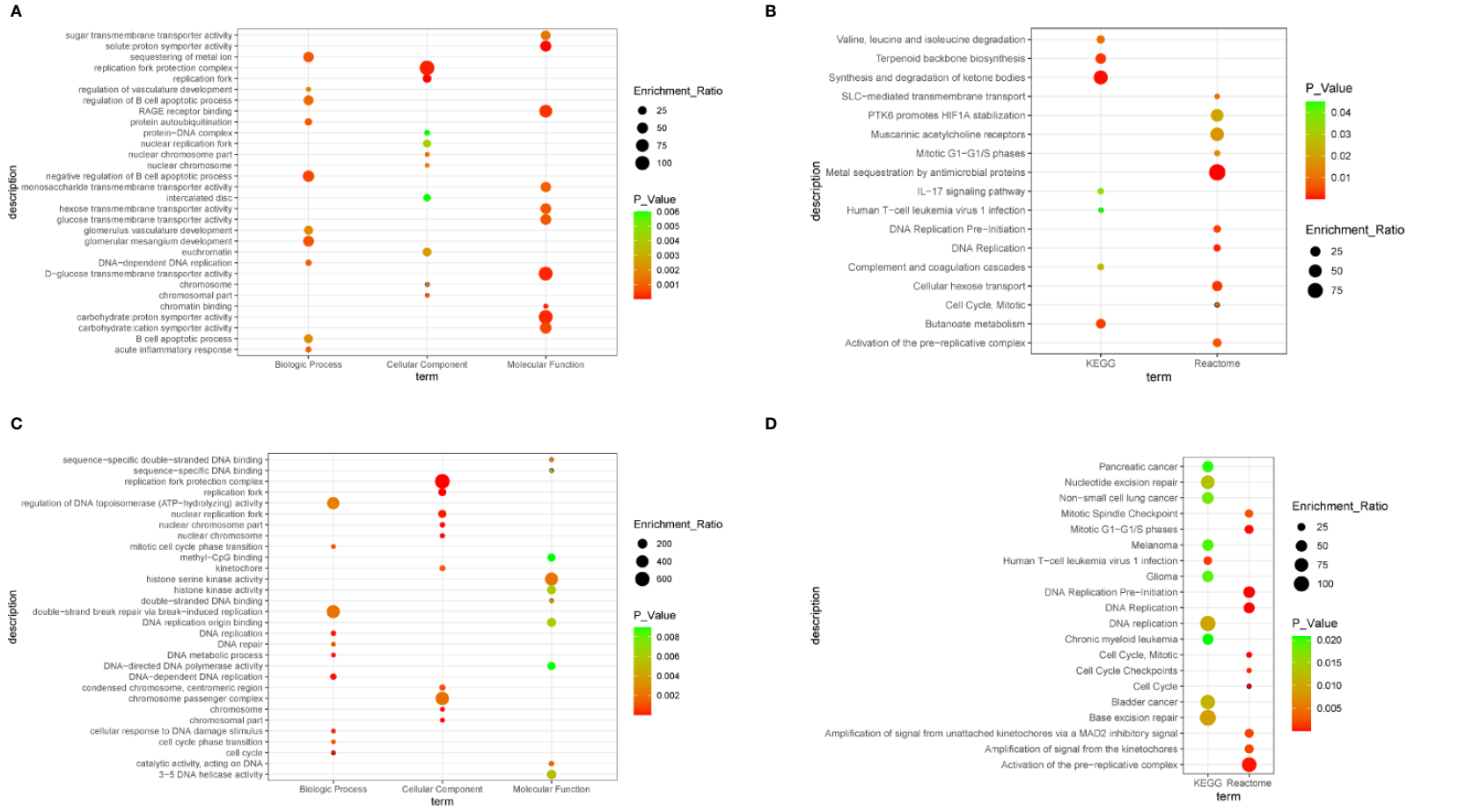
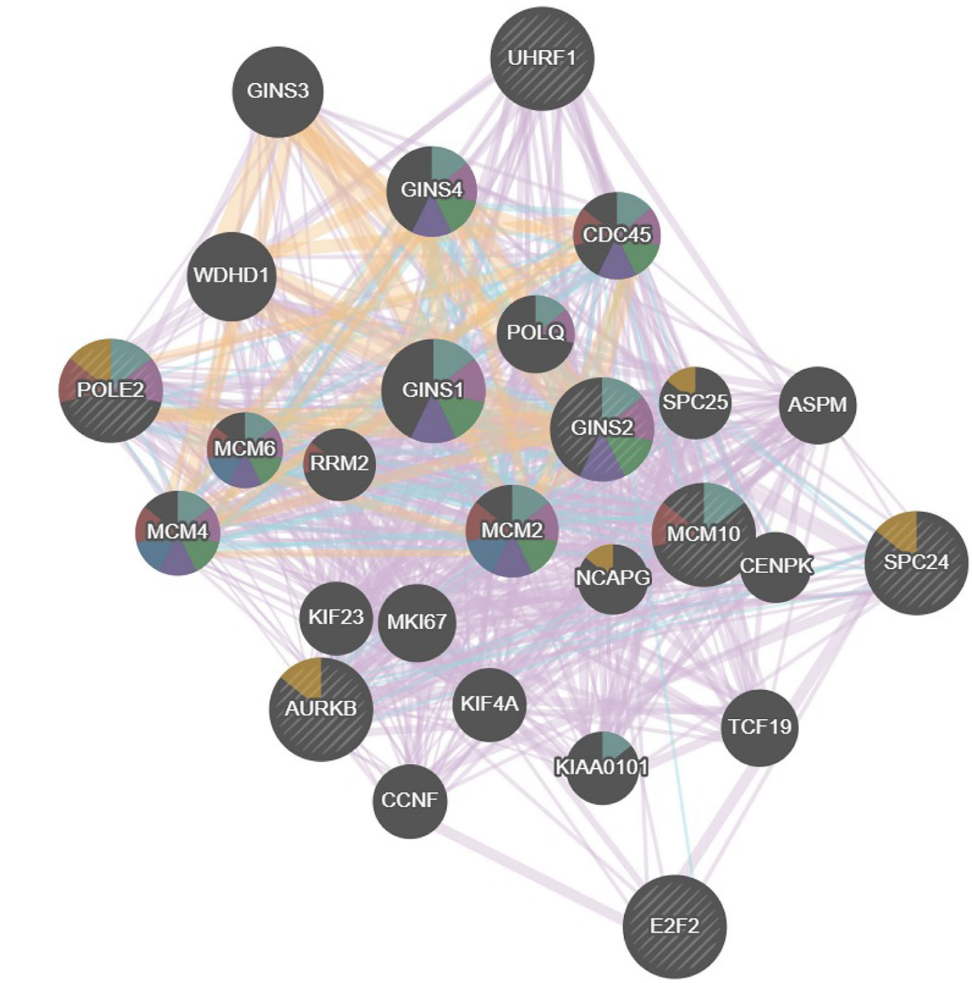


FIGURE 3 | Bubble plot. **(A)** The significantly enriched gene ontology (GO) terms of differentially expressed genes (DEGs), with P value < 0.05. **(B)** Gene networks identified through Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome analysis of DEGs, with P value < 0.05. **(C)** The significantly enriched GO terms of hub genes, with P value < 0.05. **(D)** Gene networks identified through KEGG and Reactome analysis of hub genes, with P value < 0.05.



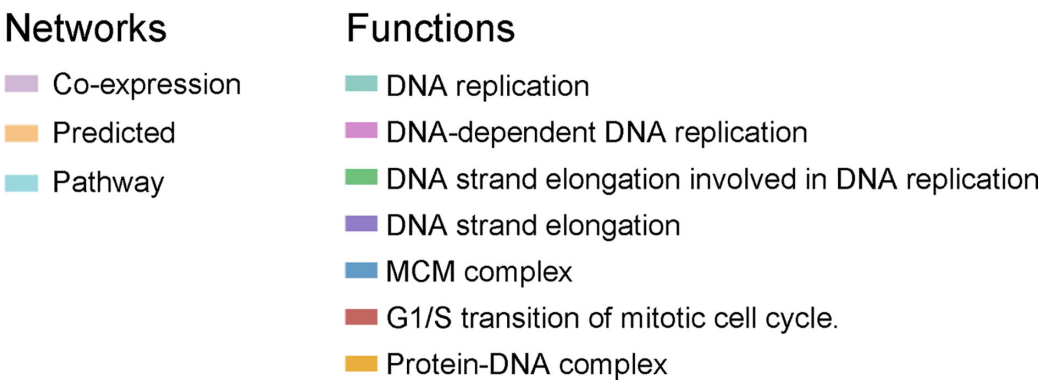


FIGURE 4 | Interaction network. A network of the hub genes and their co-expressed genes was analyzed using GeneMANIA.

acquired lapatinib resistance. Furthermore, the specific prediction model revealed that the distributions of patients' races, pathologic tumor stages, histological types, and molecular subtypes were significantly different between low-IC50 and high-IC50 groups.

MCM10, a highly conservative mini-chromosome maintenance protein, is involved in the initiation of eukaryotic genome replication. In a recent study, MCM10 induced migration and invasion of breast cancer *via* the Wnt/β-catenin pathway (Yang and Wang, 2019). SPC24, a component in the kinetochore

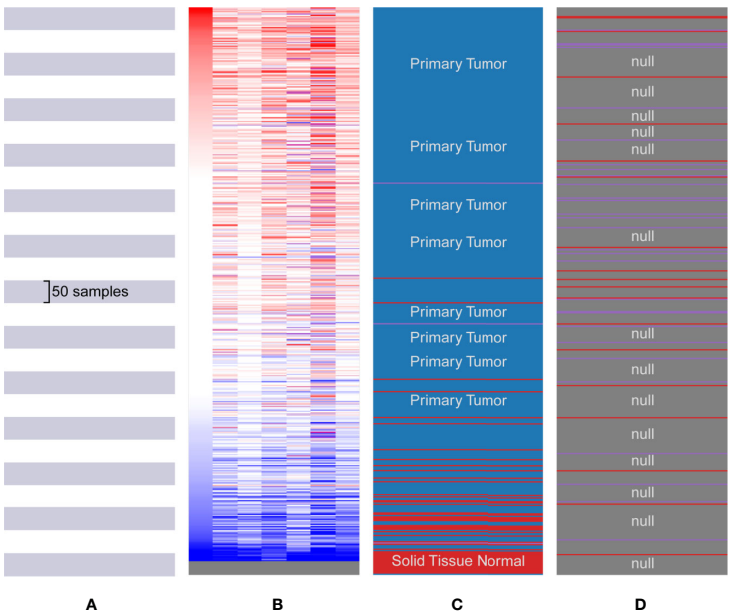


FIGURE 5 | Biological process analysis of the hub genes. **(A)** The Cancer Genome Atlas (TCGA) samples. **(B)** Upregulation of genes is marked in red; downregulation of genes is marked in blue. **(C)** Hierarchical clustering of hub genes was constructed using UCSC. The samples under the red bar are normal solid tissues and the samples under the blue bar are primary tissues. **(D)** Positive metastatic breast cancinoma estrogen receptor are marked in purple; negative in red.

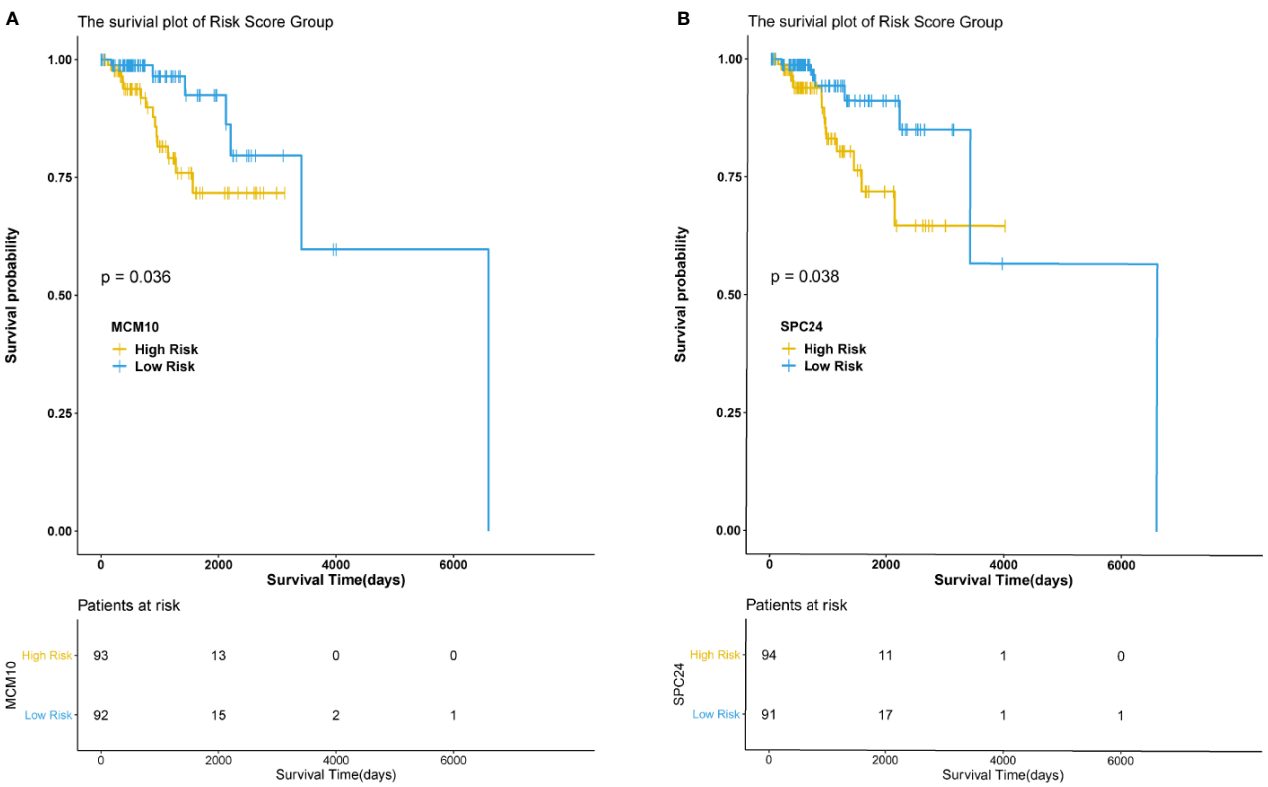


FIGURE 6 | Survival analysis of the hub genes. P < 0.05 was considered statistically signifificant. **(A)** Overall survival by low and high MCM10 expression. **(B)** Overall survival by low and high SPC24 expression.

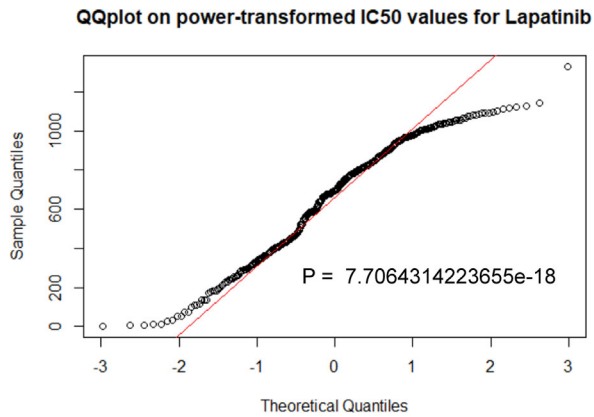


FIGURE 7 | The prediction model was performed by R package "pRRophetic". P < 0.05 was considered statistically significant.

microtubule interface, is associated with tumorigenic transformation (Zhu et al., 2015). Additionally, SPC24—which monitors the PI3K/AKT kinase pathway—is overexpressed in breast cancer, implying its importance in clinical treatment (Zhou et al., 2018). Although E2F2 was not found to be of significance in survival analysis, it could be a gene of interest. E2F2, a member of the E2F family, is typically repressed by the retinoblastoma protein pRB. E2F transcription factors exist in the CDK4/6-RB1 pathway, and this pathway is often dysregulated in hormone receptor-positive breast cancer (Spring et al., 2020). Many researches have reported that E2F1-3 transcripts are highly expressed in HER2-positive tumors (Andrechek, 2015; Wu et al., 2015). Nikolai et al. found that the potential for combined targeting of HER2 and CDK signaling pathways may be a prospective strategy (Nikolai et al., 2016). Therefore, CDK4/6 inhibitors may overcome resistance to lapatinib.

Overall, seven hub genes were analyzed, and results showed that these genes were concentrated mainly on DNA replication and cell cycle. Most interestingly, after analyzing the hub genes, we found that several signaling pathways may be related with ALR. Thus, many targeted drugs could be expected to reverse ALR.

CONCLUSION

In conclusion, the overexpression of *AURKB*, *GINS2*, *MCM10*, *UHRF1*, *POLE2*, *SPC24*, and *E2F2* in HER2-positive breast cancer patients with ALR showed that these hub genes could be potential prognostic biomarkers in such patients. Survival analysis revealed that high *MCM10* and *SPC24* expression were negative prognostic factors in patients with acquired lapatinib

TABLE 2 | Clinicopathologic characteristics of patients in different risk groups in TCGA BRCA cohort.

Characteristics	Whole cohort(n = 664)	Low-IC50(n = 332)	High-IC50(n = 332)	P value
Age				0.55
< 50 years	192 (28.9%)	92 (27.7%)	100 (30.1%)	
≥50 years	472 (71.1%)	240 (72.3%)	232 (69.9%)	
Gender				0.70
Female	7 (1.1%)	4 (1.2%)	3 (0.9%)	
Male	657 (98.9%)	328 (98.8%)	329 (99.1%)	
Race				0.03
Asian	38 (5.7%)	16 (4.8%)	22 (6.6%)	
Black	137 (20.6%)	56 (8.4%)	81 (24.4%)	
White	438 (66.0%)	232 (69.9%)	206 (62.1%)	
Pathologic tumor stage				0.0003
Stage I	111 (16.7%)	63 (19.0%)	48 (14.5%)	
Stage II	387 (58.3%)	168 (50.6%)	219 (66.0%)	
Stage III	147 (22.1%)	92 (27.7%)	55 (16.6%)	
Stage IV	9 (1.4%)	6 (1.8%)	3 (0.9%)	
Histological type				0.0004
Infiltrating Ductal Carcinoma	477 (71.8%)	223 (67.2%)	254 (76.5%)	
Infiltrating Lobular Carcinoma	126 (19.0%)	84 (25.3%)	42 (12.7%)	
Medullary Carcinoma	6 (0.9%)	0 (0)	6 (1.8%)	
Mucinous Carcinoma	10 (1.5%)	3 (0.9%)	7 (2.1%)	
Mixed Histology	15 (2.3%)	9 (2.7%)	6 (1.8%)	
Metaplastic Carcinoma	8 (1.2%)	3 (0.9%)	5 (1.5%)	
Other	21 (3.2%)	10 (3.0%)	11 (3.3%)	
Molecular subtype				<0.0001
HER2+, HR-	13 (2.0%)	5 (1.5%)	8 (2.4%)	
HER2+, HR+	64 (9.6%)	45 (13.6%)	19 (5.7%)	
TNBC	165 (24.9%)	27 (8.1%)	138 (41.6%)	
Luminal	395 (59.5%)	246 (74.1%)	149 (44.9%)	

TCGA, The Cancer Genome Atlas; BRCA, breast cancer; IC50, half-maximal inhibitory concentration; HR, hormone receptor; TNBC, triple-negative breast cancer.
Patients with information unavailable on race (51 patients, 7.7%), pathologic tumor stage (10 patients, 1.5%), histological type (1 patient, 0.1%), molecular subtype (27 patients, 4.0%) were excluded from the comparison.

resistance. Future preclinical and translational studies should be directed at defining mechanisms involved in ALR and a combination of targeted agents.

DATA AVAILABILITY STATEMENT

The data analyzed in this paper were obtained from the microarray datasets GSE16179, GSE38376, and GSE51889 from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). Gene expression data and clinicopathologic data of breast cancer patients were downloaded from The Cancer Genome Atlas (TCGA) at the UCSC Cancer Genomics Browser (http://xena.ucsc.edu/).

AUTHOR CONTRIBUTIONS

YY designed the study. SB and YC conducted all statistical analyses and drafted the manuscript. FY, CS, MY, WL, XH, JL, and HW revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Andrechek, E. R. (2015). HER2/Neu tumorigenesis and metastasis is regulated by E2F activator transcription factors. *Oncogene* 34, 217–225. doi: 10.1038/onc.2013.540

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* 25, 25–29. doi: 10.1038/75556

Chen, Y. J., Yeh, M. H., Yu, M. C., Wei, Y. L., Chen, W. S., Chen, J. Y., et al. (2013). Lapatinib-induced NF-kappaB activation sensitizes triple-negative breast cancer cells to proteasome inhibitors. *Breast Cancer Res.* 15, R108. doi: 10.1186/bcr3575

Chin, C. H., Chen, S. H., Wu, H. H., Ho, C. W., Ko, M. T., and Lin, C. Y. (2014). cytoHubba: identifying hub objects and sub-networks from complex interactome. *BMC Syst. Biol.* 8 (Suppl 4), S11. doi: 10.1186/1752-0509-8-S4-S11

Davis, S., and Meltzer, P. S. (2007). GEOquery: a bridge between the Gene Expression Omnibus (GEO) and BioConductor. *Bioinformatics* 23, 1846–1847. doi: 10.1093/bioinformatics/btm254

D'Amato, V., Raimondo, L., Formisano, L., Giuliano, M., De Placido, S., Rosa, R., et al. (2015). Mechanisms of lapatinib resistance in HER2-driven breast cancer. *Cancer Treat Rev.* 41, 877–883. doi: 10.1016/j.ctrv.2015.08.001

Edgar, R., Domrachev, M., and Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 30, 207–210. doi: 10.1093/nar/30.1.207

Garnett, M. J., Edelman, E. J., Heidorn, S. J., Greenman, C. D., Dastur, A., Lau, K. W., et al. (2012). Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature* 483, 570–575. doi: 10.1038/nature11005

Geeleher, P., Cox, N., and Huang, R. S. (2014a). pRRophetic: an R package for prediction of clinical chemotherapeutic response from tumor gene expression levels. *PLoS One* 9, e107468. doi: 10.1371/journal.pone.0107468

Geeleher, P., Cox, N. J., and Huang, R. S. (2014b). Clinical drug response can be predicted using baseline gene expression levels and in vitro drug sensitivity in cell lines. *Genome Biol.* 15, R47. doi: 10.1186/gb-2014-15-3-r47

Geyer, C. E., Forster, J., Lindquist, D., Chan, S., Romieu, C. G., Pienkowski, T., et al. (2006). Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N. Engl. J. Med.* 355, 2733–2743. doi: 10.1056/NEJMoa064320

Gradishar, W. J. (2013). Emerging approaches for treating HER2-positive metastatic breast cancer beyond trastuzumab. *Ann. Oncol.* 24, 2492–2500. doi: 10.1093/annonc/mdt217

Jassal, B., Matthews, L., Viteri, G., Gong, C., Lorente, P., Fabregat, A., et al. (2020). The reactome pathway knowledgebase. *Nucleic Acids Res.* 48, D498–D503. doi: 10.1093/nar/gkz1031

Kanehisa, M. (2002). The KEGG database. *Novartis Found Symp.* 247, 91–101. doi: 10.1002/0470857897.ch8

Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M., et al. (2002). The human genome browser at UCSC. *Genome Res.* 12, 996–1006. doi: 10.1101/gr.229102

Komurov, K., Tseng, J. T., Muller, M., Seviour, E. G., Moss, T. J., Yang, L., et al. (2012). The glucose-deprivation network counteracts lapatinib-induced toxicity in resistant ErbB2-positive breast cancer cells. *Mol. Syst. Biol.* 8, 596. doi: 10.1038/msb.2012.25

Liao, Y., Wang, J., Jaehnig, E. J., Shi, Z., and Zhang, B. (2019). WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. *Nucleic Acids Res.* 47, W199–W205. doi: 10.1093/nar/gkz401

Liu, L., Greger, J., Shi, H., Liu, Y., Greshock, J., Annan, R., et al. (2009). Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. *Cancer Res.* 69, 6871–6878. doi: 10.1158/0008-5472.CAN-08-4490

Moasser, M. M., and Krop, I. E. (2015). The Evolving Landscape of HER2 Targeting in Breast Cancer. *JAMA Oncol.* 1, 1154–1161. doi: 10.1001/jamaoncol.2015.2286

Nikolai, B. C., Lanz, R. B., York, B., Dasgupta, S., Mitsiades, N., Creighton, C. J., et al. (2016). HER2 Signaling Drives DNA Anabolism and Proliferation through SRC-3 Phosphorylation and E2F1-Regulated Genes. *Cancer Res.* 76, 1463–1475. doi: 10.1158/0008-5472.Can-15-2383

Oh, D. Y., and Bang, Y. J. (2020). HER2-targeted therapies - a role beyond breast cancer. *Nat. Rev. Clin. Oncol.* 17, 33–48. doi: 10.1038/s41571-019-0268-3

Parakh, S., Gan, H. K., Parslow, A. C., Burvenich, I. J. G., Burgess, A. W., and Scott, A. M. (2017). Evolution of anti-HER2 therapies for cancer treatment. *Cancer Treat Rev.* 59, 1–21. doi: 10.1016/j.ctrv.2017.06.005

Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., et al. (2000). Molecular portraits of human breast tumours. *Nature* 406, 747–752. doi: 10.1038/35021093

Smoot, M. E., Ono, K., Ruscheinski, J., Wang, P. L., and Ideker, T. (2011). Cytoscape 2.8: new features for data integration and network visualization. *Bioinformatics* 27, 431–432. doi: 10.1093/bioinformatics/btq675

Spring, L. M., Wander, S. A., Andre, F., Moy, B., Turner, N. C., and Bardia, A. (2020). Cyclin-dependent kinase 4 and 6 inhibitors for hormone receptor-positive breast cancer: past, present, and future. *Lancet* 395, 817–827. doi: 10.1016/S0140-6736(20)30165-3

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., et al. (2015). STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 43, D447–D452. doi: 10.1093/nar/gku1003

Warde-Farley, D., Donaldson, S. L., Comes, O., Zuberi, K., Badrawi, R., Chao, P., et al. (2010). The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res.* 38, W214–W220. doi: 10.1093/nar/gkq537

Wu, L., de Bruin, A., Wang, H., Simmons, T., Cleghorn, W., Goldenberg, L. E., et al. (2015). Selective roles of E2Fs for ErbB2-and Myc-mediated mammary tumorigenesis. *Oncogene* 34, 119–128. doi: 10.1038/onc.2013.511

Yang, W. D., and Wang, L. (2019). MCM10 facilitates the invaded/migrated potentials of breast cancer cells via Wnt/beta-catenin signaling and is positively interlinked with poor prognosis in breast carcinoma. *J. Biochem. Mol. Toxicol.* 33, e22330. doi: 10.1002/jbt.22330

Zhou, J., Pei, Y., Chen, G., Cao, C., Liu, J., Ding, C., et al. (2018). SPC24 Regulates breast cancer progression by PI3K/AKT signaling. *Gene* 675, 272–277. doi: 10.1016/j.gene.2018.07.017

Zhu, P., Jin, J., Liao, Y., Li, J., Yu, X. Z., Liao, W., et al. (2015). A novel prognostic biomarker SPC24 up-regulated in hepatocellular carcinoma. *Oncotarget* 6, 41383–41397. doi: 10.18632/oncotarget.5510

Zhu, G. Z., Yang, Y. L., Zhang, Y. J., Liu, W., Li, M. P., Zeng, W. J., et al. (2017). High Expression of AHSP, EPB42, GYPC and HEMGN Predicts Favorable Prognosis in FLT3-ITD-Negative Acute Myeloid Leukemia. *Cell Physiol. Biochem.* 42, 1973–1984. doi: 10.1159/000479837

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PKM2–c-Myc–Survivin Cascade Regulates the Cell Proliferation, Migration, and Tamoxifen Resistance in Breast Cancer

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The M2 isoform of pyruvate kinase (PKM2), as a key glycolytic enzyme, plays important roles in tumorigenesis and chemotherapeutic drug resistance. However, the intricate mechanism of PKM2 as a protein kinase regulating breast cancer progression and tamoxifen resistance needs to be further clarified. Here, we reported that PKM2 controls the expression of survivin by phosphorylating c-Myc at Ser-62. Functionally, PKM2 knockdown suppressed breast cancer cell proliferation and migration, which could be rescued by overexpression of survivin. Interestingly, we found that the level of PKM2 expression was upregulated in the tamoxifen resistant breast cancer cells MCF-7/TAMR, and knockdown of PKM2 sensitized the cells to 4-hydroxytamoxifen (4OH-T). In addition, the elevated level of PKM2 correlates with poor relapse-free survival in breast cancer patients treated with tamoxifen. Overall, our findings demonstrated that PKM2–c-Myc–survivin cascade regulated the proliferation, migration and tamoxifen resistance of breast cancer cells, suggesting that PKM2 represents a novel prognostic marker and an attractive target for breast cancer therapeutics, and that PKM2 inhibitor combined with tamoxifen may be a promising strategy to reverse tamoxifen resistance in breast cancer patients.

Keywords: breast cancer cells, M2 isoform of pyruvate kinase, tamoxifen resistance, c-Myc, survivin

INTRODUCTION

Pyruvate kinase isoform M2 (PKM2), one of the isoenzymes of pyruvate kinase (PK) (Yang and Lu, 2013), is a key glycolytic enzyme overexpressed in cancer cells (Luo and Semenza, 2012), which controls the terminal rate-limiting step of glycolysis by catalyzing the transform of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) (Hamanaka and Chandel, 2011). Previous reports suggested that PKM2 affects cell proliferation, migration, invasion, apoptosis, and cell cycle progression of tumors, including breast cancer, prostate

cancer, myeloma, liver cancer, lung cancer, and pancreatic cancer (Stetak et al., 2007; Christofk et al., 2008; Yang et al., 2012; Jiang et al., 2014; Christensen et al., 2015; Azoitei et al., 2016; Matsuda et al., 2016; Shen et al., 2016; Guo et al., 2017). Over the years, there have been increasing evidence pointing to the non-glycolytic function of PKM2 in tumor cells. For instance, PKM2 binds to and transactivates Y333-phosphorylated β-catenin to promote tumor cell proliferation (Yang et al., 2011); PKM2 promotes tumorigenesis by directly phosphorylating histone H3 at threonine 11 under EGFR activation (Yang et al., 2012); A recent study has demonstrated that nuclear PKM2 activates transcription of MEK5 by phosphorylating STAT3 at tyrosine 705 in colon cancer cells (Gao et al., 2012). However, the role of PKM2 as a protein kinase in the regulation of tumor progression in breast cancer remains to be further identified. In addition, studies have shown that PKM2 is highly correlated with drug resistance. For example, down-regulation of PKM2 by shikonin, an inhibitor of PKM2, re-sensitized the drug resistant bladder cancer cells to cisplatin (Wang et al., 2018); PKM2 expressions were positively associated with gefitinib resistance in colorectal cancer cells, and PKM2 knockdown increased gefitinib efficacy (Li et al., 2015). It has been demonstrated recently that NAMPT promotes tamoxifen resistance *via* regulation of the PKM2 translocation (Ge et al., 2019). However, the specific mechanism and role of PKM2 in regulating breast cancer tamoxifen resistance remains unknown.

c-Myc is one of the most activated oncogenes and is associated with the initiation and progression of human cancer (Dang, 1999; Nesbit et al., 1999). c-Myc was defined as an oncoprotein associated with DNA replication, transcription or RNA splicing. It has been demonstrated that c-Myc can regulate the transcription of survivin (encoded by the gene *BIRC5*), an essential member of the inhibitor of apoptosis protein (IAP) family, playing an important role in tumorigenesis (Cosgrave et al., 2006; Fang et al., 2009; Papanikolaou et al., 2011; Chen et al., 2016; Garg et al., 2016; Haque et al., 2017). Studies have shown that survivin is dramatically overexpressed in various of tumors, such as breast cancer, colon cancer, and lung cancer, and promotes the proliferation and metastasis of tumor cells (Kawasaki et al., 1998; Monzo et al., 1999; Suzuki et al., 2000; Tanaka et al., 2000; Marioni et al., 2006; McKenzie et al., 2010; Kedinger et al., 2013; Ma et al., 2016; Yang et al., 2016; Liu et al., 2019). Furthermore, the elevated survivin expression in cancer patients reveals a poor prognosis and high mortality rate (Ma et al., 2016). Survivin is usually expressed in tumor tissue, but infrequently measured in normal differentiated adult tissues. Therefore, survivin is a prospective target for the diagnosis and therapy of cancer.

In this study, we investigated the action of PKM2 as a protein kinase in the regulation of proliferation and migration of breast cancer cells. We demonstrated for the first time that PKM2 regulated the expression of survivin by interacting with c-Myc and phosphorylating c-Myc at Ser-62. In addition, we found that PKM2 was upregulated in tamoxifen resistant breast cancer cells, and PKM2 downregulation enhanced cell sensitivity to tamoxifen in both of MCF-7 and MCF-7/TAMR cells. Therefore, targeting PKM2–c-Myc–survivin pathway may provide a new strategy for inhibiting breast cancer cell proliferation and migration and for reversing tamoxifen resistance.

MATERIALS AND METHODS

Cell Lines and Culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 were cultured in Dulbecco's modified Eagle's medium (DMEM)/High glucose medium supplemented with 10% fetal bovine serum (Gibco). The tamoxifen resistant cell line MCF-7/TAMR was purchased from China Medical University. The MCF-7/TAMR cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.4 μM 4OH-Tamoxifen. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Reagents and Antibodies

4-hydroxytamoxifen (4OH-T) was purchased from Sigma. MG132 and CHX were purchased from Selleck. Rabbit monoclonal antibodies against PKM2, c-Myc, and survivin were purchased from Cell Signaling Technologies. Anti-phospho-c-Myc (Ser62) was purchased from Abcam. Anti-β-actin, GST, and Flag antibodies were purchased from Proteintech.

Transfection

The siRNA duplexes targeting PKM2 and c-Myc were purchased from Ribobio. Non-targeting siRNA was used as a control. Transfection of siRNA was accomplished according to the manufacturer's protocol. In brief, cells in the exponential phase of growth were plated in 6-well plates at 1×10^5 cells per well, grown for 24 h, then transfected with siRNA using Lipofectamine 2000 (Invitrogen) and OPTI-MEM reduced serum medium. For plasmid transfection, cells were transfected with a mixture of GST–PKM2 or His-c-Myc plasmid and Lipofectamine 2000 (Invitrogen) in OPTI-MEM reduced serum medium.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from MCF-7 and MDA-MB-231 cells using Trizol reagent (Biotech) according to the manufacturer's instructions. The quality, quantity, and integrity of RNA were measured by Nanodrop2000 spectrophotometer. The TaqMan high-capacity cDNA Kit (Takara) was used for the reverse transcription of mRNA. GAPDH acted as an internal control relative to survivin. Primers were designed and synthesized by Sangon Biotech.

Western Blot and Immunoprecipitation

Cells were washed with PBS and lysed by RIPA buffer (Beyotime, Haimen, China) supplemented with a phosphatase inhibitor cocktail and a protease inhibitor cocktail (Selleck). The concentrations of protein were quantified with a BCA protein assay kit (Beyotime, Haimen, China). The proteins were isolated

by 10% SDS-PAGE and transferred to a PVDF membrane. The PVDF membranes were incubated with primary antibody in 5% skim milk with Tween 20 for 1 h at room temperature. Membranes were then incubated with anti-rabbit or anti-mouse secondary antibody at room temperature for 1 h. Detection was completed by chemiluminescence using an ECL reagent. For immunoprecipitation under denaturing conditions, proteins were extracted using regular immunoprecipitation. The beads were washed, and then resolved by 10% SDS-PAGE. The proteins were visualized by Western Blot.

Protein Half-Life Assay

To examine c-Myc protein stability, siRNA targeting PKM2 was transfected into MCF-7 and MDA-MB-231cells. The cells were treated with cycloheximide (CHX; 20 μg/ml; Amresco) and were harvested at the indicated time points for immunoblotting.

Cell Viability Assay

Cell viability was measured by the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's protocol. Briefly, cells were cultured in 96-well plates at 5×10^3 cells per well. After treatment, cells were incubated with 10 μl CCK-8 reagent for 2 h at 37°C with 5% CO_2. The results were measured at 450 nm wave length.

EdU Assay

Proliferating cells were stained using the Cell Light EdU DNA Cell Proliferation Kit (Ribobio). Cells were exposed with 50 μmol/L of EdU for 2 h at 37°C. After fixing with 4% paraformaldehyde for 15 min, cells were treated with 0.5% Triton X-100 for 30 min and washed with PBS three times. Then, cells were exposed to 100 μl 1× Apollo® reaction cocktail for 30 min and incubated with 5 μg/ml of Hoechst 33342 to stain the cell nucleus for 30 min. Images were visualized under a fluorescent microscope.

Wound Healing Assay

After siRNA and plasmid were transfected into MCF-7 and MDA-MB-231 cells, the cells were seeded in 6-well plates at 2×10^5 cells per well and grown overnight. A wound was then made in the cell culture by scratching on the cell layer with a sharp tip, then incubation for a further 48 h in serum-free medium. The gap created by the wound was then detected under a microscope to offer an indication of the wound-healing capability of the cells.

Migration and Invasion Assays

Migration and invasion were evaluated using 24-well chemotaxis chambers (Costar, #3422, 8 μm pore size). The cells were washed twice with phosphate-buffered saline, resuspended in 100 μl serum-free medium and added into the upper chambers. The lower chambers were filled with 600 μl medium containing 10% fetal bovine serum. For the migration assay, after incubation for 24 h, the cells that had migrated through the membrane were stained and counted. For the invasion assay, the cells were incubated for 48 h in the upper chamber coated with a mixture of serum-free medium and Matrigel (3:1; BD Biosciences).

Cell Apoptosis Assays

MCF-7 cells transfected with siRNA were cultured for 72 h and harvested by centrifugation. Cell apoptosis assay was performed by Annexin V-FITC/PI Staining Kit (Mbchem) according to the manufacturer's instructions.

Soft Agar Colony Formation Assay

MCF-7/TAMR cells transfected with si-PKM2 or si-NT were seeded on top of 1.2% agar in the RPMI-1640 medium containing 10% FBS with 0.7% agar (Bioweste) in 24-well plate. After 10 days, the clones were observed with a microscope and photographed. Three independent experiments were quantified using Image J. The silencing effects were detected by Western Blot.

Kaplan–Meier Survival Analysis

The relapse-free survival of patients with tamoxifen-exposed ER + breast cancer stratified by PKM2 expression levels (low and high) were evaluated using Kaplan–Meier analysis from a large publicly available clinical breast cancer microarray online database and web tool (http://kmplot.com/analysis/).

Data Analysis

Data were analyzed by unpaired two-tailed Student's t-test. All experiments were performed at least three times. Differences between groups were considered statistically significant at $p < 0.05$. Graphpad Prism software (version 6.0) was used for analysis.

RESULTS

PKM2 Promotes Cell Proliferation and Migration, and Its High Expression Is Correlated With Poor Prognosis in Human Breast Cancer

Firstly, we compared the expressions of PKM2 between normal and breast cancer tissues using a large publicly available online database-GEPIA (http://gepia.cancer-pku.cn/). As shown in **Figure 1A**, PKM2 was significantly up-regulated in breast cancer tissues compared to normal tissues. We further analyzed the expressions of PKM2 in different types of breast cancer and found that PKM2 was significantly up-regulated in Basal-like, ER+, HER2+ breast cancer tissues compared to normal tissues (**Figure 1B**). Then, we further analyzed the expressions of PKM2 in ER+ and triple negative breast cancer by TCGA and found that there was no significant difference in the levels of PKM2 between ER+ and triple negative breast cancer tissues (**Supplemental Figure 1A**). We also measured the protein expressions of PKM2 in breast cancer cell lines. Consistently, PKM2 has similar expression in ER+ and ER− breast cancer cells (**Supplemental Figure 1B**). Next, we analyzed the survival rate using GEPIA. The overall survival was lower in patients with high PKM2 expression, suggesting that PKM2 overexpression indicates a high risk of recurrence in breast cancer patients (**Figure 1C**). To further investigate the potential oncogenic role of PKM2 in breast cancer, we knocked down PKM2 and measured the cell viability and proliferation ability in MCF-7 and MDA-MB-231 cells. **Figures 1D, E** show that PKM2

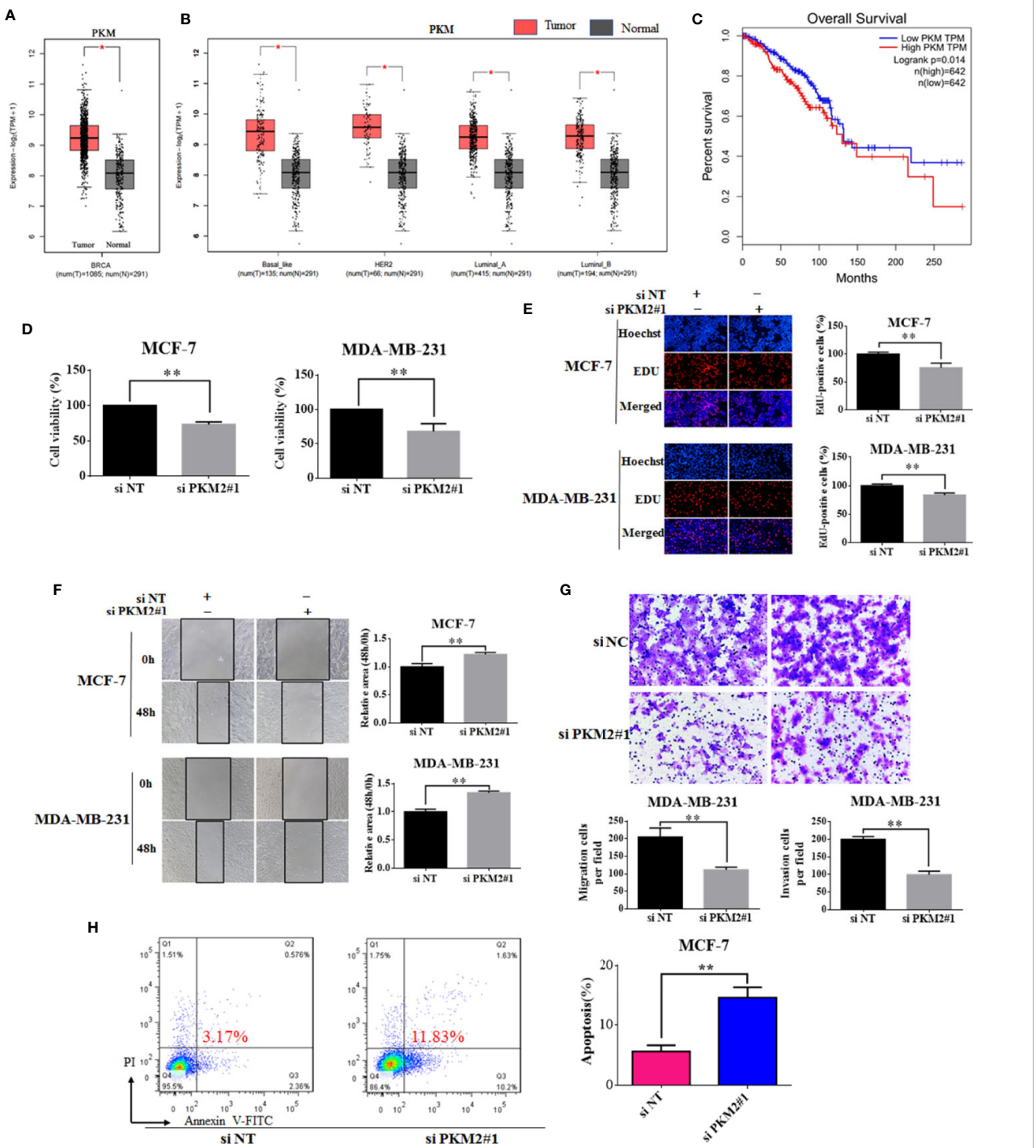

FIGURE 1 | PKM2 promotes cell proliferation and migration, and its high expression is correlated with poor prognosis in human breast cancer. **(A)** GEPIA analysis showed the expression levels of PKM2 between breast cancer and normal tissues. **(B)** GEPIA analysis showed the expression levels of PKM2 in breast cancer subtype tissues compared to normal tissues. **(C)** Survival analysis with auto select best cutoff values of PKM2 expression for breast cancer from TCGA datasets. The MCF-7 or MDA-MB-231 cells were transfected with siPKM2 or non-targeting siRNA. **(D)** Cell viability was measured by CCK-8 reagent. **(E)** Cell proliferation ability was measured by EdU. Magnification, ×200. **(F)** Migration ability was detected by wound healing assay, the black line indicates the edge of migrating cells at a given time point. Data were shown as the mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.01$). **(G)** MDA-MB-231 cells were transfected with non-targeting siRNA or PKM2 siRNA. The migration (left) and invasion (right) ability of cells were measured by transwell assays. Migration cells were incubated for 24 h, invasion cells were incubated for 48 h in the upper chambers coated with Matrigel (*$p < 0.05$, **$p < 0.01$). **(H)** MCF-7 cells were transfected with non-targeting siRNA or PKM2 siRNA. Indicated cells were stained with Annexin V/PI, and the percentage of apoptotic cells was assessed by flow cytometry (*$p < 0.05$, **$p < 0.01$).

knockdown markedly reduced the viability and proliferation of breast cancer cells. To explore the roles of PKM2 in breast cancer metastasis, we detected the effect of PKM2 on cell migration by wound healing assay. As shown in **Figure 1F**, knockdown of PKM2 markedly reduced the migration of breast cancer cell lines MCF-7 and MDA-MB-231 (**Figure 1F**). In addition, PKM2 knockdown also significantly reduced the migration and invasion of MDA-MB-231 cells using transwell assays (**Figure 1G**). We further examined the potential role of PKM2 in regulating cellular apoptosis. There was increased cellular apoptosis in PKM2 knockdown cells as compared with siRNA control cells (**Figure 1H**). These data suggest that PKM2 is necessary for cell proliferation and migration, and PKM2 may be an important prognostic factor in breast cancer patients.

PKM2 Promotes Breast Cancer Progression Through Increasing Survivin mRNA and Protein Expressions

Next, we are interested in the molecular mechanism by which PKM2 promotes cell proliferation. PKM2 was predicted to be positively associated with survivin in mRNA level using a publicly online database-GEPIA (**Figure 2A**). Survivin is well known as a member of the inhibitor of apoptosis protein family, which is crucial for the proliferation and migration of breast cancer cells (Tanaka et al., 2000). We found that knockdown of PKM2 led to a significant decrease in survivin expressions at both protein and mRNA (**Figures 2B, D**). Conversely, ectopic overexpression of PKM2 remarkably increased survivin expressions at both protein and mRNA (**Figures 2C, E**). Collectively, our results suggest that PKM2 promotes the expression of survivin by regulating transcription. Next, we further investigated whether survivin mediated the regulatory effect of PKM2 on breast cancer progression. MCF-7 or MDA-MB-231 cells were transfected with PKM2 siRNA, followed by transfection with GFP-survivin plasmid, then the cell viability, proliferation ability, and migration capacity were tested. We found that the inhibition of cell viability, proliferation and migration by PKM2 knockdown was rescued by survivin overexpression in MCF-7 and MDA-MB-231 cells (**Figures 3A–C**). Together, these experiments suggest that PKM2 promotes breast cancer cell proliferation and migration through increasing survivin transcription.

PKM2 Activates Transcription of Survivin Through c-Myc

We next sought to understand how PKM2 regulates survivin expression. Sequence analyses did not show any known DNA binding domain/motifs in PKM2 (Gao et al., 2012). One of the possibilities is that PKM2 may regulate the activation of a particular transcription factor (Harris et al., 2012). c-Myc is a known transcription factor of survivin (Cosgrave et al., 2006; Fang et al., 2009). Consistent with the previous reports, we verified the regulatory effect of c-Myc on survivin. As shown in **Figures 4A, C**, c-Myc knockdown led to reduced protein and mRNA levels of survivin in MCF-7 and MDA-MB-231 cells.

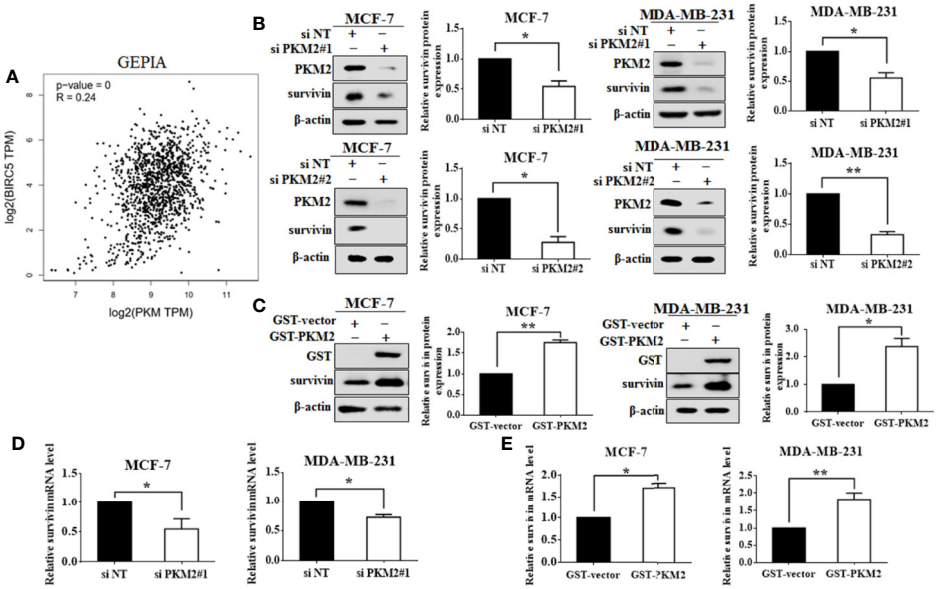

FIGURE 2 | PKM2 regulates survivin expression. **(A)** The correlation between PKM2 and survivin predicted by GEPIA. **(B)** MCF-7 or MDA-MB-231 cells were transfected with either the non-targeting siRNA or PKM2 siRNA. PKM2 and survivin protein levels were measured by immunoblotting. β-actin served as a loading control. **(C)** MCF-7 or MDA-MB-231 cells were transfected with either the empty vector plasmid or GST-PKM2 plasmid. GST and survivin protein levels were measured by immunoblotting. β-actin served as a loading control. **(D)** MCF-7 or MDA-MB-231 cells were transfected with either the non-targeting siRNA or PKM2 siRNA. PKM2 and survivin mRNA levels were detected by qRT-PCR. GAPDH served as a loading control. **(E)** MCF-7 or MDA-MB-231 cells transfected with either the empty vector plasmid or GST-PKM2 plasmid, PKM2 and survivin mRNA levels were detected by qRT-PCR. GAPDH served as a loading control. Data were shown as the mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.01$).

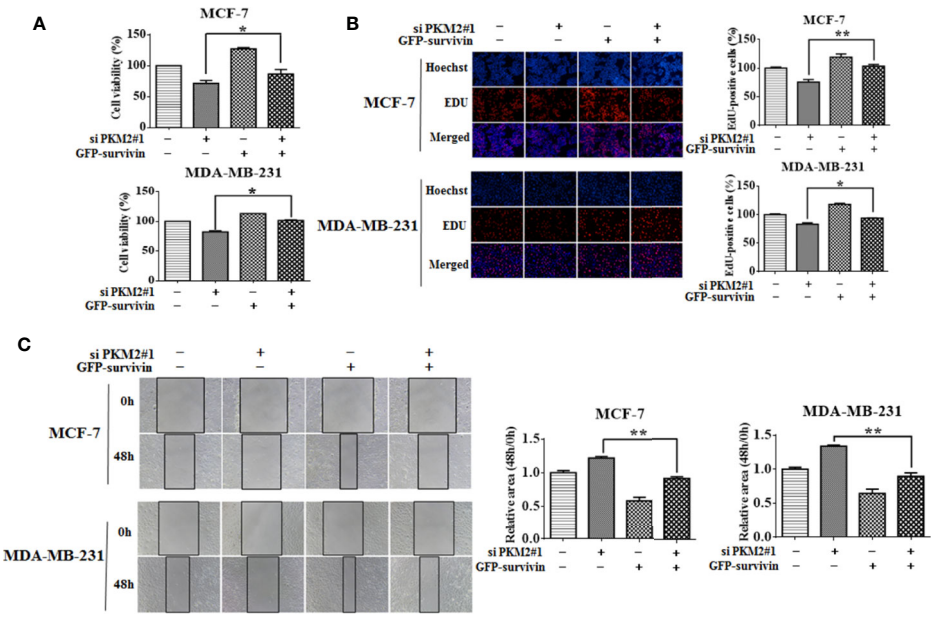

FIGURE 3 | PKM2 promotes breast cancer progression through survivin. MCF-7 or MDA-MB-231 cells were transfected with either the non-targeting siRNA or PKM2 siRNA, followed by transfection with either the empty vector plasmid or GFP-survivin plasmid. **(A)** Cell viability was measured by CCK-8 reagent. **(B)** Cell proliferation ability was measured by EdU. Magnification, ×200. **(C)** Migration ability was detected by wound healing assay, the black line indicates the edge of migrating cells at a given time point. Data were shown as the mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.01$).

Overexpression of c-Myc increased survivin expression at both mRNA and protein levels (**Figures 4B, D**). We next tested whether PKM2 activated the transcription of survivin through c-Myc. MCF-7 and MDA-MB-231 cells were transfected with PKM2 siRNA, and then transfected with His-c-Myc plasmid. The decrease of survivin at protein and mRNA levels caused by knockdown of PKM2 could be rescued by exogenous c-Myc (**Figures 4E, F**). These results show that PKM2 promotes the transcription of survivin through c-Myc.

PKM2 Interacts With c-Myc and Stabilizes c-Myc by Phosphorylating It at Ser62

We next examined the relationship between PKM2 and c-Myc. After transfected with PKM2 siRNA, a significant decrease of c-Myc protein expression can be observed in MCF-7 and MDA-MB-231 cells (**Figure 5A**), and the increase of c-Myc protein level can be observed after transfected with exogenous PKM2 (**Figure 5B**). Moreover, we found that the expression of p-c-Myc (Ser-62) was decreased or increased when transfected with PKM2 siRNA or GST-PKM2 plasmid in MCF-7 and MDA-MB-231 cells. It has been previously demonstrated that the phosphorylation of c-Myc on Ser-62 results in its stabilization (Seo et al., 2008). Thus, we wanted to know whether the regulation of PKM2 on c-Myc phosphorylation will stabilize c-Myc protein. We next examined the degradation rate of the c-Myc protein by CHX assay in MCF-7 and MDA-MB-231 cells. As shown in **Figure 5C**, PKM2 knockdown significantly shortened the half-life of c-Myc both in MCF-7 and MDA-MB-231 cells. In line with the results in **Figure 5C**, we found that MG132, a proteasome inhibitor, could rescue the down-regulation of c-Myc in the cells with knockdown of PKM2 expression (**Figure 5D**). Next, we further examined the physical interaction between PKM2 and c-Myc proteins. 293T cells transfected with Flag-PKM2 plasmid and His-c-Myc plasmid were subjected to immunoprecipitation with an anti-His antibody, and Flag-PKM2 was detected. Similarly, His-c-Myc could be detected in immunoprecipitation complexes when the anti-flag antibody was used for immunoprecipitation (**Figure 5E**). Additionally, MCF-7 and MDA-MB-231 cells were transfected with GST-vector or GST-PKM2, then were subjected to immunoprecipitation with an anti-GST antibody, and c-Myc was presented in anti-GST co-IPs from cells transfected with GST-PKM2, but not in the cells transfected with GST-vector (**Figure 5F**). Similarly, MCF-7 and MDA-MB-231 cells were transfected with His-vector or His-c-Myc, then were subjected to immunoprecipitation with an anti-His antibody, and PKM2 was presented in anti-His co-IPs from cells transfected with His-c-Myc (**Figure 5G**), suggesting the interaction between PKM2 and c-Myc. These results for the first time brought our attention that phosphorylation of c-Myc at Ser-62 by PKM2 promoted the stability of c-Myc.

Suppression of PKM2 Enhanced Tamoxifen Sensitivity in MCF-7 and MCF-7/TAMR Cells

We further investigated whether PKM2 could be related to the regulation of tamoxifen sensitivity in MCF-7 cells. As shown

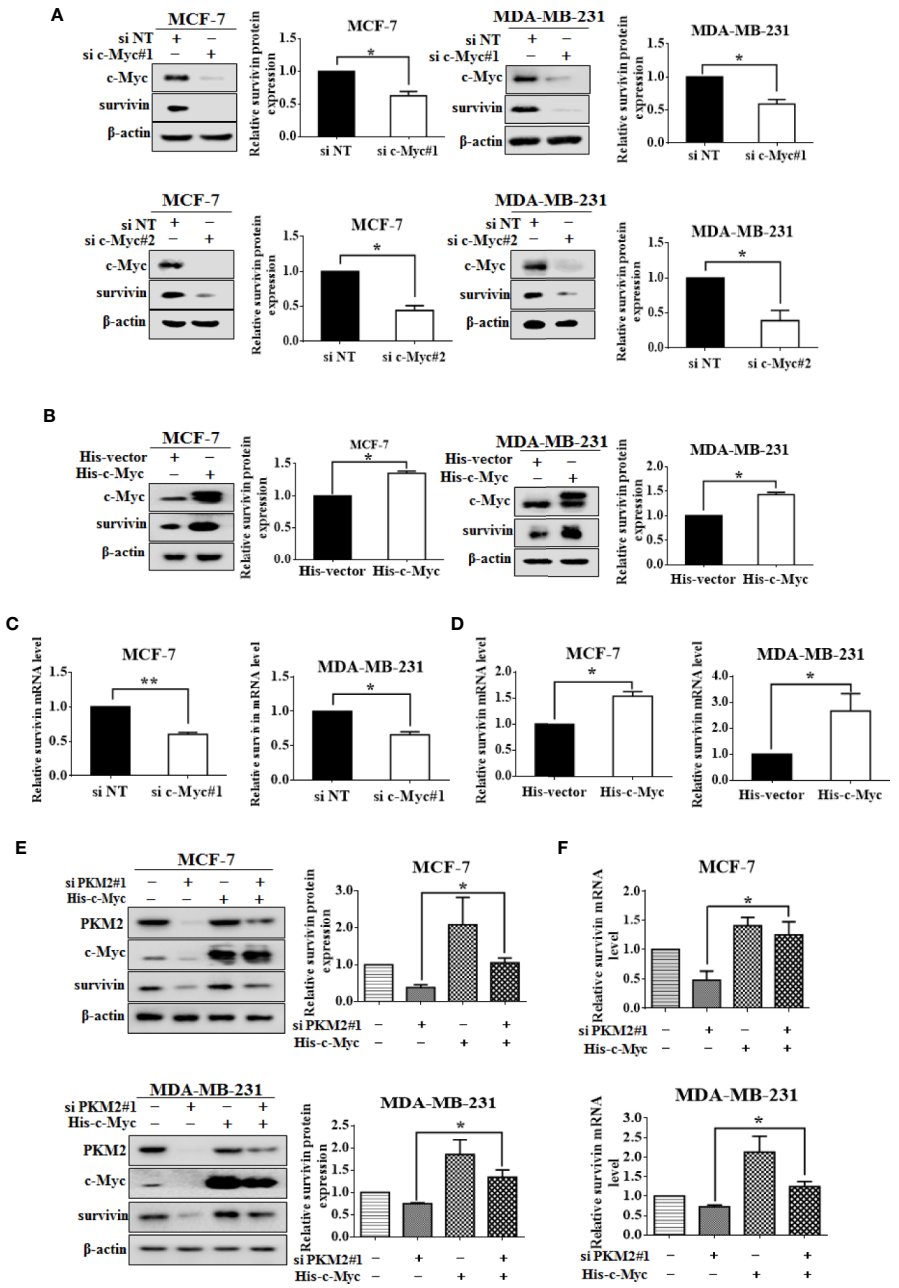

FIGURE 4 | c-Myc regulates PKM2-mediated survivin expression. **(A)** MCF-7 or MDA-MB-231 cells were transfected with either the non-targeting siRNA or c-Myc siRNA. c-Myc and survivin protein levels were measured by immunoblotting. *β*-actin served as a loading control. **(B)** MCF-7 or MDA-MB-231 cells were transfected with either the empty vector plasmid or His-c-Myc plasmid. c-Myc and survivin protein levels were measured by immunoblotting. *β*-actin served as a loading control. **(C)** MCF-7 or MDA-MB-231 cells were transfected with either the non-targeting siRNA or c-Myc siRNA. Survivin mRNA level was detected by qRT-PCR. GAPDH served as a loading control. **(D)** MCF-7 or MDA-MB-231 cells were transfected with either the empty vector plasmid or His-c-Myc plasmid. Survivin mRNA level was detected by qRT-PCR. GAPDH served as a loading control. **(E, F)** MCF-7 or MDA-MB-231 cells were respectively transfected with either the non-targeting siRNA or PKM2 siRNA, and then transfected with either the empty vector plasmid or His-c-Myc plasmid. **(E)** Survivin protein level was detected by immunoblotting. *β*-actin served as a loading control. **(F)** Survivin mRNA level was detected by qRT-PCR. GAPDH served as a loading control. Data were shown as the mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.01$).

in **Figures 6A, B**, PKM2 knockdown decreased the cell viability and proliferation in MCF-7 cells with 4OH-Tamoxifen treatment. Furthermore, we found that the expressions of PKM2, c-Myc and survivin were upregulated in MCF-7/TAMR cells as compared to MCF-7 cells (**Figure 6C**). Silencing of PKM2 resulted in a prominent decrease in the levels of c-Myc, p-c-Myc (Ser62) and survivin in MCF-7/TAMR cells (**Figure 6D**). To further investigate the role of

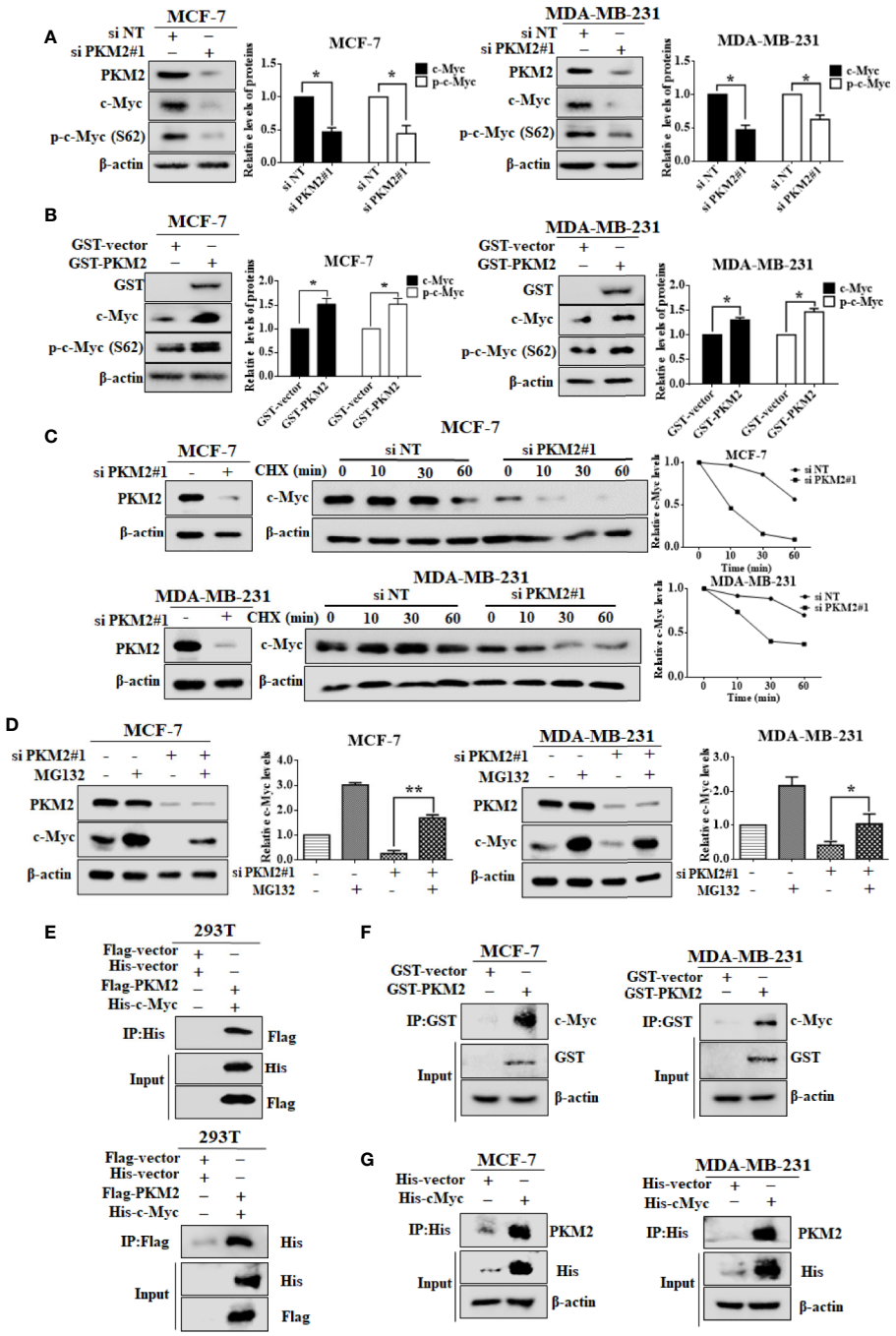

FIGURE 5 | PKM2 enhances c-Myc stability by inducing the phosphorylation of c-Myc. **(A)** MCF-7 or MDA-MB-231 cells were transfected with either the non-targeting siRNA or PKM2 siRNA. PKM2, c-Myc and p-c-Myc (Ser62) protein levels were measured by immunoblotting. *β*-actin served as a loading control. **(B)** MCF-7 or MDA-MB-231 cells were transfected with either the empty vector plasmid or GST-PKM2 plasmid. PKM2, c-Myc and p-c-Myc (Ser62) protein levels were measured by immunoblotting. *β*-actin served as a loading control. Data were shown as the mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.01$). **(C)** sMCF-7 or MDA-MB-231 cells were transfected with either the non-targeting siRNA or PKM2 siRNA, and were treated with CHX (10 μg/ml) for the indicated time, and immunoblotting analysis was applied to detect the expression levels of c-Myc. *β*-actin served as a loading control. **(D)** MCF-7 and MDA-MB-231 cells were transfected with either the non-targeting siRNA or PKM2 siRNA, and were treated with MG132 (20 μM) before extracting proteins. Western blotting was used to analyze PKM2 and c-Myc proteins in MCF-7 and MDA-MB-231 cells. *β*-Actin was used as a loading control. **(E)** 293T cells were transfected with Flag-PKM2 plasmid and His-c-Myc plasmid, and then subjected to immunoprecipitation with anti-His and anti-flag antibody. The lysates and immunoprecipitation were then analyzed. **(F)** MCF-7 or MDA-MB-231 cells were transfected with either the empty vector plasmid or GST-PKM2 plasmid, and then subjected to immunoprecipitation with an anti-GST antibody. The lysates and immunoprecipitation were analyzed. **(G)** MCF-7 or MDA-MB-231 cells were transfected with either the empty vector plasmid or His-c-Myc plasmid, and then subjected to immunoprecipitation with an anti-His antibody. The lysates and immunoprecipitation were analyzed.

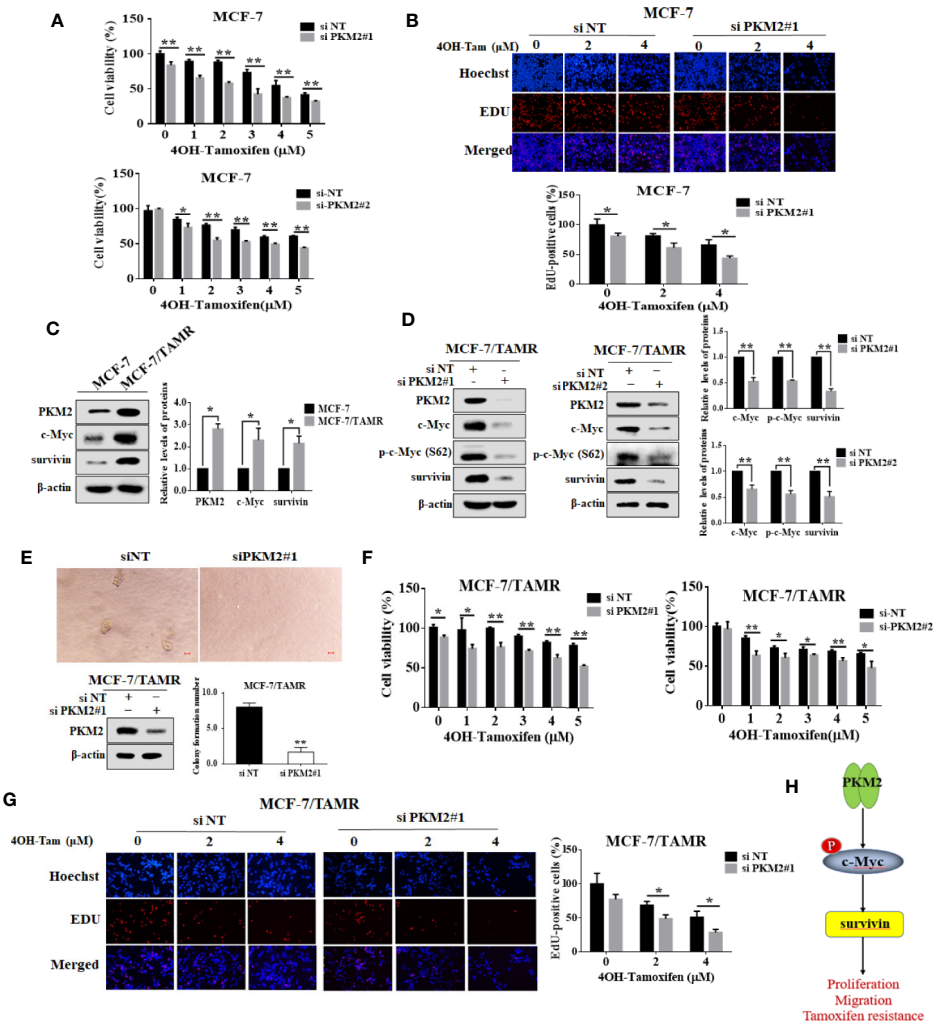

FIGURE 6 | PKM2 downregulation enhanced cell sensitivity to tamoxifen in MCF-7 and MCF-7/TAMR cells. The MCF-7 cells were transfected with siPKM2 or non-targeting siRNA. Then the cells were treated with 4-OHT for 72 h at the indicated concentration periods (0–5 μM). **(A)** Cell viability was measured by CCK-8 reagent. **(B)** Cell proliferation ability was measured by EdU. Magnification, ×200. **(C)** Western blotting was used to analyze PKM2, c-Myc and survivin proteins in tamoxifen resistant and their parental cells. *β*-Actin was used as a loading control. **(D)** PKM2 siRNA-treated and control siRNA-treated MCF-7/TAMR cells were treated for 48 h. Western blot was performed with indicated antibodies. **(E)** The anchor-independent cell growth ability of MCF-7/TAMR cells transfected with PKM2 siRNA was detected by soft agar clone formation assay. MCF-7/TAMR cells were cultured for 10 days. The clone size greater than 50 μm is considered a clone formation. The MCF-7/TAMR cells **(F, G)** were transfected with siPKM2 or non-targeting siRNA. Then the cells were treated with 4-OHT for 72 h at the indicated concentration periods (0–5 μM). **(F)** Cell viability was measured by CCK-8 reagent. **(G)** Cell proliferation ability was measured by EdU. Magnification, ×200. **(H)** A schematic model of PKM2–c-Myc–survivin axis leading to proliferation, migration and tamoxifen resistance. The results are reported as the mean ± SD of triplicate measurements; *P < 0.05, **P < 0.01, t-test, siNT vs. siPKM2.

PKM2 in MCF-7/TAMR cell proliferation, the effect of PKM2 on the anchorage-independent cell growth was measured by soft agar colony formation assay. As shown in **Figure 6E**, the size and number of clones were significantly decreased after PKM2 was silenced. We next asked whether silencing the expression of PKM2 could re-sensitize tamoxifen resistant breast cancer cells to tamoxifen treatment. **Figures 6F, G** showed that the cells became more sensitive to tamoxifen after knocking down PKM2. This result indicates that PKM2-c-Myc-survivin pathway participated in the regulation of tamoxifen resistance (**Figure 6H**), and knockdown of PKM2 enhanced cell sensitivity to tamoxifen in MCF-7 and MCF-7/TAMR cells.

Elevated Levels of PKM2, Survivin, and c-Myc Correlate With Poor Relapse-Free Survival in Patients With ER+ Breast Cancer Undergoing Tamoxifen Therapy

To examine the clinical relevance of our result, we investigated three publicly available microarray datasets, which include the relapse free survival of patients in ER+ positive breast cancer

patients treated with tamoxifen. In these datasets, we analyzed the survival rates applying the Kaplan–Meier method by a log-rank test, which indicate that PKM2, survivin and c-Myc overexpression confer a high risk of relapse in breast cancer patients treated with tamoxifen (**Figures 7A–C**). These data suggest that the PKM2, survivin and c-Myc expression levels may be important prognostic factors for tamoxifen treatment in breast cancer patients.

DISCUSSION

Breast cancer is the most common malignancy and the second leading cause of cancer-related mortality among women (Urun et al., 2015). Tamoxifen, a selective ER modulator, competitively restraints the binding of estradiol to ER, as a result inhibiting the ER-mediated transcription of kinds target genes to repress the proliferation of cancer cells. Although it is effective in adjuvant and first-line treatment of advanced ESR-positive breast cancer, development of resistance to tamoxifen remains a serious clinical problem (Nass and Kalinski, 2015). Therefore, it is imperative to find novel targets in breast cancer progression and improve breast cancer response to tamoxifen therapy. In this study, we reported for the first time that PKM2-c-Myc-survivin signaling cascade promoted breast cancer cell proliferation, migration, tamoxifen resistance (**Figure 6H**), and inhibition of PKM2 not only blocked cancer progression, but also enhanced tamoxifen efficacy in MCF-7 and MCF-7 resistant cells.

Accumulating evidence has demonstrated the important role of PKM2 in promoting cancer progression (Zheng et al., 2018; Liu et al., 2019). Consistently, we presented evidence that PKM2 promoted breast cancer cell proliferation and migration, and PKM2 overexpression predicted poor prognosis in breast cancer patients. These results revealed that PKM2 is a potential target for the treatment of breast cancer. It has been previously shown that glycolytic enzyme PKM2 which PKM2/NF-κB/miR-148a/152 feedback circuit can regulate breast cancer cells growth and angiogenesis (Yan et al., 2017), but the regulatory mechanism of PKM2 as a protein kinase on breast cancer cell proliferation and migration remains to be further explored. It was reported that the PKM2–β-catenin interaction leaded to increased binding of β-catenin to the promoter region of c-Myc (Yang et al., 2011). Our study provided new insight in the mechanistic regulation of PKM2 in c-Myc, that PKM2 interacted with c-Myc and regulated c-Myc phosphorylation, providing the first evidence that c-Myc may be a novel substrate of PKM2. We found that inhibition of PKM2 decreased c-Myc phosphorylation, resulting in down-regulating c-Myc protein expression by promoting its degradation. As an oncoprotein, c-Myc promotes cancer progression by increasing the transcription of substrate genes involved in the control of cell proliferation or growth. It has been reported that c-Myc can promote PKM2 mRNA expression by upregulation of heterogeneous nuclear ribonucleoprotein (hnRNP) transcription (David et al., 2010). Consistent with the report, we found that overexpression of c-Myc increased the expression of PKM2 compared with PKM2 knockdown cells, indicating that there is a positive feedback loop between PKM2 and c-Myc. Database analyzation and experimental results revealed the positive regulation of PKM2 on survivin transcription. As a transcription factor, c-Myc regulates survivin transcription. We verified that overexpression of c-Myc abrogated the decrease of mRNA and protein levels of survivin induced by PKM2 inhibition. Taken together, we concluded that PKM2 regulated survivin through c-Myc. Our results revealed that PKM2–c-Myc–survivin cascade promotes the proliferation and migration of breast cancer cells, serving as a potential therapeutic strategy in breast cancer.

Accumulating evidence indicates that PKM2 is highly correlated with drug resistance (Li et al., 2015; Wang et al., 2018). However, no clear evidence reveals the role of PKM2 in

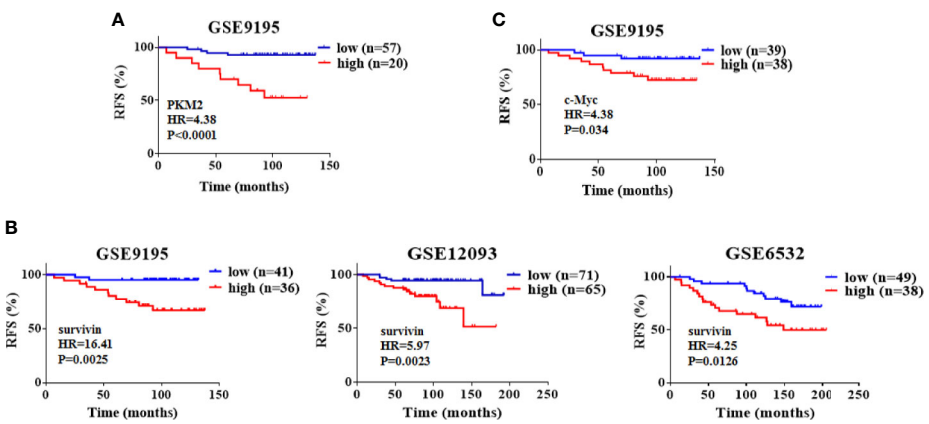

FIGURE 7 | Elevated levels of PKM2, survivin and c-Myc correlate with poor relapse-free survival in patients with ER+ breast cancer undergoing tamoxifen therapy. **(A)** Kaplan–Meier analysis with auto select best cutoff values of PKM2 expression for ER+ breast cancer patients treated with tamoxifen from GEO datasets. **(B)** Kaplan–Meier analysis with auto select best cutoff values of survivin expression for ER+ breast cancer patients treated with tamoxifen from GEO datasets. **(C)** Kaplan–Meier analysis with auto select best cutoff values of c-Myc expression for ER+ breast cancer patients treated with tamoxifen from GEO datasets. P-values were calculated by the log–rank test.

the development of tamoxifen resistance. Our findings showed that PKM2 was upregulated in the tamoxifen resistant breast cancer cells compared to sensitive cells. Inhibition of PKM2 significantly decreased c-Myc and survivin expressions. As previously reported, c-Myc and survivin were related with the development of tamoxifen resistance. It was reported that aspirin can down-regulate c-Myc protein expression to overcome tamoxifen resistance (Cheng et al., 2017). Wen-Tsung Huang demonstrated that survivin was overexpressed in MCF-7/TAMR cells as compared to MCF-7 cells, and down-regulation of survivin restored the sensitivity of MCF-7/TAMR cells to tamoxifen. Our data showed that down-regulation of PKM2 not only rendered MCF-7 cells more sensitivity to tamoxifen, but also significantly overcame tamoxifen resistance in MCF-7/TAMR cells. The implication of PKM2 in enhancing tamoxifen sensitivity was further verified in breast cancer patients that the high expression of PKM2 confers a high risk of recurrence or relapse in patients treated with tamoxifen. Collectively, PKM2 may serve as a unique therapeutic target for overcoming tamoxifen resistance in breast cancers. This also provides a hint that PKM2 inhibitors combined with endocrine drugs may be a new strategy for the treatment of tamoxifen resistance in breast cancer patients.

Taken together, the present study demonstrated for the first time that PKM2-dependent c-Myc-Ser-62 phosphorylation stabilized c-Myc, thereby increasing survivin expression, which is required for breast cancer cell proliferation and migration. Inhibition of PKM2 blocked breast cancer progression and sensitized breast cancer cells to tamoxifen, indicating that PKM2 inhibitor may be an effective combination treatment in breast cancer patients treated with tamoxifen. Altogether, PKM2 represents a strong predictor for poor prognosis and drug resistance in breast cancer and targeting PKM2–c-Myc–survivin cascade could be a novel therapeutic strategy for breast cancer treatment, even tamoxifen resistant breast cancer.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PY and A-XL designed the study. PY, A-XL, X-SC, MT, and H-YW performed the experiments. PY, A-XL, X-LW, YZ, and K-SW collected, analyzed, and interpreted the data. A-XL and YC prepared the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.550469/full#supplementary-material

SUPPLEMENTAL FIGURE 1 | (A) GEPIA analysis showed the expression levels of PKM2 between ER+ and triple negative breast cancer tissues. **(B)** Western Blot was used to analyze PKM2 proteins in MCF-7, MDA-MB-231, and BT549 cells. β-Actin was used as a loading control.

REFERENCES

Azoitei, N., Becher, A., Steinestel, K., Rouhi, A., Diepold, K., Genze, F., et al. (2016). PKM2 promotes tumor angiogenesis by regulating HIF-1alpha through NF-kappaB activation. *Mol. Cancer* 15, 3. doi: 10.1186/s12943-015-0490-2

Chen, X., Duan, N., Zhang, C., and Zhang, W. (2016). Survivin and Tumorigenesis: Molecular Mechanisms and Therapeutic Strategies. *J. Cancer* 7 (3), 314–323. doi: 10.7150/jca.13332

Cheng, R., Liu, Y. J., Cui, J. W., Yang, M., Liu, X. L., Li, P., et al. (2017). Aspirin regulation of c-myc and cyclinD1 proteins to overcome tamoxifen resistance in estrogen receptor-positive breast cancer cells. *Oncotarget* 8 (18), 30252–30264. doi: 10.18632/oncotarget.16325

Christensen, D. R., Calder, P. C., and Houghton, F. D. (2015). GLUT3 and PKM2 regulate OCT4 expression and support the hypoxic culture of human embryonic stem cells. *Sci. Rep.* 5, 17500. doi: 10.1038/srep17500

Christofk, H. R., Vander Heiden, M. G., Harris, M. H., Ramanathan, A., Gerszten, R. E., Wei, R., et al. (2008). The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* 452 (7184), 230–233. doi: 10.1038/nature06734

Cosgrave, N., Hill, A. D., and Young, L. S. (2006). Growth factor-dependent regulation of survivin by c-myc in human breast cancer. *J. Mol. Endocrinol.* 37 (3), 377–390. doi: 10.1677/jme.1.02118

Dang, C. V. (1999). c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol. Cell Biol.* 19 (1), 1–11. doi: 10.1128/mcb.19.1.1

David, C. J., Chen, M., Assanah, M., Canoll, P., and Manley, J. L. (2010). HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* 463 (7279), 364–368. doi: 10.1038/nature08697

Fang, Z. H., Dong, C. L., Chen, Z., Zhou, B., Liu, N., Lan, H. F., et al. (2009). Transcriptional regulation of survivin by c-Myc in BCR/ABL-transformed cells: implications in anti-leukaemic strategy. *J. Cell Mol. Med.* 13 (8B), 2039–2052. doi: 10.1111/j.1582-4934.2008.00549.x

Gao, X., Wang, H., Yang, J. J., Liu, X., and Liu, Z. R. (2012). Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. *Mol. Cell* 45 (5), 598–609. doi: 10.1016/j.molcel.2012.01.001

Garg, H., Suri, P., Gupta, J. C., Talwar, G. P., and Dubey, S. (2016). Survivin: a unique target for tumor therapy. *Cancer Cell Int.* 16, 49. doi: 10.1186/s12935-016-0326-1

Ge, X., Zhao, Y., Dong, L., Seng, J., Zhang, X., and Dou, D. (2019). NAMPT regulates PKM2 nuclear location through 14-3-3zeta: Conferring resistance to tamoxifen in breast cancer. *J. Cell Physiol.* 234 (12), 23409–23420. doi: 10.1002/jcp.28910

Guo, M., Zhao, X., Yuan, X., Jiang, J., and Li, P. (2017). MiR-let-7a inhibits cell proliferation, migration, and invasion by down-regulating PKM2 in cervical cancer. *Oncotarget* 8 (17), 28226–28236. doi: 10.18632/oncotarget.15999

Hamanaka, R. B., and Chandel, N. S. (2011). Cell biology. Warburg effect and redox balance. *Science* 334 (6060), 1219–1220. doi: 10.1126/science.1215637

Haque, R., Song, J., Haque, M., Lei, F., Sandhu, P., Ni, B., et al. (2017). c-Myc-Induced Survivin Is Essential for Promoting the Notch-Dependent T Cell Differentiation from Hematopoietic Stem Cells. *Genes (Basel)* 8 (3), 97. doi: 10.3390/genes8030097

Harris, I., McCracken, S., and Mak, T. W. (2012). PKM2: a gatekeeper between growth and survival. *Cell Res.* 22 (3), 447–449. doi: 10.1038/cr.2011.203

Jiang, Y. H., Wang, Y. G., Wang, T., Hawke, D. H., Zheng, Y. H., Li, X. J., et al. (2014). PKM2 phosphorylates MLC2 and regulates cytokinesis of tumour cells. *Nat. Commun.* 5, 1–14. doi: 10.1038/ncomms6566

Kawasaki, H., Altieri, D. C., Lu, C. D., Toyoda, M., Tenjo, T., and Tanigawa, N. (1998). Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Res.* 58 (22), 5071–5074. doi: 10.1061/(ASCE)0733-9496(2009)135:1(48)

Kedinger, V., Meulle, A., Zounib, O., Bonnet, M. E., Gossart, J. B., Benoit, E., et al. (2013). Sticky siRNAs targeting survivin and cyclin B1 exert an antitumoral effect on melanoma subcutaneous xenografts and lung metastases. *BMC Cancer* 13, 338. doi: 10.1186/1471-2407-13-338

Li, Q., Zhang, D., Chen, X., He, L., Li, T., Xu, X., et al. (2015). Nuclear PKM2 contributes to gefitinib resistance via upregulation of STAT3 activation in colorectal cancer. *Sci. Rep.* 5, 16082. doi: 10.1038/srep16082

Liu, B. W., Wang, T. J., Li, L. L., Zhang, L., Liu, Y. X., Feng, J. Y., et al. (2019). Oncoprotein HBXIP induces PKM2 via transcription factor E2F1 to promote cell proliferation in ER-positive breast cancer. *Acta Pharmacol. Sin.* 40 (4), 530–538. doi: 10.1038/s41401-018-0015-9

Luo, W., and Semenza, G. L. (2012). Emerging roles of PKM2 in cell metabolism and cancer progression. *Trends Endocrinol. Metab.* 23 (11), 560–566. doi: 10.1016/j.tem.2012.06.010

Ma, W. H., Liu, Y. C., Xue, M. L., Zheng, Z., and Ge, Y. L. (2016). Downregulation of survivin expression exerts antitumoral effects on mouse breast cancer cells in vitro and in vivo. *Oncol. Lett.* 11 (1), 159–167. doi: 10.3892/ol.2015.3870

Marioni, G., Bertolin, A., Giacomelli, L., Marchese-Ragona, R., Savastano, M., Calgaro, N., et al. (2006). Expression of the apoptosis inhibitor protein Survivin in primary laryngeal carcinoma and cervical lymph node metastasis. *Anticancer Res.* 26 (5B), 3813–3817.

Matsuda, S., Adachi, J., Ihara, M., Tanuma, N., Shima, H., Kakizuka, A., et al. (2016). Nuclear pyruvate kinase M2 complex serves as a transcriptional coactivator of arylhydrocarbon receptor. *Nucleic Acids Res.* 44 (2), 636–647. doi: 10.1093/nar/gkv967

McKenzie, J. A., Liu, T., Goodson, A. G., and Grossman, D. (2010). Survivin enhances motility of melanoma cells by supporting Akt activation and {alpha}5 integrin upregulation. *Cancer Res.* 70 (20), 7927–7937. doi: 10.1158/0008-5472.CAN-10-0194

Monzo, M., Rosell, R., Felip, E., Astudillo, J., Sanchez, J. J., Maestre, J., et al. (1999). A novel anti-apoptosis gene: Re-expression of survivin messenger RNA as a prognosis marker in non-small-cell lung cancers. *J. Clin. Oncol.* 17 (7), 2100–2104. doi: 10.1200/JCO.1999.17.7.2100

Nass, N., and Kalinski, T. (2015). Tamoxifen resistance: from cell culture experiments towards novel biomarkers. *Pathol. Res. Pract.* 211 (3), 189–197. doi: 10.1016/j.prp.2015.01.004

Nesbit, C. E., Tersak, J. M., and Prochownik, E. V. (1999). MYC oncogenes and human neoplastic disease. *Oncogene* 18 (19), 3004–3016. doi: 10.1038/sj.onc.1202746

Papanikolaou, V., Iliopoulos, D., Dimou, I., Dubos, S., Kappas, C., Kitsiou-Tzeli, S., et al. (2011). Survivin regulation by HER2 through NF-kappaB and c-myc in irradiated breast cancer cells. *J. Cell Mol. Med.* 15 (7), 1542–1550. doi: 10.1111/j.1582-4934.2010.01149.x

Seo, H. R., Kim, J., Bae, S., Soh, J. W., and Lee, Y. S. (2008). Cdk5-mediated phosphorylation of c-Myc on Ser-62 is essential in transcriptional activation of cyclin B1 by cyclin G1. *J. Biol. Chem.* 283 (23), 15601–15610. doi: 10.1074/jbc.M800987200

Shen, Y., Chen, M., Huang, S., and Zou, X. (2016). Pantoprazole inhibits human gastric adenocarcinoma SGC-7901 cells by downregulating the expression of pyruvate kinase M2. *Oncol. Lett.* 11 (1), 717–722. doi: 10.3892/ol.2015.3912

Stetak, A., Veress, R., Ovadi, J., Csermely, P., Keri, G., and Ullrich, A. (2007). Nuclear translocation of the tumor marker pyruvate kinase M2 induces programmed cell death. *Cancer Res.* 67 (4), 1602–1608. doi: 10.1158/0008-5472.CAN-06-2870

Suzuki, A., Ito, T., Kawano, H., Hayashida, M., Hayasaki, Y., Tsutomi, Y., et al. (2000). Survivin initiates procaspase 3/p21 complex formation as a result of interaction with Cdk4 to resist Fas-mediated cell death. *Oncogene* 19 (10), 1346–1353. doi: 10.1038/sj.onc.1203429

Tanaka, K., Iwamoto, S., Gon, G., Nohara, T., Iwamoto, M., and Tanigawa, N. (2000). Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. *Clin. Cancer Res.* 6 (1), 127–134.

Urun, Y., Utkan, G., Yalcin, B., Akbulut, H., Onur, H., Oztuna, D. G., et al. (2015). The role of cardiac biomarkers as predictors of trastuzumab cardiotoxicity in patients with breast cancer. *Exp. Oncol.* 37 (1), 53–57. doi: 10.31768/2312-8852.2015.37(1):53-57

Wang, Y., Hao, F., Nan, Y., Qu, L., Na, W., Jia, C., et al. (2018). PKM2 Inhibitor Shikonin Overcomes the Cisplatin Resistance in Bladder Cancer by Inducing Necroptosis. *Int. J. Biol. Sci.* 14 (13), 1883–1891. doi: 10.7150/ijbs.27854

Yan, X. L., Zhang, X. B., Ao, R., and Guan, L. (2017). Effects of shRNA-Mediated Silencing of PKM2 Gene on Aerobic Glycolysis, Cell Migration, Cell Invasion, and Apoptosis in Colorectal Cancer Cells. *J. Cell Biochem.* 118 (12), 4792–4803. doi: 10.1002/jcb.26148

Yang, W., and Lu, Z. (2013). Regulation and function of pyruvate kinase M2 in cancer. *Cancer Lett.* 339 (2), 153–158. doi: 10.1016/j.canlet.2013.06.008

Yang, W., Xia, Y., Ji, H., Zheng, Y., Liang, J., Huang, W., et al. (2011). Nuclear PKM2 regulates beta-catenin transactivation upon EGFR activation. *Nature* 480 (7375), 118–122. doi: 10.1038/nature10598

Yang, W., Xia, Y., Hawke, D., Li, X., Liang, J., Xing, D., et al. (2012). PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. *Cell* 150 (4), 685–696. doi: 10.1016/j.cell.2012.07.018

Yang, R., Liu, M., Liang, H., Guo, S., Guo, X., Yuan, M., et al. (2016). miR-138-5p contributes to cell proliferation and invasion by targeting Survivin in bladder cancer cells. *Mol. Cancer* 15 (1), 82. doi: 10.1186/s12943-016-0569-4

Zheng, B., Geng, L., Zeng, L., Liu, F., and Huang, Q. (2018). AKT2 contributes to increase ovarian cancer cell migration and invasion through the AKT2-PKM2-STAT3/NF-kappaB axis. *Cell Signal* 45, 122–131. doi: 10.1016/j.cellsig.2018.01.021

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Immune Checkpoint Blockade Improves Chemotherapy in the PyMT Mammary Carcinoma Mouse Model

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Despite the success of immune checkpoint blockade in cancer, the number of patients that benefit from this revolutionary treatment option remains low. Therefore, efforts are being undertaken to sensitize tumors for immune checkpoint blockade, which includes combining immune checkpoint blocking agents such as anti-PD-1 antibodies with standard of care treatments. Here we report that a combination of chemotherapy (doxorubicin) and immune checkpoint blockade (anti-PD-1 antibodies) induces superior tumor control compared to chemotherapy and immune checkpoint blockade alone in the murine autochthonous polyoma middle T oncogene-driven (PyMT) mammary tumor model. Using whole transcriptome analysis, we identified a set of genes that were upregulated specifically upon chemoimmunotherapy. This gene signature and, more specifically, a condensed four-gene signature predicted favorable survival of human mammary carcinoma patients in the METABRIC cohort. Moreover, PyMT tumors treated with chemoimmunotherapy contained higher levels of cytotoxic lymphocytes, particularly natural killer cells (NK cells). Gene set enrichment analysis and bead-based ELISA measurements revealed increased IL-27 production and signaling in PyMT tumors upon chemoimmunotherapy. Moreover, IL-27 signaling improved NK cell cytotoxicity against PyMT cells *in vitro*. Taken together, our data support recent clinical observations indicating a benefit of chemoimmunotherapy compared to monotherapy in breast cancer and suggest potential underlying mechanisms.

Keywords: cancer, immunotherapy, chemotherapy, immune checkpoint, cytotoxic lymphocytes

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INTRODUCTION

The idea to engage the immune system in the fight against cancer was already proposed in the early twentieth century but was then mainly disregarded (1). Over a century later, the discovery of immune checkpoints as brakes of the immune system and the possibility to unleash those brakes to fight cancer was rewarded with the Nobel Prize in Physiology in 2018 as a new principle for cancer immunotherapy (Press release: The Nobel Prize in Physiology or Medicine 2018). Allison, Honjo and their coworkers discovered and elucidated the function of the negative costimulatory

molecules cytotoxic T lymphocyte–associated protein 4 (CTLA-4) and programmed death 1 (PD-1), respectively (2, 3). The blockade of those inhibitory checkpoint receptors by neutralizing monoclonal antibodies is now well-known as immune checkpoint blockade and is already broadly used in the clinic. Since the first clinical trial using immune checkpoint inhibitors in 2000, there have been numerous clinical trials with either anti-CTLA-4 or anti-PD-1 as single agent drugs. To date there have been at least 500 clinical studies with PD-1 blockers conducted on at least 20 cancer types (4). Anti-PD-1 drugs are now approved for a variety of highly immunogenic cancer types, including non-small cell lung cancer, renal cell carcinoma, hodgkin's lymphoma, and metastatic melanoma. Remarkably, PD-1 blockade has shown positive results in all mentioned malignancies, measured by the overall response rate (5). However, a significant proportion of patients does not respond to immunotherapy (6). Indeed, in patients with metastatic breast cancer, single-drug anti-PD-1 therapy has shown little efficacy, due to a lower mutational load and a lower abundance of tumor-infiltrating lymphocytes (TILs) (7). Therefore, new strategies are needed to enhance the efficacy of anti-PD-1 treatment in breast cancer. In the last few years, approaches to combine PD-1 blockade with conventional treatments such as chemotherapy have shown promising results even as first-line treatment in triple-negative metastatic breast cancer [(8), NCT02425891]. It is important to note, that chemotherapy still represents the preferred standard of systemic treatment for metastatic breast cancer and remains one of the most efficient ways to improve patient outcome by decreasing tumor burden and metastasis (9). However, major limitations of chemotherapy remain, foremost non-specific toxicity, and tumor chemoresistance (10, 11). Interestingly, a recent study suggested an involvement of PD-1 signaling in the acquisition of chemoresistance and therefore emphasized the rationale for a combinatorial chemoimmunotherapy in the clinical setting (12). In addition, chemotherapy was also shown to increase the immune infiltrate and inhibit immunosuppressive components in the tumor microenvironment, which in turn can improve immune checkpoint blockade. Taken together, these findings substantiate combinatorial chemoimmunotherapy as a reasonable approach to fight breast cancer. In this study, we therefore analyzed the impact of combinatorial chemotherapy and immune checkpoint blockade in the PyMT mammary carcinoma mouse model (13), since previous studies using this model failed to show effectiveness of anti-PD-1 monotherapy (14, 15).

MATERIALS AND METHODS

Animal Experiments

Female mice expressing the polyoma virus middle T oncoprotein (PyMT) under the Mouse Mammary Tumor Virus (MMTV) promoter in a C57BL/6 background were used. In the PyMT model, mice spontaneously develop tumors in each mammary gland starting from 8 weeks after birth. Mice were divided into four groups according to treatment (anti-PD-1, IgG1, doxorubicin (DOX) + anti-PD-1, and DOX + IgG1). For animals receiving immune checkpoint blockade only, treatment was initiated (day 0) once the first tumor reached a size of 0.6 cm in diameter. Antibodies were administrated i.p. at a concentration of 20 mg/kg (on day 0) and 10 mg/kg (on day 6, 12, 18). All mice received either anti-mouse PD-1 antibody (4H2, Ono Pharmaceutical, Osaka, Japan) or anti-mouse IgG1 (BioXcell/Hölzel Diagnostik, Cologne, Germany) diluted in sterile 0.9% NaCl. In the model with the combination of chemotherapy and immune checkpoint blockade treatment started once the first mammary tumor reached a size of 1 cm in diameter. Doxorubicin (Cell Pharm, Bad-Vilbel, Germany) diluted in sterile 0.9% NaCl was administrated i.p. (5 mg/kg) once a week for 5 weeks. One day after doxorubicin administration, mice were treated with 10 mg/kg of either anti-mouse PD-1 antibody (4H2, Ono Pharmaceutical) or anti-mouse IgG1 (BioXCell). Mice were monitored three times a week for up to 5 weeks after initial treatment. Tumor size was determined by tumor palpating. The tumor volume was calculated using the formula: $V = \text{length} \times \text{width}^2 \times \pi/6$. For all animal experiments the guidelines of the Hessian animal care and use committee were followed (approval numbers: FU1127, FU1191).

Flow Cytometry

Tumor single cell suspensions were generated using the Tumor Dissociation Kit and the gentleMACS™ Dissociator (both from Miltenyi Biotec, Bergisch Gladbach, Germany) using standard protocols. The following anti-mouse antibodies were used for staining of single cell suspensions: anti-CD3-PE-CF594, anti-CD4-BV711, anti-CD8-BV650, anti-CD11c-BV711, anti-CD19-APC-Cy7, anti-CD45-AlexaFluor700, anti-CD49f-PE-CF594, anti-CD146-AlexaFluor488, anti-CD326-BV711, anti-Ly6C-PerCP-Cy5.5, anti-NK1.1-BV510 (all from BD Biosciences, Heidelberg, Germany), anti-CD31-PE-Cy7, anti-CD117-APC-eFluor780 (both from eBioscience, San Diego, USA), anti-CD90.2-PE, anti-MHC-II-APC (both from Miltenyi Biotec), anti-CD11b-BV605, anti-CD324-AlexaFluor647, anti-F4/80-PE-Cy7, anti-GITR-FITC, anti-Ly6G-APC-Cy7, anti-SiglecH-FITC, and anti-γδTCR-APC (all from Biolegend, San Diego, USA). NK/PyMT cell co-culture samples were stained with the following antibodies. anti-CD25-PE-Cy7, anti-CD69-BV605, anti-CD107a-PE and anti-NK1.1-APC (all from Biolegend). Samples were acquired with a LSR II/Fortessa™ flow cytometer (BD Biosciences) and analyzed using FlowJo software V10 (BD Biosciences). All antibodies and secondary reagents were titrated to determine optimal concentrations. CompBeads (BD Bioscience) were used for single-color compensation to create multi-color compensation matrices. For gating, fluorescence minus one (FMO) controls were used. The instrument calibration was controlled daily using Cytometer Setup and Tracking (CS&T) beads (BD Bioscience).

RNA Sequencing

Total RNA was isolated from snap frozen PyMT tumors using the peqGOLD Total RNA Kit (VWR International, Darmstadt, Germany). RNA samples were analyzed on a 2100 Bioanalyzer using Agilent RNA 6000 Nano chip (both from Agilent Technologies, Santa Clara, USA). Library preparation

was performed using the SMARTer® Stranded Total RNA Sample Prep Kit–HI (Takara Bio Europe, Saint-Germain-en-Laye, France). Quantity and quality of the cDNA libraries were determined by Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Dreieich, Germany) and Agilent High Sensitivity DNA chip (Agilent Technologies). Libraries were sequenced on a NextSeq 500 sequencer (single end, 75 cycles) using V2 chemistry (Illumina, San Diego, USA). Sequencing data were analyzed using the SeqBox software (16). In brief, after adapter trimming with skewer (17), the software used STAR (18) to map the reads to the mouse reference genome (mm10) and RSEM (19) for gene and isoform-level quantification, which allows the differential expression analysis by DESeq2 (20).

Analysis of Publicly Available Human Mammary Carcinoma Datasets

The METABRIC data set (21) was used to determine patient survival according to the gene signatures obtained from the PyMT mouse model upon combinatorial chemoimmunotherapy.

Phenoptics™ Immunofluorescence Analysis

Tumors were zinc-fixed, paraffin-embedded, and subsequently stained in a fluorescent multiplex immunohistochemistry staining using the Opal™ 7-Color Fluorescent Immunohistochemistry (IHC) Kits (Akoya, Marlborough, USA). The following anti-mouse antibodies were used: anti-αSMA (Sigma-Aldrich, Schnelldorf, Germany F3777), anti-DIO2 (Elabscience, Houston, USA, E-A-13198), anti-GSN (Biozol, Eching, Germany, BOB-PA2109), anti-MMP3 (Santa Cruz, Heidelberg, Germany, sc-21732), anti-Pan-Cytokeratin (Abcam, ab27988), anti-PD-L1 (Cell signaling, D5V38), and anti-PDK4 (Antibodies-online, Aachen, Germany, ABIN3028963) in an automated staining using the BOND RX Automated IHC Research Stainer (Leica Biosystems, Nussloch, Germany). Stained tumor sections were scanned using Vectra® 3 automated quantitative pathology imaging system and analyzed using inForm® software V2.3 (both Akoya). Marker expression in the cytoplasm was quantified with the inForm® software using a positivity or 4-bin (0–3+) scoring algorithm (22). For the latter spectrally unmixed fluorescence signals in the cytoplasm of epithelial or stromal cells were grouped into four bins based on signal distribution (0 = lowest signal, 3 = highest signal), indicating differences in protein expression.

Gene Set Enrichment Analysis

Using gene expression values (expression >0.1 log2 TPM values after DESeq2) between individual treatment groups as an input, enriched biological processes were identified using Gene Set Enrichment Analysis (GSEA) version 4.0.0 (23).

Protein Quantification

Tumor interstitial fluids were obtained by manual cryopulverization and subsequent incubation with 1:2 tumor weight/volume of 2 × PBS for 3 h at 4°C under rotation. The LEGENDplex™ mouse inflammation panel (Biolegend) was used to determine cytokines levels in the tumor supernatants. To quantify protein levels in NK/PyMT cell co-culture supernatants, ELISA kit for PRF1 (Abbexa, Cambridge, UK, abx258736) as well as the mouse IFN-γ Flex Set (BD Bioscience, 558296) were utilized according to the manufacturer's instructions. Bead-based array samples were acquired by flow cytometry and analyzed using FlowJo V10.

Cytotoxicity Assay

NK cells were isolated from spleens of either wildtype (WT) or IL-27 receptor α (IL-27Rα) KO mice using the EasySep™ Mouse NK Cell Isolation Kit (STEMCELL™ Technologies, Vancouver, Canada). NK cells used as effector cells were co-cultured for 4 h at 37°C with PyMT target cells at different effector cell-target cell ratios, as indicated. Both NK cells and PyMT cells were labeled with different fluorescent dyes (PKH67 & PKH26, Sigma-Aldrich) and dead PyMT cells were identified using 7-AAD staining (Miltenyi Biotec). Living (7-AAD-negative) PyMT cells were subsequently determined via flow cytometry.

Quantitative PCR

RNA was isolated as described above followed by cDNA transcription using the Sensiscript® cDNA synthesis kit (Qiagen, Hilden, Germany). The following murine primers were used: *Cd25*, sense: 5′-CAAGAACGGCACCATCCTAAA-3′, anti-sense: 5′-TCCTAAGCAACGCATATAGACCA-3′; *Cd69,* sense: 5′-AAGCGATATTCTGGTG AACTGG-3′, anti-sense: 5′-ATTTGCCCATTTCCATGTCTGA-3′; *Prf1*, sense: 5′-CTG CCACTCGGTCAGAATG-3′, anti-sense: 5′-CGGAGGGTAGTCACATCCAT-3′. *Rps27a* served as internal control. Data were analyzed using QuantStudio™ (Thermo Fisher Scientific).

Statistics

Data are presented as means ± SEM. Statistical comparisons between two groups were performed using either two-way ANOVA, Mann-Whitney test or unpaired two-tailed Student's *t*-test as indicated. For the latter two data were pre-analyzed to determine normal distribution and equal variance with D'Agostino–Pearson omnibus normality test. Differences in patient survival were analyzed using Log-rank (Mantel–Cox) test. Statistical analysis was performed with GraphPad Prism V8. Differences were considered significant at $p < 0.05$. Asterisks indicate significant differences between experimental groups ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

RESULTS

Doxorubicin Chemotherapy Improves the Response to PD-1 Blockade

We and others previously observed that anti-PD1 therapy was poorly effective in the PyMT mouse model of mammary carcinoma (14, 15). Sensitizing non-responsive tumors for immune checkpoint blockade is a major goal in current immunotherapy. Therefore, we asked whether a combinatorial approach consisting of doxorubicin (DOX) chemotherapy and anti-PD-1 antibody administration has an enhanced efficacy in reducing tumor growth compared to anti-PD-1 monotherapy.

Tumors in the PyMT mouse model arise spontaneously starting 8 weeks after birth. A therapeutic setting was employed, where treatment was initiated once a tumor diameter of 0.6 cm (anti-PD-1 alone) or 1 cm (DOX/anti-PD-1) had been reached. The smaller initial size in case of anti-PD-1 monotherapy was chosen to allow monitoring tumor growth over 4 weeks without reaching ethical endpoints of tumor size. Mice received intraperitoneal (i.p.) injections with either a PD-1-blocking antibody (10–20 mg/kg) or an IgG1 isotype control antibody (10–20 mg/kg) alone or with preceding DOX administration i.p. (5 mg/kg) (**Figures 1A,B**). Although anti-PD-1 monotherapy significantly slowed progression of primary tumors compared

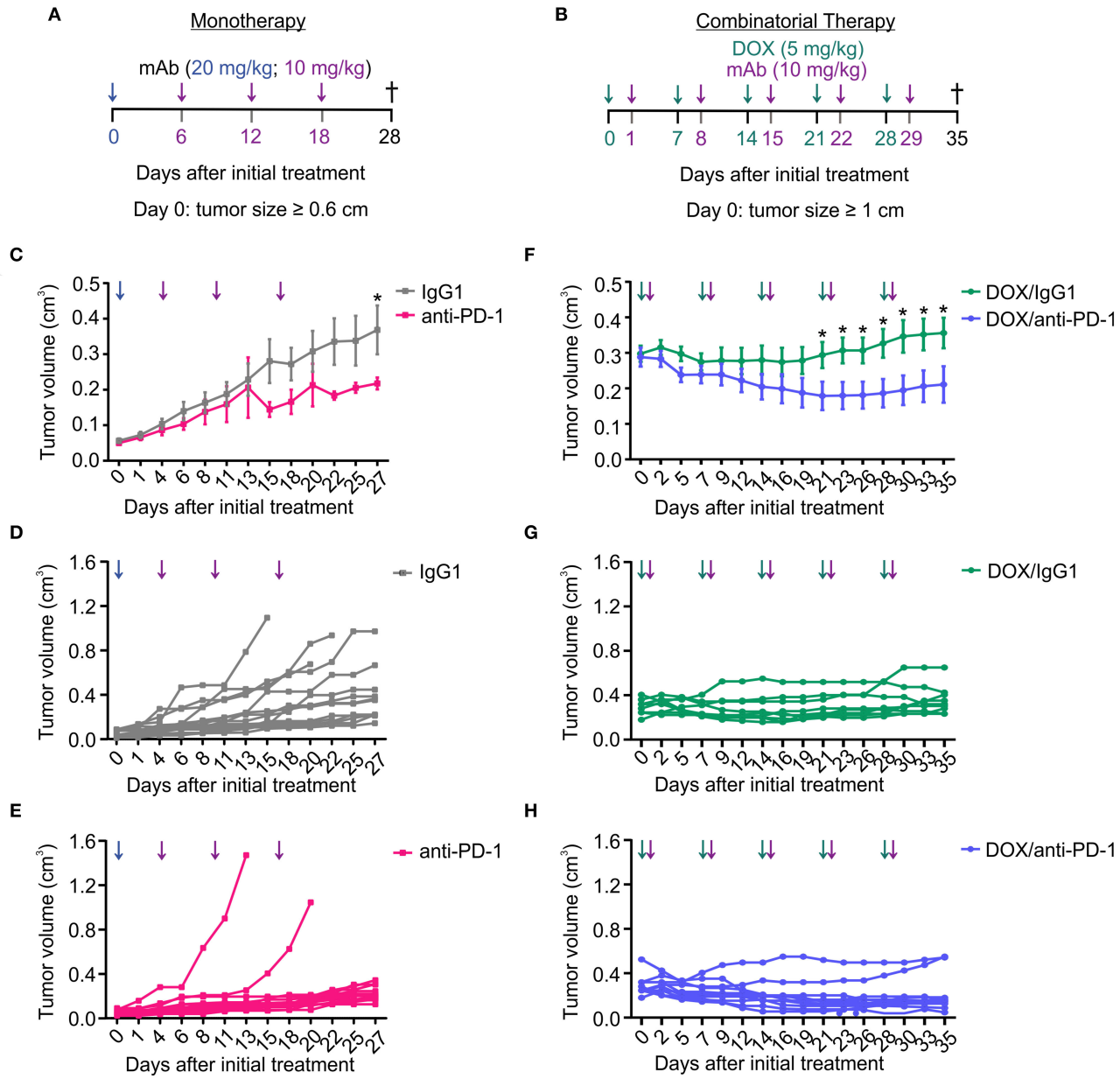

FIGURE 1 | Combination of chemotherapy and PD-1 blockade improves tumor control in the PyMT model. Treatment regimens for anti-PD-1 monotherapy and doxorubicin (DOX) plus anti-PD-1 combinatorial therapy. **(A)** Treatment regimen of monotherapy. PyMT tumors were treated with either anti-PD-1 or isotype control (IgG1) antibody (i.p.) every 6 days for 18 days (day 0 = 20 mg/kg; day 6, 12, 18 = 10 mg/kg) once the first tumor reached a size of 0.6 cm in diameter. **(B)** Treatment regimen of combinatorial therapy. PyMT tumors were treated with 5 mg/kg doxorubicin (DOX) (i.p.) and with 10 mg/kg of either anti-PD-1 or isotype control (IgG1) antibody (i.p.) once weekly for 5 weeks once the first tumor reached a size of 1 cm in diameter. **(C,F)** Cumulative tumor volume (length × width² × π/6) of primary tumors upon **(C)** monotherapy ($n = 17$ each) and **(F)** combinatorial therapy (DOX/IgG1: $n = 11$, DOX/anti-PD-1: $n = 10$) over time are shown, as well as the individual tumor volumes for **(D,E)** monotherapy and **(G,H)** chemoimmunotherapy. Data are means ± SEM, p-values were calculated using unpaired t-test; $^*p < 0.05$.

to the IgG1 control, this effect was modest, and we did not observe tumor regression (**Figures 1C–E**). In contrast, combinatorial therapy with DOX and anti-PD-1 antibody not only markedly suppressed tumor progression but also significantly reduced tumor volumes from day 21 onwards when compared to the DOX/IgG1 control (**Figures 1F–H**). Although tumor reduction was also observed upon DOX/IgG1 administration at least in some tumors, the majority of DOX/IgG1 treated tumors either responded poorly or relapsed toward the end of the study (**Figure 1G**). Notably, only two DOX/anti-PD-1 mice showed tumor progression (**Figure 1H**). In conclusion, these results show that, in the PyMT tumor model, the efficacy of anti-PD-1 treatment is enhanced by DOX chemotherapy as indicated by a partial tumor remission upon combinatorial chemoimmunotherapy.

Increased NK Cell Infiltrate Upon Combinatorial Chemotherapy and PD-1 Blockade

We wondered whether the increased susceptibility to chemoimmunotherapy was associated with increases in PD-L1 expression in tumors upon chemotherapy. Therefore, PyMT tumor sections of all four treatment groups were stained for PD-L1 and DAPI (nuclei) using Phenoptics™ multiplex IHC staining (**Figure 2A**). Interestingly, PD-L1 expression scoring with the inForm® software using a 4-bin scoring algorithm revealed no alteration in PD-L1 expression in tumors of the different treatment regimens (**Figure 2B**). Thus, alterations in PD-L1 expression did not account for improved tumor control due to chemoimmunotherapy. Next, multicolor flow cytometry analysis of tumor single-cell suspensions of all four treatment groups was performed at the experimental endpoint to investigate cellular alterations potentially increasing efficacy of the combinatorial therapy (**Supplementary Figure 1**). Flow cytometry revealed no differences in CD45+ immune cell abundance between the different treatments (**Figure 2C**). Administration of the neutralizing anti-PD-1 antibody induced an efficient depletion of PD-1 on CD4+ and CD8+ T cells as compared to the corresponding IgG1 control, while chemotherapy *per se* did not alter the abundance of PD-1-expressing T cells within the total immune cell population (**Figure 2D**). Within the CD45+ immune cell population, dendritic cell (DC) levels were unchanged, whereas monocyte and resident macrophage abundance was reduced upon chemotherapy. For monocytes, this reduction was even accentuated when the anti-PD1 antibody was applied (fold change monocytes: IgG1:DOX/IgG1 = 0.32, anti-PD-1:DOX/anti-PD-1 = 0.012; resident macrophages: IgG1:DOX/IgG1 = 0.55, anti-PD-1:DOX/anti-PD-1 = 0.47) (**Figure 2E**). Furthermore, neutrophil and tumor-associated macrophage (TAM) frequencies decreased after DOX/anti-PD-1 administration compared to anti-PD-1 monotherapy (fold change neutrophils: 0.49; TAMs: 0.65). Although B cell and NKT cell numbers in the lymphoid cell lineage were unaltered, overall T cell levels including CD4+ T cell and Treg frequencies increased after chemotherapy (fold change T cells: IgG1:DOX/IgG1 = 1.79, anti-PD-1:DOX/anti-PD-1 = 2.77; CD4+: IgG1:DOX/IgG1 = 3.62, anti-PD-1:DOX/anti-PD-1 = 3.43; Tregs: IgG1:DOX/IgG1 = 5.24, anti-PD-1:DOX/anti-PD-1 = 2.35) (**Figure 2F**). Moreover, chemotherapy in combination with anti-PD-1 administration enhanced CD8+ T abundance compared to anti-PD-1 monotherapy by 2.2-fold. Most interestingly, γδ T cell and NK cell levels were elevated upon combinatorial DOX/anti-PD-1 therapy as compared to monotherapy or DOX/IgG1 administration (fold change γδ T cells: anti-PD-1:DOX/anti-PD-1 = 3.22, DOX/IgG1:DOX/anti-PD-1 = 2.4; NK cells: anti-PD-1:DOX/anti-PD-1 = 4.54, DOX/IgG1:DOX/anti-PD-1 = 2.24). Taken together, flow cytometry data did not provide a clear explanation on the cellular mechanisms responsible for the increased efficacy of chemoimmunotherapy. However, increased cytotoxic lymphocyte levels, including NK cells, upon combinatorial DOX/anti-PD-1 therapy emerged as a promising lead.

Gene Signatures Predict Survival of Human Mammary Carcinoma Patients

To gain explanations for increased NK cell frequencies upon chemoimmunotherapy and to gain further insights into potential other mechanisms explaining the success of DOX/anti-PD-1 combinatorial therapy vs. monotherapy, whole transcriptome RNA-Seq was performed. For this purpose, mRNA was isolated from whole PyMT tumors, sequenced using NextSeq 500 and data were analyzed using DESeq2 (differentially regulated genes: adjusted $p < 0.1$; $\log_2$ fold change in expression >1). Only 4 genes were found to be significantly altered between the IgG1 control and the anti-PD-1 monotherapy group, and 19 genes were altered when comparing the DOX/IgG1 with the DOX/anti-PD-1 group, while 93 genes were differently regulated between the DOX/IgG1 and the IgG1 group (**Supplementary Table 1, Supplementary Figure 2**). 43 genes were found to be differentially expressed, comparing anti-PD-1 treatment to the combination of DOX and anti-PD-1 (**Figure 3A**). There was no meaningful overlap between the different gene signatures (**Supplementary Figure 3**), indicating that each treatment group was characterized by a unique response pattern. Out of the 43 genes altered when comparing anti-PD-1 to chemoimmunotherapy, 21 were upregulated upon DOX/anti-PD-1 administration relative to anti-PD-1 monotherapy, whereas 22 were downregulated. To test the validity of these gene signatures, we analyzed if they would hold predictive value in human mammary carcinoma. Therefore, mean expression values of genes either up- or downregulated in our model were obtained from the METABRIC data set (21). These mean expression values were then compared with clinical data in the same dataset (**Figures 3B–H**). Patients were grouped into quartiles based on the unranked mean expression of the different gene signatures and survival rates of patients with low expression (<25% percentile) were compared to those with high expression (>75% percentile). Strikingly, analyzing the METABRIC dataset revealed that patients expressing low levels of genes downregulated in PyMT tumors treated with chemoimmunotherapy showed improved survival (**Figure 3B**). This was even more pronounced for patients expressing high levels of genes that were upregulated

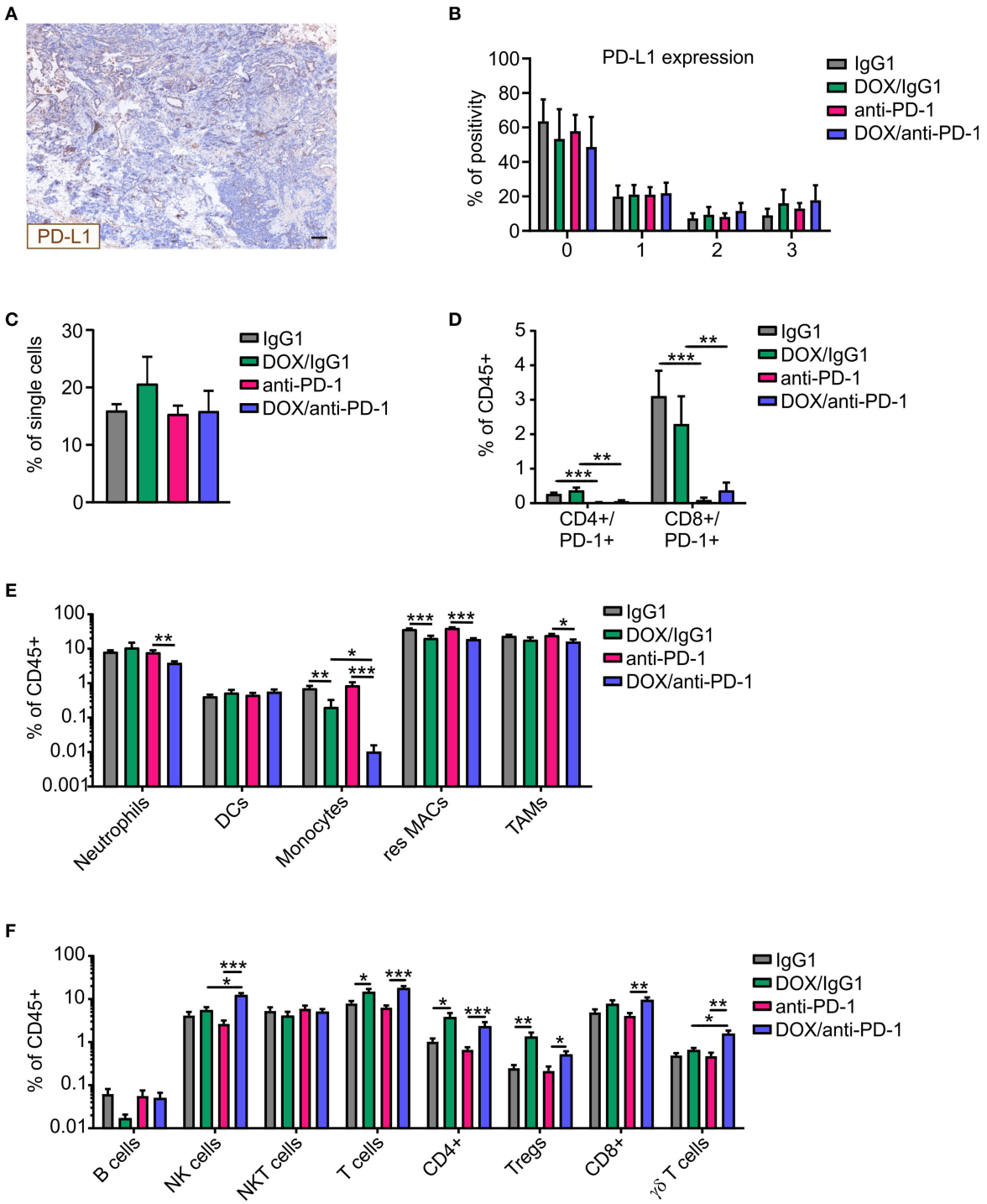

FIGURE 2 | Altered immune cell composition in PyMT tumors upon chemoimmunotherapy. PyMT tumor-bearing animals were treated with either anti-PD-1 or doxorubicin (DOX) plus anti-PD-1 and the corresponding IgG1 antibody control. PyMT tumor sections ($n \geq 4$) were stained for PD-L1 as well as for DAPI (nuclei) and analyzed using Phenoptics™. **(A)** Representative image shows PD-L1 expression for a doxorubicin (DOX) plus anti-PD-1 section. Scale bar: 100 μm. **(B)** Quantification of PD-L1 percentage positivity using the inForm® software with a 4-bin scoring algorithm (0, lowest expression; 3, highest expression). The relative frequencies of **(C)** CD45+ immune cells within total single cells and **(D)** CD4+/PD-1+ and CD8+/PD-1+ T cells **(E)** myeloid cell subsets as well as **(F)** lymphoid cell subsets relative to total CD45+ immune cells are displayed (IgG1: $n = 17$, anti-PD-1: $n = 16$, DOX/IgG1: $n = 10$, DOX/anti-PD-1: $n = 9$). Data are means ± SEM, p-values were calculated using unpaired t-test or Mann–Whitney test according to D'Agostino & Pearson omnibus normality test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

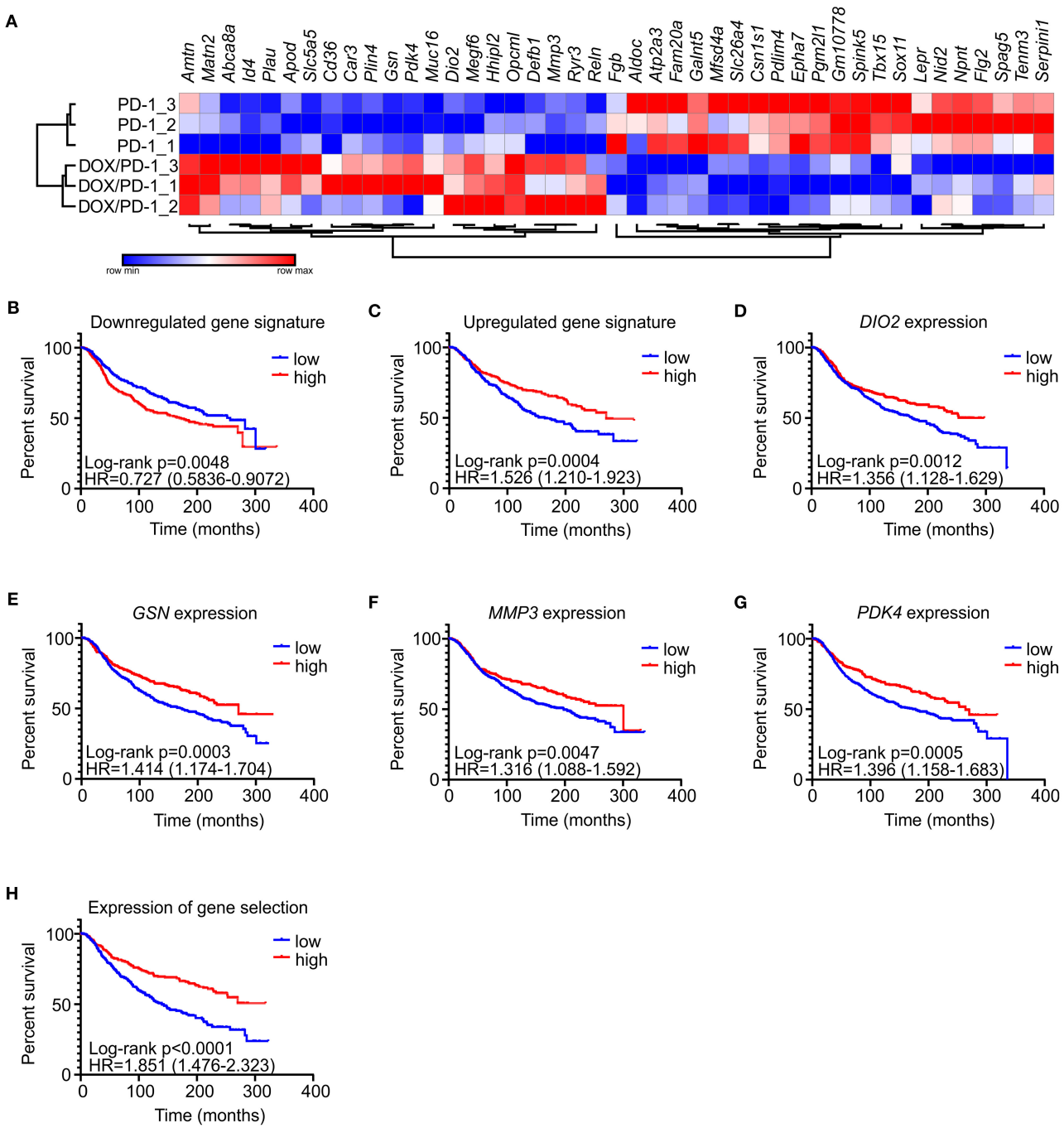

FIGURE 3 | Gene signatures of PyMT tumors treated with DOX/anti-PD-1 combinatorial therapy predict human mammary carcinoma patient survival. **(A)** Comparative transcriptome analysis of PyMT tumors upon anti-PD-1 treatment and doxorubicin (DOX) plus anti-PD-1 therapy ($n = 3$ each). Transcriptomes were generated by RNA seq. The heat map shows differentially expressed genes between both groups. **(B–H)** The METABRIC dataset (21) was analyzed for a correlation with gene signatures derived from PyMT tumors treated with DOX/anti-PD-1 compared to anti-PD-1 monotherapy. Patients were grouped into quartiles based on unranked mean expression of up- or downregulated genes and survival was analyzed. Survival rates of patients expressing high (>75% percentile) or low (<25% percentile) levels of the signature genes were compared. Shown are survival rates of patients expressing the gene set that was **(B)** downregulated or **(C)** upregulated upon DOX/anti-PD-1 therapy. The survival rates of patients expressing individual predictive upregulated genes **(D–G)**, or mean expression of these genes **(H)** are displayed. *p*-values were calculated using log-rank test.

in PyMT tumors treated with combinatorial therapy (**Figure 3C**). Hence, patient prognosis improved if they showed high expression of genes that were upregulated upon DOX/anti-PD-1 treatment and inversely also improved if they showed low expression levels of genes that were downregulated upon DOX/anti-PD-1 treatment in the PyMT model. Since the

difference in patient survival was more notable when using the upregulated gene signature, all upregulated genes were further analyzed on their individual impact on patient survival in the METABRIC dataset. Amongst all upregulated genes, four genes were found to be individually associated with improved patient survival, namely type II iodothyronine deiodinase (*DIO2*), gelsolin (*GSN*), matrix metalloproteinase 3 (*MMP3*) and pyruvate dehydrogenase kinase 4 (*PDK4*) (**Figures 3D–G**). Accordingly, a gene signature consisting of these four genes more accurately discriminated patients with improved or reduced survival prognosis when compared to the gene signature of all 21 upregulated genes (**Figure 3H**). DIO2 processes the hormone thyroxine (T4) to the more potent triiodothyronine (T3) to enhance growth, development and metabolism (24). DIO2 was overexpressed in brain tumors (oligoastrocytoma, glioblastoma, oligodendroglioma, pituitary tumors) and in thyroid adenoma (24), and in endometrial and colorectal cancer high expression was associated with a favorable prognosis (25). PDK4 regulates glucose metabolism and mitochondrial respiration and can have oncogenic or tumor suppressive effects depending on cancer type. In hepatocellular carcinoma downregulation of PDK4 is associated with poor prognosis (26), and PDK4 downregulation in lung cancer promoted cell proliferation and tumor growth (27), while high PDK4 expression was correlated with poor patient outcome in breast cancer (28). GSN and MMP3 are both involved in extracellular matrix (ECM) remodeling. GSN is a ubiquitous actin filament-severing protein (29), whose tumor-suppressive functions on various cancer types when highly expressed were previously noted (30, 31). In colon cancer, for instance, overexpression of GSN reduces proliferation and invasion of colon carcinoma cells (32) and in breast cancer downregulation of GSN correlates with malignant progression (33). MMP3 degrades several components of the ECM. Previous studies attributed oncogenic effects to MMP3 (34, 35), and high expression of MMP3 is considered unfavorable in pancreatic, pulmonary, and mammary carcinoma (36).

Histological Validation of Predictive Genes Confirms Transcriptome Analyses

To validate the impact of these four selected target genes upon chemoimmunotherapy at protein level, PyMT tumor sections of all four treatment groups were stained using Phenoptics™ multiplex IHC staining. Therefore, tumor sections were stained for the four specific prognostic markers, as well as for Pan-Cytokeratin (Pan CK) as an epithelial/tumor marker, alpha-smooth muscle actin (α-SMA) as a stromal marker and were counterstained with DAPI (**Figures 4A–D**). Tumor tissues were segmented into stromal and epithelial compartments and the four markers were quantified within these two tumor fractions, respectively, using the inForm® software with a 4-bin scoring algorithm (**Figures 4E–L**). Spectrally unmixed fluorescence signals in the cytoplasm of epithelial or stromal cells were grouped into four bins based on signal distribution (0 = lowest signal, 3 = highest signal), indicating differences in protein expression. The distribution within the four bins was calculated accordingly. These analyses revealed that DIO2 expression was significantly elevated in both the epithelial and the stromal compartment of DOX/anti-PD-1 treated tumors compared to anti-PD-1 only treated tumors, as represented by decreased levels in the first bin (lowest expression) and enhanced levels in the fourth bin (highest expression) (**Figures 4E,F**). In contrast, GSN expression was unchanged throughout the different treatments and bins (**Figures 4G,H**). MMP3 expression decreased in the chemoimmunotherapy group in the first bin in both the stromal and epithelial compartment (**Figures 4I,J**), indicating that tumors administered with DOX/anti-PD-1 showed enhanced protein levels of MMP3 as compared to anti-PD-1 treated tumors. Finally, PDK4 signals were solely increased in the epithelial section of tumors treated with DOX/anti-PD-1 combination therapy (**Figures 4K,L**). Overall, the histology data generally supported our findings at the transcriptome level, since three out of four markers that were transcriptionally upregulated upon chemoimmunotherapy, and were predictive in human mammary carcinoma patients were also elevated at protein level. The individual function of these proteins in the context of tumor control remains to be determined.

IL-27 Is Induced Upon Chemoimmunotherapy and Enhances NK Cell Activation and Cytotoxicity Toward PyMT Tumor Cells

In addition to the histological analysis, transcriptome data were also used for gene set enrichment analyses (GSEA) to identify gene sets that were differentially regulated between individual treatment groups within the Molecular Signatures Database (normalized enrichment score $\geq$1.6, $p \leq 0.05$, FDR $q \leq 0.25$). DOX monotherapy (+ IgG1) induced the most prominent changes (58 gene sets induced) when compared to IgG1 treatment alone, with a number of pathways being induced by DOX treatment that suggest changes in intracellular signaling events (**Supplementary Table 2**). When performing GSEA to compare pathways between the DOX/anti-PD-1 and the anti-PD-1 group, we found that 13 gene sets enriched upon (**Figure 5A**; **Supplementary Table 2**). Amongst the gene sets most significantly enriched in the DOX/anti-PD-1 group were IL-12 family signaling, as well as individual pathways within this cytokine family, namely IL-12 and IL-27 signaling (**Figures 5B,C**). These GSEA results raised the question whether IL-12 or IL-27 protein levels were altered in PyMT tumors when comparing chemoimmunotherapy and anti-PD-1 monotherapy. Therefore, tumor interstitial fluids of all initial four treatment groups were analyzed via the LEGENDplex™ Mouse Inflammation Panel, determining protein levels of 13 different cytokines (**Figure 5D**). While most cytokine levels were not significantly altered, chemotherapy reduced IL-17A levels as well as GM-CSF levels. However, most interestingly, whereas IL-12p70 amounts were rather, although not significantly, decreased, IL-27 levels were elevated upon chemotherapy plus anti-PD-1 treatment compared to anti-PD-1 monotherapy. Since these data suggested an involvement of IL-27 signaling in the anti-tumor efficacy of chemoimmunotherapy and flow cytometry analysis revealed enhanced NK cells frequencies upon this combinatorial

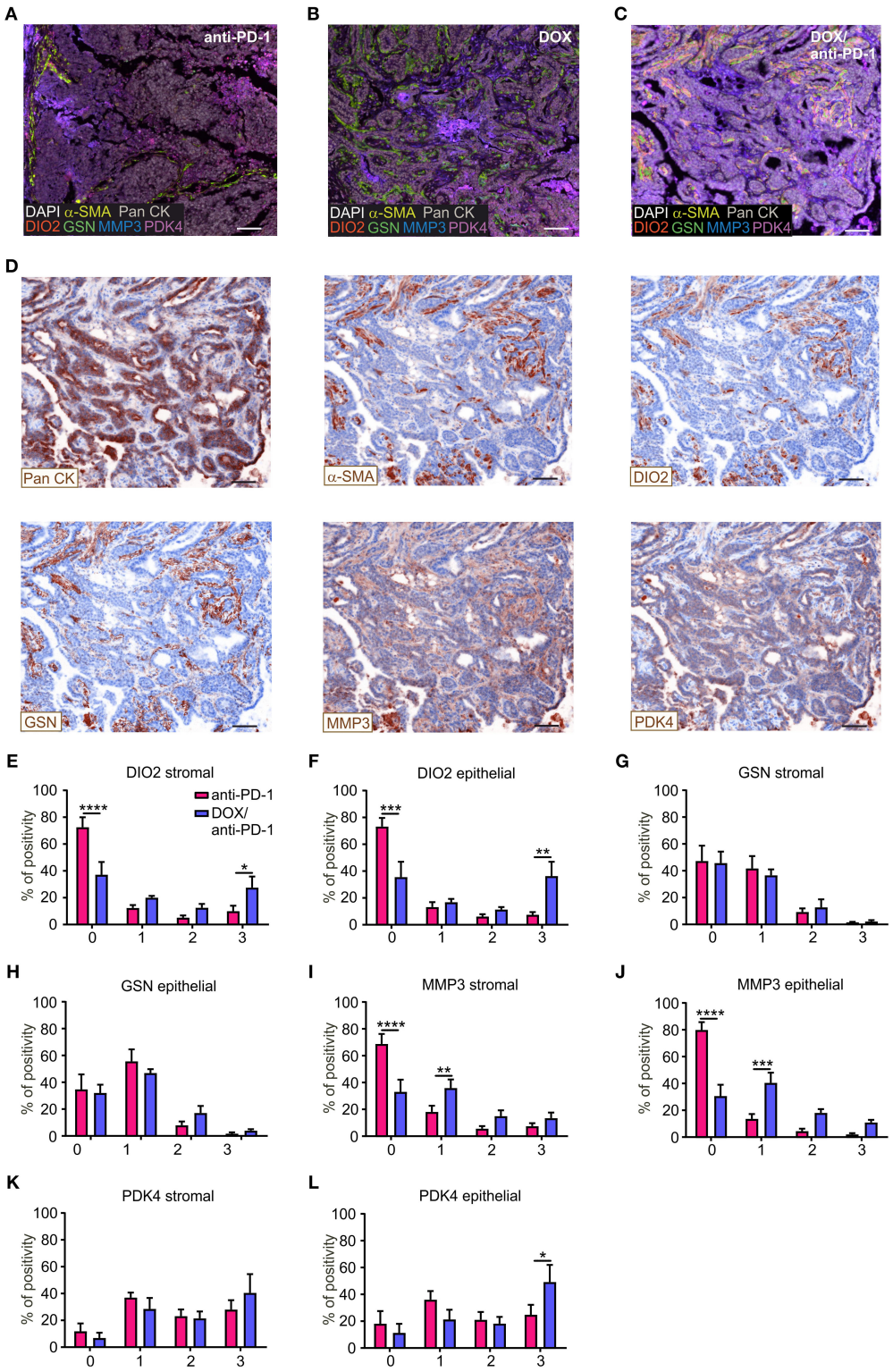

FIGURE 4 | Histological validation of predictive genes. PyMT tumor sections ($n = 6$ each) were stained for DIO2, GSN, MMP3, PDK4, Pan-Cytokeratin (Pan CK; epithelial marker), α-SMA (stromal marker), DAPI (nuclei), and analyzed using Phenoptics™. Representative images show combined expression of all markers for **(A)** anti-PD-1, **(B)** doxorubicin (DOX), and **(C)** DOX plus anti-PD-1 treated tumors as well as **(D)** the expression of single markers for the DOX/anti-PD-1 section. Scale bars: 100 μm. **(E–L)** Quantification of marker percentage positivity using the inForm® software and a 4-bin scoring algorithm (0, lowest expression; 3, highest expression). Data are means ± SEM, p-values were calculated using two-way ANOVA with uncorrected Fisher's LSD test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

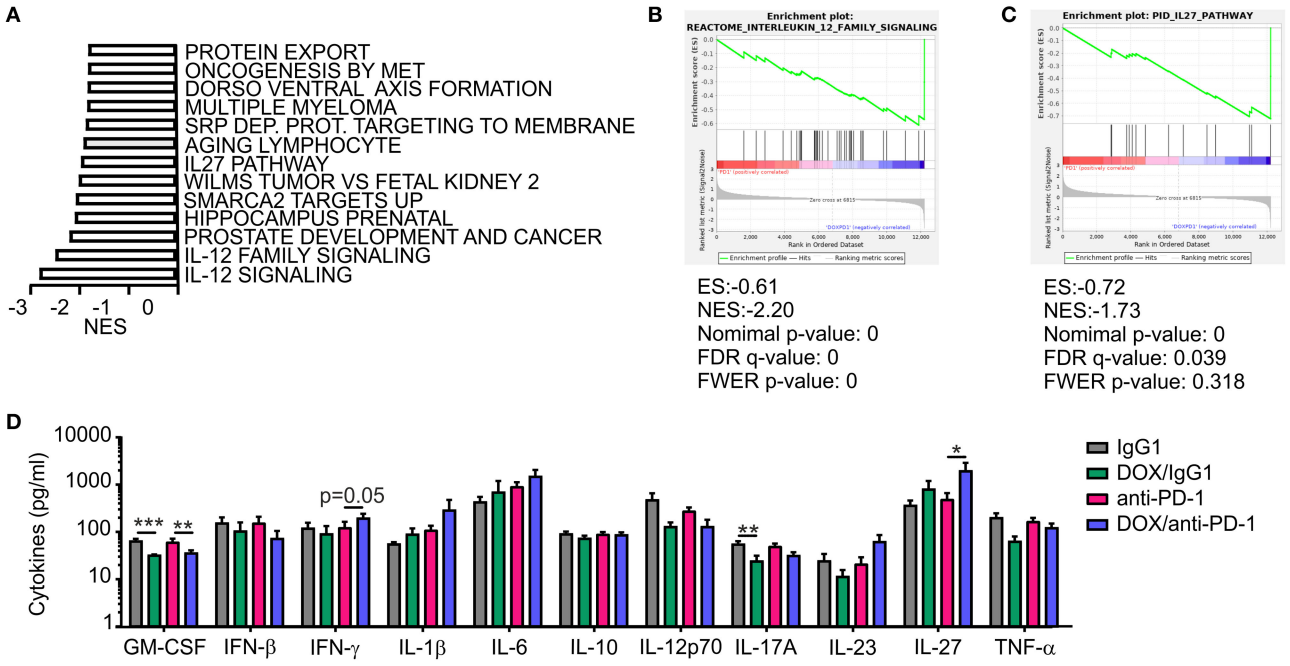

FIGURE 5 | Increased IL-27 expression in PyMT tumors upon DOX/anti-PD-1 therapy. **(A–C)** Gene set enrichment analysis (GSEA) was performed using transcriptome data of tumors from PyMT mice treated with doxorubicin (DOX) plus anti-PD-1 compared to anti-PD-1 monotherapy. **(A)** Significantly ($p < 0.05$; false discovery rate (FDR) <0.25) enriched pathways upon DOX/anti-PD-1 therapy compared to anti-PD-1 monotherapy are shown. NES, normalized enrichment score. Selected enrichment plots for **(B)** IL-12 family signaling and **(C)** IL-27 pathway are displayed. ES, enrichment score; FDR, false discovery rate; FWER, familywise error rate; NES, normalized enrichment score. **(D)** Quantification of cytokine levels in PyMT tumors upon DOX/anti-PD-1 or anti-PD-1 monotherapy and the corresponding IgG1 controls using LEGENDplex™ are displayed. Data are means ± SEM, *p*-values were calculated using unpaired *t*-test or Mann–Whitney test according to D'Agostino & Pearson omnibus normality test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

treatment, we wondered whether IL-27 would directly affect NK cell cytotoxicity. Therefore, we performed a NK cell cytotoxicity assay using NK cells from spleens of either wild type (WT) or IL-27 receptor α (IL-27Rα) KO mice as effector cells that were co-cultured with PyMT target cells at different effector cell-target cell ratios. NK cells and PyMT cells were labeled with different fluorescent dyes and live vs. dead PyMT cells were identified by 7-AAD staining. Analyzing tumor cell viability in the cytotoxicity assay demonstrated a significantly decreased cytotoxicity of NK cells derived from IL-27Ra KO mice toward PyMT tumor cells at a target cell-effector cell ratio of 1:10 when compared to the WT NK cells (**Figure 6A**). At other ratios, no significant differences in cytotoxicity were observed. To further explore the effect of IL-27 on NK cell cytotoxicity, the assay was repeated at the 1:10 ratio, with or without the addition of 20 ng/ml recombinant murine IL-27. The data again indicated a decreased cytotoxicity of IL-27Ra KO NK cells toward PyMT tumor cells and, more importantly, revealed an enhanced cytotoxicity of WT NK cells, but not IL-27Ra KO NK cells, when supplemented with recombinant IL-27 (**Figure 6B**). These data suggest that IL-27 produced upon chemoimmunotherapy has the capacity to increase NK cell cytotoxicity toward PyMT tumor cells. Next, we asked how IL-27 may improve NK cell effector functions. To elucidate this, the NK cell cytotoxicity was repeated using WT NK cells and PyMT cells in co-culture without (CTRL) or supplemented with 20 ng/ml recombinant murine IL-27. Afterwards, the co-cultured cells were stained for NK1.1, CD25, CD69, and CD107a to determine their activation status (**Figure 6C**). Flow cytometry analysis indeed revealed tendencies for increased mean fluorescence intensities (MFI) upon IL-27 addition for all three activation markers, reaching significance for CD107. To further characterize NK cell effector functions, the protein levels for the NK cell-derived cytolytic protein perforin (PRF1) as well as for IFN-γ as another activation marker were quantified in the co-culture supernatants (**Figure 6D**). While IFN-γ was not detectable, PRF1 protein levels did not differ significantly upon IL-27 supplementation. These data suggest that PRF1 release *per se* was not the driver of IL-27-dependent NK cell activation in the *in vitro* assay. However, PRF1 levels were also determined in PyMT tumor interstitial fluids of all treatment groups (**Figure 6E**). This analysis revealed that DOX administration in general enhanced PRF1 amounts, which was significant upon DOX/anti-PD-1 treatment as opposed to anti-PD-1 monotherapy. Next, the mRNA expression levels of *Cd25*, *Cd69*, and *Prf1* in whole tumors of both anti-PD-1 groups were analyzed (**Figure 6F**). Although mRNA expression of *Prf1* was not significantly changed, a tendency for elevated levels was found, corresponding to protein data (**Figure 6E**). Notably, mRNA expression of both activation markers *Cd25* and *Cd69* was increased in tumors treated with chemoimmunotherapy as

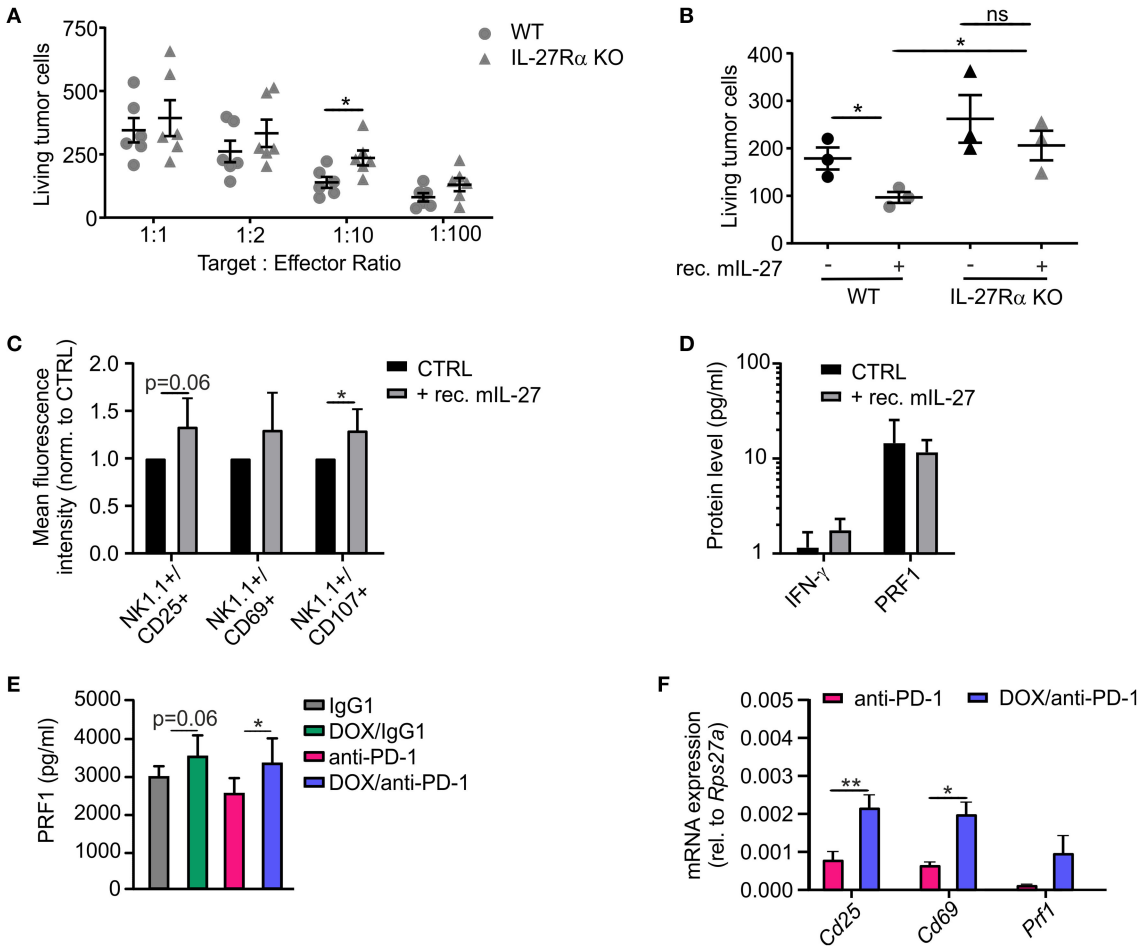

FIGURE 6 | IL-27 improves NK cell activation and cytotoxicity toward PyMT cells. **(A,B)** NK cells were isolated from spleens of wildtype (WT) or IL-27 receptor α KO (IL-27Rα KO) mice and co-cultured with PyMT tumor cells for 4 h at 37°C. Afterwards, living PyMT cells were determined by flow cytometry using 7-AAD staining. PyMT tumor cell viability **(A)** dependent on addition of WT or IL-27Rα KO NK cells at different target: effector ratios ($n = 6$ each) and **(B)** at a target: effector ratio of 1:10 with or without addition of 20 ng/ml recombinant murine IL-27 ($n = 3$ each) are shown. **(C,D)** NK cells were isolated from WT spleens and co-cultured with PyMT tumor cells for 4 h at 37°C at a target: effector ratio of 1:10 with or without (CTLR) addition of 20 ng/ml recombinant murine IL-27 ($n = 5$ each). **(C)** NK cells were subsequently stained for expression of NK1.1, CD25, CD69, and CD107 and analyzed by flow cytometry. The mean fluorescence intensities normalized to the CTRL are shown. **(D)** Quantification of NK activation markers on protein level in the co-culture supernatants is displayed. **(E,F)** mRNA and interstitial fluid were extracted from whole PyMT tumors of all treatment regimens. **(E)** Quantification of PRF1 protein levels in interstitial fluid and **(F)** NK activation markers on mRNA level is shown. Data are means ± SEM, *p*-values were calculated using unpaired *t*-test or Mann–Whitney test according to D'Agostino & Pearson omnibus normality test or one sample *t*-test, $*p < 0.05$, $**p < 0.01$. N.d., not detectable; ns, not significant.

compared to those treated with anti-PD-1 alone. While these molecular alterations in PyMT tumors cannot be attributed exclusively to NK cells, they support a milieu containing activated lymphocytes upon chemoimmunotherapy in PyMT tumors compared to anti-PD-1 monotherapy.

DISCUSSION

While immune checkpoint blockade such as anti-PD-1 and anti-PD-L1 treatment proved to be impressively effective across a wide range of cancer types (37–39) only a small fraction of breast cancer patients benefits from anti-PD-(L)1 monotherapy (5, 40). Consequently, an obvious approach to improve response rates is the combination of immune checkpoint blockade and standard regimens such as chemotherapy. Indeed, our study showed an enhanced efficacy of anti-PD-1 administration plus DOX chemotherapy in reducing the growth in PyMT tumors compared to monotherapy. In line with our findings, preclinical studies demonstrated the efficacy of anti-PD-(L)1 plus different chemotherapy agents in murine colon and lung adenocarcinoma models (41, 42). Interestingly, a recent clinical phase 3 study (IMpassion130) assessing the efficacy and safety of atezolizumab (anti-PD-L1 antibody) plus nab-paclitaxel (chemotherapy) in patients with unresectable, locally advanced or metastatic triple-negative breast cancer (TNBC) reported a clinically

meaningful overall survival benefit with chemoimmunotherapy in patients with PD-L1 immune cell-positive disease (43). These findings are supported by another recent phase 2 clinical trial (TONIC trial) (44). Patients suffering from metastatic TNBC were treated with nivolumab (anti-PD-1 antibody) without or with additional irradiation, cyclophosphamide, cisplatin, or DOX treatment. In this cohort, the objective response rate was highest in patients treated with nivolumab in combination with chemotherapy, particularly with DOX (44). This was attributed to the induction of T cell cytotoxicity pathways and an inflammatory gene signature including JAK-STAT and TNF-α signaling after DOX treatment in responders. Our data confirm the potential advantage of DOX in combination with anti-PD-1 treatment. This may extend beyond TNBC since the PyMT model is considered closely resembling the situation in human HER2-positive mammary tumors. Moreover, our RNA-Seq approach identified genes related to IL-12/IL-27 signaling, which also includes JAK-STAT pathway genes and molecules involved in triggering cytotoxic lymphocytes, which is another similarity to the TONIC trial.

In an attempt to identify the immune cell subsets that could have mediated the anti-tumor effect upon DOX/anti-PD-1 treatment, we detected elevated levels of NK cells when comparing PyMT mice receiving DOX/anti-PD-1 treatment to mice receiving monotherapy. CD8+ T cells and γδ T cells were elevated in the DOX/anti-PD-1 group compared to the group receiving anti-PD-1 as single agent, again indicating a sensitizing effect of chemotherapy. These lymphocyte subsets are known for their ability to effectively kill tumor cells (45, 46). We focused on NK cells given their specific induction only in the combination therapy group. It has been shown that the cytolytic functions of NK cells can be markedly improved by immune checkpoint blockade or chemotherapy (47, 48). We observed an involvement of IL-27 signaling in the more efficient chemoimmunotherapy compared to monotherapy. Importantly, we were able to demonstrate an IL-27-dependent higher cytotoxicity of NK cells toward PyMT tumor cells. Supporting our results, previous studies have identified IL-27 as an NK cell activator by promoting their cell viability and cytolytic activity in several cancer models (49). Moreover, IL-27 has been shown to enhance the activation and proliferation of CD8+ T cells (50) and to trigger anti-tumor functions in γδ T cells (51), thus also affecting T cell subsets that were elevated upon DOX/anti-PD-1 treatment in our study. Taken together, our data suggest an involvement of IL-27 and cytotoxic lymphocytes such as NK cells in the efficacy of chemoimmunotherapy in the PyMT model. An individual contribution of these immune cell subsets may be tested in the future by cell depletion approaches.

Chemotherapy with DOX in the PyMT alone was not sufficient to induce lasting tumor control. Our mouse model thus mimicked the situation in cancer patients, where its use as a single drug is hampered by tumor resistance. Drug resistance mechanisms have predominantly been tested in 2D or 3D cell culture (11). Therefore, transcriptomic data from our chemoresistance model comparing DOX therapy to the IgG1 control group might be of interest for future studies in this direction. There was a pattern of increased signaling through the Hedgehog pathway, through Ras and GPCRs such as sphingosine-1-phosphate receptors (S1PRs) upon treatment with DOX when chemoresistance was established. These signaling pathways were all prominently connected to tumor growth in the past (52–54), which may provide an explanation why chemotherapy in this model failed. Indeed, we recently described that blocking S1PR4 signaling improved chemotherapy response and prevented tumor relapse in the PyMT model (55).

Despite of the promising results combinatorial chemoimmunotherapy has shown, the individual clinical outcome for breast cancer patients remains difficult to predict. Our data reveal a gene signature with potential prognostic value. This gene signature consists of four genes that were upregulated in the DOX/anti-PD-1 group relative to the anti-PD-1 monotherapy group, namely *DIO2, PDK4, GSN*, and *MMP3*. Not all of the proteins were previously associated with a positive prognosis in cancer. The association of DIO2 overexpression in endometrial and colorectal cancer with a favorable prognosis (25) is in accordance with our findings in breast cancer. Also the observation that downregulation of GSN in breast cancer promoted malignant transformation (33) agrees with our study. PDK4 on the contrary was connected to poor patient outcome in breast cancer (28). This study utilized TCGA data as opposed to METABRIC data used in our study and a different cut-off strategy based on the number of cases designated as PDK4-positive. By simply dividing patients in upper and lower quartiles and using a database with more cases, we observed a positive correlation of PDK4 expression with survival in breast cancer patients. Also MMP3 expression was connected to promoting rather than restricting mammary carcinoma (36). This discrepancy to our study is not necessarily contradictory, since this study did not observe any differences in patient outcome regarding overall survival, but in distant metastasis-free survival (DMFS). Here, the prognostic value was also strongly dependent on tumor subtype and grade. It was stated that in HER2-positive tumors, such as PyMT tumors (13), an association of MMP3 expression with DMFS was not significant.

Clearly, studies investigating protein expression, activity, and cellular localization of these four markers in the tumor microenvironment are required to determine their precise impact on tumor development. It is important to note that the predictive value of our four gene signature was independent of treatment (hormone, radio-, or chemotherapy) in the METABRIC cohort. There was also no difference in the expression of the four genes irrespective of whether patients did or did not receive chemotherapy, while patients receiving hormone or radiotherapy actually expressed lower levels of these genes. Thus, the four gene signature predicts survival independent of prior standard of care treatment. It will be interesting to see how its expression is affected in patients receiving immune checkpoint blockade in the future. Importantly, to the best of our knowledge, an impact of these proteins in anti-tumor immunity or lymphocyte function has not been reported, indicating that the success of sensitizing for immune checkpoint blockade may be determined, at least partially, independently of a direct impact on cytotoxic lymphocytes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI under accession number GSE149479.

ETHICS STATEMENT

The animal study was reviewed and approved by Hessian animal care and use committee were followed (approval numbers: FU1127 and FU1191).

AUTHOR CONTRIBUTIONS

ES-F, CO, and AW conceptualized and designed research. ES-F, AF, and AW developed methodology. ES-F, AF, CO, MB, and AH performed experiments and acquired data. ES-F, TS, and AW analyzed and interpreted results. KT and BB provided technical and material support. BB and AW supervised research and all authors participated in writing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.01771/full#supplementary-material

REFERENCES

1. Coley WB. The treatment of inoperable sarcoma by bacterial toxins (the mixed toxins of the *Streptococcus erysipelas* and the *Bacillus prodigiosus*). *Proc R Soc Med*. (1910) 3:1–48. doi: 10.1177/003591571000301601
2. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med*. (1995) 182:459–65. doi: 10.1084/jem.182.2.459
3. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J*. (1992) 11:3887–95. doi: 10.1002/j.1460-2075.1992.tb05481.x
4. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science*. (2018) 359:1350–5. doi: 10.1126/science.aar4060
5. Wu X, Gu Z, Chen Y, Chen B, Chen W, Weng L, et al. Application of PD-1 blockade in cancer immunotherapy. *Comput Struct Biotechnol J*. (2019) 17:661–74. doi: 10.1016/j.csbj.2019.03.006
6. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. (2012) 366:2443–54. doi: 10.1056/NEJMoa1200690
7. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. *Nature*. (2013) 500:415–21. doi: 10.1038/nature12477
8. Planes-Laine G, Rochigneux P, Bertucci F, Chrétien A-S, Viens P, Sabatier R, et al. PD-1/PD-L1 targeting in breast cancer: the first clinical evidences are emerging. a literature review. *Cancers*. (2019) 11:1033. doi: 10.3390/cancers11071033
9. Beil DR, Wein LM. Sequencing surgery, radiotherapy and chemotherapy: insights from a mathematical analysis. *Breast Cancer Res Treat*. (2002) 74:279–86. doi: 10.1023/A:1016357311845
10. Carvalho C, Santos RX, Cardoso S, Correia S, Oliveira PJ, Santos MS, et al. Doxorubicin: the good, the bad and the ugly effect. *Curr Med Chem*. (2009) 16:3267–85. doi: 10.2174/092986709788803312
11. Lovitt CJ, Shelper TB, Avery VM. Doxorubicin resistance in breast cancer cells is mediated by extracellular matrix proteins. *BMC Cancer*. (2018) 18:41. doi: 10.1186/s12885-017-3953-6
12. Black M, Barsoum IB, Truesdell P, Cotechini T, Macdonald-Goodfellow SK, Petroff M, et al. Activation of the PD-1/PD-L1 immune checkpoint confers tumor cell chemoresistance associated with increased metastasis. *Oncotarget*. (2016) 7:10557–67. doi: 10.18632/oncotarget.7235
13. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am J Pathol*. (2003) 163:2113–26. doi: 10.1016/S0002-9440(10)63568-7
14. Sekar D, Govene L, Del Río ML, Sirait-Fischer E, Fink AF, Brüne B, et al. Downregulation of BTLA on NKT cells promotes tumor immune control in a mouse model of mammary carcinoma. *Int J Mol Sci*. (2018) 19:752. doi: 10.3390/ijms19030752
15. Bos PD, Plitas G, Rudra D, Lee SY, Rudensky AY. Transient regulatory T cell ablation deters oncogene-driven breast cancer and enhances radiotherapy. *J Exp Med*. (2013) 210:2435–66. doi: 10.1084/jem.20130762
16. Beccuti M, Cordero F, Arigoni M, Panero R, Amparore EG, Donatelli S, et al. SeqBox: RNAseq/ChIPseq reproducible analysis on a consumer game computer. *Bioinformatics*. (2018) 34:871–2. doi: 10.1093/bioinformatics/btx674
17. Jiang H, Lei R, Ding SW, Zhu S. Skewer: a fast and accurate adapter trimmer for next-generation sequencing paired-end reads. *BMC Bioinformatics*. (2014) 15:182. doi: 10.1186/1471-2105-15-182
18. Dobin A, Gingeras TR. Mapping RNA-seq reads with STAR. *Curr Protoc Bioinformatics*. (2015) 51:11.14.1–19. doi: 10.1002/0471250953.bi1114s51
19. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. (2011) 12:323. doi: 10.1186/1471-2105-12-323
20. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. (2014) 15:550. doi: 10.1186/s13059-014-0550-8
21. Curtis C, Shah SP, Chin S-F, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. (2012) 486:346–52. doi: 10.1038/nature10983
22. Kramer AS, Latham B, Diepeveen LA, Mou L, Laurent GJ, Elsegood C, et al. InForm software: a semi-automated research tool to identify presumptive human hepatic progenitor cells, and other histological features of pathological significance. *Sci Rep*. (2018) 8:3418. doi: 10.1038/s41598-018-21757-4
23. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. (2005) 102:15545–50. doi: 10.1073/pnas.0506580102
24. Casula S, Bianco AC. Thyroid hormone deiodinases and cancer. *Front Endocrinol.* (2012) 3:74. doi: 10.3389/fendo.2012.00074

25. Uhlen M, Zhang C, Lee S, Sjöstedt E, Fagerberg L, Bidkhori G, et al. A pathology atlas of the human cancer transcriptome. *Science*. (2017) 357:eaan2507. doi: 10.1126/science.aan2507
26. Yang C, Wang S, Ruan H, Li B, Cheng Z, He J, et al. Downregulation of PDK4 increases lipogenesis and associates with poor prognosis in hepatocellular carcinoma. *J Cancer*. (2019) 10:918–26. doi: 10.7150/jca.27226
27. Li G, Li M, Hu J, Lei R, Xiong H, Ji H, et al. The microRNA-182-PDK4 axis regulates lung tumorigenesis by modulating pyruvate dehydrogenase and lipogenesis. *Oncogene*. (2017) 36:989–98. doi: 10.1038/onc.2016.265
28. Guda MR, Asuthkar S, Labak CM, Tsung AJ, Alexandrov I, Mackenzie MJ, et al. Targeting PDK4 inhibits breast cancer metabolism. *Am J Cancer Res*. (2018) 8:1725–38.
29. Burtnick LD, Koepf EK, Grimes J, Jones EY, Stuart DI, McLaughlin PJ, et al. The crystal structure of plasma gelsolin: implications for actin severing, capping, and nucleation. *Cell*. (1997) 90:661–70. doi: 10.1016/S0092-8674(00)80527-9
30. Tanaka M, Müllauer L, Ogiso Y, Fujita H, Moriya S, Furuuchi K, et al. Gelsolin: a candidate for suppressor of human bladder cancer. *Cancer Res*. (1995) 55:3228–32.
31. Müllauer L, Fujita H, Ishizaki A, Kuzumaki N. Tumor-suppressive function of mutated gelsolin in ras-transformed cells. *Oncogene*. (1993) 8:2531–6.
32. Li WX, Yang MX, Hong XQ, Dong TG, Yi T, Lin SL, et al. Overexpression of gelsolin reduces the proliferation and invasion of colon carcinoma cells. *Mol Med Rep*. (2016) 14:3059–65. doi: 10.3892/mmr.2016.5652
33. Winston JS, Asch HL, Zhang PJ, Edge SB, Hyland A, Asch BB. Downregulation of gelsolin correlates with the progression to breast carcinoma. *Breast Cancer Res Treat*. (2001) 65:11–21. doi: 10.1023/A:1006446108411
34. Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, et al. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell*. (1999) 98:137–46. doi: 10.1016/S0092-8674(00)81009-0
35. Lochter A, Galosy S, Muschler J, Freedman N, Werb Z, Bissell MJ. Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J Cell Biol*. (1997) 139:1861–72. doi: 10.1083/jcb.139.7.1861
36. Mehner C, Miller E, Nassar A, Bamlet WR, Radisky ES, Radisky DC. Tumor cell expression of MMP3 as a prognostic factor for poor survival in pancreatic, pulmonary, and mammary carcinoma. *Genes Cancer*. (2015) 6:480–9. doi: 10.18632/genesandcancer.90
37. Topalian SL, Sznol M, McDermott DF, Kluger HM, Carvajal RD, Sharfman WH, et al. Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. *J Clin Oncol*. (2014) 32:1020–30. doi: 10.1200/JCO.2013.53.0105
38. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. *N Engl J Med*. (2015) 372:2018–28. doi: 10.1056/NEJMoa1501824
39. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med*. (2015) 372:311–9. doi: 10.1056/NEJMoa1411087
40. Emens LA, Cruz C, Eder JP, Braiteh F, Chung C, Tolaney SM, et al. Long-term clinical outcomes and biomarker analyses of atezolizumab therapy for patients with metastatic triple-negative breast cancer: a phase 1 study. *JAMA Oncol*. (2019) 5:74–82. doi: 10.1001/jamaoncol.2018.4224
41. Cubas R, Moskalenko M, Cheung J, Yang M, McNamara E, Xiong H, et al. Chemotherapy combines effectively with anti-PD-L1 treatment and can augment antitumor responses. *J Immunol*. (2018) 201:2273–86. doi: 10.4049/jimmunol.1800275
42. Pfirschke C, Engblom C, Rickelt S, Cortez-Retamozo V, Garris C, Pucci F, et al. Immunogenic chemotherapy sensitizes tumors to checkpoint blockade therapy. *Immunity*. (2016) 44:343–54. doi: 10.1016/j.immuni.2015.11.024
43. Schmid P, Rugo HS, Adams S, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab plus nab-paclitaxel as first-line treatment for unresectable, locally advanced or metastatic triple-negative breast cancer (IMpassion130): updated efficacy results from a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol*. (2020) 21:44–59. doi: 10.1016/S1470-2045(19)30689-8
44. Voorwerk L, Slagter M, Horlings HM, Sikorska K, van de Vijver KK, Maaker M, et al. Immune induction strategies in metastatic triple-negative breast cancer to enhance the sensitivity to PD-1 blockade: the TONIC trial. *Nat Med*. (2019) 25:920–8. doi: 10.1038/s41591-019-0432-4
45. Martínez-Lostao L, Anel A, Pardo J. How do cytotoxic lymphocytes kill cancer cells? *Clin Cancer Res*. (2015) 21:5047–56. doi: 10.1158/1078-0432.CCR-15-0685
46. Lawand M, Déchanet-Merville J, Dieu-Nosjean MC. Key features of gamma-delta T-cell subsets in human diseases and their immunotherapeutic implications. *Front Immunol*. (2017) 8:761. doi: 10.3389/fimmu.2017.00761
47. Oyer JL, Gitto SB, Altomare DA, Copik AJ. PD-L1 blockade enhances anti-tumor efficacy of NK cells. *Oncoimmunology*. (2018) 7:e1509819. doi: 10.1080/2162402X.2018.1509819
48. Zingoni A, Fionda C, Borrelli C, Cippitelli M, Santoni A, Soriani A. Natural killer cell response to chemotherapy-stressed cancer cells: role in tumor immunosurveillance. *Front Immunol*. (2017) 8:1194. doi: 10.3389/fimmu.2017.01194
49. Matsui M, Kishida T, Nakano H, Yoshimoto K, Shin-Ya M, Shimada T, et al. Interleukin-27 activates natural killer cells and suppresses NK-resistant head and neck squamous cell carcinoma through inducing antibody-dependent cellular cytotoxicity. *Cancer Res*. (2009) 69:2523–30. doi: 10.1158/0008-5472.CAN-08-2793
50. Schneider R, Yaneva T, Beauseigle D, El-Khoury L, Arbour N. IL-27 increases the proliferation and effector functions of human naïve CD8+ T lymphocytes and promotes their development into Tc1 cells. *Eur J Immunol*. (2011) 41:47–59. doi: 10.1002/eji.201040804
51. Morandi F, Prigione I, Airoldi I. Human TCRγδ+ T cells represent a novel target for IL-27 activity. *Eur J Immunol*. (2012) 42:1547–52. doi: 10.1002/eji.201142241
52. Downward J. Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer*. (2003) 3:11–22. doi: 10.1038/nrc969
53. Pyne NJ, Pyne S. Sphingosine 1-phosphate and cancer. *Nat Rev Cancer*. (2010) 10:489–503. doi: 10.1038/nrc2875
54. Wu F, Zhang Y, Sun B, McMahon AP, Wang Y. Hedgehog signaling: from basic biology to cancer therapy. *Cell Chem Biol*. (2017) 24:252–80. doi: 10.1016/j.chembiol.2017.02.010
55. Olesch C, Sirait-Fischer E, Berkefeld M, Fink AF, Susen RM, Ritter B, et al. S1PR4 ablation reduces tumor growth and improves chemotherapy via CD8+ T cell expansion. *J Clin Invest*. (2020) 136928. doi: 10.1172/JCI136928

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An Arthropod Hormone, Ecdysterone, Inhibits the Growth of Breast Cancer Cells via Different Mechanisms

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Ecdysterone (Ecdy) is a hormone found in arthropods, which regulates their development. It is also synthesized by a number of plants to combat insect pests. It provides a number of beneficial pharmacological effects including the anabolic and adaptogenic ones. Ecdysterone is widely marketed as food supplement to enhance the physical performance of athletes. In addition to the estrogen receptor beta (ERbeta)-dependent anabolic effect of Ecdy in muscles, the molecular mechanisms of the plethora of other Ecdy-induced pharmacological effects remain unknown. The aim of this study was to investigate the pharmacological effect of ecdysterone on human breast cancer cell lines of different molecular subtypes. Surprisingly, in contrast to the anabolic effect on muscle tissues, we have revealed a tumor suppressive effect of Ecdy on a panel of breast cancer cell lines studied. Using the SeaHorse-based energy profiling, we have demonstrated that Ecdy dampened glycolysis and respiration, as well as greatly reduced the metabolic potential of triple negative breast cancer cell lines. Furthermore, we have revealed that Ecdy strongly induced autophagy. As part of the combined treatment, based on the Combination Index (CI) and Dose Reduction Index (DRI), Ecdy synergized with doxorubicin to induce cell death in several breast cancer cell lines. In contrast, Ecdy had only minor effect on non-transformed human fibroblasts. Collectively, our results indicate that ecdysterone can be considered as a new potential adjuvant for genotoxic therapy in treatment of breast cancer patients.

Keywords: ecdysterone, breast cancer, doxorubicin, autophagy, energy metabolism, synergism, doxorubicin, triple negative breast cancer, combination index, dose reduction index, 2-deoxyglucose, extracellular acidification rate, oxygen consumption rate, multiple drug resistance

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INTRODUCTION

Ecdysteroids constitute a class of steroid hormones found in arthropods, which regulate their development including molting and reproduction. Co-evolution of plants and its pathogens and animals, including insects, has generated a plethora of different biochemical pathways allowing plants to synthesize various protective compounds that defend them from various environmental insults.

Thus, Rausher (2001) about 6% of plant species (ferns, angiosperms and gymnosperms) synthesize ecdysteroids (phytoecdysteroids) as protective mechanisms against insects (Dinan,

2001). To date, 517 different ecdysteroids derived from both plants and insects have been described and listed in the ecdysteroid database (EcdyBase, www.ecdybase.org).

A number of studies have shown that ecdysteroids partake in different biological activities within humans (Lafont and Dinan, 2003; Dinan et al., 2009; Isenmann et al., 2019). However, the pharmacological potential of the vast majority of ecdysteroids remains to be elucidated. The most studied one is 20-Hydroxyecdysone or Ecdysterone (Ecdy). Extracts of Ecdy produced by *Leuzea carthamoides* are widely marketed as various diet supplements for athletes.

Early pharmacological experiments have shown that it has a low toxicity in mammals (LD 50 > 9 g/kg) (Ogawa et al., 1974; Lafont and Dinan, 2003). The maximum recommended dose of Ecdy for athletes is 500–1,000 mg per day (Dinan and Lafont, 2006). The positive pharmacological effects of ecdysterone on humans are well documented and include: anabolic, anti-diabetic, neuron protective, anti-angiocardiopathological, immune-stimulating, antidepressant to name a few (for a comprehensive review, see (Lafont and Dinan, 2003; Dinan et al., 2009; Bajguz et al., 2015).

Ecdysterone attracts the most attention as a natural anabolic and adaptogenic compound. It is widely marketed as a "natural anabolic agent" to athletes, in the form of dietary supplements which increase strength and muscle mass during resistance training, to reduce fatigue, and to ease recovery (Isenmann et al., 2019). A number of papers have shown an ecdysterone-mediated increase in sport performance among both mice and humans (Azizov and Seĭfulla, 1998; Gorelick-Feldman et al., 2008; Parr et al., 2015; Isenmann et al., 2019). These studies demonstrated the anabolic effect of ecdysterone and its benefical effects to athletes, and contributed to ecdysterone being considered as a potential enhancement substance in anti-doping control (Parr et al., 2020). Since December 2019, ecdysterone is in the focus of WADA (World Anti-doping Agency) investigations.

In insects, ecdysterone acts in nanomolar concentrations through ecdysone nuclear receptors (EcR). However, this compound does not display any hormonal activity in humans because they have no EcRs, nor it interacts with androgen or glucocorticoid receptors. However, ecdysterone was shown *in vitro* to stimulate ERbeta, which is involved in skeletal muscle hypertrophy (Parr et al., 2014).

Considering the diversity of ecdysterone-mediated pharmacological activities in the human organism, including different tissues (see Dinan and Lafont, 2006), it seems that the ERbeta-signaling pathway is not the only molecular mechanism which is utilized by ecdysterone. However, other molecular mechanisms underlying the broad spectrum of ecdysterone-mediated pharmacological effects remain elusive.

Unlike for other hormone-like compounds, very little information is available about the effects of ecdysterone on cancer cells and malignancies. It is interesting to note that despite the reported anabolic properties of ecdysterone regarding sport performance, several studies described ecdysterone-induced sensitization of cancer cells to genotoxic drugs and reduction of tumors in mice (Konovalova et al., 2002; Martins et al., 2015).

Here, we provide evidence that ecdysterone can inhibit the proliferation of breast cancer cells. Mechanistically, it down-regulates the metabolic potential of cancer cells and induces autophagy. Moreover, ecdysterone strongly sensitizes breast cancer cells to doxorubicin, resulting in a significant reduction of the effective dose of doxorubicin. Importantly, the effect of ecdysterone on non-transformed human fibroblasts was minimal.

MATERIALS AND METHODS

Cell Lines and Reagents

All the cell lines used in this study (MCF7, MDA-MB-231, MDA-MB-468, DF2 and WI-38) were purchased from ATCC. Cells were grown in DMEM media supplemented with 10% fetal bovine serum, 100 μg/ml gentamycin, and 2 mM L-glutamine. To grow MCF7 cells the medium was also supplemented with 10 μg/ml insulin (NM Penfild, Denmark). Cells were grown at 37°C in 5% CO_2 atmosphere.

Ecdysterone (95% purity, Frog Tech, Russia) was dissolved in DMSO. Thus, DMSO was used as a control for all experiments with ecdysterone (0 μM Ecdy). Doxorubicin (98% purity, Sigma, United States) and 2-DG (98% purity, Sigma, United States) were dissolved in water.

MTT Assay

For MTT experiments, 10,000 cells were planted overnight in each well of a 96-well plate. 10 wells per sample were used. A day after, ecdysterone or (and) doxorubicin were added in the required concentrations for 48 h. For cells treated with ecdysterone, DMSO was used as a control. Then 10 μL of 5 mg/ml Thiazolyl Blue (Paneko, Russia) solution was added to each well and cells were kept for 3,5 h at 37°C in CO_2 incubator. After removing the thiazol-containing medium, 150 μL isopropyl alcohol (supplemented with 40 mM HCl and 0,1% NP-40) was added to dissolve the MTT-formazan salt. The absorbance at 570 and 630 nm (reference) was measured using BioRad iMark microplate reader (BioRad, United States). Results are represented as the mean ± SD.

Colony-formation Assay

To perform colony-formation assay, 1,000 cells were planted per well on a 6-well plate, in triplicates. 24 h later, the cells were treated with 0, 100, 150, 250, 350 or 500 μM ecdysterone for 96 h. After treatment, fresh media was added, and cells were grown for 10 days. After the indicated time, cells were fixed with acetic acid/methanol (1:7, v/v) and stained with 0.5% crystal violet. The number of colonies was calculated. Results are represented as the mean ± SD of three biological replicates.

Proliferation Assay

About 25,000 cells were seeded in 12-well plates and incubated with different amounts of 0–750 μM Ecdy for 4 days. Following the incubation, cells were trypsinized, stained with trypan blue and calculated using hemocytometer. Six replicates were used for analysis. Results are calculated as the mean ± SD; $^*p < 0.05$.

Cell Cycle Analysis

Flow cytometry analysis of cell cycle distribution was done essentially as described previously (Lezina et al., 2014). A day after seeding, cells were treated with ecdysterone (0, 250, 500, or 750 μM) for 48 h in triplicates. After harvesting, cells were washed once with PBS, and fixed in 70% ethanol at −20°C for 1 h. The 30 min staining of DNA content was carried out by using 50 μg/ml of PI (Invitrogen, United States) and 1 μg/ml RNase A (ThermoFischer). Samples were analyzed by CytoFLEX (Beckman Coulter, United States) flow cytometer. Results were processed by CyteExpert software (Beckman Coulter, United States).

SeaHorse Energy Profiling

To perform the energy profiling using SeaHorse apparatus, 30,000 cells were seeded to each well (except for the background wells) of a 24-well SeaHorse plate (Agilent, United States) overnight. Four wells were used per sample. Then, 0–1,000 μM Ecdy was added for 48 h. 12 h before analysis, the Sensor Cartridge was equilibrated in XF Calibrant (Agilent, United States) at 37°C in a non-CO_2 incubator. SeaHorse XF Energy Phenotype kit (Agilent, United States) was applied for assay. SeaHorse XF base medium was supplemented with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose, pH 7.4. Stressor mix consisting of olygomycin and FCCP (both Agilent, United States) was used to achieve final concentrations 1 and 2 μM in wells, respectively. Assay was run in the XFe24 Analyser device (Agilent, United States) in accordance with the manufacturer's instructions. Data were normalized using total protein quantification by BCA assay (ThermoFischer, United States) and processed by SeaHorse XF Cell Energy Phenotype Test Report Generator (Agilent, United States). Results are represented as the mean ± SEM.

Analysis of Apoptosis and Total Cell Death

Flow cytometric determination of cell death including apoptosis was carried out by using annexin V-FITC/(PI or 7-AAD) double staining. To analyze the influence of ecdysterone on cell death, annexin V–FITC/PI kit (BD Biosciences, United States) was used, whereas in studies of combined treatments (doxorubicin and ecdysterone) annexin V–FITC/7AAD (ThermoFischer, United States) was applied in accordance with the corresponding manufacturer's protocols. Cells were treated for 48 h with ecdysterone (0, 250, 500, and 750 μM) and doxorubicin (0, 0.15, and 0.25 μM) separately or in combination. A minimum of 5,000 cells were analyzed by CytoFLEX (Beckman Coulter, United States) flow cytometer using corresponding channels in three independent experiments. Values of the median were used for calculation. Results were represented as the mean ± SEM of three experiments.

Measurement of LysoTracker Intensity

A day after seeding, cells were treated by ecdysterone (0, 250, 500 or 750 μM) for 48 h in triplicates. Before analysis, cells were treated with 75 nM LysoTracker Red DND-99 (ThermoFischer, United States) for 2 h at 37°C in a CO_2 incubator. Then cells were washed in PBS, detached with trypsin and analyzed by flow cytometry (CytoFlex, Beckman Coulter, United States). Values of the median were used for calculation. Results were represented as the mean ± SEM of three experiments.

Analysis of Autophagic Flux

Autophagic flux was revealed by blocking autophagy using chloroquine followed by western-blot with anti-LC3 and p62 antibodies, as well as immunofluorescence (staining with anti-LC3 antibodies). The next day after being planted in Petri dishes or glass cover slips, cells were treated with 0–1,500 μM Ecdy for 32 h followed by a co-treatment with the same concentrations of Ecdy and 50 μM chloroquine to block autophagy for 16 h. Then cells were subjected to either western-blot or immunofluorescence.

Western-Blot

Cell lysates were prepared using RIPA buffer (150 mM NaCl; 50 mM Tris–HCl pH 8.0; 0.5%NP-40; 1 mM PMSF, protease inhibitor cocktail). After protein quantification by BCA assay (ThermoFischer, United States), 20 ug of Laemli-diluted cell lysates were loaded on 17% SDS-PAGE, run on TRIS-Glycine running buffer, followed by transfer to a nitrocellulose membrane (Bio-RAD, United States). Following 1 h blocking in PBST-diluted 5% nonfat milk, membranes were incubated with primary antibodies: LC3B (1:1,000, #2775S, Cell Signaling, United States), p62 (1:1,000, #5114, Cell Signaling, United States) or β-actin (1:5000, A3854, Sigma-Aldrich, United States). After PBST washing, secondary anti-mouse or anti-rabbit antibodies (1:10,000; Sigma-Aldrich, United States) conjugated with horseradish peroxidase were used. ECL system (ThermoScientific, United States) and ChemiDoc Touch Imager (Bio-Rad, United States) were applied for detection.

Immunofluorescence

Cells grown on glass cover slips were fixed with 4% PFA in PBS for 15 min and then washed three times in PBS, followed by 60 min incubation in permeabilization blocking solution (5% BSA, 0.3% Triton X-100 in PBS) at room temperature. Then, cells were stained with anti-LC3B antibodies (1:200, #2775S, Cell Signaling, United States) in permeabilization blocking solution for 16 h at 4°C, washed three times in PBS, incubated with the secondary antibody in permeabilization blocking solution (goat anti-rabbit, AlexaFluor488, Invitrogen, United States) for 1 h at room temperature and washed three times in PBS. Slides were mounted using ProLong Gold Antifate Mountant with DAPI (P36931, Invitrogen). Images were analyzed by confocal microscope (Olympus, FV3000, Germany).

Assessment of Drug Synergy

IC50 and drug synergy were obtained using results of MTT-assay. IC50 was calculated using AAT Bioquest IC50 online calculator https://www.aatbio.com/tools/ic50-calculator. Drug interaction was assessed by Chou-Talalay algorithms (CompuSyn software, http://www.combosyn.com/; (Chou and Talalay, 1984; Chou, 2006). Results were represented as CI (Combination Index) plots and a Table which includes values for CI and DRI (Dose Reduction Index). CI < 1 attests synergistic action of drugs; DRI estimates the extent to which the dose of one or more agents in the combination can be reduced to achieve

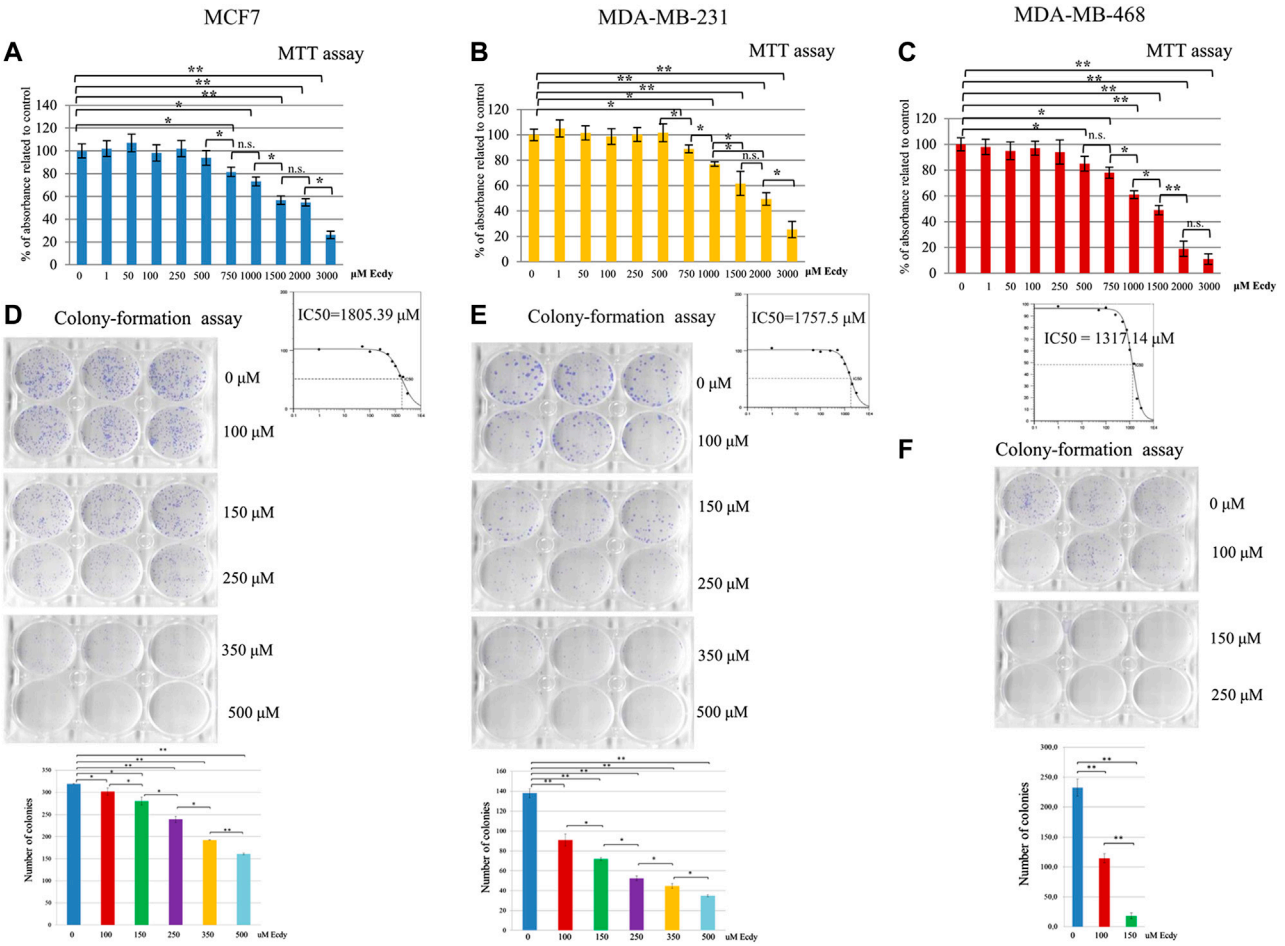

FIGURE 1 | Ecdysterone inhibits breast cancer cell lines. Results of MTT-test for: **(A)** MCF7, **(B)** MDA-MB-231, and **(C)** MDA-MB-468 cells treated with increasing concentrations (0–3,000 μM) of Ecdy for 48 h. Results are depicted as the mean ± SD. *$p < 0.05$; **$p < 0.01$; n.s. – not significant. Corresponding IC50 plots are displayed below the MTT diagrams. IC50 was calculated using AAT Bioquest IC50 online calculator https://www.aatbio.com/tools/ic50-calculator. Results of the colony-formation assay for **(D)** MCF7, **(E)** MDA-MB-231, and **(F)** MDA-MB-468 treated with indicated concentration of Ecdy. Photographs of plates and quantification diagrams are shown. Results are displayed as the mean ± SEM of three experiments. *$p < 0.05$; **$p < 0.01$.

effect levels that are comparable with those achieved with single agents.

Statistical Analysis

All results are demonstrated as mean ± standard deviation (SD) or standard error of the mean (SEM) of at least three biological replicates. Statistical significance was analyzed using the Student's *t*-test: *$p < 0.05$; **$p < 0.01$; n.s – not significant.

RESULTS

Ecdysterone inhibits Proliferation and Induces Death of Breast Cancer Cell Lines

Several papers describe the ecdysterone-mediated tumor suppressive effect on some cancer cells (Konovalova et al., 2002; Martins et al., 2015). We evaluated the effect of this drug on three human breast cancer cell lines with different molecular properties: MCF7 (luminal, $ER^+Pr^+Her2^-$), MDA-MB-231 (TNBC) and MDA-MB-468 (TNBC).

As ecdysterone is reported to exert anabolic activity in muscle tissue, which should facilitate the proliferation, we decided to carry out MTT assay to see if ecdysterone affects the proliferation of cancer cell lines studied. To do this, we have used a broad range of concentrations ranging from one to 3,000 μM. Results shown in **Figures 1A–C** clearly demonstrate that in our case the treatment with Ecdy has down-regulated all three cell lines starting with a concentration of 250–750 μM. No increase in cell proliferation was detected.

We have also carried out colony-formation assay. As this analysis implies the growth of colonies from single cells and they are very sensitive to any treatment, we have chosen a lower concentration of Ecdy. Results shown in **Figures 1D–F** confirm that the treatment with Ecdy inhibits the growth of all three cell lines. Moreover, photographs of plates with colonies clearly showed that not only the number of colonies, but also their size, was significantly reduced upon treatment with Ecdy.

The analysis of the cell cycle has shown that ecdysterone affected to varying degrees the cell cycle distributions of MCF7 and MDA-MB-231 cells, but had no effect on MDA-MB-468 cells (**Figures**

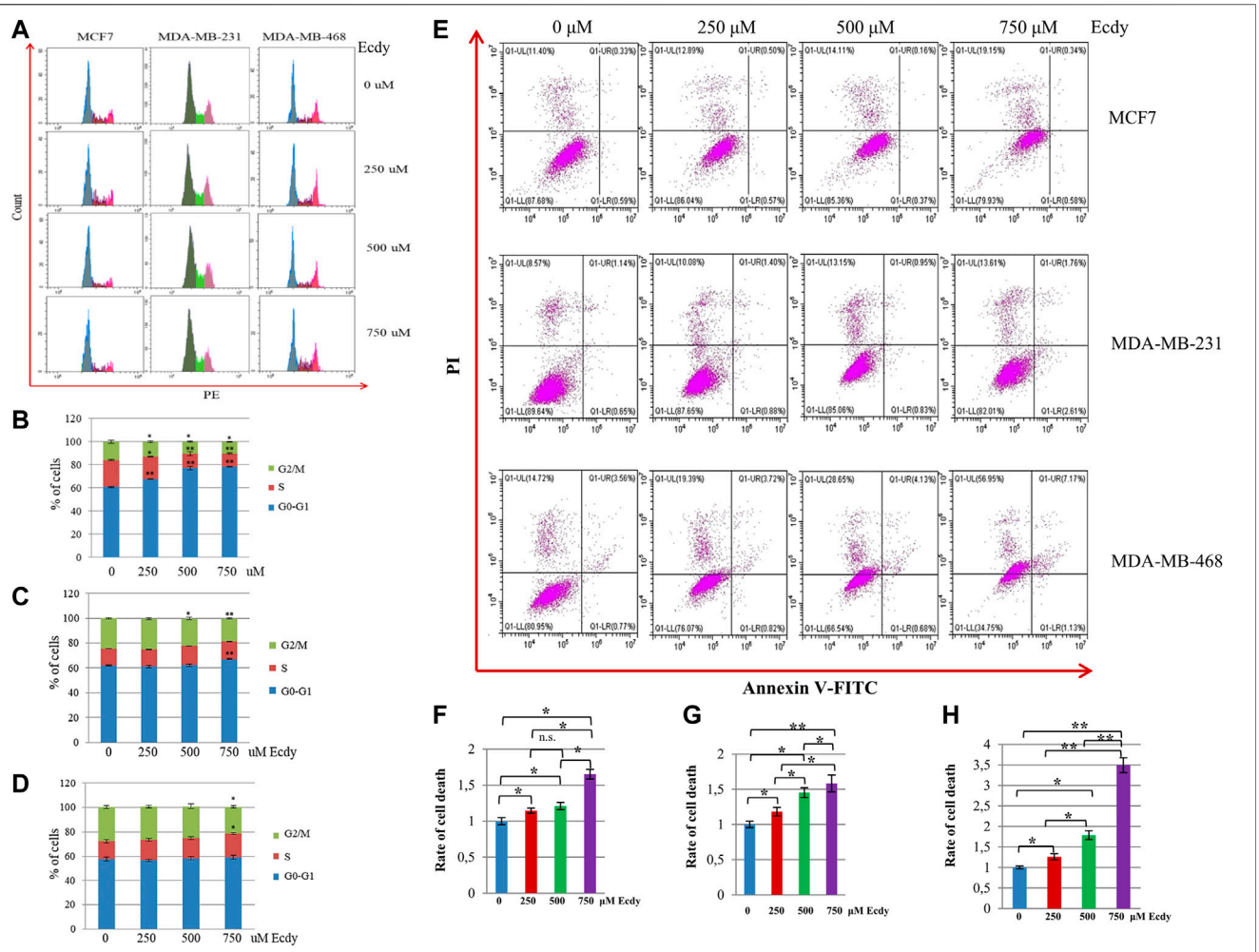

FIGURE 2 | Ecdysterone alters the cell cycle and induces cell death depending on the cancer cell line. **(A)** Cell cycle profiles (flow cytometry) of breast cancer cell lines treated with 0, 250, 500 or 750 μM Ecdy for 48 h. Cell cycle analyses are shown for MCF7 **(B)**, MDA-MB-231 **(C)**, and MDA-MB-468 **(D)** cells. Results are displayed as the mean ± SEM of three experiments. *$p < 0.05$; **$p < 0.01$. **(E)** Annexin V-FITC/Propidium iodide (PI) profile of the same breast cancer cell lines treated with 0, 250, 500 or 750 μM Ecdy for 48 h (flow cytometry). Rates of cell death related to DMSO-treated cells (0 μM Ecdy) calculated for **(F)** MCF7, **(G)** MDA-MB-231, and **(H)** MDA-MB-468 cells are represented as diagrams. Results are calculated as mean ± SEM of three experiments. *$p < 0.05$; **$p < 0.01$; n.s – not significant.

2A–D). It significantly increased the number of MCF7 cells in the G1-phase (**Figure 2B**). To a lesser extent, the same was true for MDA-MB-231 cells (**Figure 2C**). However, MDA-MB-468 cells were insensitive to Ecdy-induced alterations in the cell cycle (**Figure 2D**).

We also determined if ecdysterone affected the level of cell death. Annexin V/PI staining followed by flow cytometry (**Figure 2E**) demonstrated that ecdysterone increased the rate of cell death up to 1.6 fold for both MCF7 and MDA-MB-231 cells (**Figures 2F,G**), and 3.5 times in the case of MDA-MB-468 cells (**Figure 2H**). It is important to notice that although Ecdy elevated overall cell death in all cell lines, it did not increase the population of Annexin V-positive cells, suggesting that Ecdy elicited death of MCF7 cells via mechanism(s) other than apoptosis (**Figure 2E**). In contrast, Ecdy increased the population of Annexin V-positive (apoptotic) cells by 30% for MDA-MB-231 and by 48% for MDA-MB-468 cell line (**Figure 2E**).

These data suggest that the ability of ecdysterone to induce cell cycle arrest or elicit cell death of breast cancer cells presumably depends on the genetic background of a particular cell line.

Ecdysterone Down-Regulates Energy Metabolism of Breast Cancer Cells

As ecdysterone possesses anabolic properties in muscle tissue, we were interested to see whether it alters the energy metabolism of the breast cancer cell lines. To do this, we employed the SeaHorse energy profiling using Energy Phenotype kit.

Energy profiling of MDA-MB-231 cells treated with 0, 250, 500, 750 or 1,000 μM of ecdysterone has shown ecdysterone-mediated inhibition of respiration (basic OCR). For instance, 500 and 750 μM Ecdy decreased respiration by approximately 21 and 31%, respectively (**Figure 3A**). At the same time, ecdysterone did not alter glycolysis (basic ECAR, **Figure 3B**) but significantly decreased the metabolic potential (both stressed respiration (stressed OCR) and glycolysis (stressed ECAR), **Figures 3A,B**). Thus, 500 μM Ecdy mitigated stressed OCR by 23% and stressed ECAR by 18%. Stressed OCR and ECAR reflect the metabolic potential of cells–percentage increase of stressed OCR over the

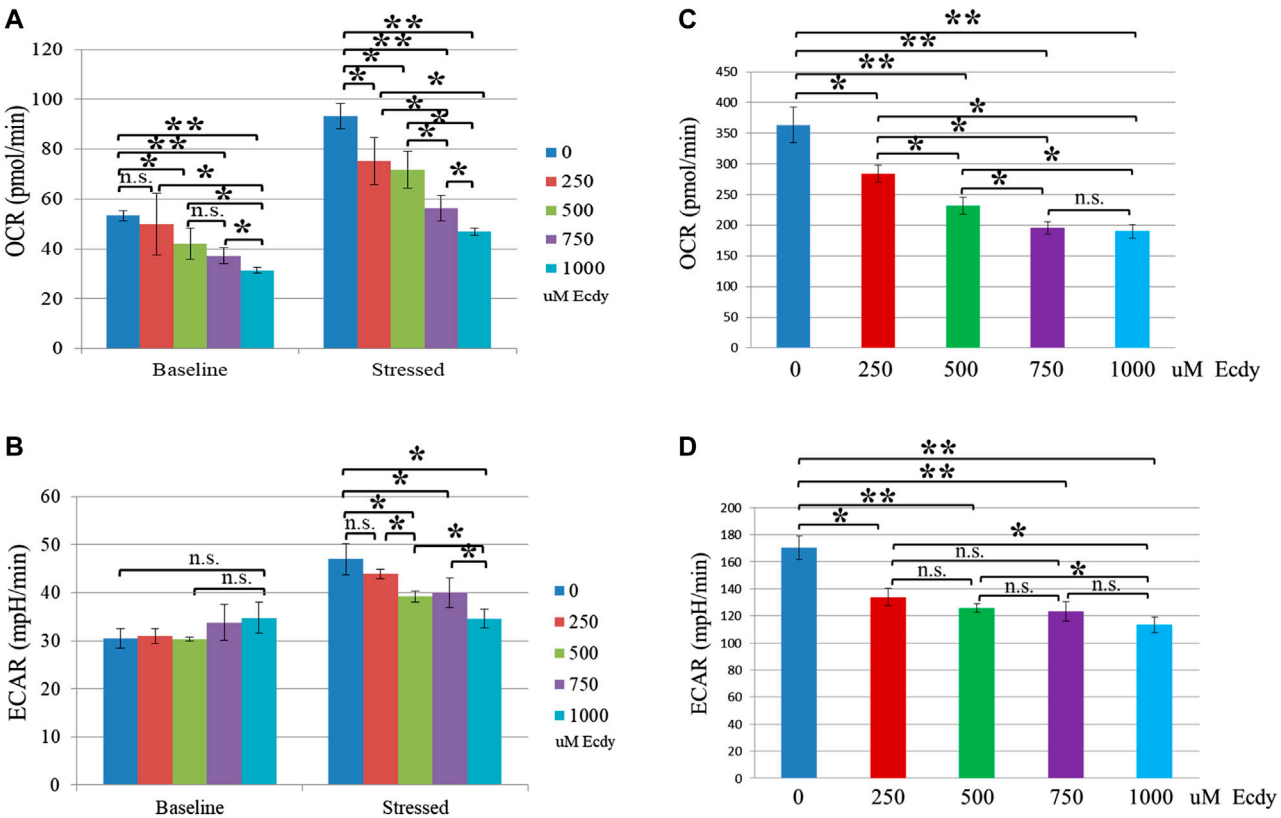

FIGURE 3 | Ecdysterone attenuates energy metabolism of TNBC breast cancer cell lines. SeaHorse energy profiling (Energy Phonotype kit) of **(A,B)** MDA-MB-231 and **(C,D)** MDA-MB-468 cancer cell lines treated with 0, 250, 500, 750 or 1,000 μM Ecdy for 48 h. OCR–oxygen consumption rate (respiration), ECAR–extracellular acidification rate (glycolysis). Baseline–base (normal) respiration (OCR) or glycolysis (ECAR); Stressed - OCR and ECAR of cells under an induced energy demand (in the presence of stressor compounds–FCCP and oligomycin). Stressed OCR and ECAR reflect metabolic potential–percentage increase of stressed OCR over baseline OCR, and stressed ECAR over baseline ECAR. Metabolic Potential is the measure of cells' ability to meet an energy demand via respiration and glycolysis. Results are shown as mean ± SEM of three experiments. *$p < 0.05$; **$p < 0.01$; n.s – not significant.

baseline OCR, and stressed ECAR over the baseline ECAR. Metabolic Potential (MP) is the measure of cells' ability to meet the energy demand via respiration and glycolysis. Thereby, these results suggest that ecdysterone greatly reduced the capacity of MDA-MB-231 cells for metabolic adaptation.

In the case of MDA-MB-468 cell line, the same treatment with ecdysterone has led to an inhibition of both the baseline respiration and of glycolysis (**Figures 3C,D**). Even 250 μM Ecdy dampened respiration and glycolysis by 22%, whereas 750 μM Ecdy inhibited them further by 47 and 28%, respectively. Taken together, these data suggest Ecdy was able to attenuate the level of energy metabolism in TNBC breast cancer cells.

Ecdysterone Induces Autophagy

Tang and colleagues (Tang et al., 2018) has reported that ecdysterone promotes autophagy in osteoporotic rats. We have carried out staining in three ecdysterone-treated (0, 250, 500, and 750 μM, 48 h) breast cancer cell lines with LysoTracker, a fluorescent dye, reflecting the number of lysosomes which can be indicative of autophagy (Chikte et al., 2014). Flow cytometry analysis has shown the strong increase of LysoTracker fluorescence in all three cell lines in order of increase the ecdysterone concentration (**Figures 4A–C**).

To further study whether ecdysterone affects the autophagic flux the breast cancer cell lines were incubated with different concentrations of ecdysterone (0, 250, 500, and 750 μM) for 32 h followed by 16 h of incubation with both ecdysterone and 50 μM chloroquine to block the autophagic-mediated degradation. Treated cells were analyzed by western-blot with LC3 and p62 antibodies or by immunofluorescence microscopy to evaluate the LC3 staining. Results of both immunoblotting and immunofluorescence (**Figures 4D–G**; **Supplementary Figure S1**) demonstrate that in the case of all three lines, ecdysterone strongly induced autophagy.

Taken together, these data strongly suggest that ecdysterone induces autophagy in all breast cancer cells concomitantly with the increasing ecdysterone concentration.

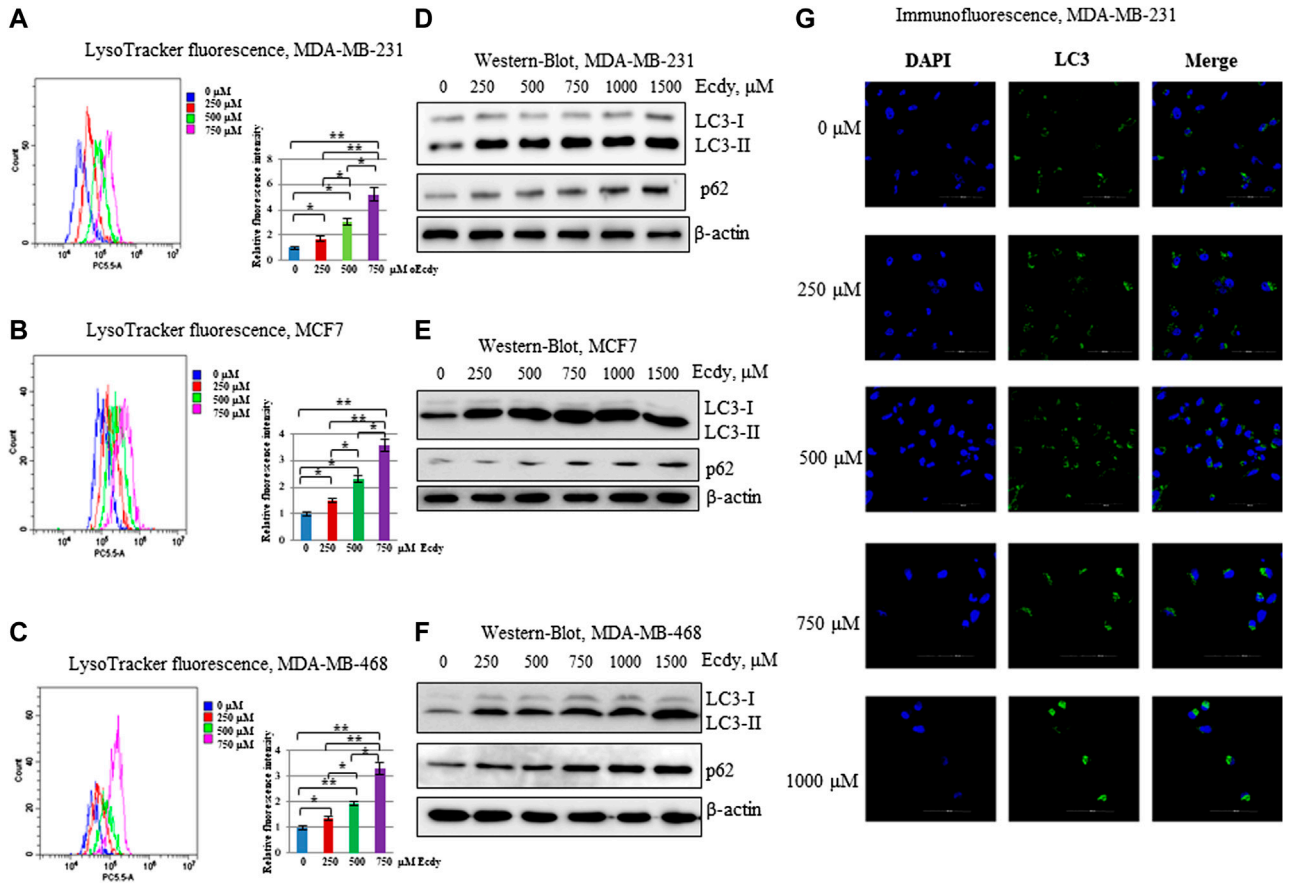

FIGURE 4 | Ecdysterone induces autophagy. **(A)** MDA-MB-231, **(B)** MCF7, **(C)** MDA-MB-468 cells were treated with 0, 250, 500, 750 μM Ecdy for 48 h followed by LysoTracker staining and flow cytometry analysis. The fluorescence of LysoTracker reflects the amount of lysosomes. Flow cytometry plots with median fluorescence and diagrams with fluorescence intensity related to DMSO-treated cells (0 μM Ecdy) are depicted. Results are calculated as the mean ± SEM of three experiments. *$p < 0.05$; **$p < 0.01$. **(D)** MDA-MB-231, **(E)** MCF7, **(F)** MDA-MB-468 cell lines were treated with 0, 250, 500, 750, 1,000, and 1,500 μM Ecdy for 32 h and then co-treated with the same concentrations of Ecdy and 50 μM chloroquine to block autophagy for 16 h followed by western-blot analysis for LC3 and p62 reflecting autophagy flux. **(G)** MDA-MB-231 cells treated as above were stained with DAPI and anti-LC3 antibodies and analyzed by immunofluorescence microscopy.

Ecdysterone synergizes With Doxorubicin to Down-Regulate Breast Cancer Cell Lines

Given that ecdysterone mediated the inhibition of growth of all breast cancer cell lines, we decided to examine its effect on cancer cells a part of the combined treatment with doxorubicin, a genotoxic drug which is widely applied as chemotherapeutics. To this end, we carried out MTT-assay using ecdysterone (250, 500, or 750 μM) and doxorubicin (0.15 or 0.25 μM) alone or in combination. To determine its synergistic effect we calculate the combination Index (CI) and dose reduction index (DRI) using Chou-Talalay algorithms (Chou and Talalay, 1984; Chou, 2006).

The results shown in **Figures 5A–C** demonstrate that ecdysterone significantly sensitizes all breast cancer cell lines to the treatment with doxorubicin. CI plots (**Figures 5D–F**) and **Table 1** show that all three Ecdy concentrations have a pronounced synergistic (CI ranges 0.47–0.89) interaction with doxorubicin. In turn, the use of Ecdy allowed the reduction of the effective dose of doxorubicine from 1.4 to 17.9 times (**Table 1**), depending on the particular cell line. It is important to notice that in most cases the addition of even 250 μM ecdysterone was sufficient to down-regulate the growth of breast cancer cells 1.5–2 times more efficiently than the corresponding concentrations of doxorubicin (**Figures 5A–C**).

These data clearly demonstrate the ability of ecdysterone to synergize with doxorubicin to down-regulate the proliferation of breast cancer cells.

Ecdysterone Significantly Enhances Doxorubicin- and 2-DG-Induced Cell Death

The observed synergistic effect of doxorubicin and ecdysterone likely results from cell death. To directly check this hypothesis, we treated the cell lines with ecdysterone (250 μM) and doxorubicin (0.15 or 0.25 μM) alone or in combination, followed by staining with Annexin V/7AAD and flow cytometry analysis. Since we have already shown that ecdysterone down-regulated the metabolic potential, we decided to apply 2-deoxyglucose (2-DG), a promising inhibitor of glycolysis, which currently

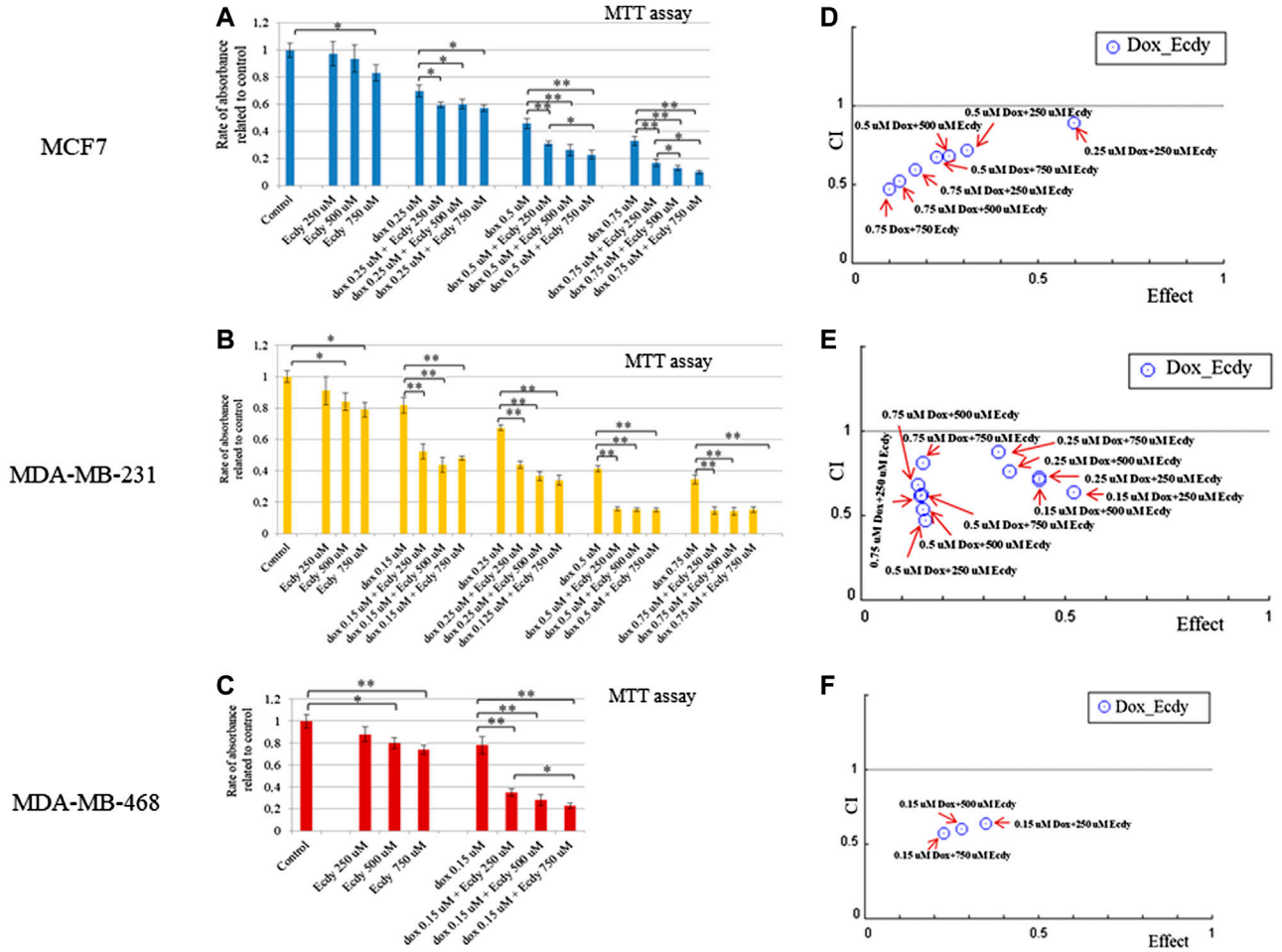

FIGURE 5 | Ecdysterone acts synergistically with doxorubicin to inhibit breast cancer cells. Results of MTT-test demonstrating the inhibition of **(A)** MCF7, **(B)** MDA-MB-231 **(C)** MDA-MB-468 cell lines by doxorubicin or ecdy along and in combination. Results are represented as the mean ± SD. *$p < 0.05$; **$p < 0.01$. Combination Index (CI) plots which reflect the distribution of CIs for different combinations of Dox/Ecdy concentrations, depending on their effects (rate of absorbance related to control) for: **(D)** MCF7, **(E)** MDA-MB-231 **(F)** MDA-MB-468 cells. CI plots were calculated using Chou-Talalay algorithms (CompuSyn software, http://www.combosyn.com/; Chou, 2006). CI < 1 attests at the synergistic action of drugs; DRI estimates the extent to which the dose of doxorubicin in the combination with the indicated dose of Ecdy can be reduced to achieve effect levels that are comparable to those achieved with single agents.

undergoes clinical trials. We also treated cells with either 2-DG (10 mM) or ecdysterone (250 μM) alone or in combination.

Figures 6A–F and **Supplementary Figures S2A–C** demonstrate that the combined treatment (either doxorubicin or 2-DG with 250 μM Ecdy) both increased the level of cell death by several times relative to control, or in comparison to the treatment with an individual drug. Accordingly, co-treatment with doxorubicin and ecdysterone elevated the level of apoptosis in MCF7 cells by 23% and in MDA-MB-231 cells by 3.15 times, respectively, compared to doxorubicin alone.

Co-treatment of Ecdy with 2-DG also significantly enhanced both apoptosis and total cell death (**Figures 6C–F**). It increased the rate of cell death by 21% for MCF7 and 17% for MDA-MB-231 cells.

We also repeated the previously described treatment of several breast cancer cells with combinations of doxorubicin (0.25 or 0.15 μM, respectively) and ecdysterone (250, 500, and 750 μM) followed by immunofluorescence microscopy to detect the release of cytochrome C from the mitochondria upon apoptosis. Taken together, results shown in **Figure 7** and **Supplementary Figures S2, S3** confirm that treatment with Ecdy significantly enhanced the doxorubicin-induced release of cytochrome C in all cancer cell lines.

Taken together, these data suggest that ecdysterone mediates strong synergy with doxorubicin in attenuation of the proliferation of breast cancer cells.

Ecdysterone Has Minimal Effects on Non-Transformed Human Fibroblasts

To compare the effects of ecdysterone on cancerous vs. non-cancerous cells, we decided to test normal non-transformed human fibroblasts, DF2 and WI-38 cell lines after the treatment with Ecdy.

TABLE 1 | Synergistic effect of ecdysterone and doxorubicin calculated using Chou-Talalay algorithms (Chou and Talalay, 1984; Chou, 2006).

	Dose dox, μM	Dose ecdy, μM	Effect	CI	Dox_DRI
MCF7	0,25	250	0,59	0,89	1,36
	0,25	500	0,6	1,07	3,04
	0,25	750	0,57	1,13	2,17
	0,5	250	0,31	0,72	11,66
	0,5	500	0,26	0,68	6,63
	0,5	750	0,23	0,67	4,88
	0,75	250	0,17	0,59	17,86
	0,75	500	0,13	0,52	10,58
	0,75	750	0,1	0,47	8,26
	Dose dox, μM	**Dose ecdy, μM**	**Effect**	**CI**	**Dox_DRI**
MDA-MB-231	0,15	250	0,52	0,64	2,61
	0,15	500	0,44	0,72	3,32
	0,15	750	0,48	1,04	2,93
	0,25	250	0,44	0,72	1,98
	0,25	500	0,37	0,77	2,45
	0,25	750	0,34	0,88	2,65
	0,5	250	0,16	0,47	2,64
	0,5	500	0,15	0,54	2,76
	0,5	750	0,15	0,62	2,79
	0,75	250	0,14	0,61	1,89
	0,75	500	0,14	0,68	1,95
	0,75	750	0,15	0,81	1,84
	Dose dox, μM	**Dose ecdy, μM**	**Effect**	**CI**	**Dox_DRI**
MDA-MB-468	0,15	250	0,35	0,64	1,69
	0,15	500	0,28	0,6	1,85
	0,15	750	0,23	0,57	1,99

CI, Combination Index, Dox_DRI, Dose Reduction Index for doxorubicin.

First, we assessed the effect of Ecdy on proliferation of breast cancer cell lines and non-transformed human fibroblasts. As shown in **Supplementary Figure S4A,B**, Ecdy down-regulated the proliferation of cancer cell lines significantly more robustly compared to normal human fibroblasts at all concentrations tested.

To study whether ecdysterone elicits effects on autophagy in non-transformed fibroblasts similar to what we have observed in breast cancer cells, we employed the previously described flow cytometry analysis with LysoTracker DND-99 Red combined with western blotting for LC3 and p62. Results shown in **Supplementary Figure S6** demonstrate that Ecdy induced autophagy in fibroblasts to a much lesser extent than in breast cancer cells (**Figure 4**).

We have also investigated the effect of combined treatment with Ecdy and doxorubicin on DF2 and WI38 fibroblasts (**Supplementary Figure S7**). To this end, we have employed previously described Annexin V-FITC/7-AAD staining followed by flow cytometry. Surprisingly, the minimal effective concentration of Ecdy (250 uM) in combination with doxorubicin that induced death of breast cancer cells, had almost no effect on DF2 and WI38 fibroblasts (**Figure 6**; **Supplementary Figure S2**).

Finally, we have carried out MTT assay on DF2 and WI38 normal human fibroblasts as well as mouse embryonic fibroblasts (MEFs) treated with either Ecdy alone or in combination with doxorubicin. **Supplementary Figure S8** demonstrates that in contrast to breast cancer cell lines (**Figure 5**), an increased ecdysterone concentration up-regulated the survival of WI-38 and MEFs cells (**Supplementary Figures S7B,C**). In the case of DF2 cells, the combined treatment (doxorubicin + ecdysterone) displayed even a small protection of these cells from doxorubicin (**Supplementary Figure S7A**). Regarding WI-38 and MEFs (**Figures 7B,C**), the same combined treatment has incomparable low inhibitory effect in contrast to breast cancer cells (**Figure 5**).

Taken together, our experiments show that ecdysterone significantly down-regulates cancer cells with no or little effect on fibroblasts.

DISCUSSION

Ecdysterone is a hormone found in arthropods, yet is also synthesized by a number of plants to combat insect pests by disrupting their development, molting, and reproduction. Unlike insects, mammals do not harbor any homologs of ecdysterone nuclear receptor (EcR). However, ecdysterone possesses a variety of beneficial pharmacological effects on humans, including anabolic and adaptogenic ones (Báthori et al., 2008). Ecdysterone is marketed as a diet supplement to enhance the physical performance of athletes, and recently became the focus of WADA investigations (https://www.wada-ama.org/en/resources/research/ecdysteroids-as-non-conventional-anabolic-agents-pharmacodynamics; Parr et al., 2020). Numerous studies have documented oncogenic properties of male steroid hormones and its derivatives on several human organs, including testis, liver, breast, and others (Sirianni et al., 2012; Salerno et al., 2018). Therefore, it is important to assess all biologically active supplements for their potential side effects including the tumorigenic one.

Although positive ecdysterone-mediated pharmacological influence on organisms is well documented (Lafont and Dinan, 2003), we decided to examine possible pharmacological effects of ecdysterone on proliferation of human breast cancer cell lines of different molecular subtypes. Surprisingly, despite the fact that anabolic properties of ecdysterone in muscles have been reported (Parr et al., 2015; Isenmann et al., 2019), we have not observed ecdysterone-mediated growth stimulation of cancer cells. Instead, in our MTT experiments administration of ecdysterone caused the attenuation of cell growth of breast cancer cells starting from the concentration of 250–750 μM. Apparently, Ecdy can negatively regulate cancer cells through various mechanisms because it inhibited the cell cycle and induced death to a different extend depending on the particular cellular background. While Ecdy significantly affected the cell cycle distribution of MCF7 cells, it had almost no effect on cell cycle of MDA-MB-468 cells. Furthermore, it elicited a two-fold increase in cell death of the MDA-MB-468 cells relative to MCF7. In contrast to cancer cells, ecdysterone displayed a significantly less inhibitory impact on proliferation of human non-transformed fibroblasts compared to cancer cells.

Metabolic reprogramming is a known hallmark of cancer cells, in which they manifest diverse metabolic phenotypes to maintain their proliferation and to combat anticancer therapies (Shuvalov

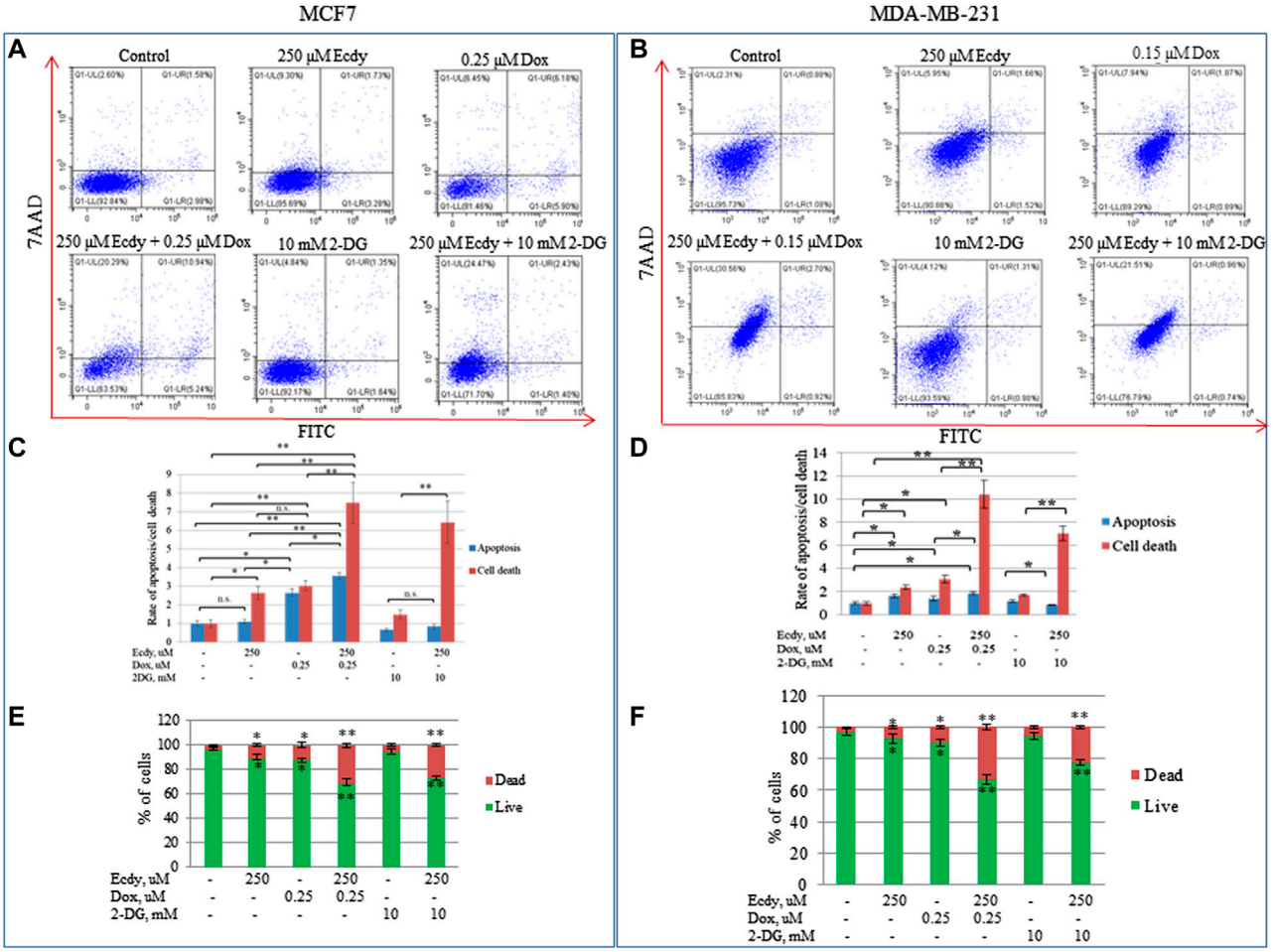

FIGURE 6 | Ecdysterone synergizes with doxorubicin and 2-DG to induce apoptosis and death of breast cancer cell lines. Annexin V-FITC/7-AAD profiles of **(A)** MCF7, **(B)** MDA-MB-231 breast cancer cells treated with doxorubicin, ecdysterone, and 2-DG alone or in combination for 48 h (flow cytometry). **(C,D)** Diagrams show the rate of apoptosis/total cell death for the same cell lines based on Annexin V-FITC/7-AAD data. **(E,F)** Diagrams show percent of live/dead cells. Results are calculated as the mean ± SEM of three experiments. *$p < 0.05$; **$p < 0.01$; n.s – not significant.

et al., 2017; Sun et al., 2020). Among breast cancers, the TNBC subtype has the worst prognosis with questioned targeted therapies. Therefore, we decided to assess the influence of Ecdy on the energy metabolism of two TNBC cancer cell lines. The SeaHorse energy profiling has shown that Ecdy significantly dampened respiration, as well as the metabolic potential of MDA-MB-231 cells, and strongly reduced both respiration and glycolysis in MD-MB-468 cells. Moreover, we have revealed that Ecdy sensitizes breast cancer cell lines to 2-DG which is in accordance with Ecdy-mediated down-regulation of energy metabolism. 2-DG is a promising inhibitor of glycolysis, which decreases the energy of the cancer cells thus making chemotherapy and other treatments more effective. It underwent clinical trials and most likely is useful for the treatment of breast cancer including TNBC (Wokoun et al., 2017; Lucantoni et al., 2018; O'Neill et al., 2019).

Although Ecdy only weakly inhibits proliferation of fibroblasts in the proliferation assay, it does activate fibroblasts in MTT assay which may result from their metabolic activation. The effect of Ecdy on metabolism of different cancer and non-cancer cells should be studied in further details.

The modulation of cancer-specific metabolic adaptations weakens the malignant cells and widens the therapeutic window for effective treatment of TNBC patients (Lanning et al., 2017; Wang et al., 2020). Ecdy-mediated negative regulation of the energy metabolism in TNBC cells can be potentially important for the treatment of this most dangerous sub-type of breast malignancy.

Ecdy can promote autophagy upon the onset of osteoporosis in rats (Tang et al., 2018). In addition, Ecdy protects from degeneration human nucleus pulposus cells, which form the inner core of the vertebral disc (Wen et al., 2019). This effect is mediated by Ecdy-dependent induction of autophagy, which counteracts the effect of apoptosis. In line with these observations, we have demonstrated that Ecdy strongly induced autophagy in breast cancer cells, in contrast to non-transformed human fibroblasts. Although autophagy can play dual roles in both tumor promotion and suppression (Yun and

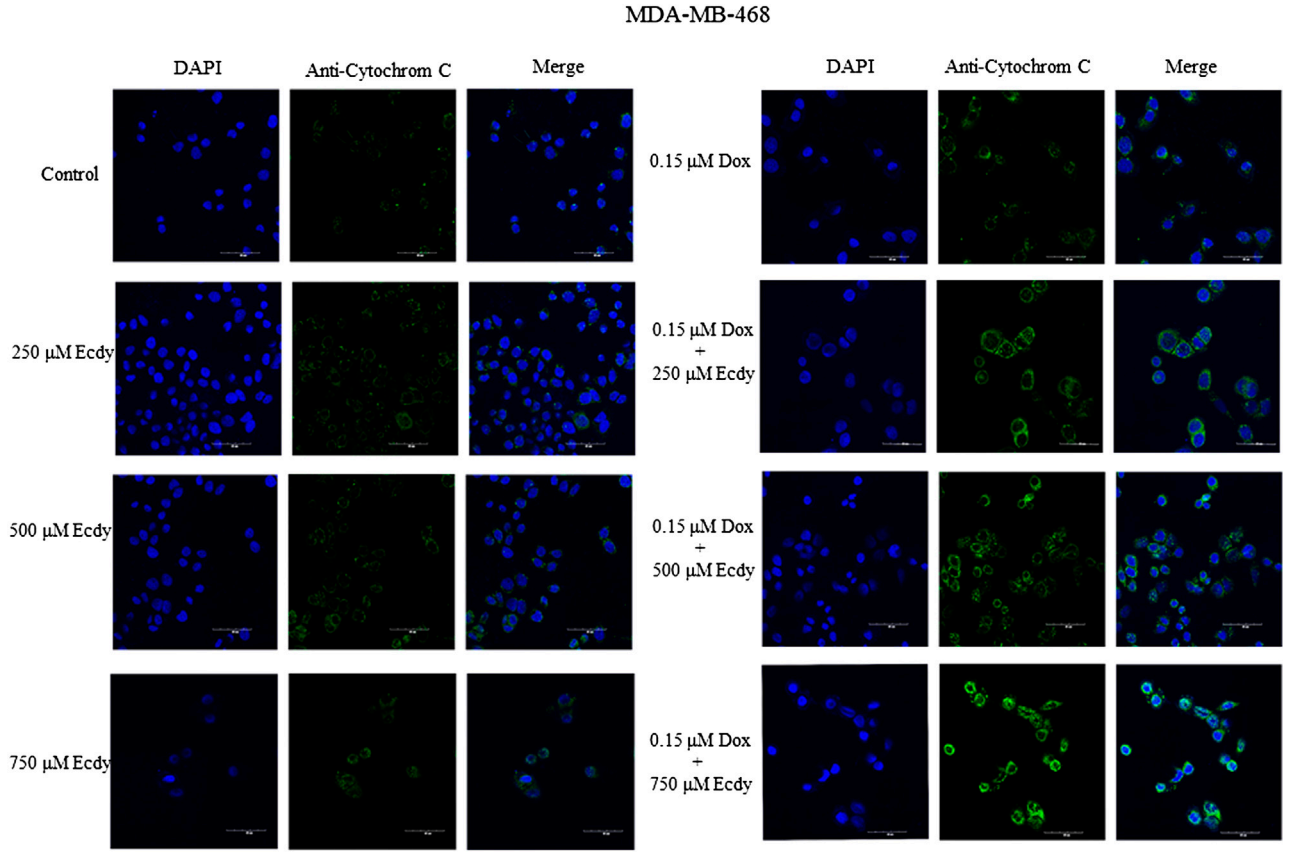

FIGURE 7 | Ecdysterone synergizes with doxorubicin to induce apoptosis in MDA-MB-468 cells. Confocal microscopy showing an increase in cytochrome C release upon combined treatment with doxorubicin and Ecdy. MDA-MB-468 cells treated with 0.15 µM doxorubicin or 250–750 µM Ecdy for 48 h alone or in combination were stained with DAPI and anti-cytochrome C antibodies and subsequently analyzed by confocal microscopy.

Lee, 2018), in terms of chemotherapy autophagy is usually considered as a mechanism of drug-resistance against therapeutics. For example, doxorubicin-induced autophagy is involved in the development of chemoresistance, and the inhibition of autophagy effectively overcomes doxorubicin resistance in a variety of cancers (Zhou et al., 2019).

Surprisingly, despite its positive effect on autophagy, Ecdy displayed a strong synergistic effect (CI ranges 0, 47-0, 89) with doxorubicin, which significantly enhances doxorubicin-induced cell death (DRI ranges 1, 4-17, 9 times) of breast cancer cells according to Chou-Talalay algorithms (Chou and Talalay, 1984; Chou, 2006). Notably, Ecdy strongly enhanced the action of doxorubicin in concentrations (250, 500, and 750 µM), which are sufficient to inhibit energy metabolism and induce autophagy. It is important to note that when Ecdy was used alone, it failed to significantly down-regulate the proliferation of cancer cells. Noteworthy, Ecdy was not able to sensitize non-cancerous (fibroblast) cells to doxorubicin as it was observed for breast cancer cells. Our results are in accordance with other studies (Konovalova et al., 2002; Martins et al., 2012; Martins et al., 2015) that have shown that ecdysterone made both drug-resistant and non-drug-resistant cancer cells more susceptible to doxorubicin treatment. Furthermore, Ecdy was shown to significantly stimulate the chemotherapeutic effect of cisplatin in mice models (Konovalova et al., 2002). Taken together, these data suggests that in moderate concentrations, Ecdy sensitizes cancer cells to treatments with chemotherapeutic agents and thus can potentially serve as an adjuvant therapeutic.

Furthermore, since Ecdy enhances the ability to cope with stress and enhances resistance to tiredness (Báthori et al., 2008; Dinan et al., 2009), it seems beneficial to administer it as part of cytotoxic therapy with doxorubicin. The latter produces multiple severe side effects including the cumulative cardiotoxicity, acute nausea and vomiting, gastrointestinal disturbances, alopecia baldness, and neurologic disturbances (Carvalho et al., 2009). However, additional experiments aimed at the elucidation of effectiveness of Ecdy and its toxicity to organs and tissues are required to assess the therapeutic potential of ecdysterone as an adjuvant therapy to treat breast cancer.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

NB and OS designed experiments and wrote the manuscript. OS carried out majority of experiments. OF and AD participated in MTT assays. AP had part in SeaHorse experiments. ET participated in IF studies. GJ contributed to western-blot experiments. All authors participated in preparation of the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.561537/full#supplementary-material

REFERENCES

Azizov, A., and Seĭfulla, R. (1998). The effect of elton, leveton, fitoton and adapton on the work capacity of experimental animals. *Eksp. Klin. Farmakol.* 61, 61–63.

Bajguz, A., Bąkała, I., and Talarek, M. (2015). "Ecdysteroids in plants and their pharmacological effects in vertebrates and humans," in *Studies in Natural Products Chemistry* (New York, NY: Elsevier), 121–145.

Báthori, M., Tóth, N., Hunyadi, A., Márki, A., and Zador, E. (2008). Phytoecdysteroids and anabolic-androgenic steroids–Structure and effects on humans. *Curr. Med. Chem.* 15, 75–91. doi:10.2174/092986708783330674

Carvalho, C., Santos, R., Cardoso, S., Correia, S., Oliveira, P., Santos, M., et al. (2009). Doxorubicin: The good, the bad and the ugly effect. *Curr. Med. Chem.* 16, 3267–3285. doi:10.2174/092986709788803312

Chikte, S., Panchal, N., and Warnes, G. (2014). Use of LysoTracker dyes: A flow cytometric study of autophagy. *Cytometry* 85, 169–178. doi:10.1002/cyto.a.22312

Chou, T.-C., and Talalay, P. (1984). Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzym. Regul.* 22, 27–55. doi:10.1016/0065-2571(84)90007-4

Chou, T.-C. (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol. Rev.* 58, 621–681. doi:10.1124/pr.58.3.10

Dinan, L. (2001). Phytoecdysteroids: Biological aspects. *Phytochemistry* 57, 325–339. doi:10.1016/s0031-9422(01)00078-4

Dinan, L., Harmatha, J., Volodin, V., and Lafont, R. (2009). "Phytoecdysteroids: Diversity, biosynthesis and distribution," in *Ecdysone: Structures and functions* (New York, NY: Springer), 3–45. doi:10.1002/arch.20334

Dinan, L., and Lafont, R. (2006). Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. *J. Endocrinol.* 191, 1–8. doi:10.1677/joe.1.06900

Gorelick-Feldman, J., Maclean, D., Ilic, N., Poulev, A., Lila, M. A., Cheng, D., et al. (2008). Phytoecdysteroids increase protein synthesis in skeletal muscle cells. *J. Agric. Food Chem.* 56, 3532–3537. doi:10.1021/jf073059z

Isenmann, E., Ambrosio, G., Joseph, J. F., Mazzarino, M., De La Torre, X., Zimmer, P., et al. (2019). Ecdysteroids as non-conventional anabolic agent: Performance enhancement by ecdysterone supplementation in humans. *Arch. Toxicol.* 93, 1807–1816. doi:10.1007/s00204-019-02490-x

Konovalova, N., Mitrokhin, Iu. I., Volkova, L., Sidorenko, L., and Todorov, I. N. (2002). [Ecdysterone modulates antitumor activity of cytostatics and biosynthesis of macromolecules in tumor-bearing animals]. *Izv. Akad. Nauk. Seriia Biol.* 6, 650–658. [in Russian].

Lafont, R., and Dinan, L. (2003). Practical uses for ecdysteroids in mammals including humans: and update. *J. Insect Sci.* 3, 7. doi:10.1093/jis/3.1.7

Lanning, N. J., Castle, J. P., Singh, S. J., Leon, A. N., Tovar, E. A., Sanghera, A., et al. (2017). Metabolic profiling of triple-negative breast cancer cells reveals metabolic vulnerabilities. *Canc. Metabol.* 5, 6. doi:10.1186/s40170-017-0168-x

Lezina, L., Aksenova, V., Ivanova, T., Purmessur, N., Antonov, A. V., Tentler, D., et al. (2014). KMTase Set7/9 is a critical regulator of E2F1 activity upon genotoxic stress. *Cell Death Differ.* 21, 1889–1899. doi:10.1038/cdd.2014.108

Lucantoni, F., Dussmann, H., and Prehn, J. H. (2018). Metabolic targeting of breast cancer cells with the 2-deoxy-D-glucose and the mitochondrial bioenergetics inhibitor MDIVI-1. *Front. Cell Dev. Biol.* 6, 113. doi:10.3389/fcell.2018.00113

Martins, A., Tóth, N., Ványolós, A., Béni, Z., Zupkó, I., and Molnár, J. (2012). Significant activity of ecdysteroids on the resistance to doxorubicin in mammalian cancer cells expressing the human ABCB1 transporter. *J. Med. Chem.* 55, 5034–5043. doi:10.1021/jm300424n

Martins, A., Sipos, P., Dér, K., Csábi, J., Miklos, W., Berger, W., et al. (2015). Ecdysteroids sensitize MDR and non-MDR cancer cell lines to doxorubicin, paclitaxel, and vincristine but tend to protect them from cisplatin. *BioMed Res. Int.* 2015, 895360. doi:10.1155/2015/895360

Ogawa, S., Nishimoto, N., and Matsuda, H. (1974). "Pharmacology of ecdysones in vertebrates," in *Invertebrate endocrinology and hormonal heterophylly*. Editor W. J. Burdette (New York Inc: Springer-Verlag), 341–344.

O'neill, S., Porter, R. K., Mcnamee, N., Martinez, V. G., and O'driscoll, L. (2019). 2-Deoxy-D-Glucose inhibits aggressive triple-negative breast cancer cells by targeting glycolysis and the cancer stem cell phenotype. *Sci. Rep.* 9, 3788. doi:10.1038/s41598-019-39789-9

Parr, M., Botrè, F., Naß, A., Hengevoss, J., Diel, P., and Wolber, G. (2015). Ecdysteroids: A novel class of anabolic agents? *Biol. Sport* 32, 169. doi:10.5604/20831862.1144420

Parr, M. K., Ambrosio, G., Wuest, B., Mazzarino, M., De La Torre, X., Sibilia, F., et al. (2020). Targeting the administration of ecdysterone in doping control samples. *Forensic Toxicol.* 38, 172–184. doi:10.1007/s11419-019-00504-y

Parr, M. K., Zhao, P., Haupt, O., Ngueu, S. T., Hengevoss, J., Fritzemeier, K. H., et al. (2014). Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone. *Mol. Nutr. Food Res.* 58, 1861–1872. doi:10.1002/mnfr.201300806

Rausher, M. D. (2001). Co-evolution and plant resistance to natural enemies. *Nature* 411, 857–864. doi:10.1038/35081193

Salerno, M., Cascio, O., Bertozzi, G., Sessa, F., Messina, A., Monda, V., et al. (2018). Anabolic androgenic steroids and carcinogenicity focusing on Leydig cell: a literature review. *Oncotarget* 9 (27), 19415–19426. doi:10.18632/oncotarget.24767

Shuvalov, O., Petukhov, A., Daks, A., Fedorova, O., Vasileva, E., and Barlev, N. A. (2017). One-carbon metabolism and nucleotide biosynthesis as attractive targets for anticancer therapy. *Oncotarget* 8, 23955–23977. doi:10.18632/oncotarget.15053

Sirianni, R., Cipolloni, R., Capparelli, C., Chimento, A., Panza, S., Catalano, S., Lanzino, M., et al. (2012). Nandrolone and stanozolol upregulate aromatase

expression and further increase IGF-I-dependent effects on MCF-7 breast cancer cell proliferation. *Mol. Cell. Endocrinol.* 363, 100–110. doi:10.1016/j.mce.2012.08.002

Sun, X., Wang, M., Wang, M., Yu, X., Guo, J., Sun, T., et al. (2020). Metabolic reprogramming in triple-negative breast cancer. *Front. Oncol.* 10, 428. doi:10.3389/fonc.2020.00428

Tang, Y. H., Yue, Z. S., Xin, D. W., Zeng, L. R., Xiong, Z. F., Hu, Z. Q., et al. (2018). β-ecdysterone promotes autophagy and inhibits apoptosis in osteoporotic rats. *Mol. Med. Rep.* 17, 1591–1598. doi:10.3892/mmr.2017.8053

Wang, Z., Jiang, Q., and Dong, C. (2020). Metabolic reprogramming in triple-negative breast cancer. *Cancer Biol. Med.* 17, 44–59. doi:10.20892/j.issn.2095-3941.2019.0210

Wen, F., Yu, J., He, C. J., Zhang, Z. W., and Yang, A. F. (2019). β-ecdysterone protects against apoptosis by promoting autophagy in nucleus pulposus cells and ameliorates disc degeneration. *Mol. Med. Rep.* 19, 2440–2448. doi:10.3892/mmr.2019.9861

Wokoun, U., Hellriegel, M., Emons, G., and Gründker, C. (2017). Co-treatment of breast cancer cells with pharmacologic doses of 2-deoxy-D-glucose and metformin: Starving tumors. *Oncol. Rep.* 37, 2418–2424. doi:10.3892/or.2017.5491

Yun, C., and Lee, S. (2018). The roles of autophagy in cancer. *Int. J. Mol. Sci.* 19, 3466. doi:10.3390/ijms19113466

Zhou, Y., Niu, W., Luo, Y., Li, H., Xie, Y., Wang, H., et al. (2019). p53/Lactate dehydrogenase A axis negatively regulates aerobic glycolysis and tumor progression in breast cancer expressing wild-type p53. *Canc. Sci.* 110, 939–949. doi:10.1111/cas.13928

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GLOSSARY

Ecdy ecdysterone

Dox doxorubicin

ERbeta Estrogen Receptor beta

TNBC Triple Negative Breast Cancer

CI Combination Index

DRI Dose Reduction Index

2-DG 2-deoxyglucose

DMSO dimethyl sulfoxide

FCCP Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

PI Propidium iodide

7-AAD 7-Aminoactinomycin D

ECAR Extracellular Acidification Rate

OCR Oxygen Consumption Rate

MDR Multiple Drug Resistance

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REVIEW
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Mechanisms of CDK4/6 Inhibitor Resistance in Luminal Breast Cancer

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As a new-generation CDK inhibitor, a CDK4/6 inhibitor combined with endocrine therapy has been successful in the treatment of advanced estrogen receptor–positive (ER+) breast cancer. Although there has been overall progress in the treatment of cancer, drug resistance is an emerging cause for breast cancer–related death. Overcoming CDK4/6 resistance is an urgent problem. Overactivation of the cyclin-CDK-Rb axis related to uncontrolled cell proliferation is the main cause of CDK4/6 inhibitor resistance; however, the underlying mechanisms need to be clarified further. We review various resistance mechanisms of CDK4/6 inhibitors in luminal breast cancer. The cell signaling pathways involved in therapy resistance are divided into two groups: upstream response mechanisms and downstream bypass mechanisms. Finally, we discuss possible strategies to overcome CDK4/6 inhibitor resistance and identify novel resistance targets for future clinical application.

Keywords: luminal breast cancer, endocrine resistance, upstream response signaling, downstream bypass signaling, CDK4/6 inhibitor

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INTRODUCTION

Breast cancer (BC) is a common women-related malignant tumor disease in developed countries. Estrogen receptor–positive (ER-positive) breast cancer represents approximately 70% of all BC (Goldhirsch et al., 2011; Malvezzi et al., 2013). ER-positive breast cancer can be further stratified into pathological subtypes, such as ductal or mixed ductal and lobular, mucinous, and tubular carcinomas, which are referred to as luminal breast cancer (Ignatiadis and Sotiriou, 2013). Luminal breast tumors are highly heterogeneous in terms of histology and response to treatment. Luminal A and B are two main ER-positive breast cancer subtypes, based on different gene expression profiles, prognosis, and clinical therapy responses (Sotiriou and Pusztai, 2009).

The difference between luminal A and B is mainly related to the expression of hormone receptors. Luminal B tumors have lower levels of ER expression, lower or no levels of progesterone receptor (PR) expression, but higher tumor grade and higher Ki-67–positive staining than luminal A tumors (Goldhirsch et al., 2011; Creighton, 2012). Endocrine therapy, such as ER downregulators, selective ER modulators, and aromatase inhibitors, is considered to be the primary treatment for luminal A and luminal B. However, in the clinic, the main therapy for luminal B is chemotherapy, due to the

lower sensitivity of these patients to endocrine treatment or drug resistance (Rouzier et al., 2005; Ignatiadis et al., 2012). In fact, endocrine resistance is an unavoidable problem in clinical therapy of luminal tumors. Development of new therapy methods to avert endocrine resistance is an urgent challenge in clinical medicine (Anurag et al., 2020).

It is well known that the cell cycle is driven by cyclin-dependent kinases (CDKs), such as CDK4 and CDK6, which are also closely associated with tumor initiation and progression Yu et al., 2006; Choi et al., 2012). The activity of cyclin D and CDK4/6 complexes is considered to play the major role in tumor cell proliferation driven by estrogen, especially in breast cancer (Filmus et al., 1994). In recent years, it has been established that targeting the cell cycle for anticancer treatment is a rational option that could be combined with endocrine therapy.

CDK inhibitors, which target overactive CDK activities in tumor cells, have been widely used in preclinical or clinical trials. In the clinic, three CDK4/6 inhibitors, namely, palbociclib (Fry et al., 2004), ribociclib (Infante et al., 2016), and abemaciclib (Patnaik et al., 2016), have been successfully used in combination with other endocrine therapy drugs for ER-positive and human epidermal growth factor receptor-2 (HER2)–negative advanced breast cancer treatment (Ribnikar et al., 2019); in addition, significant overall survival (OS) benefits have been confirmed at ESMO2019 conference.

Despite the fact that the new guidelines for the therapy of advanced breast cancer includes a CDK4/6 inhibitor combined with endocrine treatment as the first- or second-line drug in most countries, most patients eventually develop acquired drug resistance to CDK4/6 inhibitors (Konecny et al., 2011). Several factors affect the effectiveness of CDK4/6 inhibitors, such as continuous expression of G1-S-phase cyclins and gene mutations in key cell signaling pathways (Herrera-Abreu et al., 2016). Research on the molecular mechanisms or clinical strategies to overcome CDK4/6 inhibitor resistance is ongoing (Pandey et al., 2019; Portman et al., 2019). Therefore, the major emerging consideration in treatment of advanced luminal breast cancer is now CDK4/6 inhibitor resistance.

In this review, we discuss three CDK4/6 inhibitors with different clinical trial results and various resistance mechanisms, aiming to help identify novel clinical therapeutic targets to improve endocrine therapy resistance and provide possible strategies to overcome resistance to CDK4/6 inhibitors in advanced luminal breast cancer.

CDK4/6 Inhibitors in Luminal Breast Cancer

In malignant cells, overactive CDK activities are targeted by CDK inhibitors. The major barrier limiting CDK inhibitors from further development is the lack of selectivity, due to similar structures among CDKs (Shapiro, 2006; Michaud et al., 2010). In the meantime, some biocomputing technologies, such as computer-aided (Kalra et al., 2017) and pharmacological (Tadesse et al., 2018; Yin et al., 2018) approaches, have been employed to develop a new-generation CDK inhibitor with higher selectivity. Recently, there has been great progress in CDK inhibitor design, especially the design of CDK4/6 inhibitors, which have been successfully used in clinical trials.

ATP-binding domains are the main drug targets of CDK4/6 inhibitors to block cell cycle G1-S transition (Asghar et al., 2015). Three third-generation CDK inhibitors, palbociclib, ribociclib, and abemaciclib, have higher specificity to CDK4/6 than other members of the CDK family and have been translated into clinical use against advanced luminal breast cancer. The phase III MONALEESA-3 trial used a combination of ribociclib and fulvestrant in advanced ER+/HER2 breast cancer demonstrated an increased PFS (progression-free survival) (Slamon et al., 2018) and an improved OS compared with fulvestrant alone (Slamon et al., 2019). The phase III MONARCH-plus trial with abemaciclib and nonsteroidal aromatase inhibitor (NSAI) or fulvestrant treatment showed improved PFS in predominantly Chinese postmenopausal women with ER+/HER2 breast cancer (Jiang et al., 2019a). Moreover, in the phase III MONARCH HER trial, triple treatment with abemaciclib, trastuzumab (Herceptin), and fulvestrant showed better therapy outcomes than trastuzumab plus chemotherapy in ER+/HER2+ patients. In addition, phase II/III trials of the three CDK4/6 inhibitors in combination with letrozole, tamoxifen, fulvestrant, and herceptin in the first-/second-line setting have already been completed (**Table 1**).

Resistance Mechanisms of CDK4/6 Inhibitor

CDK4/6 inhibitors are not a panacea due to the therapy resistance. It has been reported in the PALOMA-2 trial that more than 30% patients experienced recurrence of their cancer within 2 years of CDK4/6 inhibitor treatment (Finn et al., 2016), indicating that palbociclib combined with endocrine therapy may affect CDK inhibitor sensitivity and allow tumor cells to return to a proliferative phenotype. However, whether the mechanism of endocrine therapy resistance is associated with the inhibition of cell cycle or activation of other "bypass" signaling pathways is not fully understood. We summarized the molecular mechanisms of CDK4/6 inhibitor resistance below (**Figure 1**).

CyclinD1-CDK4/6–Rb Pathway

Upregulation of cyclin–CDK activity promotes the cell cycle and proliferation (Hanahan and Weinberg, 2011). The retinoblastoma (Rb) protein acts as a gatekeeper to prevent the cell cycle from progressing from G1 phase into S phase. CDK4/6 forms heterodimers with D-type cyclins (particularly D1) to phosphorylate Rb. As a result, Rb protein phosphorylation leads to the release of transcription factor E2Fs, which activates the DNA transcriptional program for cell cycle G1/S.

In luminal breast cancer, the development of resistance to endocrine therapy is associated with the function and integrity of Rb (Musgrove et al., 2011). Fortunately, the low incidence of Rb gene deletion/mutation (3.9%) in luminal-like breast tumors offers the possibility for CDK4/6 inhibition (Ciriello et al., 2015). This viewpoint has been solidified by a study that showed that the effects of clinical therapy in luminal breast cancer were not sensitive to palbociclib when Rb expression is absent (Dean et al., 2012). In addition, the function of Rb can also be regulated by E2F transcriptional factors. Malorni et al.

TABLE 1 | CDK4/6 inhibitors for the treatment of advanced luminal breast cancer in phase II/III trials.

Clinical trial	Regimen	Phase	Patients	PFS (months)	ORR	Hazard ratio	References
First line							
PALOMA-1	Letrozole + palbociclib/Letrozole	II	165	10.2 vs. 20.2	39 vs. 55%	0.49	Finn et al. (2015)
PALOMA-2	Letrozole + palbociclib/Letrozole	III	666	14.5 vs. 24.8	44 vs. 55%	0.58	Finn et al. (2016)
MONALEESA-2	Letrozole ± ribociclib	III	668	14.7 vs. 26.0	37 vs. 53%	0.57	Hortobagyi et al. (2018)
MONARCH-3	NSAI ± abemaciclib	III	493	14.7 vs. 28.2	44 vs. 59%	0.54	Goetz et al. (2017)
MONALEESA-7	NSAI/Tamoxifen + OFS ± ribociclib	III	672	13.0 vs. 23.8	36 vs. 51%	0.55	Tripathy et al. (2018)
Second line							
PALOMA-3	Fulvestrant ± palbociclib	III	521	4.6 vs. 9.5	11.1 vs. 25%	0.46	Cristofanilli et al. (2016)
MONARCH-1	Abemaciclib monotherapy	II	132	6.0	20%	—	Dickler et al. (2017)
MONARCH-2	Fulvestrant ± abemaciclib	III	669	9.3 vs. 16.4	21 vs. 48	0.55	Sledge et al. (2017)
MONALEESA-3	Fulvestrant ± ribociclib	III	725	12.8 vs. 20.5	29 vs. 41%	0.59	Slamon et al. (2018)
MONARCH-plus	NSAI ± abemaciclib	III	306	14.73 vs. NE	30.3 vs. 56%	0.499	Jiang et al. (2019a)
	Fulvestrant ± ribociclib	III	157	5.59 vs. 11.47	7.5 vs. 38.5%	0.376	
MONARCH HER	Herceptin + abemaciclib + fulvestrant	III	79	8.32 vs. 0.65 vs. 5.69	32.9 vs. 13.9 vs.	0.673	Tolaney et al. (2019)
	Herceptin + abemaciclib	III	79		13.9%	0.943	
	Herceptin + chemo	III	79			—	

PFS, progression free survival; NSAI, nonsteroidal aromatase inhibitors; OFS, ovarian function suppression; ORR, objective response rate; NE indicates that the value could not be estimated.

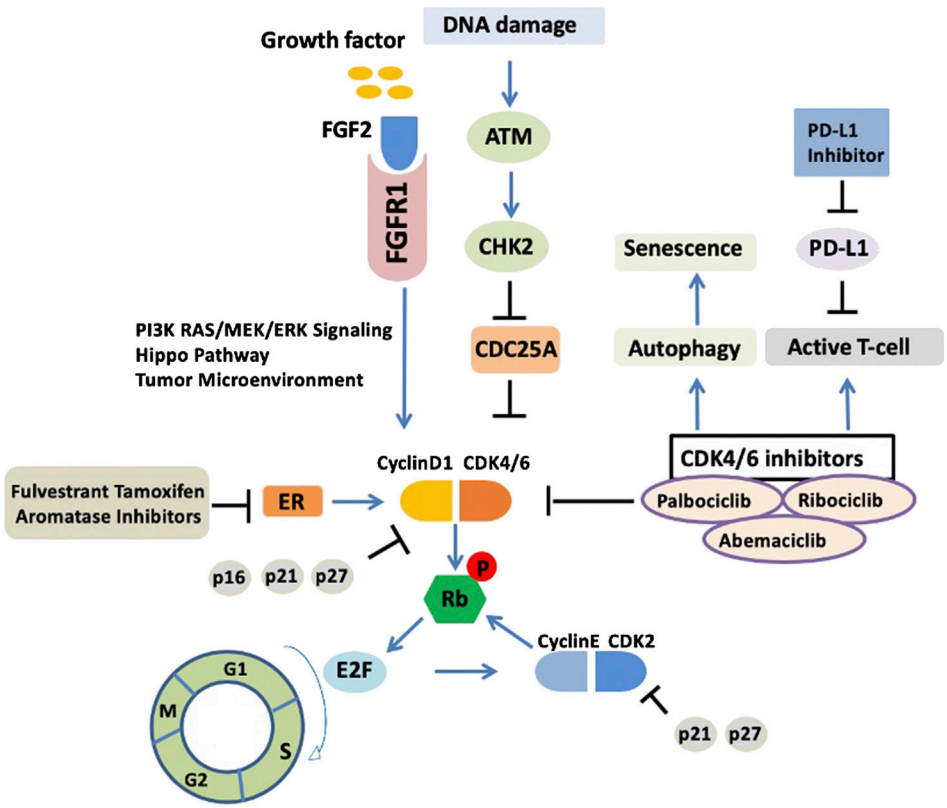

FIGURE 1 | Upstream response and downstream bypass signaling mechanisms of CDK4/6 inhibitor resistance. Current molecular mechanisms of CDK4/6 inhibitor resistance are highlighted. ER, estrogen receptor; CDK, cyclin dependent kinases; ATM, ataxia telangiectasia mutated; CHK2, checkpoint kinase 2; Rb, retinoblastoma protein.

indicated that the expression of both E2F1 and E2F2 could cause loss of Rb and predict the sensitivity of cell lines to palbociclib in luminal breast cancer (Malorni et al., 2016).

CDK4/6 Overexpression

Overexpression of CDK4 or CDK6 is the main mechanism of resistance to CDK4/6 inhibitors. Studies have shown that

increased expression of CDK6 reduced the response of CDK4/6 inhibitors in luminal cell line models. At the same time, knockdown of CDK6 rescued the therapy sensitivity, which indicated that CDK6-mediated drug resistance may be independent of CDK4 expression (Yang et al., 2017). In addition, either high or low expression of CDK4 has been detected in CDK4/6 inhibitor–resistant breast cancer cells (Bollard et al., 2017). Therefore, whether the expression of CDK4 is associated with CDK4/6 inhibitor resistance requires further investigation.

p16 Amplification

As a member of the INK4 family, p16 is a natural inhibitor of CDK4 and plays a vital role in the regulation of the cell cycle (Serrano et al., 1993). In general, p16 severs as a tumor suppressor and targets the CDK4/6 complex in dysregulatory cells depending on the function of Rb (Medema et al., 1995). For example, Dean JL et al. reported that the resistance to CDK4/6 inhibitors was caused by the absence of Rb, regardless of p16 expression (Dean et al., 2012). On the other hand, the expression level of p16 affected the effectiveness of CDK4/6 inhibition. Overexpression of p16-mediated resistance to CDK4/6 inhibitors in the absence of Rb (Witkiewicz et al., 2011) and low expression of p16 did not rescue the clinical benefit in Rb-positive luminal breast cancer patients in the phase II palbociclib monotherapy trial (DeMichele et al., 2015). The potential mechanism is that p16 overexpression suppresses the activity of CDK4 and expression of cyclin D1 (Witkiewicz et al., 2011), which are the main targets of CDK4/6 inhibitors, thus leading to reduced or no effects of CDK4/6 inhibition (Elvin et al., 2017). Whether p16 amplification and loss of Rb work together in CDK4/6 inhibitor resistance is not clearly understood. Further studies revealing the mechanistic association between p16 and Rb might be beneficial to avert acquired resistance to CDK4/6 inhibitors.

ATM-CHK2 Activation

Deficiency of mismatch repair may lead to the endocrine therapy resistance in luminal breast cancer through the abrogation of CHK2-mediated inhibition of CDK4. A recent study showed that defects in single-strand break repair in luminal breast cancer can drive endocrine therapy resistance and is closely associated with the ATM-CHK2-CDC25A pathway (Anurag et al., 2018). ATM, as a DNA damage sensor, activates CHK2, which in turn phosphorylates CDC25A at S123 for degradation. Importantly, as a phosphatase, CDC25A could inhibit the phosphorylation of CDK4/6. The CDK4/6 complex activity could be reactivated with the "on state" of CDC25A. Therefore, the cross talk between the CDK4/6–Rb and ATM–CHK2–CDC25A axes is very important. Moreover, recently, Haricharan et al. demonstrated that for the efficacy of endocrine agents in luminal tumors, both ATM and CHK2 are required; inactivation of either of these negative cell cycle regulators prevents cell cycle arrest upon ER inhibition (Haricharan et al., 2017).

Loss of ER Expression

In luminal breast cancer, activation of ER is the major driver of CDK4/6. Selective ER-related endocrine therapy, such as ER downregulators (fulvestrant), ER modulators (tamoxifen), and aromatase inhibitors (AIs), have been combined with CDK4/6 inhibitors and broadly used in the treatment of advanced ER-positive breast cancer. The expression level of cyclin D1 could be upregulated by ER (Du et al., 2014). Resistance to CDK4/6 inhibitors may be related to the decrease in cyclin D1 due to the loss of ER (Gong et al., 2017b). For instance, resistance to the CDK4/6 inhibitor abemaciclib occurred in preclinical trials and was associated with the loss of cyclin D1 and ER/PR expression. In addition, studies showed that CDK6 overexpression diminished the responsiveness to ER antagonism and mediated the resistance to CDK4/6 inhibitors by decreasing the expression of ER and PR (Yang et al., 2017). Moreover, tumor biopsy specimens from patients associated with changes in ER/PR levels showed resistance to CDK4/6 inhibitors mediated by low ER/PR expression. Moreover, it also has been indicated that luminal tumors are resistant to endocrine therapy when they have an activating *ESR1* mutation; however, CDK4/6 inhibitors take effect regardless of *ESR1* mutation status (Fribbens et al., 2016).

Activation of PI3K–AKT–mTOR Signaling

The PI3K–AKT–mTOR signaling pathway is involved in tumor cell growth, survival, and metastasis. In luminal breast cancer, ER transcriptional activity could be enforced by the activation of PI3K–AKT–mTOR signaling, which drives endocrine therapy resistance (Miller et al., 2011). Furthermore, activation of the PI3K–AKT–mTOR pathway can also promote the stability of the CDK4/6 complex, thus reversing the effects of CDK4/6 inhibition (Miller et al., 2011). A recent study indicated that loss of PTEN expression could mediate CDK4/6 inhibitor resistance by increasing AKT activation and decreasing the expression of p27, which leads to the excessive activation of CDK4 and CDK2 (Costa et al., 2020). CDK4 in lysosome activates mTORC1 and is also associated with cancer progression (Martinez-Carreres et al., 2019). Moreover, it has been reported that CDK4/6 inhibitors preferred to activate PI3K–AKT–mTOR pathway than ER signaling (Takeshita et al., 2018), with the reactivation of E2F (Jansen et al., 2017). Therefore, the therapeutic trial, endocrine therapy backbone combined with PI3K and mTOR inhibition, and CDK4/6 inhibitors can be combined.

Strategies that inhibit PI3K and mTOR activities have been shown to restore sensitivity to endocrine therapy. Everolimus, a mTOR inhibitor, was the first drug developed to overcome endocrine therapy resistance in combination with AI (Pronzato, 2017). Some other mTORC1/2 inhibitors also restored the sensitivity of CDK4/6 inhibitors in resistant cells by suppressing Rb phosphorylation (Michaloglou et al., 2018). PI3K inhibitors, such as alpelisib, combined with fulvestrant prolonged PFS among patients with mutated PIK3CA in advanced luminal breast cancer who had previously received endocrine therapy (Andre et al., 2019). In addition, PI3K inhibitors have been implicated in the prevention of early CDK4/6 inhibitor adaptions by decreasing the expression of cyclin D1 (Herrera-Abreu et al., 2016). In the future, a combination of a PI3K–AKT–mTOR pathway inhibitor and a CDK4/6 inhibitor may be a valuable therapeutic strategy.

Upregulation of FGFR Pathway

The fibroblast growth factor receptor (FGFR) pathway is involved the proliferation and survival in luminal breast cancer (Sahores et al., 2018). Like other mitogenic pathways, FGFR is relevant in the crosslinking of cyclin D and CDK4/6. Of the five FGFRs, FGFR1 is associated with CDK4/6 inhibitor resistance. FGFR1 activates the PI3K–AKT–mTOR and RAS–MEK–ERK signaling pathways (Turner et al., 2010). In the clinic, FGFR1 overexpression mediated resistance to palbociclib or ribociclib when combined with endocrine therapy (fulvestrant) (Formisano et al., 2017). This could be reversed by the FGFR tyrosine kinase inhibitor (TKI) lucitanib (Formisano et al., 2019). FGFR2 amplification has also been reported in metastatic luminal breast cancer and the response to an mTOR inhibitor (Wein et al., 2017). In addition, FGF2 could also activate FGFR signaling and mediate endocrine therapy resistance in preclinical research (Turner et al., 2010). A previous study showed that the FGFR2 inhibitor formononetin had a strong inhibitory effect on angiogenesis and tumor growth (Wu et al., 2015). Therefore, targeting FGFR1/2 in luminal breast cancer may be a viable option combined with the inhibition of CDK4/6 to overcome CDK4/6 inhibitor resistance.

Alterations of Hippo Pathway

The Hippo pathway is closely related to the development and progression of breast cancer and has emerged as a linchpin in breast cancer therapy resistance (Gujral and Kirschner, 2017) (Shi et al., 2015). Hippo pathway effectors, such as YAP, TAZ, and TEAD, have been employed as drug targets to hit other signaling pathways (Dey et al., 2020). In ovarian cancer, YAP expression is associated with PI3K inhibitor resistance (Muranen et al., 2016). TEADs have also been shown to be a mediator of CDK6 induction (Xie et al., 2013). Importantly, alterations in the Hippo pathway are related to CDK4/6 inhibitor resistance. In the latter clinical case, loss of *FAT1* is associated with CDK4/6 inhibitor resistance caused by YAP/TAZ nuclear localization and CDK6 overexpression in ER-positive breast cancer (Li et al., 2018). Therefore, targeting the Hippo pathway offers a new therapeutic strategy against CDK4/6 inhibitor resistance.

Downstream Bypass Signaling Mechanisms

The molecular mechanisms responsible for resistance to CDK4/6 inhibitors are diverse and complicated, and the current knowledge is far from complete. Recently, several new "bypass" signaling pathway mechanisms on CDK4/6 inhibitor adaption have been discovered.

Activation of CDK2 Signaling

Cyclin E–CDK2 complexes can also inactivate Rb by phosphorylating Rb and releasing transcriptional factor E2F to initiate the cell cycle. However, as the "second wave" that phosphorylates Rb, the efficiency of this process is subsequent to CDK4/6 complexes. Excessive activation of the CDK2 pathway mediates resistance to CDK4/6 inhibitors because released E2F reverse targets cyclin E2, stabilizing the cyclin E2–CDK2 complexes and reducing CDK4/6 inhibition (Caldon et al., 2009). The abnormal expression of cyclin E1/2-CDK2 and persistent activation of E2F are associated with resistance to CDK4/6 inhibitors (Taylor-Harding et al., 2015). For instance, *CCNE1* gene amplification also induces resistance in the CDK4/6 single agent model; *CCNE2* gene amplification has been found in patients in whom palbociclib treatment failed (Hortobagyi et al., 2016). Moreover, in the clinic, lower *CCNE1* messenger RNA expression is often associated with improved palbociclib efficacy in ER-positive metastatic breast cancer (Turner et al., 2019). Activity of cyclin E1–CDK2 complexes could be suppressed by $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ (Martin et al., 2017); therefore, the development of CDK2 inhibitors have the potency and advantage as bypass signals to reduce CDK4/6 inhibitor resistance by the inhibition of cyclin E1/2–CDK2 (Caldon et al., 2012).

Autophagy

Autophagy is generally thought of as a cell survival mechanism. The activation of autophagy induces cell cycle arrest and cell senescence (Glick et al., 2010). Targeting autophagy is an available strategy for novel drug development and tumor treatment. Autophagy inhibition is relevant to the efficacy of anti–breast cancer drugs (Chittaranjan et al., 2014). An accumulation of evidence suggests that autophagy activation is involved in resistance to CDK4/6 inhibitors. Studies have shown that breast cancer cells activate autophagy in response to palbociclib, possibly through the inhibition of cyclin D1 expression, and the combination of autophagy and CDK4/6 inhibitors induces irreversible growth inhibition and senescence *in vitro* (Vijayaraghavan et al., 2017b). More work is being done to increase the efficacy of CDK4/6 inhibitors by inhibiting autophagy, which may help avert CDK4/6 inhibitor resistance.

Immune Evasion

The adaptive immune response plays a role in CDK4/6 inhibitor efficacy. CDK4/6 inhibitors promote tumor immunogenicity, and the effects of CDK4/6 inhibitors targeting both tumor T cells and regulatory T cells are associated with reduced activity of E2F transcription factors and DNA methyltransferase (Goel et al., 2017). In addition, CDK4/6 inhibitors enhance antitumor immunity by increasing T-cell activation and promoting T cells to kill tumor cells (Deng et al., 2018). Moreover, immunotherapeutic approaches combined with CDK4/6 inhibitors could achieve better therapeutic effects. CDK4/6 inhibitors increase the expression of PD-L1 (programmed cell death ligand 1), thus inducing the inflammatory microenvironment and improving tumor immunogenicity (Minton, 2017; Schaer et al., 2018). Therefore, CDK4/6 inhibitors combined with a PD-L1 immune checkpoint inhibitor can improve the effect of tumor immunotherapy. Currently, there are several ongoing clinical trials of immune checkpoint antibodies, including pembrolizumab and avelumab (Anurag et al., 2020). However, immune evasion or alterations in the immune microenvironment eventually leads to CDK4/6 inhibitor resistance (Goel et al., 2017; Teh and Aplin, 2019).

TABLE 2 | Possible strategies to overcome resistance to CDK4/6 inhibitors in ER-positive BC.

Resistance study	Potential mechanisms	Possible strategies	References
Cell cycle genes	Rb, cyclin D1, cyclin E	Intact Rb, *CCNE1* amplification	Turner et al. (2019)
	CDK4, CDK6	Knockdown of CDK4 and CDK6	Yang et al. (2017)
	p16, p21, p27	Intact Rb and knockdown of *p16*	Dean et al. (2012), Elvin et al. (2017)
Crosstalk pathways	ATM-CHK2	ATM inhibitor Ku60019	Haricharan et al. (2017), Anurag et al. (2018), Lang et al. (2018)
	PI3K/AKT/mTOR	PI3K-AKT-mTOR inhibitors	Costa et al. (2020)
	ER	Selective ER-related endocrine therapy	Fribbens et al. (2016)
	FGFR	FGFR2 inhibitor formononetin	Wu et al. (2015)
	Hippo	*FAT1*, verteporfin, CA3, VGLL4 peptide	Liu-Chittenden et al. (2012), Li et al. (2018), Song et al. (2018), Smith et al. (2019)
	CDK2	Flavopiridol, AT7519, dinaciclib	Tan et al. (2004), Squires et al. (2009), Parry et al. (2010)
	Autophagy	NAPI, ATG7, chloroquine	Liang et al. (2016), Gong et al. (2017a), Cui et al. (2018)
Combination therapy	Endocrine therapy	Fulvestrant, tamoxifen and AI	Turner et al. (2017)
	PI3K/mTOR inhibitor	Alpelisib, everolimus	Pronzato (2017), Andre et al. (2019)
	Immune checkpoint inhibitor	Pembrolizumab, atezolizumab, nivolumab	Kok et al. (2018), Schmid et al. (2019), Schneeweiss et al. (2019)
	Epigenetic inhibitor	Romidepsin, vorinostat, tucidinostat	Robertson et al. (2013), Bian et al. (2018), Jiang et al. (2019b)

NAPI, nanoparticle autophagy inhibitors.

In terms of mechanism, immune evasion may be associated with the abnormal expression of immune-related regulators, such as IFN-α and IFN-β, and change in tumor microenvironment of CDK4/6 inhibitor–resistant breast tumors (Vijayaraghavan et al., 2017a). Future investigations using tumor-infiltrating lymphocyte analyses are needed to better understand CDK4/6 inhibitor resistance mechanisms of immune evasion.

Epigenetic Alterations

Histone deacetylases (HDACs) can increase CDK4/6 inhibition efficacy and mediate cell cycle arrest by upregulating p21 expression in CDK4/6 inhibitor resistant tumors (Lee et al., 2018). Even though the mechanism is not very clear, HDAC inhibition works synergistically with CDK4/6 inhibitors in luminal breast cancer. Cornell et al. demonstrated that miR-432-5p–mediated suppression of the TGF-β signaling pathway via SMAD4 knockdown and increased CDK6 expression, thus conferring transmissible and reversible CDK4/6 inhibitor adaptation (Cornell et al., 2019). In addition, a recent study showed that LncRNA TROJAN could mediate resistance to CDK4/6 inhibitors by increasing CDK2 activation in ER+ breast cancer (Jin et al., 2020). Analysis of patient plasma exosomes may identify emerging resistance mechanisms.

Strategies to Overcome CDK4/6 Inhibitor Resistance

In the clinic, treatment effectiveness is based on the improved survival of patients. Currently, endocrine targeted therapy and chemotherapy are common options for the treatment of luminal breast cancer. CDK4/6 inhibitors have been used in advanced ER-positive breast cancer patients with antimitosis, but they eventually develop resistance to the CDK4/6 inhibitors (Franco et al., 2014; Yoshida et al., 2016). In the past 5 years, endocrine therapy combined with PI3K and mTOR inhibitors and CDK4/6 inhibitors has gradually become a new therapeutic strategy. Several studies have confirmed that CDK4/6 inhibitors combined with PI3K inhibitors (Vora et al., 2014) or mTORC1/2 inhibitors could reverse resistance (Michaloglou et al., 2018). Furthermore, studies have shown that CDK4/6 inhibitors may increase tumor immunogenicity, which provides a rationale for combination regimens composed of CDK4/6 inhibitors and immunotherapies. Therefore, CDK4/6 inhibitors combined with other clinical therapies might be a cautious approach to overcome therapy resistance. We summarized possible strategies to overcome resistance to CDK4/6 inhibitors in **Table 2**.

Potential Biomarkers for Predicting CDK4/6 Inhibitor Resistance

Whether CDK4/6 inhibition is truly suitable for patients with advanced ER-positive breast cancer and whether resistance develops are being studied in a number of preclinical studies and models. Rb may be a biomarker. It has been demonstrated that fully functional Rb is required for the effective use of CDK4/6 inhibitors in the clinic (Karakas et al., 2016). However, not all Rb+/ER+ patients would benefit from CDK4/6 inhibitor therapy, even though the mutation of Rb is very rare (3.9%) in ER-positive breast cancer. The utility of Rb as biomarker combined with low-molecular-weight cyclin E1 (LMWE) is associated with CDK4/6 inhibitor sensitivity (Hunt et al., 2017). A cohort of 109 patients with Rb-/LMWE+ had shorter PFS when treated with palbociclib plus endocrine therapy (Vijayaraghavan et al., 2017b). Although cyclin D1 plays a vital role in CDK4/6 inhibition, unfortunately, *CCND1* amplification as single biomarker for CDK4/6 inhibitor sensitivity needs to be refined further. In the PALOMA-1 study, patients treated with palbociclib plus letrozole had no beneficial outcomes regardless of *CCND1* status (Finn et al., 2015). Moreover, CDK4 phosphorylation status shows the potential as a biomarker to predict the sensitivity to palbociclib but needs further clinical observation (Raspe et al., 2017).

CONCLUSION

The development of CDK4/6 inhibitors has been a significant advancement in luminal breast cancer therapy. In other breast cancer subtypes, such as triple negative breast cancer, clinical trials of CDK4/6 inhibitors in combination with anti-androgen inhibitors are still ongoing. However, resistance to CDK4/6 inhibitors in clinical treatment is an unavoidable problem. Although CDK4/6 inhibitor resistance has been well investigated and different mechanisms have been revealed, systematic and comprehensive clinical trials are required to develop new strategies to overcome CDK4/6 inhibitor resistance. Therefore, further efforts to investigate much more precise resistance mechanisms to CDK4/6 inhibitors or to develop more successful CDK inhibitors are needed in order to explore new therapeutic approaches to avert or overcome resistance.

AUTHOR CONTRIBUTIONS

ZL: conceptual design and article writing; WZ: work drawing; JZ: conceptual design; YZ: make keyword tables; QX: work drawing; SL: conceptual design and article writing; CC: conceptual design and article writing

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REFERENCES

Andre, F., Ciruelos, E., Rubovszky, G., Campone, M., Loibl, S., and Rugo, H. S. (2019). Alpelisib for PIK3CA-mutated, hormone receptor-positive advanced breast cancer. *N. Engl. J. Med.* 380(20), 1929–1940. doi:10.1056/NEJMoa1813904

Anurag, M., Punturi, N., Hoog, J., Bainbridge, M. N., Ellis, M. J., and Haricharan, S. (2018). Comprehensive profiling of DNA repair defects in breast cancer identifies a novel class of endocrine therapy resistance drivers. *Clin. Canc. Res.* 24 (19), 4887–4899. doi:10.1158/1078-0432.CCR-17-3702

Anurag, M., Haricharan, S., and Ellis, M. J. (2020). CDK4/6 inhibitor biomarker research: are we barking up the wrong tree? *Clin. Canc. Res.* 26 (1), 3–5. doi:10.1158/1078-0432.CCR-19-3119

Asghar, U., Witkiewicz, A. K., Turner, N. C., and Knudsen, E. S. (2015). The history and future of targeting cyclin-dependent kinases in cancer therapy. *Nat. Rev. Drug Discov.* 14 (2), 130–146. doi:10.1038/nrd4504

Bian, X., Liang, Z., Feng, A., Salgado, E., and Shim, H. (2018). HDAC inhibitor suppresses proliferation and invasion of breast cancer cells through regulation of miR-200c targeting CRKL. *Biochem. Pharmacol.* 147, 30–37. doi:10.1016/j.bcp.2017.11.008

Bollard, J., Miguela, V., Ruiz de Galarreta, M., Venkatesh, A., Bian, C. B., and Roberto, M. P. (2017). Palbociclib (PD-0332991), a selective CDK4/6 inhibitor, restricts tumour growth in preclinical models of hepatocellular carcinoma. *Gut* 66 (7), 1286–1296. doi:10.1136/gutjnl-2016-312268

Caldon, C. E., Sergio, C. M., Schutte, J., Boersma, M. N., Sutherland, R. L., and Carroll, J. S. (2009). Estrogen regulation of cyclin E2 requires cyclin D1 but not c-Myc. *Mol. Cell Biol.* 29 (17), 4623–4639. doi:10.1128/MCB.00269-09

Caldon, C. E., Sergio, C. M., Kang, J., Muthukaruppan, A., Boersma, M. N., and Stone, A. (2012). Cyclin E2 overexpression is associated with endocrine resistance but not insensitivity to CDK2 inhibition in human breast cancer cells. *Mol. Canc. Therapeut.* 11 (7), 1488–1499. doi:10.1158/1535-7163.MCT-11-0963

Chittaranjan, S., Bortnik, S., Dragowska, W. H., Xu, J., Abeysundara, N., and Leung, A. (2014). Autophagy inhibition augments the anticancer effects of epirubicin treatment in anthracycline-sensitive and -resistant triple-negative breast cancer. *Clin. Canc. Res.* 20 (12), 3159–3173. doi:10.1158/1078-0432.CCR-13-2060

Choi, Y. J., Li, X., Hydbring, P., Sanda, T., Stefano, J., and Christie, A. L. (2012). The requirement for cyclin D function in tumor maintenance. *Canc. Cell* 22 (4), 438–451. doi:10.1016/j.ccr.2012.09.015

Ciriello, G., Gatza, M. L., Beck, A. H., Wilkerson, M. D., Rhie, S. K., and Pastore, A. (2015). Comprehensive molecular portraits of invasive lobular breast cancer. *Cell* 163 (2), 506–519. doi:10.1016/j.cell.2015.09.033

Cornell, L., Wander, S. A., Visal, T., Wagle, N., and Shapiro, G. I. (2019). MicroRNA-mediated suppression of the TGF-beta pathway confers transmissible and reversible CDK4/6 inhibitor resistance. *Cell Rep.* 26 (10), 2667–2680.e7. doi:10.1016/j.celrep.2019.02.023

Costa, C., Wang, Y., Ly, A., Hosono, Y., Murchie, E., and Walmsley, C. S. (2020). PTEN loss mediates clinical cross-resistance to CDK4/6 and PI3Kalpha inhibitors in breast cancer. *Canc. Discov.* 10 (1), 72–85. doi:10.1158/2159-8290.CD-18-0830

Creighton, C. J. (2012). The molecular profile of luminal B breast cancer. *Biologics* 6, 289–297. doi:10.2147/BTT.S29923

Cristofanilli, M., Turner, N. C., Bondarenko, I., Ro, J., Im, S. A., and Masuda, N. (2016). Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. *Lancet Oncol.* 17 (4), 425–439. doi:10.1016/S1470-2045(15)00613-0

Cui, Z., Zhang, Y., Xia, K., Yan, Q., Kong, H., and Zhang, J. (2018). Nanodiamond autophagy inhibitor allosterically improves the arsenical-based therapy of solid tumors. *Nat. Commun.* 9 (1), 4347. doi:10.1038/s41467-018-06749-2

Dean, J. L., McClendon, A. K., Hickey, T. E., Butler, L. M., Tilley, W. D., and Witkiewicz, A. K. (2012). Therapeutic response to CDK4/6 inhibition in breast cancer defined by *ex vivo* analyses of human tumors. *Cell Cycle* 11 (14), 2756–2761. doi:10.4161/cc.21195

DeMichele, A., Clark, A. S., Tan, K. S., Heitjan, D. F., Gramlich, K., and Gallagher, M. (2015). CDK 4/6 inhibitor palbociclib (PD0332991) in Rb+ advanced breast cancer: phase II activity, safety, and predictive biomarker assessment. *Clin. Canc. Res.* 21 (5), 995–1001. doi:10.1158/1078-0432.CCR-14-2258

Deng, J., Wang, E. S., Jenkins, R. W., Li, S., Dries, R., and Yates, K. (2018). CDK4/6 inhibition augments antitumor immunity by enhancing T-cell activation. *Canc. Discov.* 8 (2), 216–233. doi:10.1158/2159-8290.Cd-17-0915

Dey, A., Varelas, X., and Guan, K. L. (2020). Targeting the Hippo pathway in cancer, fibrosis, wound healing and regenerative medicine. *Nat. Rev. Drug Discov.* 19 (7), 480–494. doi:10.1038/s41573-020-0070-z

Dickler, M. N., Tolaney, S. M., Rugo, H. S., Cortes, J., Dieras, V., and Patt, D. (2017). MONARCH 1, A phase II study of abemaciclib, a CDK4 and CDK6 inhibitor, as a single agent, in patients with refractory HR(+)/HER2(−) metastatic breast cancer. *Clin. Canc. Res.* 23 (17), 5218–5224. doi:10.1158/1078-0432.CCR-17-0754

Du, C., Li, Z., Wang, S., Zhou, Z., Wang, J., and Dong, J. (2014). Tongshu capsule down-regulates the expression of estrogen receptor alpha and suppresses human breast cancer cell proliferation. *PLoS One* 9 (8), e104261. doi:10.1371/journal.pone.0104261

Elvin, J. A., Gay, L. M., Ort, R., Shuluk, J., Long, J., and Shelley, L. (2017). Clinical benefit in response to palbociclib treatment in refractory uterine leiomyosarcomas with a common CDKN2A alteration. *Oncologist* 22 (4), 416–421. doi:10.1634/theoncologist.2016-0310

Filmus, J., Robles, A. I., Shi, W., Wong, M. J., Colombo, L. L., and Conti, C. J. (1994). Induction of cyclin D1 overexpression by activated ras. *Oncogene* 9 (12), 3627–3633.

Finn, R. S., Crown, J. P., Lang, I., Boer, K., Bondarenko, I. M., and Kulyk, S. O. (2015). The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. *Lancet Oncol.* 16 (1), 25–35. doi:10.1016/S1470-2045(14)71159-3

Finn, R. S., Martin, M., Rugo, H. S., Jones, S., Im, S. A., and Gelmon, K. (2016). Palbociclib and letrozole in advanced breast cancer. *N. Engl. J. Med.* 375 (20), 1925–1936. doi:10.1056/NEJMoa1607303

Formisano, L., Stauffer, K. M., Young, C. D., Bhola, N. E., Guerrero-Zotano, A. L., and Jansen, V. M. (2017). Association of FGFR1 with ERalpha maintains ligand-independent ER transcription and mediates resistance to estrogen deprivation in ER(+) breast cancer. *Clin. Canc. Res.* 23 (20), 6138–6150. doi:10.1158/1078-0432.CCR-17-1232

Formisano, L., Lu, Y., Servetto, A., Hanker, A. B., Jansen, V. M., and Bauer, J. A. (2019). Aberrant FGFR signaling mediates resistance to CDK4/6 inhibitors in ER+ breast cancer. *Nat. Commun.* 10 (1), 1373. doi:10.1038/s41467-019-09068-2

Franco, J., Witkiewicz, A. K., and Knudsen, E. S. (2014). CDK4/6 inhibitors have potent activity in combination with pathway selective therapeutic agents in models of pancreatic cancer. *Oncotarget* 5 (15), 6512–6525. doi:10.18632/oncotarget.2270

Fribbens, C., O'Leary, B., Kilburn, L., Hrebien, S., Garcia-Murillas, I., and Beaney, M. (2016). Plasma ESR1 mutations and the treatment of estrogen receptor-positive advanced breast cancer. *J. Clin. Oncol.* 34 (25), 2961–2968. doi:10.1200/JCO.2016.67.3061

Fry, D. W., Harvey, P. J., Keller, P. R., Elliott, W. L., Meade, M., and Trachet, E. (2004). Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. *Mol. Canc. Therapeut.* 3 (11), 1427–1438.

Glick, D., Barth, S., and Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *J. Pathol.* 221 (1), 3–12. doi:10.1002/path.2697

Goel, S., DeCristo, M. J., Watt, A. C., BrinJones, H., Sceneay, J., and Li, B. B. (2017). CDK4/6 inhibition triggers anti-tumour immunity. *Nature* 548 (7668), 471–475. doi:10.1038/nature23465

Goetz, M. P., Toi, M., Campone, M., Sohn, J., Paluch-Shimon, S., and Huober, J. (2017). Monarch 3: abemaciclib as initial therapy for advanced breast cancer. *J. Clin. Oncol.* 35 (32), 3638–3646. doi:10.1200/JCO.2017.75.6155

Goldhirsch, A., Wood, W. C., Coates, A. S., Gelber, R. D., Thurlimann, B., and Senn, H. J. (2011). Strategies for subtypes–Dealing with the diversity of breast cancer: highlights of the St. Gallen international expert consensus on the primary therapy of early breast cancer 2011. *Ann. Oncol.* 22 (8), 1736–1747. doi:10.1093/annonc/mdr304

Gong, C., Hu, C., Gu, F., Xia, Q., Yao, C., and Zhang, L. (2017a). Co-delivery of autophagy inhibitor ATG7 siRNA and docetaxel for breast cancer treatment. *J. Contr. Release* 266, 272–286. doi:10.1016/j.jconrel.2017.09.042

Gong, X., Litchfield, L. M., Webster, Y., Chio, L. C., Wong, S. S., and Stewart, T. R. (2017b). Genomic aberrations that activate D-type cyclins are associated with enhanced sensitivity to the CDK4 and CDK6 inhibitor abemaciclib. *Canc. Cell* 32 (6), 761–776.e6. doi:10.1016/j.ccell.2017.11.006

Gujral, T. S., and Kirschner, M. W. (2017). Hippo pathway mediates resistance to cytotoxic drugs. *Proc. Natl. Acad. Sci. U. S. A.* 114 (18), E3729–E3738. doi:10.1073/pnas.1703096114

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144 (5), 646–674. doi:10.1016/j.cell.2011.02.013

Haricharan, S., Punturi, N., Singh, P., Holloway, K. R., Anurag, M., and Schmelz, J. (2017). Loss of MutL disrupts CHK2-dependent cell-cycle control through CDK4/6 to promote intrinsic endocrine therapy resistance in primary breast cancer. *Canc. Discov.* 7 (10), 1168–1183. doi:10.1158/2159-8290.CD-16-1179

Herrera-Abreu, M. T., Palafox, M., Asghar, U., Rivas, M. A., Cutts, R. J., and Garcia-Murillas, I. (2016). Early adaptation and acquired resistance to CDK4/6 inhibition in estrogen receptor-positive breast cancer. *Canc. Res.* 76 (8), 2301–2313. doi:10.1158/0008-5472.CAN-15-0728

Hortobagyi, G. N., Stemmer, S. M., Burris, H. A., Yap, Y. S., Sonke, G. S., and Paluch-Shimon, S. (2016). Ribociclib as first-line therapy for HR-positive, advanced breast cancer. *N. Engl. J. Med.* 375 (18), 1738–1748. doi:10.1056/NEJMoa1609709

Hortobagyi, G. N., Stemmer, S. M., Burris, H. A., Yap, Y. S., Sonke, G. S., and Paluch-Shimon, S. (2018). Updated results from MONALEESA-2, a phase III trial of first-line ribociclib plus letrozole versus placebo plus letrozole in hormone receptor-positive, HER2-negative advanced breast cancer. *Ann. Oncol.* 29 (7), 1541–1547. doi:10.1093/annonc/mdy155

Hunt, K. K., Karakas, C., Ha, M. J., Biernacka, A., Yi, M., and Sahin, A. A. (2017). Cytoplasmic cyclin E predicts recurrence in patients with breast cancer. *Clin. Canc. Res.* 23 (12), 2991–3002. doi:10.1158/1078-0432.CCR-16-2217

Ignatiadis, M., Singhal, S. K., Desmedt, C., Haibe-Kains, B., Criscitiello, C., and Andre, F. (2012). Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. *J. Clin. Oncol.* 30 (16), 1996–2004. doi:10.1200/JCO.2011.39.5624

Ignatiadis, M., and Sotiriou, C. (2013). Luminal breast cancer: from biology to treatment. *Nat. Rev. Clin. Oncol.* 10 (9), 494–506. doi:10.1038/nrclinonc.2013.124

Infante, J. R., Cassier, P. A., Gerecitano, J. F., Witteveen, P. O., Chugh, R., and Ribrag, V. (2016). A phase I study of the cyclin-dependent kinase 4/6 inhibitor ribociclib (LEE011) in patients with advanced solid tumors and lymphomas. *Clin. Canc. Res.* 22 (23), 5696–5705. doi:10.1158/1078-0432.CCR-16-1248

Jansen, V. M., Bhola, N. E., Bauer, J. A., Formisano, L., Lee, K. M., and Hutchinson, K. E. (2017). Kinome-wide RNA interference screen reveals a role for PDK1 in acquired resistance to CDK4/6 inhibition in ER-positive breast cancer. *Canc. Res.* 77 (9), 2488–2499. doi:10.1158/0008-5472.CAN-16-2653

Jiang, Z., Hu, X., Zhang, Q., Sun, T., Yin, Y., and Li, H. (2019a). MONARCHplus: a phase III trial of abemaciclib plus nonsteroidal aromatase inhibitor (NSAI) or fulvestrant (F) for women with HR+/HER2− advanced breast cancer (ABC). *Ann. Oncol.* 30, v863. doi:10.1093/annonc/mdz394.014

Jiang, Z., Li, W., Hu, X., Zhang, Q., Sun, T., and Cui, S. (2019b). Tucidinostat plus exemestane for postmenopausal patients with advanced, hormone receptor-positive breast cancer (ACE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol.* 20 (6), 806–815. doi:10.1016/S1470-2045(19)30164-0.

Jin, X., Ge, L. P., Li, D. Q., Shao, Z. M., Di, G. H., and Xu, X. E. (2020). LncRNA TROJAN promotes proliferation and resistance to CDK4/6 inhibitor via CDK2 transcriptional activation in ER+ breast cancer. *Mol. Canc.* 19 (1), 87. doi:10.1186/s12943-020-01210-9

Kalra, S., Joshi, G., Munshi, A., and Kumar, R. (2017). Structural insights of cyclin dependent kinases: implications in design of selective inhibitors. *Eur. J. Med. Chem.* 142, 424–458. doi:10.1016/j.ejmech.2017.08.071

Karakas, C., Biernacka, A., Bui, T., Sahin, A. A., Yi, M., and Akli, S. (2016). Cytoplasmic cyclin E and phospho-cyclin-dependent kinase 2 are biomarkers of aggressive breast cancer. *Am. J. Pathol.* 186 (7), 1900–1912. doi:10.1016/j.ajpath.2016.02.024

Kok, M., Voorwerk, L., Horlings, H., Sikorska, K., Vijver, K. v. d., and Slagter, M. (2018). Adaptive phase II randomized trial of nivolumab after induction treatment in triple negative breast cancer (TONIC trial): final response data stage I and first translational data. *J. Clin. Oncol.* 36 (Suppl. 15), 1012. doi:10.1200/JCO.2018.36.15_suppl.1012

Konecny, G. E., Winterhoff, B., Kolarova, T., Qi, J., Manivong, K., and Dering, J. (2011). Expression of p16 and retinoblastoma determines response to CDK4/6 inhibition in ovarian cancer. *Clin. Canc. Res.* 17 (6), 1591–1602. doi:10.1158/1078-0432.CCR-10-2307

Lang, L., Hou, Y., Chen, Y., Tu, G., Tao, J., and Yang, D. (2018). ATM-mediated phosphorylation of cortactin involved in actin polymerization promotes breast cancer cells migration and invasion. *Cell. Physiol. Biochem.* 51 (6), 2972–2988. doi:10.1159/000496048

Lee, J., Lim, B., Pearson, T., Tripathy, D., Ordentlich, P., and Ueno, N. (2018). Abstract P5-21-15: the synergistic antitumor activity of entinostat (MS-275) in combination with palbociclib (PD 0332991) in estrogen receptor-positive and triple-negative breast cancer. *Canc. Res.* 78 (Suppl. 4), 893–905.e8. doi:10.1158/1538-7445.Sabcs17-p5-21-15

Li, Z., Razavi, P., Li, Q., Toy, W., Liu, B., and Ping, C. (2018). Loss of the FAT1 tumor suppressor promotes resistance to CDK4/6 inhibitors via the Hippo pathway. *Canc. Cell* 34 (6), 893–905.e8. doi:10.1016/j.ccell.2018.11.006

Liang, D. H., Choi, D. S., Ensor, J. E., Kaipparettu, B. A., Bass, B. L., and Chang, J. C. (2016). The autophagy inhibitor chloroquine targets cancer stem cells in triple negative breast cancer by inducing mitochondrial damage and impairing DNA break repair. *Canc. Lett.* 376 (2), 249–258. doi:10.1016/j.canlet.2016.04.002

Liu-Chittenden, Y., Huang, B., Shim, J. S., Chen, Q., Lee, S. J., and Anders, R. A. (2012). Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. *Genes Dev.* 26 (12), 1300–1305. doi:10.1101/gad.192856.112

Malorni, L., Piazza, S., Ciani, Y., Guarducci, C., Bonechi, M., and Biagioni, C. (2016). A gene expression signature of retinoblastoma loss-of-function is a predictive biomarker of resistance to palbociclib in breast cancer cell lines and is prognostic in patients with ER positive early breast cancer. *Oncotarget* 7 (42), 68012–68022. doi:10.18632/oncotarget.12010

Malvezzi, M., Bertuccio, P., Levi, F., La Vecchia, C., and Negri, E. (2013). European cancer mortality predictions for the year 2013. *Ann. Oncol.* 24 (3), 792–800. doi:10.1093/annonc/mdt010

Martin, L.-A., Pancholi, S., Ribas, R., Gao, Q., Simigdala, N., and Nikitorowicz-Buniak, J. (2017). Abstract P3-03-09: resistance to palbociclib depends on multiple targetable mechanisms highlighting the potential of drug holidays and drug switching to improve therapeutic outcome. *Canc. Res.* 77 (Suppl. 4), P3-03-09. doi:10.1158/1538-7445.Sabcs16-p3-03-09

Martinez-Carreres, L., Puyal, J., Leal-Esteban, L. C., Orpinell, M., Castillo-Armengol, J., and Giralt, A. (2019). CDK4 regulates lysosomal function and mTORC1 activation to promote cancer cell survival. *Canc. Res.* 79 (20), 5245–5259. doi:10.1158/0008-5472.CAN-19-0708

Medema, R. H., Herrera, R. E., Lam, F., and Weinberg, R. A. (1995). Growth suppression by p16ink4 requires functional retinoblastoma protein. *Proc. Natl. Acad. Sci. U.S.A.* 92 (14), 6289–6293. doi:10.1073/pnas.92.14.6289

Michaloglou, C., Crafter, C., Siersbaek, R., Delpuech, O., Curwen, J. O., and Carnevalli, L. S. (2018). Combined inhibition of mTOR and CDK4/6 is required for optimal blockade of E2F function and long-term growth inhibition in estrogen receptor-positive breast cancer. *Mol. Canc. Therapeut.* 17 (5), 908–920. doi:10.1158/1535-7163.MCT-17-0537

Michaud, K., Solomon, D. A., Oermann, E., Kim, J. S., Zhong, W. Z., and Prados, M. D. (2010). Pharmacologic inhibition of cyclin-dependent kinases 4 and 6 arrests the growth of glioblastoma multiforme intracranial xenografts. *Cancer Res.* 70 (8), 3228–3238. doi:10.1158/0008-5472.CAN-09-4559

Miller, T. W., Rexer, B. N., Garrett, J. T., and Arteaga, C. L. (2011). Mutations in the phosphatidylinositol 3-kinase pathway: role in tumor progression and therapeutic implications in breast cancer. *Breast Cancer Res.* 13 (6), 224. doi:10.1186/bcr3039

Minton, K. (2017). Tumour immunology: cell cycle inhibitors boost tumour immunogenicity. *Nat. Rev. Immunol.* 17 (9), 529. doi:10.1038/nri.2017.104

Muranen, T., Selfors, L. M., Hwang, J., Gallegos, L. L., Coloff, J. L., and Thoreen, C. C. (2016). ERK and p38 MAPK activities determine sensitivity to PI3K/mTOR inhibition via regulation of MYC and YAP. *Canc. Res.* 76 (24), 7168–7180. doi:10.1158/0008-5472.CAN-16-0155

Musgrove, E. A., Caldon, C. E., Barraclough, J., Stone, A., and Sutherland, R. L. (2011). Cyclin D as a therapeutic target in cancer. *Nat. Rev. Canc.* 11 (8), 558–572. doi:10.1038/nrc3090

Pandey, K., An, H. J., Kim, S. K., Lee, S. A., Kim, S., and Lim, S. M. (2019). Molecular mechanisms of resistance to CDK4/6 inhibitors in breast cancer: a review. *Int. J. Canc.* 145 (5), 1179–1188. doi:10.1002/ijc.32020

Parry, D., Guzi, T., Shanahan, F., Davis, N., Prabhavalkar, D., and Wiswell, D. (2010). Dinaciclib (SCH 727965), a novel and potent cyclin-dependent kinase inhibitor. *Mol. Canc. Therapeut.* 9 (8), 2344–2353. doi:10.1158/1535-7163.MCT-10-0324

Patnaik, A., Rosen, L. S., Tolaney, S. M., Tolcher, A. W., Goldman, J. W., and Gandhi, L. (2016). Efficacy and safety of abemaciclib, an inhibitor of CDK4 and CDK6, for patients with breast cancer, non-small cell lung cancer, and other solid tumors. *Canc. Discov.* 6 (7), 740–753. doi:10.1158/2159-8290.CD-16-0095

Portman, N., Alexandrou, S., Carson, E., Wang, S., Lim, E., and Caldon, C. E. (2019). Overcoming CDK4/6 inhibitor resistance in ER-positive breast cancer. *Endocr. Relat. Canc.* 26 (1), R15–R30. doi:10.1530/ERC-18-0317.

Pronzato, P. (2017). Role of everolimus in the treatment of metastatic HER2-negative/HR-positive breast cancer. *Future Oncol.* 13 (16), 1371–1384. doi:10.2217/fon-2017-0098

Raspe, E., Coulonval, K., Pita, J. M., Paternot, S., Rothe, F., and Twyffels, L. (2017). CDK4 phosphorylation status and a linked gene expression profile predict sensitivity to palbociclib. *EMBO Mol. Med.* 9 (8), 1052–1066. doi:10.15252/emmm.201607084

Ribnikar, D., Volovat, S. R., and Cardoso, F. (2019). Targeting CDK4/6 pathways and beyond in breast cancer. *Breast* 43, 8–17. doi:10.1016/j.breast.2018.10.001

Robertson, F. M., Chu, K., Boley, K. M., Ye, Z., Liu, H., and Wright, M. C. (2013). The class I HDAC inhibitor Romidepsin targets inflammatory breast cancer tumor emboli and synergizes with paclitaxel to inhibit metastasis. *J. Exp. Therapeut. Oncol.* 10 (3), 219–233.

Rouzier, R., Perou, C. M., Symmans, W. F., Ibrahim, N., Cristofanilli, M., and Anderson, K. (2005). Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin. Canc. Res.* 11 (16), 5678–5685. doi:10.1158/1078-0432.CCR-04-2421

Sahores, A., May, M., Sequeira, G. R., Fuentes, C., Jacobsen, B., and Lanari, C. (2018). Targeting FGFR with BGJ398 in breast cancer: effect on tumor growth and metastasis. *Curr. Cancer Drug Targets* 18 (10), 979–987. doi:10.2174/1568009618666171214114706

Schaer, D. A., Beckmann, R. P., Dempsey, J. A., Huber, L., Forest, A., and Amaladas, N. (2018). The CDK4/6 inhibitor abemaciclib induces a T cell inflamed tumor microenvironment and enhances the efficacy of PD-L1 checkpoint blockade. *Cell Rep.* 22 (11), 2978–2994. doi:10.1016/j.celrep.2018.02.053

Shapiro, G. I. (2006). Cyclin-dependent kinase pathways as targets for cancer treatment. *J. Clin. Oncol.* 24 (11), 1770–1783. doi:10.1200/JCO.2005.03.7689

Schmid, P., Cortés, J., Dent, R., Pusztai, L., McArthur, H. L., and Kuemmel, S. (2019). LBA8_PR–KEYNOTE-522: phase III study of pembrolizumab (pembro) + chemotherapy (chemo) vs placebo (pbo) + chemo as neoadjuvant treatment, followed by pembro vs pbo as adjuvant treatment for early triple-negative breast cancer (TNBC). *Ann. Oncol.* 30, v851–v934. /doi:10.1093/annonc/mdz394.003

Schneeweiss, A., Rugo, H. S., Winer, E. P., Barrios, C. H., Iwata, H., and Dieras, V. (2019). IMpassion130: expanded safety analysis from a P3 study of atezolizumab (A) + nab-paclitaxel (nP) in patients (pts) with treatment (tx)-naïve, locally advanced or metastatic triple-negative breast cancer (mTNBC). *J. Clin. Oncol.* 37 (Suppl. 15), 1068. doi:10.1200/JCO.2019.37.15_suppl.1068

Serrano, M., Hannon, G. J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 366 (6456), 704–707. doi:10.1038/366704a0

Shi, P., Feng, J., and Chen, C. (2015). Hippo pathway in mammary gland development and breast cancer. *Acta Biochim. Biophys. Sin.* 47 (1), 53–59. doi:10.1093/abbs/gmu114

Slamon, D. J., Neven, P., Chia, S., Fasching, P. A., De Laurentiis, M., and Im, S. A. (2018). Phase III randomized study of ribociclib and fulvestrant in hormone receptor-positive, human epidermal growth factor receptor 2-negative advanced breast cancer: MONALEESA-3. *J. Clin. Oncol.* 36 (24), 2465–2472. doi:10.1200/JCO.2018.78.9909

Slamon, D. J., Neven, P., Chia, S., Fasching, P. A., De Laurentiis, M., and Im, S.-A. (2019). Overall survival with ribociclib plus fulvestrant in advanced breast cancer. *N. Engl. J. Med.* 382 (6), 514–524. doi:10.1056/NEJMoa1911149

Sledge, G. W., Jr., Toi, M., Neven, P., Sohn, J., Inoue, K., and Pivot, X. (2017). MONARCH 2: abemaciclib in combination with fulvestrant in women with HR+/HER2− advanced breast cancer who had progressed while receiving endocrine therapy. *J. Clin. Oncol.* 35 (25), 2875–2884. doi:10.1200/JCO.2017.73.7585

Smith, S. A., Sessions, R. B., Shoemark, D. K., Williams, C., Ebrahimighaei, R., and McNeill, M. C. (2019). Antiproliferative and antimigratory effects of a novel YAP–TEAD interaction inhibitor identified using in silico molecular docking. *J. Med. Chem.* 62 (3), 1291–1305. doi:10.1021/acs.jmedchem.8b01402

Song, S., Xie, M., Scott, A. W., Jin, J., Ma, L., and Dong, X. (2018). A novel YAP1 inhibitor targets CSC-enriched radiation-resistant cells and exerts strong antitumor activity in esophageal adenocarcinoma. *Mol. Canc. Therapeut.* 17 (2), 443–454. doi:10.1158/1535-7163.MCT-17-0560

Sotiriou, C., and Pusztai, L. (2009). Gene-expression signatures in breast cancer. *N. Engl. J. Med.* 360 (8), 790–800. doi:10.1056/NEJMra0801289

Squires, M. S., Feltell, R. E., Wallis, N. G., Lewis, E. J., Smith, D. M., and Cross, D. M. (2009). Biological characterization of AT7519, a small-molecule inhibitor of cyclin-dependent kinases, in human tumor cell lines. *Mol. Canc. Therapeut.* 8 (2), 324–332. doi:10.1158/1535-7163.MCT-08-0890

Tadesse, S., Bantie, L., Tomusange, K., Yu, M., Islam, S., and Bykovska, N. (2018). Discovery and pharmacological characterization of a novel series of highly selective inhibitors of cyclin-dependent kinases 4 and 6 as anticancer agents. *Br. J. Pharmacol.* 175 (12), 2399–2413. doi:10.1111/bph.13974

Takeshita, T., Yamamoto, Y., Yamamoto-Ibusuki, M., Tomiguchi, M., Sueta, A., and Murakami, K. (2018). Clinical significance of plasma cell-free DNA mutations in PIK3CA, AKT1, and ESR1 gene according to treatment lines in ER-positive breast cancer. *Mol. Canc.* 17 (1), 67. doi:10.1186/s12943-018-0808-y

Tan, A. R., Yang, X., Berman, A., Zhai, S., Sparreboom, A., and Parr, A. L. (2004). Phase I trial of the cyclin-dependent kinase inhibitor flavopiridol in combination with docetaxel in patients with metastatic breast cancer. *Clin. Canc. Res.* 10 (15), 5038–5047. doi:10.1158/1078-0432.CCR-04-0025

Taylor-Harding, B., Aspuria, P. J., Agadjanian, H., Cheon, D. J., Mizuno, T., and Greenberg, D. (2015). Cyclin E1 and RTK/RAS signaling drive CDK inhibitor resistance via activation of E2F and ETS. *Oncotarget* 6 (2), 696–714. doi:10.18632/oncotarget.2673

Teh, J. L. F., and Aplin, A. E. (2019). Arrested developments: CDK4/6 inhibitor resistance and alterations in the tumor immune microenvironment. *Clin. Canc. Res.* 25 (3), 921–927. doi:10.1158/1078-0432.CCR-18-1967

Tolaney, S. M., Wardley, A. M., Zambelli, S., Hilton, J., Troso-Sandoval, T., and Ricci, F. (2019). MonarcHER: a randomized phase II study of abemaciclib plus trastuzumab with or without fulvestrant versus trastuzumab plus standard-of-care chemotherapy in women with HR+, HER2+ advanced breast cancer (ABC). *Ann. Oncol.* 30, v861–v862. doi:10.1093/annonc/mdz394.012

Tripathy, D., Im, S. A., Colleoni, M., Franke, F., Bardia, A., and Harbeck, N. (2018). Ribociclib plus endocrine therapy for premenopausal women with hormone-receptor-positive, advanced breast cancer (MONALEESA-7): a randomised phase 3 trial. *Lancet Oncol.* 19 (7), 904–915. doi:10.1016/S1470-2045(18)30292-4

Turner, N., Pearson, A., Sharpe, R., Lambros, M., Geyer, F., and Lopez-Garcia, M. A. (2010). FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. *Canc. Res.* 70 (5), 2085–2094. doi:10.1158/0008-5472.CAN-09-3746

Turner, N. C., Neven, P., Loibl, S., and Andre, F. (2017). Advances in the treatment of advanced oestrogen-receptor-positive breast cancer. *Lancet* 389 (10087), 2403–2414. doi:10.1016/S0140-6736(16)32419-9

Turner, N. C., Liu, Y., Zhu, Z., Loi, S., Colleoni, M., and Loibl, S. (2019). Cyclin E1 expression and palbociclib efficacy in previously treated hormone receptor-positive metastatic breast cancer. *J. Clin. Oncol.* 37 (14), 1169–1178. doi:10.1200/JCO.18.00925

Vijayaraghavan, S., Doostan, I., Carey, J. P. W., and Keyomarsi, K. (2017a). Abstract 2060: characterizing acquired resistance to palbociclib in breast cancer. *Canc. Res.* 77 (Suppl. 13), 2060. doi:10.1158/1538-7445.Am2017-2060

Vijayaraghavan, S., Karakas, C., Doostan, I., Chen, X., Bui, T., and Yi, M. (2017b). CDK4/6 and autophagy inhibitors synergistically induce senescence in Rb positive cytoplasmic cyclin E negative cancers. *Nat. Commun.* 8, 15916. doi:10.1038/ncomms15916

Vora, S. R., Juric, D., Kim, N., Mino-Kenudson, M., Huynh, T., and Costa, C. (2014). CDK 4/6 inhibitors sensitize PIK3CA mutant breast cancer to PI3K inhibitors. *Canc. Cell* 26 (1), 136–149. doi:10.1016/j.ccr.2014.05.020

Wein, L., Savas, P., Van Geelen, C., Caramia, F., Moodie, K., and Joshi, S. (2017). FGFR2 amplification in metastatic hormone-positive breast cancer and response to an mTOR inhibitor. *Ann. Oncol.* 28 (8), 2025–2027. doi:10.1093/annonc/mdx194

Witkiewicz, A. K., Knudsen, K. E., Dicker, A. P., and Knudsen, E. S. (2011). The meaning of p16(ink4a) expression in tumors: functional significance, clinical associations and future developments. *Cell Cycle* 10 (15), 2497–2503. doi:10.4161/cc.10.15.16776

Wu, X. Y., Xu, H., Wu, Z. F., Chen, C., Liu, J. Y., and Wu, G. N. (2015). Formononetin, a novel FGFR2 inhibitor, potently inhibits angiogenesis and tumor growth in preclinical models. *Oncotarget* 6 (42), 44563–44578. doi:10.18632/oncotarget.6310

Xie, Q., Chen, J., Feng, H., Peng, S., Adams, U., and Bai, Y. (2013). YAP/TEAD-mediated transcription controls cellular senescence. *Canc. Res.* 73 (12), 3615–3624. doi:10.1158/0008-5472.CAN-12-3793

Yang, C., Li, Z., Bhatt, T., Dickler, M., Giri, D., and Scaltriti, M. (2017). Acquired CDK6 amplification promotes breast cancer resistance to CDK4/6 inhibitors and loss of ER signaling and dependence. *Oncogene* 36 (16), 2255–2264. doi:10.1038/onc.2016.379

Yin, L., Li, H., Liu, W., Yao, Z., Cheng, Z., and Zhang, H. (2018). A highly potent CDK4/6 inhibitor was rationally designed to overcome blood brain barrier in gliobastoma therapy. *Eur. J. Med. Chem.* 144, 1–28. doi:10.1016/j.ejmech.2017.12.003

Yoshida, A., Lee, E. K., and Diehl, J. A. (2016). Induction of therapeutic senescence in vemurafenib-resistant melanoma by extended inhibition of CDK4/6. *Cancer Res.* 76 (10), 2990–3002. doi:10.1158/0008-5472.CAN-15-2931

Yu, Q., Sicinska, E., Geng, Y., Ahnstrom, M., Zagozdzon, A., and Kong, Y. (2006). Requirement for CDK4 kinase function in breast cancer. *Cancer Cell* 9 (1), 23–32. doi:10.1016/j.ccr.2005.12.012

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REVIEW
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Progress in the Understanding of the Mechanism of Tamoxifen Resistance in Breast Cancer

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Tamoxifen is a drug commonly used in the treatment of breast cancer, especially for postmenopausal patients. However, its efficacy is limited by the development of drug resistance. Downregulation of estrogen receptor alpha (ERα) is an important mechanism of tamoxifen resistance. In recent years, with progress in research into the protective autophagy of drug-resistant cells and cell cycle regulators, major breakthroughs have been made in research on tamoxifen resistance. For a better understanding of the mechanism of tamoxifen resistance, protective autophagy, cell cycle regulators, and some transcription factors and enzymes regulating the expression of the estrogen receptor are summarized in this review. In addition, recent progress in reducing resistance to tamoxifen is reviewed. Finally, we discuss the possible research directions into tamoxifen resistance in the future to provide assistance for the clinical treatment of breast cancer.

Keywords: tamoxifen, breast cancer, cell cycle regulators, autophagy, resistance

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INTRODUCTION

Breast cancer is the most common cancer in women (Bray et al., 2018), and endocrine therapy plays an important role in breast cancer treatment (Rugo et al., 2016). More than 60% of breast cancers are estrogen-receptor (ER) positive (Lopez-Tarruella and Schiff, 2007; Vargo-Gogola and Rosen, 2007). Tamoxifen is an antagonist of ERα66, and it is commonly used in the treatment of ER-positive breast cancers (Binkhorst et al., 2012); however, the efficacy is not satisfactory because of the development of tamoxifen resistance. RTKs (receptor tyrosine kinases) and the activation of the PI3K-PTEN/AKT/mTOR pathway caused by the overexpression of RTKs are thought to be closely related to resistance to tamoxifen (Hosford and Miller, 2014; Yin et al., 2014).

On the other hand, ERα36, a 36 kDa truncated isoform of ERα66 located on the cytoplasmic membrane of breast cancer (Lv et al., 2015; Omarjee et al., 2017), has been reported to be related to the drug resistance and metastasis of cancer cells (Zhang and Wang, 2013; Yin et al., 2014; Omarjee et al., 2017). Tamoxifen can activate ERα36, which in turn activates MAPK, AKT, and other signaling pathways, leading to tamoxifen resistance (Tong et al., 2010).

In recent years, a large body of evidence has shown that protective autophagy, cell cycle regulators, and some transcription factors play a key role in tamoxifen resistance, such as KLF4 regulating drug resistance by regulating MAPK and the discovery of LEM4 (Gao et al., 2018; Jia et al., 2018). Scientists have proposed many methods to reduce drug resistance through these mechanisms and have made great progress.

In this review, the development of tamoxifen resistance in breast cancer is discussed, with special emphasis on the effects of some newly discovered enzymes and transcription factors on tamoxifen resistance, the protective autophagy of cells, and the latest progress in cell cycle regulators.

The Role of Receptor Tyrosine Kinases (RTKs) in Tamoxifen Resistance

RPTKs are a class of enzyme-linked receptors that have been found to come in many kinds, including epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, macrophage colony stimulating factor (M-CSF), insulin and insulin-like growth factor-1 (IGF-1) receptor, vascular endothelial growth factor (VEGF) receptor, and hepatocyte growth factor (HGF) receptor. The PI3K/AKT/mTOR signaling pathway is one of the important mechanisms of tamoxifen resistance, and HER2 activates PI3K as a member of the EGFR family (Mansouri et al., 2018a). It has been proven that high expression of *p*-AKT is associated with a worse prognosis, and inhibiting the expression of AKT is beneficial for sensitizing drug-resistant cells (Block et al., 2012; Karlsson et al., 2019). In addition, activation of the PI3K/AKT pathway is not just associated with tamoxifen resistance. Recent studies have shown that activation of the PI3K/AKT pathway can cause tamoxifen-resistant (TAM-R) cells to develop drug resistance to DNA-damaging chemotherapy by upregulating BARD1 and BRCA1 (Zhu et al., 2018), which makes the PI3K/AKT pathway particularly important in the treatment of breast cancer.

The mechanism of activation of the PI3K/AKT/mTOR pathway has also been studied by many scientists. CC chemokine ligand 2 (CCL2), which is secreted by tumor-associated macrophages (TAMs), has been found to be related to activation of the PI3K/AKT/mTOR pathway. However, NF-κB promotes the secretion of CCL2 (Li et al., 2020a). Inhibition of the PI3K/AKT pathway may be beneficial to improve the efficacy of chemotherapy and endocrine therapy for breast cancer patients. Many drugs targeting PI3K, mTOR, or AKT to overcome tamoxifen resistance have been put into use. However, due to the complexity of the PI3K/AKT/mTOR pathway, inhibiting the pathway at any level will activate compensatory mechanisms, which limits the efficacy of inhibitors (Choi et al., 2016; Lui et al., 2016). We need to study the cross-talk between these pathways in future research.

The combined use of several inhibitors may be an important way to improve tamoxifen resistance in the future. Both VEGF and HER2 are members of the RTK family. Studies have shown that the expression of VEGF in drug-resistant cells is upregulated. VEGF contributes to angiogenesis and promotes tumor growth, which is not conducive to a good prognosis of breast cancer patients (Oh et al., 2010). The MAPK/ERK pathway has been proven to contribute to tamoxifen resistance (Heckler et al., 2014; Peng et al., 2017; Yin et al., 2017), whereas VEGF overexpression in drug-resistant cells leads to increased activation of MAPK. Surprisingly, the use of VEGF inhibitors was not found to be helpful in overcoming tamoxifen resistance (Mansouri et al., 2018b), which may also be attributed to the complex network of drug resistance. There is still no evidence that VEGF is related to tamoxifen resistance.

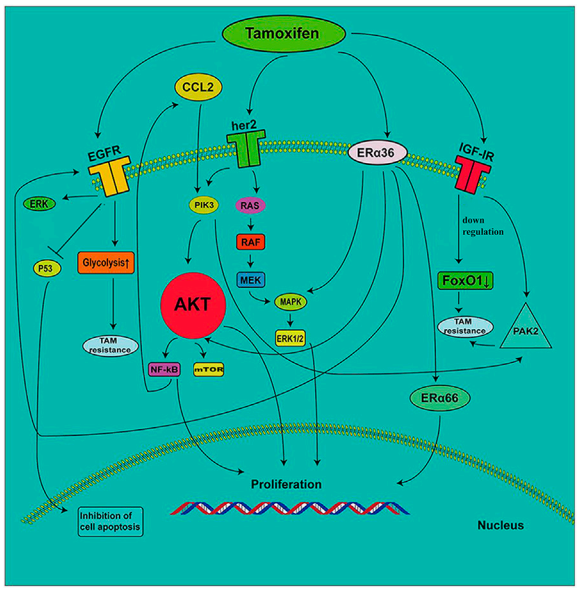

FIGURE 1 | The role of RTKs and ERα36 in the development of tamoxifen resistance. EGFR induces tamoxifen resistance by enhancing the glycolytic pathway. The increase in EGF signal transduction induces a decrease in P53 expression, which leads to the inhibition of cell proliferation. TAMs secrete CC-chemokine ligand 2 (CCL2), which activates the PI3K/AKT/mTOR pathway. NF-κB promotes the secretion of CCL2. ERα36 contributes to the upregulation of EGFR, which increases ERK phosphorylation. The decrease in IGF-1R expression leads to the inhibition of FoxO1 expression, which results in the development of tamoxifen resistance. IGF1R mediates the expression of PAK2 and leads to drug resistance.

EGFR is also thought to be related to tamoxifen resistance. Tamoxifen downregulates the expression of miR-186-3p, which leads to further upregulation of the expression of EREG, a target gene of miR-186-3p. EREG then activates EGFR even more, subsequently enhancing glycolysis and leading to tamoxifen resistance (He et al., 2019). It has been reported that the NOGO-B receptor is related to tamoxifen resistance. The NOGO-B receptor contributes to the transport of RAS, which enhances EGF signal transduction, resulting in a decrease in p53 expression and the development of drug resistance (Gao et al., 2018).

ERα36 has been reported to be associated with tamoxifen resistance (Yin et al., 2014), and ERα36 reduces the sensitivity of breast cancer cells to tamoxifen by upregulating EGFR. EGFR expression and the basal level of ERK phosphorylation are upregulated in TAM-R cells. The EGFR/ERK signaling pathway can be blocked by knocking out ERα36 (Li et al., 2020b). However, lapatinib cannot only inhibit the phosphorylation of EFGR and HER2, but also decreases the expression of ERα36 (Yin et al., 2014). Interestingly, studies have shown that cross-talk between HER2 and ERK is conducive to the development of drug resistance (Ito et al., 2012).

In addition to members of the RTK family, such as HER2, EGFR, and VEGF, some research has shown that IGFR is also

associated with tamoxifen resistance. Inhibition of IGF-1R reduces the sensitivity of cells to tamoxifen, which may be due to the inhibition of FoxO1 expression by the reduction of IGF-1R expression (Vaziri-Gohar et al., 2017). However, IGF1R signaling may be beneficial to the development of tamoxifen resistance in some aspects. P21-activated kinase 2 (PAK2) is a tamoxifen resistance inducer, while IGF1R can lead to the development of tamoxifen resistance by promoting the expression of PAK2 (Zhang et al., 2018).

In general, there is a complex network in the mechanisms of action of the RTK family, and ERα36 affects the sensitivity of breast cancer cells to tamoxifen. These signaling cascades are described in **Figure 1**. The development of inhibitors for corresponding targets based on these mechanisms is the focus of previous research. However, due to the compensatory mechanisms that appear when any specific target is inhibited, the clinical effect of improving drug resistance is not very significant. Therefore, studies on improving drug resistance by other mechanisms have emerged in recent years.

The Role of Enzymes and Transcription Factors in Tamoxifen Resistance

Based on the aforementioned mechanism, some enzymes and transcription factors also play a vital role in the complex network of ER-positive breast cancer resistance to tamoxifen. SOX9 is a transcription factor related to endocrine resistance (Jeselsohn et al., 2017; Xue et al., 2019). Histone deacetylase 5 (HDAC5), a member of the HDAC family whose main function is to remove acetyl groups, enables the deacetylation of SOX9 to facilitate its nuclear localization in TAM-R cells. Moreover, MYC plays an important role in the activation of HDAC5 transcription, and the C-MYC/HDAC5/SOX9 axis is related to tamoxifen resistance (Xue et al., 2019).

HDAC1, another member of the HDAC family, has also been reported to be associated with tamoxifen resistance. The expression of RBP2 is significantly higher in TAM-R cells than in cells sensitive to tamoxifen. The RBP2–ER–NRIP1–HDAC1 complex leads to IGF1R activation. The relationship between RBP and tamoxifen resistance is related to the PI3K/AKT pathway. RBP activates the PI3K/AKT pathway by enhancing the cross-talk between IGF1R and the HER2 receptor, which leads to drug resistance (Choi et al., 2018). Interestingly, it has also been reported that HDAC promotes the expression of ERα66 and AKT, and the use of HDAC inhibitors can inhibit the level of AKT by reducing the stability of its mRNA (Thomas et al., 2013).

Silent information regulator 2-related enzyme 1 (SIRT1) is a deacetylase dependent on nicotinamide adenine dinucleotide, which is highly expressed in a variety of tumors and has been proven to inhibit the growth of breast cancer cells (Liu et al., 2009; Kuo et al., 2013). The T-box protein Brachyury, a transcription factor, promotes the resistance of breast cancer cells to tamoxifen by inhibiting SIRT1 (Li et al., 2016). There are many different mechanisms for the effects of acetylases on tamoxifen resistance.

Estrogen regulates tumor growth by binding to ERα66 in the cytoplasm. Tamoxifen is antagonistic to ERα66. However, the use of tamoxifen has been confirmed to be involved in the upregulation of ERα36, SPhk1 (sphingosine kinase 1), and S1P (sphingosine-1-phosphate), which further activates downstream signaling pathways and causes drug resistance (Maczis et al., 2018), while the inhibition of ERα36 is beneficial to restore the sensitivity of breast cancer cells to tamoxifen.

Protein arginine N-methyltransferase 2 (PRMT2; HRMT1L1) is a member of the arginine methyltransferase family (Scott et al., 1998) that inhibits the resistance of breast cancer cells to tamoxifen by inhibiting the ERα36, PI3K, MAPK, and other signaling pathways (Shen et al., 2018).

It has been shown that the expression of hypoxia inducible factor HIF-1α contributes to the decrease in ERα, which is related to the sensitivity of endocrine therapy. HIF-1α reduces the sensitivity of breast cancer cells to tamoxifen. Interestingly, the expression of HIF-1α is related to the expression of EGFR (Jogi et al., 2019).

In contrast to HIF-1α, Spalt-like transcription factor 2 (SALL2), a transcription factor related to disease progression, enhances the sensitivity of breast cancer cells to tamoxifen, while ERα is downregulated after silencing SALL2 (Ye et al., 2019). This shows that tamoxifen is an effective endocrine therapy drug in ER-positive breast cancer patients. However, the expression of ERα is positively correlated with the sensitivity of tamoxifen therapy in ER-positive breast cancer patients. Numerous transcription factors regulate the sensitivity of breast cancer cells to tamoxifen by regulating ERα through various mechanisms. In addition, ERα also mediates the expression of glutathione S-transferase mu 3 (GSTM3) to resist cytotoxicity caused by drug therapy and to protect the drug-resistant cells.

The expression of GSTM3 was found to be higher in HER2-positive cancer cells (Lin et al., 2018). This indicates that there may be a relationship between GSTM3 and the RTK pathway, and the mechanism by which enzymes and transcription factors regulate tamoxifen resistance is also closely related to the RTK pathway. Li et al. (2018) found that the ER–c-Src–HER2 complex plays a vital role in tamoxifen resistance, while c-Cbl reverses tamoxifen resistance by inhibiting the formation of the ER–c-Src–HER2 complex. It seems that most enzymes are involved in drug resistance through the RTK pathway.

In addition, some enzymes can be used to predict the sensitivity of endocrine therapy in breast cancer. Shimoda et al. (2017) found that the expression of ASPH was upregulated in tamoxifen-resistant cells, and the upregulation depended on the PI3K and MAPK pathways. The cells with high expression of ASPH were more sensitive to tamoxifen than those with low expression of ASPH, and the results were statistically significant.

Aspartate-b-hydroxylase (ASPH) may also predict the sensitivity of breast cancer cells to tamoxifen (Shimoda et al., 2017). Gwak et al. (2017) also found that the expression of the transcription factor OCT 4 may be related to the poor efficacy of tamoxifen, and its expression level can be used to predict the sensitivity of breast cancer cells to tamoxifen. The mechanisms mentioned in this review related to tamoxifen resistance are summarized in **Table 1**.

TABLE 1 | Summary of mechanisms leading to tamoxifen resistance

Factors	Mechanism(pathway)	Expression in breast cancer	Ref
EREG	miR-186-3p/EREG/EGFR regulatory cascade	High	(He et al., 2019)
NgBR	Promote EGF signaling	High	(Gao et al., 2018)
ERα36	Promote EGFR/ERK signaling	High	(Li et al., 2020b)
	Activate HER2 expression and its cascade	High	(Mansouri et al., 2018b)
	Activate Sphkl/S1P axis	High	(Maczis et al., 2018)
IGF1R	Inhibit FoxOl expression	Low	(Vaziri-Gohar et al., 2017)
PAK2	PAK2 acts downstream of IGF1R signaling	High	(Zhang et al., 2018)
Brachyury	Downregulate SIRT1 expression	High	(Li et al., 2016)
RBP2	Activate ER-IGF1R-ErbB signaling cascade	High	(Choi et al., 2018)
Cyclin D1	Promote the progress of G1-S phase	High	(Viedma-Rodriguez et al., 2014)
LEM4	Promote the transcription of cyclin D1	High	(Gao et al., 2018)
SPY1	SPY1 binds to CDK, mediates the phosphorylation of ERK	High	(Ferraiuolo et al., 2017)
SOX9	c-MYC/HDAC5/SOX9 axis	High	(Xue et al., 2019)
SALL2	Activate AKT/mTOR signaling	Low	(Ye et al., 2019)
HIF-1α	Downregulate the expression of ERα	High	(Jogi et al., 2019)

The Discovery of LEM4 and the Association Between Cell Cycle Regulators and Resistance to Tamoxifen

As a competitive antagonist of estradiol, tamoxifen can bind to estrogen receptors in competition with estradiol and form a stable complex, which inhibits the transcription activity of the estrogen receptor and blocks breast cancer cells in G1 phase to inhibit tumor proliferation. However, tamoxifen has little effect on the cell cycle when cells are treated with tamoxifen alone (Cheng et al., 2017). Previous studies have shown that cyclin D1 and cyclin E are essential for the emergence of tamoxifen resistance in breast cancer cells. Cyclin D1 promotes the progression of the G1–S phase, and tamoxifen can reduce the expression of cyclin D1, which is highly expressed in drug-resistant cells (Viedma-Rodriguez et al., 2014). Based on these mechanisms, scientists have previously proposed many methods to overcome drug resistance, such as the cyclin-dependent kinase (CDK) 4/6 inhibitors palbociclib and ribociclib (Finn et al., 2015; Cristofanilli et al., 2016; Hortobagyi et al., 2016).

The latest research in the last 2 years found that LEM4 (LEM structural protein), which is highly expressed in breast cancer-resistant cells, promotes the transcription of cyclin D1 through ligand-independent activation of receptors. Furthermore, LEM4 interacts with CDK 4/6 and Rb to accelerate the G1–S transition (Gao et al., 2018). Therefore, LEM4 reduces the inhibitory effect of tamoxifen on the G1–S phase transition of breast cancer cells. On the other hand, the existence of LEM4 allows the estrogen receptor to undergo ligand-independent activation in the presence of tamoxifen. LEM4 is expected to be a biological index to predict tamoxifen resistance in ER-positive breast cancer, and targeting LEM4 may be a feasible research direction to overcome tamoxifen resistance in the future.

In addition, Yu et al. (2019) found that cell division cycle associated 8 (CDCA8) may be related to tamoxifen resistance. It is highly expressed in drug-resistant cells. After the CACA8 gene was knocked out, the number of drug-resistant cells in the G1 phase increased, and the drug resistance of the cells to tamoxifen decreased (Yu et al., 2019).

Ferraiuolo et al. (2017) discovered another cell cycle protein, Spy1, which mediates the phosphorylation of ERK under the condition of binding to CDK; the increase in its level is related to tamoxifen resistance.

With the progress of mechanistic research, many new treatments have emerged in recent years. Aspirin (ASA) is a kind of nonsteroidal anti-inflammatory drug that has been used in the treatment of many tumors, including rectal cancer, lung cancer, pancreatic cancer, and breast cancer (Jiang et al., 2020; Wang and Huang, 2020; Wu et al., 2020; Zhang et al., 2020), but whether it is beneficial to the survival of patients is still uncertain. However, the use of aspirin seems to be helpful in overcoming tamoxifen resistance. The expression of cyclin D1 was downregulated, and the number of cells arrested in the G0/G1 phase was increased when tamoxifen was used in combination with ASA. The combination of ASA and tamoxifen can overcome the drug resistance of ER-positive breast cancer cells to tamoxifen (Cheng et al., 2017).

Maqbool et al. (2020) synthesized a novel thiosemicarbazone, DpC. They found that the combination of DpC and tamoxifen effectively reduced cyclin D1, upregulated p27, and inhibited the proliferation of breast cancer cells, which may be helpful to overcome the drug resistance of tamoxifen.

The Latest Progress in the Relationship Between Autophagy and Resistance to Tamoxifen

Autophagy is the process by which cells engulf their excess proteins or organelles, transport them to lysosomes, and degrade their contents. Their main role is to deal with the stress-induced injury of cells (Antonioli et al., 2017). However, autophagy seems to have two opposing roles in tumor cells. On the one hand, tumor cells can undergo autophagic cell death through self-phagocytosis, after which the cytoskeleton is mostly preserved. On the other hand, autophagy can delay the apoptosis

of stressed and damaged cells, and protect their survival (Cook et al., 2011; Sun et al., 2015). Previous studies have shown that autophagy may have a strong relationship with tamoxifen resistance, and it may be an important mechanism of tamoxifen resistance (Gonzalez-Malerva et al., 2011; Nagelkerke et al., 2014), but the relationship between autophagy and tamoxifen resistance is still in the exploratory stage, and the specific mechanism is still unclear.

Recent studies have suggested that autophagy plays a very important role in cell protection. Lysosome-associated membrane protein (LAMP) is an important mediator of the process of autophagy and lysosome fusion. Autophagy was inhibited, and the cells were re-sensitized to tamoxifen after LAMP3 knockdown (Nagelkerke et al., 2014). TAM-R cells have a higher level of autophagy than tamoxifen-sensitive cells, and inhibition of autophagy will improve the efficacy of TAM (Liu et al., 2019). Wang et al. (2019) found that the expression of the H19 gene was enhanced in TAM-R cells and that H19 was significantly related to the enhancement of autophagy in breast cancer cells. Knockout of the H19 gene could make breast cancer cells re-sensitized to tamoxifen.

Why does tamoxifen enhance autophagy and lead to drug resistance? It is well known that tumor cells need a lot of energy to maintain their growth and proliferation, and a significant amount of this energy comes from enhanced glycolysis (Kim and Dang, 2006). The use of tamoxifen has been found to be related to the energy metabolism of cells. It was found that the ATP level of breast cancer cells decreased after tamoxifen treatment. Moreover, the use of tamoxifen could lead to the upregulation of the expression of MTA1, which further destroys mitochondrial function, while drug-resistant cells meet their energy needs through enhanced autophagy (Lee et al., 2018; Das et al., 2019). We speculate that the enhancement of autophagy may be the result of the increased energy demand of tumor cells and the anti-stress response of tumor cells.

Many autophagy-related genes have been discovered, and many autophagy inhibitors have been developed to inhibit tamoxifen resistance. Cheng et al. (2019) found that the use of icariin significantly increased the apoptosis of TAM-R cells; more TAM-R cells remained in the G0/G1 phase, while S phase/G2 phase cells were significantly reduced. At the same time, the expression of cyclin D1, Bcl-2, LC3-1, LC3-II, AGT5, and Beclin-1 were all downregulated. Interestingly, the expression of Beclin-1 downregulates the estrogen signal, which is beneficial to overcoming the resistance to tamoxifen (John et al., 2008). Similarly, Qi et al. (2017) found that autophagy is beneficial to the survival of breast cancer cells, while Z-ligustilide, which inhibits autophagy, may be helpful to overcome the resistance to tamoxifen in breast cancer.

SEL is an antagonist of XOP1. Combined treatment with SEL and 4-OH tamoxifen downregulated the expression of AKT and activated autophagy by blocking the glycolysis pathway, leading to cell death (Kulkoyluoglu-Cotul et al., 2019). Moreover, the degree of autophagy and the expression of autophagy-related genes can be used to judge drug resistance and select the treatment method, which may be helpful for the treatment of ER-positive breast cancer patients in the future.

The relationship between tamoxifen and energy metabolism may become a key research direction in the future, and it is of great significance to control the apoptosis and proliferation of tumor cells and to restore the sensitivity to tamoxifen.

Progress and Future Direction of Tamoxifen Resistance in Breast Cancer

Endocrine therapy is extremely important for ER-positive breast cancer patients. It mainly includes selective estrogen receptor modulators (SERMs), estrogen receptor downregulated modulators (SERDs), and aromatase inhibitors (AIS). Tamoxifen is one of the SERMs (Ali et al., 2016).

To overcome the resistance to tamoxifen, an increasing number of methods have been studied. ASA can not only reduce drug resistance by blocking G0/G1 phase-resistant cells but also by inhibiting the phosphorylation of AKT (Cheng et al., 2017). Phosphodiesterase 4D (PDE4D) can block cAMP and downstream signaling channels, making cells resistant to tamoxifen. However, the level of cAMP in cells is increased and the phosphorylation level of AKT is decreased after the use of aspirin (Mishra et al., 2018).

In addition, NF-κB has been proven to be related to the resistance of tamoxifen. Li et al. (2019) found that aspirin inhibited the activation of NF-κB signaling, which contributed to overcoming the resistance of cells to targeted therapeutic drugs. Aspirin seems to be a feasible strategy to overcome tamoxifen resistance, and it is expected to provide a new direction to breast cancer treatment. In addition to ASA combined with tamoxifen, proteasome inhibitors (PIs) combined with endocrine therapy have also been proven to be beneficial to the sensitization of tamoxifen-resistant cells (Maynadier et al., 2016; Cheng et al., 2017).

Inhibiting kinases in the RTK pathway to overcome drug resistance is also considered to be a viable approach. For example, gefitinib, perifosine, or GnRH-I and GnRH-II analogs were used to inhibit AKT expression (Block et al., 2012). Giordano et al. (2011) found that the primary bile acid chenodeoxycholic acid (CDCA) can activate the farnesoid X receptor (FXR) and inhibit the expression of HER2. Quercetin has also been found to restore the sensitivity to tamoxifen by mediating the upregulation of ERα and the downregulation of HER-2 (Wang et al., 2015).

The combination of tamoxifen and gefitinib promoted the apoptosis of drug-resistant cells. Gefitinib inhibited the downregulation of ERα by EGFR and restored the sensitivity of cells to tamoxifen to a certain extent (Jeong et al., 2019). Interestingly, another study showed that gefitinib has no effect on the activity of breast cancer-resistant cells, while neratinib, another EGFR inhibitor, induced the apoptosis of resistant cells by inhibiting the EGFR and HER2 signaling pathways (Kim et al., 2015). In addition, the use of dichloroacetate can overcome tamoxifen resistance by downregulating EGFR (Woo et al., 2016). Therefore, further study is needed on the effect of EGFR inhibitors on tamoxifen-resistant cells.

Peptidyl-prolyl isomerase Pin1 participates in the development of drug resistance by inducing E2F-4. Interestingly, all-trans retinoic acid (ATRA), an inhibitor of

TABLE 2 | Summary of recent studies of drugs that may be helpful in improving tamoxifen resistance.

Medicine	Therapeutic mechanism	Ref
ASA	block cell cycle in G0/G1 phase	(Cheng et al., 2017)
	target PDE4D/cAMP/ER stress axis	(Mishra et al., 2018)
	suppressed NF-κB signaling pathway	(Li et al., 2019)
Gefitinib/Perifosine/ analogs of GnRH-I/II	inhibit erbB and AKT signaling	(Block et al., 2012)
CDCA	inhibit HER2 expression	(Giordano et al., 2011)
Quercetin	mediate upregulation of ERα and downregulation of HER2	(Wang et al., 2015)
Neratinib	inhibit EGFR and HER2 signaling pathway	(Kim et al., 2015)
Dichloroacetate	downregulate EGFR expression	(Woo et al., 2016)
ATRA	inhibit the activation of ERK 1/2 and AKT	(Huang et al., 2019)
Resveratrol	reduce endogenous TGF-β production and reverse EMT	(Shi et al., 2013)
DpC	inhibit the expression of cyclin D1 and ERα	(Maqbool et al., 2020)

Pin1, inhibits the drug resistance of cells mainly by inhibiting the ERK 1/2 and AKT pathways (Huang et al., 2019).

Inhibition of epithelial–mesenchymal transition (EMT)-like phenomena is also a direction to take to overcome drug resistance. In addition to LDHA inhibiting EMT-like phenomena, resveratrol can also inhibit EMT by inhibiting TGF-β and overcoming tamoxifen resistance. Interestingly, EGFR activation is also related to EMT-like phenotype change, which confirms that inhibition of EMT contributes to overcome tamoxifen resistance (Zuo et al., 2011; Shi et al., 2013; Das et al., 2019). The drugs that overcome tamoxifen resistance mentioned in this review are summarized in **Table 2**.

With the advances in science and technology, some new approaches have been developed to improve tamoxifen sensitivity. For example, the application of nanotechnology (Guney Eskiler et al., 2018; Kumar et al., 2018) and the benefits of cold atmospheric plasma (CAP) in overcoming drug resistance, etc. (Lee et al., 2017).

Early judgments about the possible efficacy of endocrine therapy is of great significance in clinical treatment. Therefore, some prognostic markers suggesting tamoxifen resistance have been identified (Putluri et al., 2014; Elias et al., 2015; Gwak et al., 2017; Shimoda et al., 2017; Gong et al., 2018). The discovery of these markers is conducive to making early judgments about endocrine therapy efficacy and predictions of recurrence, which is helpful for doctors when making appropriate changes to the treatment strategy.

CONCLUSIONS AND PROSPECTS

Tamoxifen plays an important role in ER-positive breast cancer patients. However, drug resistance limits its efficacy, illustrating the importance of overcoming tamoxifen resistance in breast cancer. Most methods to overcome breast cancer resistance are based on the mechanism of drug resistance, such as inhibition of the RTK pathway, upregulation of ERα36, and blocking protective autophagy, cell cycle regulators and EMT-like phenomenon. In addition, some new methods have broadened the field of vision to overcome the drug resistance of tamoxifen. For example, some drugs combined with tamoxifen can inhibit the development of drug resistance, and the development of some new technologies is conducive to reducing the drug resistance of tamoxifen, and some prognostic markers of tamoxifen resistance have been discovered.

Research on the relationship between autophagy, cell cycle regulators, and resistance to tamoxifen has made great progress in recent years. The enhanced autophagy in drug-resistant cells is mainly due to the destruction of mitochondrial function caused by tamoxifen, and drug-resistant cells meet their energy demand through autophagy. The methods to overcome drug resistance according to the autophagy mechanism are mainly limited in the current research to the inhibition of autophagy by autophagy inhibitors.

In addition to continuing to look for better autophagy inhibitors to overcome the resistance, we hypothesized that tamoxifen combined with other drugs that protect mitochondrial function can prevent enhanced autophagy and overcome the drug resistance of tamoxifen. This is a new idea to improve the drug resistance of tamoxifen, and there is little research in this area.

Moreover, by detecting the level of autophagy and the expression of autophagy-related genes, the level of cell resistance can be judged, and treatment can be formulated and changed accordingly, which may improve the clinical treatment of breast cancer.

Targeting LEM4 is a feasible research direction to overcome tamoxifen resistance in the future. It has been proven that the high expression of LEM4 in drug-resistant cells is an important mechanism involved in the attenuation of the inhibitory effect of tamoxifen on the G1–S transition. Targeting LEM4 will play a significant role in overcoming tamoxifen resistance.

Overall, the main direction to overcome tamoxifen resistance in the future is not limited to inhibiting the expression of pathways related to tamoxifen resistance but may focus more on cyclins related to tamoxifen resistance, targeting LEM4 and inhibiting autophagy.

AUTHOR CONTRIBUTIONS

Conception: JY, JZ, and JH. Collection and assembly of data: All authors; Data analysis and interpretation: JY, JZ, KD, and RZ; Manuscript writing: JY and JZ; Final approval of manuscript: All authors.

REFERENCES

Ali, S., Mondal, N., Choudhry, H., Rasool, M., Pushparaj, P. N., Khan, M. A., et al. (2016). Current management strategies in breast cancer by targeting key altered molecular players. *Front. Oncol.* 6, 45. doi:10.3389/fonc.2016.00045

Antonioli, M., Di Rienzo, M., Piacentini, M., and Fimia, G. M. (2017). Emerging mechanisms in initiating and terminating autophagy. *Trends Biochem. Sci.* 42 (1), 28–41. doi:10.1016/j.tibs.2016.09.008

Binkhorst, L., van Gelder, T., and Mathijssen, R. H. J. (2012). Individualization of tamoxifen treatment for breast carcinoma. *Clin. Pharmacol. Ther.* 92 (4), 431–433. doi:10.1038/clpt.2012.94

Block, M., Gründker, C., Fister, S., Kubin, J., Wilkens, L., Mueller, M. D., et al. (2012). Inhibition of the AKT/mTOR and erbB pathways by gefitinib, perifosine and analogs of gonadotropin-releasing hormone I and II to overcome tamoxifen resistance in breast cancer cells. *Int. J. Oncol.* 41 (5), 1845–1854. doi:10.3892/ijo.2012.1591

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA A Cancer J. Clin.* 68 (6), 394–424. doi:10.3322/caac.21492

Cheng, R., Liu, Y.-J., Cui, J.-W., Yang, M., Liu, X.-L., Li, P., et al. (2017). Aspirin regulation of c-myc and cyclinD1 proteins to overcome tamoxifen resistance in estrogen receptor-positive breast cancer cells. *Oncotarget* 8 (18), 30252–30264. doi:10.18632/oncotarget.16325

Cheng, X., Tan, S., Duan, F., Yuan, Q., Li, Q., and Deng, G. (2019). Icariin induces apoptosis by suppressing autophagy in tamoxifen-resistant breast cancer cell line MCF-7/TAM. *Breast Cancer* 26 (6), 766–775. doi:10.1007/s12282-019-00980-5

Choi, A.-R., Kim, J.-H., Woo, Y. H., Cheon, J. H., Kim, H. S., and Yoon, S. (2016). Co-treatment of LY294002 or MK-2206 with AZD5363 attenuates AZD5363-induced increase in the level of phosphorylated AKT. *Ar* 36 (11), 5849–5858. doi:10.21873/anticanres.11170

Choi, H.-J., Joo, H.-S., Won, H.-Y., Min, K.-W., Kim, H.-Y., Son, T., et al. (2018). Role of RBP2-induced ER and IGF1R-ErbB signaling in tamoxifen resistance in breast cancer. *J. Natl. Cancer Inst.* 110 (4), 400. doi:10.1093/jnci/djx207

Cook, K. L., Shajahan, A. N., and Clarke, R. (2011). Autophagy and endocrine resistance in breast cancer. *Expet Rev. Anticancer Ther.* 11 (8), 1283–1294. doi:10.1586/era.11.111

Cristofanilli, M., Turner, N. C., Bondarenko, I., Ro, J., Im, S.-A., Masuda, N., et al. (2016). Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. *Lancet Oncol.* 17 (4), 425–439. doi:10.1016/S1470-2045(15)00613-0

Das, C. K., Parekh, A., Parida, P. K., Bhutia, S. K., and Mandal, M. (2019). Lactate dehydrogenase A regulates autophagy and tamoxifen resistance in breast cancer. *Biochim. Biophys. Acta Mol. Cell Res.* 1866 (6), 1004–1018. doi:10.1016/j.bbamcr.2019.03.004

Elias, D., Vever, H., Lænkholm, A.-V., Gjerstorff, M. F., Yde, C. W., Lykkesfeldt, A. E., et al. (2015). Gene expression profiling identifies FYN as an important molecule in tamoxifen resistance and a predictor of early recurrence in patients treated with endocrine therapy. *Oncogene* 34(15), 1919–1927. doi:10.1038/onc.2014.138

Ferraiuolo, R.-M., Tubman, J., Sinha, I., Hamm, C., and Porter, L. A. (2017). The cyclin-like protein, SPY1, regulates the ERα and ERK1/2 pathways promoting tamoxifen resistance. *Oncotarget* 8 (14), 23337–23352. doi:10.18632/oncotarget.15578

Finn, R. S., Crown, J. P., Lang, I., Boer, K., Bondarenko, I. M., Kulyk, S. O., et al. (2015). The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. *Lancet Oncol.* 16 (1), 25–35. doi:10.1016/S1470-2045(14)71159-3

Gao, A., Sun, T., Ma, G., Cao, J., Hu, Q., Chen, L., et al. (2018). LEM4 confers tamoxifen resistance to breast cancer cells by activating cyclin D-CDK4/6-Rb and ERα pathway. *Nat. Commun.* 9 (1), 4180. doi:10.1038/s41467-018-06309-8

Giordano, C., Catalano, S., Panza, S., Vizza, D., Barone, I., Bonofiglio, D., et al. (2011). Farnesoid X receptor inhibits tamoxifen-resistant MCF-7 breast cancer cell growth through downregulation of HER2 expression. *Oncogene* 30 (39), 4129–4140. doi:10.1038/onc.2011.124

Gong, C., Man, E. P. S., Tsoi, H., Lee, T. K. W., Lee, P., Ma, S.-T., et al. (2018). BQ323636.1, a novel splice variant to NCOR2, as a predictor for tamoxifen-resistant breast cancer. *Clin. Canc. Res.* 24 (15), 3681–3691. doi:10.1158/1078-0432.CCR-17-2259

Gonzalez-Malerva, L., Park, J., Zou, L., Hu, Y., Moradpour, Z., Pearlberg, J., et al. (2011). High-throughput ectopic expression screen for tamoxifen resistance identifies an atypical kinase that blocks autophagy. *Proc. Natl. Acad. Sci. USA* 108 (5), 2058–2063. doi:10.1073/pnas.1018157108

Guney Eskiler, G., Cecener, G., Dikmen, G., Egeli, U., and Tunca, B. (2018). Solid lipid nanoparticles: reversal of tamoxifen resistance in breast cancer. *Eur. J. Pharmaceut. Sci.* 120, 73–88. doi:10.1016/j.ejps.2018.04.040

Gwak, J. M., Kim, M., Kim, H. J., Jang, M. H., and Park, S. Y. (2017). Expression of embryonal stem cell transcription factors in breast cancer: oct4 as an indicator for poor clinical outcome and tamoxifen resistance. *Oncotarget* 8 (22), 36305–36318. doi:10.18632/oncotarget.16750

He, M., Jin, Q., Chen, C., Liu, Y., Ye, X., Jiang, Y., et al. (2019). The miR-186-3p/EREG axis orchestrates tamoxifen resistance and aerobic glycolysis in breast cancer cells. *Oncogene* 38 (28), 5551–5565. doi:10.1038/s41388-019-0817-3

Heckler, M. M., Thakor, H., Schafer, C. C., and Riggins, R. B. (2014). ERK/MAPK regulates ERRγ expression, transcriptional activity and receptor-mediated tamoxifen resistance in ER+ breast cancer. *FEBS J.* 281 (10), 2431–2442. doi:10.1111/febs.12797

Hortobagyi, G. N., Stemmer, S. M., Burris, H. A., Yap, Y.-S., Sonke, G. S., Paluch-Shimon, S., et al. (2016). Ribociclib as first-line therapy for HR-positive, advanced breast cancer. *N. Engl. J. Med.* 375 (18), 1738–1748. doi:10.1056/NEJMoa1609709

Hosford, S. R. and Miller, T. W. (2014). Clinical potential of novel therapeutic targets in breast cancer: CDK4/6, Src, JAK/STAT, PARP, HDAC, and PI3K/AKT/mTOR pathways. *Pharmgenomics Pers Med* 7, 203–215. doi:10.2147/PGPM.S52762

Huang, S., Chen, Y., Liang, Z.-M., Li, N.-N., Liu, Y., Zhu, Y., et al. (2019). Targeting Pin1 by all-trans retinoic acid (ATRA) overcomes tamoxifen resistance in breast cancer via multifactorial mechanisms. *Front. Cell Dev. Biol.* 7, 322. doi:10.3389/fcell.2019.00322

Ito, T., Kamijo, S., Izumi, H., Kohno, K., Amano, J., and Ito, K.-I. (2012). Alteration of Y-box binding protein-1 expression modifies the response to endocrine therapy in estrogen receptor-positive breast cancer. *Breast Canc. Res. Treat.* 133 (1), 145–159. doi:10.1007/s10549-011-1731-8

Jeong, Y., Bae, S. Y., You, D., Jung, S. P., Choi, H. J., Kim, I., et al. (2019). EGFR is a therapeutic target in hormone receptor-positive breast cancer. *Cell. Physiol. Biochem.* 53 (5), 805–819. doi:10.33594/000000174

Jeselsohn, R., Cornwell, M., Pun, M., Buchwalter, G., Nguyen, M., Bango, C., et al. (2017). Embryonic transcription factor SOX9 drives breast cancer endocrine resistance. *Proc. Natl. Acad. Sci. USA* 114 (22), E4482–E4491. doi:10.1073/pnas.1620993114

Jia, Y., Zhou, J., Luo, X., Chen, M., Chen, Y., Wang, J., et al. (2018). KLF4 overcomes tamoxifen resistance by suppressing MAPK signaling pathway and predicts good prognosis in breast cancer. *Cell. Signal.* 42, 165–175. doi:10.1016/j.cellsig.2017.09.025

Jiang, M.-J., Chen, Y.-Y., Dai, J.-J., Gu, D.-N., Mei, Z., Liu, F.-R., et al. (2020). Dying tumor cell-derived exosomal miR-194-5p potentiates survival and repopulation of tumor repopulating cells upon radiotherapy in pancreatic cancer. *Mol. Canc.* 19 (1), 68. doi:10.1186/s12943-020-01178-6

Jögi, A., Ehinger, A., Hartman, L., and Alkner, S. (2019). Expression of HIF-1α is related to a poor prognosis and tamoxifen resistance in contralateral breast cancer. *PLoS One* 14 (12), e0226150. doi:10.1371/journal.pone.0226150

John, S., Nayvelt, I., Hsu, H.-C., Yang, P., Liu, W., Das, G. M., et al. (2008). Regulation of estrogenic effects by beclin 1 in breast cancer cells. *Canc. Res.* 68 (19), 7855–7863. doi:10.1158/0008-5472.CAN-07-5875

Karlsson, E., Veenstra, C., Gårsjö, J., Nordenskjöld, B., Fornander, T., and Stål, O. (2019). PTPN2 deficiency along with activation of nuclear Akt predict endocrine resistance in breast cancer. *J. Canc. Res. Clin. Oncol.* 145 (3), 599–607. doi:10.1007/s00432-018-2810-6

Kim, J.-w. and Dang, C. V. (2006). Cancer's molecular sweet tooth and the warburg effect: figure 1. *Canc. Res.* 66 (18), 8927–8930. doi:10.1158/0008-5472.CAN-06-1501

Kim, S., Lee, J., Oh, S. J., Nam, S. J., and Lee, J. E. (2015). Differential effect of EGFR inhibitors on tamoxifen-resistant breast cancer cells. *Oncol. Rep.* 34 (3), 1613–1619. doi:10.3892/or.2015.4116

Kulkoyluoglu-Cotul, E., Smith, B. P., Wrobel, K., Zhao, Y. C., Chen, K. L. A., Hieronymi, K., et al. (2019). Combined targeting of estrogen receptor alpha and XPO1 prevent akt activation, remodel metabolic pathways and induce autophagy to overcome tamoxifen resistance. *Cancers* 11 (4), 479. doi:10.3390/cancers11040479

Kumar, M., Sharma, G., Misra, C., Kumar, R., Singh, B., Katare, O. P., et al. (2018). N-desmethyl tamoxifen and quercetin-loaded multiwalled CNTs: a synergistic approach to overcome MDR in cancer cells. *Mater. Sci. Eng. C* 89, 274–282. doi:10.1016/j.msec.2018.03.033

Kuo, S.-J., Lin, H.-Y., Chien, S.-Y., and Chen, D.-R. (2013). SIRT1 suppresses breast cancer growth through downregulation of the Bcl-2 protein. *Oncol. Rep.* 30 (1), 125–130. doi:10.3892/or.2013.2470

Lee, M.-H., Koh, D., Na, H., Ka, N.-L., Kim, S., Kim, H.-J., et al. (2018). MTA1 is a novel regulator of autophagy that induces tamoxifen resistance in breast cancer cells. *Autophagy* 14 (5), 812–824. doi:10.1080/15548627.2017.1388476

Lee, S., Lee, H., Jeong, D., Ham, J., Park, S., Choi, E. H., et al. (2017). Cold atmospheric plasma restores tamoxifen sensitivity in resistant MCF-7 breast cancer cell. *Free Radic. Biol. Med.* 110, 280–290. doi:10.1016/j.freeradbiomed.2017.06.017

Li, D., Ji, H., Niu, X., Yin, L., Wang, Y., Gu, Y., et al. (2020a). Tumor-associated macrophages secrete CC-chemokine ligand 2 and induce tamoxifen resistance by activating PI3K/Akt/mTOR in breast cancer. *Canc. Sci.* 111 (1), 47–58. doi:10.1111/cas.14230

Li, G., Zhang, J., Xu, Z., and Li, Z. (2020b). ERalpha36 as a potential therapeutic target for tamoxifen-resistant breast cancer cell line through EGFR/ERK signaling pathway. *Canc. Manag. Res.* 12, 265–275. doi:10.2147/CMAR.S226410

Li, K., Ying, M., Feng, D., Du, J., Chen, S., Dan, B., et al. (2016). Brachyury promotes tamoxifen resistance in breast cancer by targeting SIRT1. *Biomed. Pharmacother.* 84, 28–33. doi:10.1016/j.biopha.2016.09.011

Li, L., Hu, M., Wang, T., Chen, H., and Xu, L. (2019). Repositioning aspirin to treat lung and breast cancers and overcome acquired resistance to targeted therapy. *Front Oncol* 9, 1503. doi:10.3389/fonc.2019.01503

Li, W., Xu, L., Che, X., Li, H., Zhang, Y., Song, N., et al. (2018). C-Cbl reverses HER2-mediated tamoxifen resistance in human breast cancer cells. *BMC Canc.* 18 (1), 507. doi:10.1186/s12885-018-4387-5

Lin, J. H., Tu, S. H., Chen, L. C., Huang, C. C., Chang, H. L., Cheng, T. C., et al. (2018). Oestrogen receptor-regulated glutathione S-transferase mu 3 expression attenuates hydrogen peroxide-induced cytotoxicity, which confers tamoxifen resistance on breast cancer cells. *Breast Canc. Res. Treat.* 172 (1), 45–59. doi:10.1007/s10549-018-4897-5

Liu, J., Yue, W., and Chen, H. (2019). The correlation between autophagy and tamoxifen resistance in breast cancer. *Int. J. Clin. Exp. Pathol.* 12 (6), 2066–2074.

Liu, T., Liu, P. Y., and Marshall, G. M. (2009). The critical role of the class III histone deacetylase SIRT1 in cancer. *Canc. Res.* 69 (5), 1702–1705. doi:10.1158/0008-5472.CAN-08-3365

Lopez-Tarruella, S. and Schiff, R. (2007). The dynamics of estrogen receptor status in breast cancer: re-shaping the paradigm. *Clin. Canc. Res.* 13 (23), 6921–6925. doi:10.1158/1078-0432.CCR-07-1399

Lui, A., New, J., Ogony, J., Thomas, S., and Lewis-Wambi, J. (2016). Everolimus downregulates estrogen receptor and induces autophagy in aromatase inhibitor-resistant breast cancer cells. *BMC Canc.* 16, 487. doi:10.1186/s12885-016-2490-z

Lv, L., Qimin, W., Xiupeng, L., Lin, S., Huamin, Q., Lifen, W., et al. (2015). Expression and localization of estrogen receptor in human breast cancer and its clinical significance. *Cell Biochem. Biophys.* 71 (1), 63–68.

Maczis, M. A., Maceyka, M., Waters, M. R., Newton, J., Singh, M., Rigsby, M. F., et al. (2018). Sphingosine kinase 1 activation by estrogen receptor alpha36 contributes to tamoxifen resistance in breast cancer. *J. Lipid Res.* 59 (12), 2297–2307. doi:10.1194/jlr.M085191

Mansouri, S., Farahmand, L., Teymourzadeh, A., and Majidzadeh, A. K. (2018a). Clinical evidence on the magnitude of change in growth pathway activity in relation to tamoxifen resistance is required. *Curr. Cancer Drug Targets* 18 (7), 668–676. doi:10.2174/1568009617666170808110820

Mansouri, S., Feizi, N., Mahdi, A., Majidzadeh, A. K., and Farahmand, L. (2018b). A review on the role of VEGF in tamoxifen resistance. *Anticancer Agents Med. Chem.* 18 (14), 2006–2009. doi:10.2174/1871520618666180911142259

Maqbool, S. N., Lim, S. C., Park, K. C., Hanif, R., Richardson, D. R., Jansson, P. J., et al. (2020). Overcoming tamoxifen resistance in oestrogen receptor-positive breast cancer using the novel thiosemicarbazone anti-cancer agent, DpC. *Br. J. Pharmacol.* 177 (10), 2365–2380. doi:10.1111/bph.14985

Maynadier, M., Basile, I., Gallud, A., Gary-Bobo, M., and Garcia, M. (2016). Combination treatment with proteasome inhibitors and antiestrogens has a synergistic effect mediated by p21WAF1 in estrogen receptor-positive breast cancer. *Oncol. Rep.* 36 (2), 1127–1134. doi:10.3892/or.2016.4873

Mishra, R. R., Belder, N., Ansari, S. A., Kayhan, M., Bal, H., Raza, U., et al. (2018). Reactivation of cAMP pathway by PDE4D inhibition represents a novel druggable Axis for overcoming tamoxifen resistance in ER-positive breast cancer. *Clin. Canc. Res.* 24 (8), 1987–2001. doi:10.1158/1078-0432.CCR-17-2776

Nagelkerke, A., Sieuwerts, A. M., Bussink, J., Sweep, F. C., Look, M. P., Foekens, J. A., et al. (2014). LAMP3 is involved in tamoxifen resistance in breast cancer cells through the modulation of autophagy. *Endocr. Relat. Canc.* 21 (1), 101–112. doi:10.1530/ERC-13-0183

Oh, S. J., Kim, O., Lee, J. S., Kim, J. A., Kim, M. R., Choi, H. S., et al. (2010). Inhibition of angiogenesis by quercetin in tamoxifen-resistant breast cancer cells. *Food Chem. Toxicol.* 48 (11), 3227–3234. doi:10.1016/j.fct.2010.08.028

Omarjee, S., Jacquemetton, J., Poulard, C., Rochel, N., Dejaegere, A., Chebaro, Y., et al. (2017). The molecular mechanisms underlying the ERalpha-36-mediated signaling in breast cancer. *Oncogene* 36 (18), 2503–2514. doi:10.1038/onc.2016.415

Peng, W. X., Huang, J. G., Yang, L., Gong, A. H., and Mo, Y. Y. (2017). Linc-RoR promotes MAPK/ERK signaling and confers estrogen-independent growth of breast cancer. *Mol. Canc.* 16 (1), 161. doi:10.1186/s12943-017-0727-3

Putluri, N., Maity, S., Kommagani, R., Creighton, C. J., Putluri, V., Chen, F., et al. (2014). Pathway-centric integrative analysis identifies RRM2 as a prognostic marker in breast cancer associated with poor survival and tamoxifen resistance. *Neoplasia* 16 (5), 390–402. doi:10.1016/j.neo.2014.05.007

Qi, H., Jiang, Z., Wang, C., Yang, Y., Li, L., He, H., et al. (2017). Sensitization of tamoxifen-resistant breast cancer cells by Z-ligustilide through inhibiting autophagy and accumulating DNA damages. *Oncotarget* 8 (17), 29300–29317. doi:10.18632/oncotarget.16832

Rugo, H. S., Rumble, R. B., Macrae, E., Barton, D. L., Connolly, H. K., Dickler, M. N., et al. (2016). Endocrine therapy for hormone receptor-positive metastatic breast cancer: American society of clinical oncology guideline. *J. Clin. Oncol.* 34 (25), 3069–3103. doi:10.1200/JCO.2016.67.1487

Scott, H. S., Antonarakis, S. E., Lalioti, M. D., Rossier, C., Silver, P. A., and Henry, M. F. (1998). Identification and characterization of two putative human arginine methyltransferases (HRMT1L1 and HRMT1L2). *Genomics* 48 (3), 330–340. doi:10.1006/geno.1997.5190

Shen, Y., Zhong, J., Liu, J., Liu, K., Zhao, J., Xu, T., et al. (2018). Protein arginine N-methyltransferase 2 reverses tamoxifen resistance in breast cancer cells through suppression of ER-alpha36. *Oncol. Rep.* 39 (6), 2604–2612. doi:10.3892/or.2018.6350

Shi, X. P., Miao, S., Wu, Y., Zhang, W., Zhang, X. F., Ma, H. Z., et al. (2013). Resveratrol sensitizes tamoxifen in antiestrogen-resistant breast cancer cells with epithelial-mesenchymal transition features. *Int. J. Mol. Sci.* 14 (8), 15655–15668. doi:10.3390/ijms140815655

Shimoda, M., Hori, A., Wands, J. R., Tsunashima, R., Naoi, Y., Miyake, T., et al. (2017). Endocrine sensitivity of estrogen receptor-positive breast cancer is negatively correlated with aspartate-beta-hydroxylase expression. *Canc. Sci.* 108 (12), 2454–2461. doi:10.1111/cas.13416

Sun, W. L., Lan, D., Gan, T. Q., and Cai, Z. W. (2015). Autophagy facilitates multidrug resistance development through inhibition of apoptosis in breast cancer cells. *Neoplasma* 62 (2), 199–208. doi:10.4149/neo_2015_025

Thomas, S., Thurn, K. T., Raha, P., Chen, S., and Munster, P. N. (2013). Efficacy of histone deacetylase and estrogen receptor inhibition in breast cancer cells due to concerted down regulation of Akt. *PLoS One* 8 (7), e68973. doi:10.1371/journal.pone.0068973

Tong, J. S., Zhang, Q. H., Wang, Z. B., Li, S., Yang, C. R., Fu, X. Q., et al. (2010). ER-alpha36, a novel variant of ER-alpha, mediates estrogen-stimulated proliferation of endometrial carcinoma cells via the PKCdelta/ERK pathway. *PLoS One* 5 (11), e15408. doi:10.1371/journal.pone.0015408

Vargo-Gogola, T. and Rosen, J. M. (2007). Modelling breast cancer: one size does not fit all. *Nat. Rev. Canc.* 7 (9).doi:10.1038/nrc2193

Vaziri-Gohar, A., Zheng, Y., and Houston, K. D. (2017). IGF-1 receptor modulates FoxO1-mediated tamoxifen response in breast cancer cells. *Mol. Canc. Res.* 15 (4), 489–497. doi:10.1158/1541-7786.MCR-16-0176

Viedma-Rodriguez, R., Baiza-Gutman, L., Salamanca-Gomez, F., Diaz-Zaragoza, M., Martinez-Hernandez, G., Ruiz Esparza-Garrido, R., et al. (2014). Mechanisms associated with resistance to tamoxifen in estrogen receptor-positive breast cancer (review). *Oncol. Rep.* 32 (1), 3–15. doi:10.3892/or.2014.3190

Wang, B. and Huang, Y. (2020). Effect of aspirin use on neoadjuvant chemoradiotherapy for rectal cancer: a meta-analysis with trial sequential analysis. *J. Canc. Res. Clin. Oncol.* 146 (3), 2161–2171. doi:10.1007/s00432-020-03222-w

Wang, H., Tao, L., Qi, K., Zhang, H., Feng, D., Wei, W., et al. (2015). Quercetin reverses tamoxifen resistance in breast cancer cells. *J BUON* 20 (3), 707–713.

Wang, J., Xie, S., Yang, J., Xiong, H., Jia, Y., Zhou, Y., et al. (2019). The long noncoding RNA H19 promotes tamoxifen resistance in breast cancer via autophagy. *J. Hematol. Oncol.* 12 (1), 81. doi:10.1186/s13045-019-0747-0

Woo, S. H., Seo, S. K., Park, Y., Kim, E. K., Seong, M. K., Kim, H. A., et al. (2016). Dichloroacetate potentiates tamoxifen-induced cell death in breast cancer cells via downregulation of the epidermal growth factor receptor. *Oncotarget* 7 (37), 59809–59819. doi:10.18632/oncotarget.10999

Wu, Y., Yan, B., Xu, W., Guo, L., Wang, Z., Li, G., et al. (2020). Compound C enhances the anticancer effect of aspirin in HER-2-positive breast cancer by regulating lipid metabolism in an AMPK-independent pathway. *Int. J. Biol. Sci.* 16 (4), 583–597. doi:10.7150/ijbs.39936

Xue, Y., Lian, W., Zhi, J., Yang, W., Li, Q., Guo, X., et al. (2019). HDAC5-mediated deacetylation and nuclear localisation of SOX9 is critical for tamoxifen resistance in breast cancer. *Br. J. Canc.* 121 (12), 1039–1049. doi:10.1038/s41416-019-0625-0

Ye, L., Lin, C., Wang, X., Li, Q., Li, Y., Wang, M., et al. (2019). Epigenetic silencing of SALL2 confers tamoxifen resistance in breast cancer. *EMBO Mol. Med.* 11 (12), e10638. doi:10.15252/emmm.201910638

Yin, H., Zhu, Q., Liu, M., Tu, G., Li, Q., Yuan, J., et al. (2017). GPER promotes tamoxifen-resistance in ER+ breast cancer cells by reduced Bim proteins through MAPK/Erk-TRIM2 signaling axis. *Int. J. Oncol.* 51 (4), 1191–1198. doi:10.3892/ijo.2017.4117

Yin, L., Zhang, X. T., Bian, X. W., Guo, Y. M., and Wang, Z. Y. (2014). Disruption of the ER-alpha36-EGFR/HER2 positive regulatory loops restores tamoxifen sensitivity in tamoxifen resistance breast cancer cells. *PLoS One* 9 (9), e107369. doi:10.1371/journal.pone.0107369

Yu, D., Shi, L., Bu, Y., and Li, W. (2019). Cell division cycle associated 8 is a key regulator of tamoxifen resistance in breast cancer. *J Breast Cancer* 22(2), 237–247. doi:10.4048/jbc.2019.22.e29

Zhang, X. and Wang, Z. Y. (2013). Estrogen receptor-alpha variant, ER-alpha36, is involved in tamoxifen resistance and estrogen hypersensitivity. *Endocrinology* 154 (6), 1990–1998. doi:10.1210/en.2013-1116

Zhang, Y., Lv, C., Dong, Y., and Yang, Q. (2020). Aspirin-targeted PD-L1 in lung cancer growth inhibition. *Thorac Cancer* 11(6), 1587–1593. doi:10.1111/1759-7714.13433

Zhang, Y., Wester, L., He, J., Geiger, T., Moerkens, M., Siddappa, R., et al. (2018). IGF1R signaling drives antiestrogen resistance through PAK2/PIX activation in luminal breast cancer. *Oncogene* 37 (14), 1869–1884. doi:10.1038/s41388-017-0027-9

Zhu, Y., Liu, Y., Zhang, C., Chu, J., Wu, Y., Li, Y., et al. (2018). Tamoxifen-resistant breast cancer cells are resistant to DNA-damaging chemotherapy because of upregulated BARD1 and BRCA1. *Nat. Commun.* 9 (1), 1595. doi:10.1038/s41467-018-03951-0

Zuo, J. H., Zhu, W., Li, M. Y., Li, X. H., Yi, H., Zeng, G. Q., et al. (2011). Activation of EGFR promotes squamous carcinoma SCC10A cell migration and invasion via inducing EMT-like phenotype change and MMP-9-mediated degradation of E-cadherin. *J. Cell. Biochem.* 112 (9), 2508–2517. doi:10.1002/jcb.23175

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REVIEW
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The Breast Cancer Stem Cells Traits and Drug Resistance

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Drug resistance is a major challenge in breast cancer (BC) treatment at present. Accumulating studies indicate that breast cancer stem cells (BCSCs) are responsible for the BC drugs resistance, causing relapse and metastasis in BC patients. Thus, BCSCs elimination could reverse drug resistance and improve drug efficacy to benefit BC patients. Consequently, mastering the knowledge on the proliferation, resistance mechanisms, and separation of BCSCs in BC therapy is extremely helpful for BCSCs-targeted therapeutic strategies. Herein, we summarize the principal BCSCs surface markers and signaling pathways, and list the BCSCs-related drug resistance mechanisms in chemotherapy (CT), endocrine therapy (ET), and targeted therapy (TT), and display therapeutic strategies for targeting BCSCs to reverse drug resistance in BC. Even more importantly, more attention should be paid to studies on BCSC-targeted strategies to overcome the drug resistant dilemma of clinical therapies in the future.

Keywords: breast cancer stem cells, drug resistance, clinical therapy, surface markers, breast cancer stem cell signaling pathways

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INTRODUCTION

Breast cancer (BC) is one of the most common cancers diagnosed among women and ranked as the second cause of cancer-related death among women, after lung cancer (DeSantis et al., 2019; Siegel et al., 2019). There are various types of BC therapeutic strategies, such as breast surgery, radiotherapy (RT), chemotherapy (CT), endocrine therapy (ET), targeted therapy (TT), and others, which are based on the types of tumor pathologies. For example, breast-conserving/mastectomy surgery and adjuvant CT are applied to treat early BCs. Antitumor drugs are utilized alone or in combination to reduce the risk of BC recurrence. For ERα-positive and Her2-positive tumors patients, hormone therapy and targeted therapy, respectively, conduce to significant prognosis improvements. Additionally, chemotherapy is considered the best option in advanced triple-negative BC (TNBC). These treatment options have contributed to a BC death rate decline over the past three decades (DeSantis et al., 2019). Hence, therapies improvement is a milestone in BC therapy.

However, many BC patients still experience poor drug response and tumor recurrence in clinical observation (Harbeck and Gnant, 2017). Some BC cells exhibit intrinsic drug-resistance, while others are initially drug-sensitive, but acquire resistance to anticancer drugs (Abad et al., 2020). These drug failures are considered as chemoresistance in BC cells, owing to the survival of a special population of heterogeneity cells in tumors which possess drug-resistance features (Eiro et al., 2019). These heterogeneity cells are known as residual disease and can eventually lead to recurrence (**Figure 1**).

Cancer stem cells (CSCs), which were discovered and developed over the past decades, play a major role in drug-resistance and relapse of solid tumors (Reya et al., 2001; Clarke et al., 2006). Besides drug-resistance, previous studies have showed that cancer initiation (Barker et al., 2009),

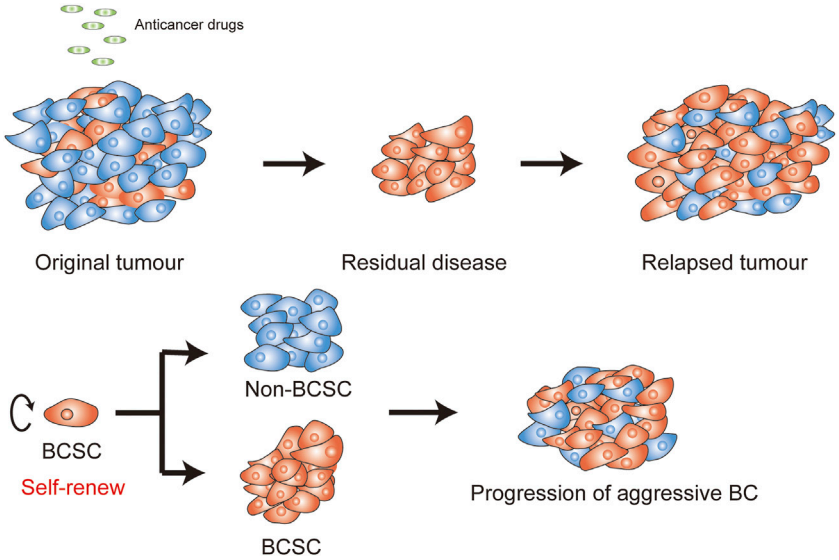

FIGURE 1 | Self-renew in BCSCs. **(A)** Anticancer drugs are often utilized for treat BC, efficiently targeting breast cancer cells (BCCs) (blue cells), but not BCSCs (orange cells). The residual stem-like cell populations can drive a more aggressive BC and trigger recurrence. **(B)** BCSCs can form a new tissue by the balance of renewal and divisions.

progression (Lytle et al., 2018), and metastasis (Oskarsson et al., 2014) could also be induced by CSCs (Nalla et al., 2019). CSCs play a similar role in BC. Al-Hajj first isolated BC stem cells (BCSCs) with specific markers (EpCAM+/CD44$^+$/CD24$^-$) which have the potential to lead to bulk tumors *in vivo* (Al-Hajj et al., 2003). Targeting BCSCs, in any hypotype of BC: luminalA, luminalB, human epithelial growth factor receptor 2 (HER2) overexpression, or TNBC, is the key therapy approach to reverse drug resistance (Dey et al., 2019). Therefore, we need to understand the role of BCSCs in drug-resistance mechanisms, which will overcome the drug-resistance problem and promote BC prognosis.

Here, first we summarize the BCSC markers and signaling pathways that are possible therapeutic targets for drug resistance. More importantly, we focus on the mechanism of resistance to specific drugs, such as anthracycline, taxane, tamoxifen, trastuzumab, among others. Lastly, novel studies about emerging therapies of reversing drug resistance by targeting BCSCs are discussed. We insist that the important breakthroughs in the field of BCSCs research will help researchers effectively find and target BC resistance mechanism and, ultimately, help patients achieve a favorable prognosis.

CENTRAL SURFACE MARKERS IN BREAST CANCER STEM CELLS

BCSCs surface biomarkers are utilized for identifying or isolating BC. However, emerging studies show that different surface markers determine different BCSCs (Dey et al., 2019; Sridharan et al., 2019); the functions of BCSCs are based on the type of markers they contain. The key surface markers of BCSCs and their functions in BC are listed in **Table 1**. Novel drugs are being designed to target these markers for regulating the activation of BCSCs in order to achieve an efficient response to anti-BC treatment (**Figure 2**). Thus, we list the central surface markers in BCSCs and their known functions in BCSCs regulation.

CD44

CD44 was initially used to isolate BCSCs from tumors. Meanwhile, BC cells with an overexpression of CD44 marker, known as BC-initiating cells (BCIC), showed tumorigenic ability *in vivo* (Ponti et al., 2005). CD44 is a cell membrane receptor for hyaluronan acid (HA) (Bourguignon et al., 2004). HA-CD44 interaction play an important role in inhibiting metastasis (Lv et al., 2018a; Bourguignon, 2019), reversing drug resistance (Liu J. et al., 2019), and suppressing invasion (Sarkar et al., 2019) among BC cells. For instance, The binding of CD44 and HA activated c-Src-Twist/miR-10b/RhoGTPase-ROK signaling, that are associated with the activation of the PI3K/AKT-dependent invasion and metastasis in cancers (Bourguignon et al., 2010). Furthermore, the high expression of CD44 is essential for BC multidrug resistance by regulation of the chemoresistance receptor through stimulation of signal transducer and activator of transcription 3 (STAT3) pathway (Louderbough and Schroeder, 2011). Moreover, the interaction of the cleaved product of CD44 (CD44ICD) and cAMP-response element binding protein (CREB) can up-regulate fructose-2,6-bisphosphatase 4 (PFKFB4) expression, which activates

TABLE 1 | The BCSCs Surface markers in significant literatures.

Surface markers	Mediated signaling	Function in BC
CD44 (Al-Hajj et al., 2003)	PI3K/AKT signaling (Ghatak et al., 2002) NF-κB signaling (Cho et al., 2015) CREB/TGF-β2 signaling (Ouhtit et al., 2018)	Forming tumors (Al-Hajj et al., 2003), promoting metastasis (Hill et al., 2006), associated with (BRCA1) mutational status (Bane et al., 2013) Predicting prognosis (Bane et al., 2013), stimulating migration (Bourguignon et al., 2003), promoting cell adhesion (Ponta et al., 2003), promoting. Cell growth, survival, and invasion (Louderbough and Schroeder, 2011)
ALDH1 (Ginestier et al., 2007)		Associated to tumor-initiating characteristics (Ginestier et al., 2007), promoting self-renewal (Ginestier et al., 2009), As target for BCSCs-targeted therapy (Angeloni et al., 2015) Predicting prognosis 11823860 (Alexe et al., 2006; van't Veer et al., 2002) promoting metastasis (Marcato et al., 2011)
CD133	IL6/Notch3 signaling (Sansone et al., 2016)	Regulation of ET-resistant (Sansone et al., 2016) promoting self-renewal (Sansone et al., 2016) BCSCs identification (Bai et al., 2018) CD133 aptamers or CD133-targeted drug delivery system for BCSCs-targeted therapy (Shigdar et al., 2013; Swaminathan et al., 2013)
EpCAM		Regulation of migration and metastasis (Baccelli et al., 2013) promoting chemoresistance (Wang T. et al., 2015)
ABCG2		Promoting BC chemoresistance, tumorigenicity and metastasis (Bai et al., 2018), Sorting BCSCs from BRCA1-mutated BC cells (Leccia et al., 2014)
GD2		Aassociated with GD3S-mediated EMT (Liang et al., 2017), promoting tumorigenicity and metastasis (Battula et al., 2017),BCSCs. identification (Bai et al., 2018)
CXCR4	SDF-1/CXCR4 signaling (Yi et al., 2014)	Promoting metastasis (Muller et al., 2001), promoting migration or invasion (Luker et al., 2012)

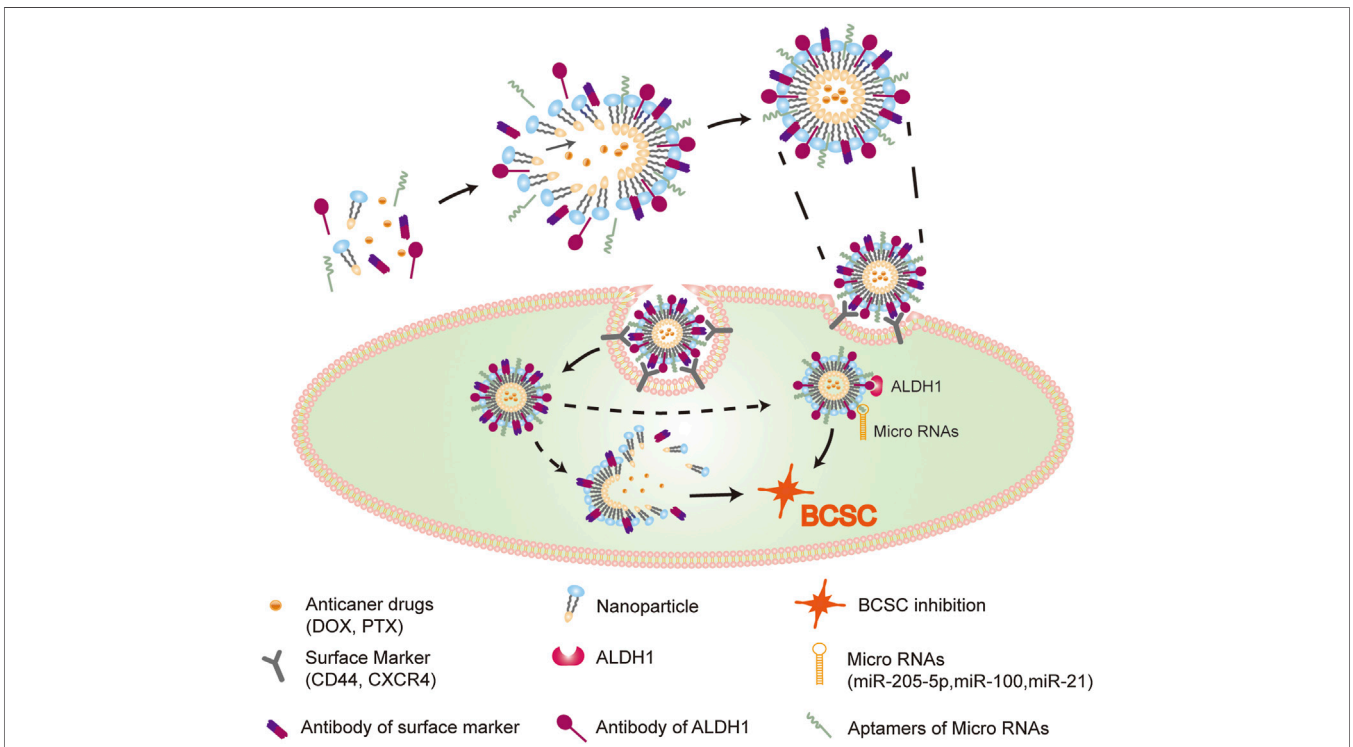

FIGURE 2 | Effective drugs delivery system in BCSCs. Nanoparticles, which modified by surface markers (CD44, CXCR4) ligands and intracellular molecular (micro RNAs, ALDH1) ligands, loaded with anti-cancer drugs, efficiently targeting BCSCs. The delivery system shows effectively reversal of drug resistance through dual inhibition of BCSCs via repressing both ex- and intracellular tumorigenic markers.

glycolysis and impoves BC stemness (Gao et al., 2018). CSCs are associated with tumor metastasis and invasion.

Conversely, CD44 is also utilized as a targeting marker of HA-drug-nanocomposite complex. The combination of HA and docetaxel (DTX), loaded in polymeric nanoparticles (NPs), improved the effect of drug delivery by targeting CD44+^{high} BC cells (Gaio et al., 2020). Similarly, a HA-NPs complex loaded with paclitaxel (PTX) was well designed to target CD44

for improvement of chemotherapeutic effects in metastatic cancer (Lv et al., 2018b). These results demonstrate the important role of CD44 in BC stemness, invasion, metastasis, and drug resistance. We should aim at significantly reversing drug resistance through the use of nano-drug combinations, improving drug efficacy, and ultimately, ensuring a favorable prognosis.

CD133

CD133, known as Prominin-1, is independently expressed on the surface of stem cells and various tissue tumor stem cells. Similar to CD44, CD133 BC cells show stem-like properties and are found to be enrich in basal-like, triple negative, HER2+ or luminal tumors (Borgna et al., 2012).

xenograft-initiating $CD44^{pos}CD49f^{high}CD133/2^{high}$ cells among ER-negative tumors were capable of forming ER-negative tumors (Meyer et al., 2010), supporting the evidence that CD133 is an identifier molecule for BCSCs with high aggressive properties.

The accumulation of $CD133^{high}$ BCSCs aggravated BC and tended to induce drug-resistance (Bousquet et al., 2017), proliferation (Brugnoli et al., 2017), vasculogenic mimicry (Liu et al., 2013), invasion, and metastasis (Bock et al., 2014). For instance, heterogeneous BC cells with CD133 marker displayed resistance to drugs and the potential to form a mass in NOD/SCID mice (Wright et al., 2008). Moreover, $CD133^{high}$ BCSCs were enriched in the tumors of hormonal therapy (HT)-resistant BC, forming metastatic luminal BC by self-renewal during HT (Sansone et al., 2016). The capability of self-renewal can be switched through re-expression of estrogen receptor (ER) by inhibition of IL6R/IL6-Notch pathways (Sansone et al., 2016). Furthermore, a ribonucleoprotein complex (LncRNA MALAT1 and HUR) down-regulated the expression of CD133+ phenotype and inhibited the stem cell properties of BCSCs, leading to tumorigenesis and metastasis failure both in MCF-7 and MDA-MB-231 (Latorre et al., 2016), revealing the indirect mechanism of CD133 and drug resistance in BC.

Recently, a novel CD133-targeting drug delivery system that uses nanoparticles loaded with drugs was reported. An anti-CD133 antibody into nanoparticles loaded with paclitaxel, increased the accumulation of paclitaxel in CD133+ cells, decreased the population of BCSCs, and inhibited the tumorigenic ability *in vivo* (Swaminathan et al., 2013). This implies that CD133-targeting will contribute to the development of BCSC-targeting therapeutics to reverse drug resistance.

EpCAM

EpCAM, a type I transmembrane glycoprotein, is known as a phenotype of epithelial tumors and is overexpressed in BCSCs (Munz et al., 2009). EpCAM can promoting BCSCs survival through the activation of Wnt/β-catenin signaling pathway (Sena and Chandel, 2012). It can also promote adhesion between epithelial cells, playing an important role in migration and metastasis. For example, $EpCAM^+$ disseminated tumor cells (DTCs), isolated from the peripheral blood of BC patients, contained a class of metastatic initiating BC cells that could cause bone, lung, and liver metastases in NOD-SCID mice (Baccelli et al., 2013). Moreover, EpCAM still plays an important role in reversing resistance. For instance, Survivin silencing, mediated by EpCAM aptamer, can make BCSCs sensitive to doxorubicin, leading to the reversal of resistance, which indicates that this novel strategy is an effective method to reverse drug resistance in BC (Wang T. et al., 2015).

ALDH1

ALDH1 is an NAD(P)+ dependent enzyme that mediates the oxidation of intracellular aldehydes into carboxylic acids. ALDH1 acts as a common marker of both normal and malignant breast stem cells, especially in BCSCs. ALDH1-high activity is an independent predictor of progression and poor survival of BC patients (Ginestier et al., 2007). Moreover, CD44+/CD24−/ALDH1+ MDA-MB-231 and CD44+/CD133−/ALDH1+ MDA-MB-468 BC cells demonstrated stronger tumorigenic and metastatic capabilities than $ALDH1^{low}CD44^{low}$ BC cells (Croker et al., 2009).

However, ALDH activity of BCSCs was mainly dependent on ALDH1A3, rather than on ALDH1A1 (Marcato et al., 2011), further enhancing the understanding of specific targets of BCSCs. The main explanation for this difference is that the expression level of ALDH1A1 in breast epithelial cells is lower than that of ALDH1A3. The strong association between LDH1A3 high expression and metastasis in BC patients was also reversed to confirm the importance of ALDH1A3 in BC. Contrarily, NOTCH signaling pathway increased ALDH1A1 Lys-353 deacetylation at a post-translational level through the induction of silent information regulator 2 (SIRT2) expression, promoting tumorigenesis and tumor growth in a BC model (Zhao et al., 2014). Conversely, inhibition of ALDH activity resulted in drug (doxorubicin/paclitaxel) resistance reversal in $ALDH^{high}$ $CD44^+$ BCSCs (Croker and Allan, 2012). Therefore, these studies reveal that ALDH1 not only can be utilized to distinguish BCSCs, but also as a potential therapeutic target for drug resistance reversal in BC. ALDH1 regulation might be useful in explaining drug resistance in further research.

CXCR4

As a specific receptor of stromal cell-derived factor-1 (SDF-1), CXC chemokine receptor 4 (CXCR4) is essential for BCSCs-related metastasis. The SDF-1/CXCR4 signaling pathway mediates the role of promoting the directional metastasis of CXCR4+ BCSCs. Both antibody neutralization and CXCR4 knockdown inhibited the proliferation of orthotopically transplanted breast tumor and metastasis (Muller et al., 2001). Non-metastatic BCSCs promote the transformation of non-BCSCs to $CXCR4^+$ BCSCs in BC tissues (Mukherjee et al., 2016). Besides, CXCR4+ BCSCs displayed decreased vimentin and increased E-cadherin, indicating the occurrence of epithelial-mesenchymal transitions (EMT). These findings demonstrate that $CXCR4^+$ BCSC triggered EMT-related metastasis.

BC metastasis is closely related to drug resistance, so CXCR4 may be a key factor of reversins drug resistance. CXCR4 is also closely related with tumor microenvironmental changes. CXCR4 is highly expressed in BC metastases; thus, I.X. found that suppressed CXCL12/CXCR4 signaling pathway or silenced

CXCR4 in BCSCs sensitizes BC to immune checkpoint blockers, inhibiting metastasis reversing drug-resistance in BC (Chen I. X. et al., 2019). In a similar mechanism, DPP-4 inhibitors were found to reverse drug-resistance via ABC transporters-mediated CXCL12/CXCR4/mTOR/TGFβ axis in BC cells (Li et al., 2020). An innovative strategy, consisting of an oncolytic virus loaded with a CXCR4 antagonist, was utilized for targeting the CXCL12/CXCR4 signaling pathway, being remarkably effective in primary and metastatic BC (Gil et al., 2013). Furthermore, the activation of SDF-1/CXCR4 signaling pathway can increase the phosphorylation of 60 proteins with migration or invasion properties in BC, which might be key mediators for CXCR4-induced BCSCs proliferation (Yi et al., 2014). These evidences emphasized CXCR4 as a therapeutic target to inhibit microenvironment-induced stemness and the appearance of metastatic phenotypes and made it possible to eradicate the activation of CXCR4-related signaling pathway, decreasing the proportion of CXCR4+ BCSCs.

ABCG2

As a known drug-resistant protein, ABCG2 is highly expressed in BC resistant cells, especially in resistance-related BCSCs. Sun found that stem-like $CD44^+CD24^{-/low}$ cells isolated from several BC cell lines, such as SK-BR-3, MDA-MB-231, and MCF-7 displayed a higher expression of ABCG2 than non-stem cells (Sun et al., 2015). Furthermore, ABCG2 is considered to be a more effective surface marker for BCSCs identification than $CD44^+CD24^-$ (Leccia et al., 2014). Moreover, several pieces of evidence have highlighted ABCG2 as a therapeutic target to overcome BC multidrug resistance. For instance, downregulation of either Rab5A or Rab21 increases surface expression of ABCG2 and efflux of intracellular drugs, reversing BC drug-resistance (Yousaf and Ali, 2020). Moreover, it has also been demonstrated that drug resistance can be reversed by ABCG2 modulators at a molecular level (Hasanabady and Kalalinia, 2016; Pena-Solorzano et al., 2017). However, few small molecule modulators have shown to be effective in preclinical trials. Therefore, the role of ABCG2 inhibitors in reversing resistance by mediating BCSCs should be re-examined and more *in vivo* evidence should be presented.

GD2

GD2, a b-series ganglioside, is another cell membrane phenotype of BCSCs. Indeed, $GD2^+$ BC cells, isolated from either BC cell lines or clinical tumor tissue in BC patients, show stemness. Meanwhile, it has been revealed that GD2+ cells, human mammary epithelial cells-derived GD2+ cells, were highly $CD44^+CD24^-$ (Battula et al., 2012). GD3 synthase, a rate-limiting enzyme, regulates the synthesis of GD2 and is considered a kind of therapeutic target for BCSCs. GD3S was positively correlated with the expression of GD2+ in BCSCs, and the low expression of GD3S not only resulted in the decreased expression of GD2+, but also disrupted EMT-mediated tumor formation ability of BC cells (Liang et al., 2017). Consistently, another study indicated that the high expression of GD3S was closely associated with the activation of nuclear factor kappa-B (NF-κB) in $GD2^+$ BCSCs. The Inhibition of NF-κB signal can significantly reduce the expression of GD3S and the proportion of $GD2^+$ BCSCs, abolishing the capability of BCSCs to metastasize (Battula et al., 2017). Based on the correlation between BCSCs, GD3S, and GD2, the development of GD3S-related signals as a novel therapeutic target may induce BCSCs to reverse drug resistance.

CENTRAL SIGNALING PATHWAYS IN BREAST CANCER STEM CELLS

As mentioned above, surface markers play an important role in maintaining the stemness of BCSCs, but they can't work independently of intercellular signaling pathways. Here, we continue to describe the activation of several key intracellular signaling pathways in BC, as a result of gene mutation, epigenetic modifications, or tumor microenvironment changes, which generate drug resistance-related BCSCs. Therefore, understanding the relevant pathways can contribute to better understand the characteristics of BCSCs and determine the research direction of reversing drug resistance targeted therapy. Major mechanisms of drug resistance in BCSCs are shown in **Figure 3**

Notch Signaling Pathway

Notch signaling pathway is one of the regulative mechanisms of BCSCs' self-renewal and survival. Cytokine IL-6 regulates Notch signaling, and the increase of IL-6 was detected in BC treated with HT, activating the Notch3 signaling in BC cells. The activation of Notch3 signaling enables BC cells to self-renew instead of the ER-dependent survival mechanism, thus impacting clinical efficacy of HT. However, inhibiting Notch signaling significantly reduced the self-renewal ability of $CD133^{high}ER^{low}$ BCSCs in HT-resistant cells (Sansone et al., 2016). Similarly, another study indicated that the combination of MK-0752 (gamma secretase inhibitors) and Tocilizumab (IL6R antagonist) remarkably decreases the proportion of BCSCs and inhibits cell proliferation or tumor growth in BC, through Notch3 signaling pathway (Wang D. et al., 2018).

Moreover, emerging evidence suggested that BCSCs mediate drug resistance in BC through Notch-related signaling pathway. For example, the activation of Notch signaling pathway promotes the appearance of stem cell phenotype in $ERα/ESR1^+$ BC cell lines and causes drug resistance to ET for BC (Gelsomino et al., 2018). Consistently, the activation of JAG1-NOTCH4 signaling pathway stimulates BCSCs activity and generates anti-estrogen resistance in BC (Simoes et al., 2015). In particular, Notch1 also mediated trastuzumab resistance in BCSCs by inhibiting PTEN expression to cause the activation of ERK1/2 signaling. Notch1-PTEN-ERK1/2 signaling might be a target for the novel therapy strategies of combining anti-Notch1 and anti-MEK/ERK to reverse trastuzumab resistance (Baker et al., 2018).

Wnt/β-Catenin Signaling Pathway

Wnt/β-catenin signaling pathway also plays an important role in BCSCs self-renewal. A previous study has shown that Wnt/β-catenin signaling pathway was deemed as a key mechanism

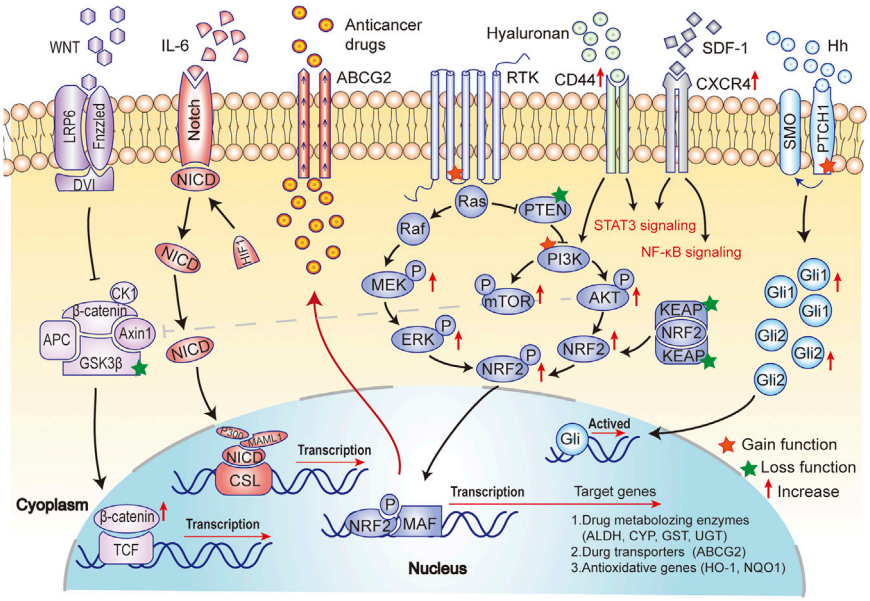

FIGURE 3 | Major mechanisms of drug resistance in BCSCs. Drug resistance is not only a result of the activation of the self-renewal (Notch and Hh signaling pathway) and anti-apoptotic (PI3K/AKT/mTOR signaling) in BCSCs, but also a consequence of the promotion of metastasis (EMT and Wnt/β-catenin signaling pathway), anti-oxidative activity (NRF2 signaling) and ATP-binding cassette (ABC) transporter (ABCG2) activity in BCSCs.

of Sam68- mediated self-renewal in BC cells (Wang L. et al., 2015). Another study displayed that Gomisin M2 remarkably inhibited BCSCs self-renewal by suppressing the Wnt/β-catenin signaling pathway (Yang Y. et al., 2019). Compared to other cells, the higher level of Wnt/β-catenin signaling pathway contributes to the high resistance level of BCSC. CWP232228, a small-molecule of Wnt/β-Catenin inhibitor, suppressed the proliferation of BCSCs by inhibiting β-catenin-mediated transcription (Jang et al., 2015). Furthermore, this result implied that Wnt/β-catenin might indirectly regulate drug resistance by BCSCs self-renewal or proliferation, promoting Wnt/β-catenin as a therapeutic target for BCSCs therapy in the future.

PI3K/AKT/mTOR Signaling Pathway

The activation of the phosphatidylinositol 3-kinase (PI3K)-related signaling pathway in BCSCs was reported in recent years and can be contribute to drug resistance in BC. Mounting evidence demonstrated that PI3K/AKT/mTOR signaling pathway has an important role on ET-resistance in ER+ BC (Droog et al., 2013; Ojo et al., 2015). PI3K/Akt/mTOR signaling pathway induces BCSCs metastasis by CD44 regulation. Moreover, the interaction of PI3k and Wnt/β-catenin signaling maintain the self-renewal and stemness abilities of BCSCs (Solzak et al., 2017).

Impressively, inhibition of PI3K/AKT/mTOR signaling pathway reduces BCSC survival and self-renewal. In other words, the inhibitors of PI3K/AKT/mTOR signaling pathway impact the activity of BCSCs. For instance, IGF-1R, a stemness marker, is associated with BC disease progression. Inhibitors of IGF-1R and its downstream PI3K/Akt/mTOR signaling pathway reduced the population of BCSCs. Therefore, IGF-1R/PI3K/Akt/mTOR signaling pathways are favorable targets for the treatment of BCSCs (Chang et al., 2013). Indeed, rapalogs, such as NVP-BEZ235 and NVPBGT226, were utilized as inhibitors of both PI3K and mTOR. Meanwhile, the inhibition of PI3K by rapalogs could stimulate GLP1-mediated stem-like features in BC cell lines, as the BCSCs generated imply rapalogs resistance in BC (Posada et al., 2017). Hence, future research is necessary to elucidate the relationship between the distinct mechanisms of drug resistance and the regulation of PI3k-mediated BCSCs.

Hedgehog Signaling Pathway

The activation of Hh signaling pathway is essential to balance tissue homeostasis and self-renewal in BC. Generally, Hh signaling pathway is up-regulated in BCSCs, which may be involved in stemness maintenance. However, there are few pieces of evidence to understand the abnormal activation of Hh signaling pathway in BCSCs. Interestingly, SHH (Sonic Hedgehog), secreted by BCSCs, regulated cancer-associated fibroblasts (CAFs) via the activation of Hh signaling (Valenti et al., 2017), promoting BCSCs proliferation and self-renewal. Furthermore, Shh (Sonic Hedgehog)-mediated Hh signaling activation gives rise to salinomycin resistance (He et al., 2015). Conversely, inhibition of the Hh signaling pathway could sensitize BCSCs to paclitaxel by cyclopamine (He et al., 2015). So far, rare inhibitors of Hh signaling pathway were applied to regulate BCSCs. Thus, further studies on the activation mechanisms of Hh signaling pathway-related stemness maintenance or resistance in BCSCs are needed to identify drugs that target Hh signaling pathway for reversing drug resistance.

RESISTANCE TO CANCER THERAPY

Resistance to Chemotherapy

Chemotherapy is an important part of BC routine treatment. Effective neoadjuvant chemotherapy helps patients to reduce tumor burden and clinical stage and provides opportunities for breast conserving surgery. Moreover, accumulating evidence indicated that advance BC patients benefit from chemotherapy. However, recent studies showed that the phenomenon of BCSCs enrichment occurs after chemotherapy in BC. Therefore, we focus on various chemotherapeutic drugs, listing the specific relationship between BCSCs and drugs, understanding the mechanism of chemotherapy resistance and summarizing the potential therapeutic strategies to reverse drug resistance.

Paclitaxel Resistance

Paclitaxel, a microtubule stabilizer, is widely used in BC clinical chemotherapy. It can keep the cells in the G2/M phase to inhibit the cell cycle (Horwitz et al., 1986). Unfortunately, paclitaxel resistance is becoming a clinical challenge in BC treatment. The mechanisms of paclitaxel resistance are the following. First, paclitaxel-resistant cells have the mutant microtubule binding sites, which can impact tubulin expression. Mutations in microtubule-related proteins (e.g., βI-tubulin (Giannakakou et al., 1997) and βIII-tubulin (Magnani et al., 2006)) contributed to paclitaxel resistance. Secondly, the expression of transporters, such as ATP-binding cassette transporter MDR-1/P-gp (ABCB1) (Genovese et al., 2017), BCRP (ABCG2) (Arnason and Harkness, 2015; Robey et al., 2018), which are required for paclitaxel resistance, is abnormal. Paclitaxel-resistant cells exhibit stem-like properties (Bumbaca and Li, 2018). Some scholars insist that tumor stem cells can resist to chemotherapy, and that a higher expression of CD44+/CD24- tumors displayed greater resistance to neoadjuvant chemotherapy (Creighton et al., 2009; Marotta et al., 2011). Recently, Tanei found that ALDH1 is enriched in chemotherapy resistance cells (Tanei et al., 2009). Interestingly, ALDH1 and CD44 were utilized as important surface markers to isolate BCSCs.

Recently, with the going research between BCSCs and paclitaxel resistance, scientists often focus on the biological metabolism of BCSCs with a unique perspective. Lee discovered that the interaction of MYC and MCL1 regulated the production of reactive oxygen species (ROS) and participated in mitochondrial oxidative phosphorylation (OXPHOS), further activating the HIF-dependent hypoxia pathway and enhancing the enrichment of BCSCs and paclitaxel resistance (Lee et al., 2017). Similarly, the laboratory of Dr. Samanta investigated and verified that, after paclitaxel or gemcitabine chemotherapy, BCSCs increased activity and expression of HIF-1α and HIF-2α through the paclitaxel-ROS-HIF-IL-6/IL-8 axis after chemotherapy (paclitaxel or gemcitabine) (Samanta et al., 2014). Consequently, HIF-mediated downstream signaling pathways will become a crucial target for paclitaxel resistance in BCSCs. Ultimately, IL-6 and IL-8, paclitaxel-induced, increased BCSCs enrichment and drug resistance through the STAT3 (Marotta et al., 2011) and TGF-β pathways (Bhola et al., 2013), respectively. Thus, the intrinsic relationship between STAT3 signaling and TGF-β pathway can also be an important target to regulate BCSCs to reverse drug resistance. In summary, paclitaxel resistance is not only related to its unique metabolic pathway, but also to the biological behavior of BCSCs.

Anthracyclines Resistance

Anthracyclines, inhibitors of topoisomerase II (TOPO II), are a broad-spectrum chemotherapy drugs, including doxorubicin and epirubicin, which are widely used in BC chemotherapy. Nevertheless, the emergence of drug resistance often caused the failure of anthracyclines chemotherapy. Emerging studies have shown that anthracyclines could exhibit different drug resistance patterns in different parts of cells (Capeloa et al., 2020): on the cell envelope, ATP-binding cassette transporter can decrease the concentration of intracellular anthracyclines (Gottesman et al., 2002; Sun et al., 2015). In the cytoplasm, alterations in apoptosis (Gyorffy et al., 2005) and autophagy (Liu et al., 2011; Sun et al., 2011) pathways impact the cytotoxic effects of anthracyclines in the cytoplasm; in the nucleus, gene mutations regulate the expression and activation of TOPO II, inhibiting the effect of anthracyclines-induced DNA damage and promoting anthracyclines resistance (Press et al., 2011; Wijdeven et al., 2015). These resistance-related proteins or pathways above are affected by metabolism. Thus, anthracyclines metabolism impacts the sensitivity of BC to anthracyclines. Many $CD44^{+}$ or CD133+ BCSCs are enriched in tumors under anthracyclines therapy in BC (Jia et al., 2016). Other studies have shown that BCSCs could effectively remove DNA damage caused by chemotherapeutic drugs (Nicolay et al., 2016), and that the dysregulation of Annexin A3 (ANXA3) changed the sensitivity of BCSCs to doxorubicin (Du et al., 2018). These evidences support the role of BCSCs in anthracyclines resistance, and further studies on the therapeutic targets of BCSCs to reverse anthracyclines resistance should be performed.

Platinum Resistance

Platinum is one of the most common drugs for advanced BC because of its DNA-damaging properties. It interacts with DNA at guanine and adenine nucleotides to form Pt–DNA nonfunctional adducts that destroy double-stranded the DNA template and inhibit the division of tumor cells. However, platinum is not considered an option if progression of disease occurs during platinum-based chemotherapy. Unfortunately, only 47% of advanced BC patients are sensitive to platinum drugs (Sledge et al., 1988). Fortunately, mounting studies show that platinum resistance is associated with BCSCs. For instance, Disulfiram could improve the cytotoxic effect of cisplatin by reversing BCSCs-mediated cisplatin resistance. Meanwhile, Disulfiram exhibited difference ability to eliminate ROS between BCSCs and non-BCSCs (Yang Z. et al., 2019). Coincidentally, more than one researcher suggested that the stem-like BC cells are modulated by ROS (Nguyen et al., 2020; Nourbakhsh et al., 2020). These results implied that ROS could affect platinum resistance by regulating BCSCs. Besides, Xu proposed that IL-6 enhances resistance to cisplatin via the activation of STAT3 pathway in BC (Xu et al., 2018).

Although STAT3 has been shown to induce BCSCs, it is unclear that IL6/STAT3 signaling pathway may affect the resistance to platinum by BCSCs modulation.

Capecitabine Resistance

Capecitabine is commonly used as a chemotherapy drug for advanced second-line BC. The cytotoxic effect of capecitabine is triggered by 5'-furan and thymidine phosphorylase. Therefore, low activity of thymidine phosphorylase led to capecitabine resistance in tumor tissues (Ishikawa et al., 1998). However, few pieces of evidence indicated the relationship between BCSCs and capecitabine resistance as the consequence of capecitabine metabolism complexity.

Based on clinical observations, multidrug resistance is the main form of chemotherapy resistance. For example, paclitaxel-resistant BC often shows resistance to anthracycline at the same time (Lee et al., 2006). The main reason is that ATP-binding box transporters take part in both paclitaxel and anthracycline metabolisms, increasing the expression of drug-resistant proteins, such as MDR-1 (Genovese et al., 2017). Meanwhile, studies found that BCSCs that have DNA mismatch repair function ability, caused resistance to both anthracycline and platinum chemotherapy, but failed to resist to paclitaxel (Fedier et al., 2001). In brief, multiple pathways in BCSCs regulated the activation of metabolism and induced resistance to multiple chemotherapeutic drugs in BC, such as paclitaxel, anthracyclines, platinum, and capecitabine. Thus, it is expected that highly effective drugs targeting BCSCs emerge as a new therapeutic strategy for multi-chemotherapeutic resistance.

Resistance to Endocrine Therapy

Endocrine therapy (ET) is a highly effective treatment for estrogen receptor (ER) positive BC by blocking ER pathway and depriving the tumor of estrogen (Howell, 2008). As a matter of fact, the ER signaling pathway is a complicated biological pathway that regulates many functions, such as cell proliferation, invasion, and angiogenesis, and is used as a crucial survival pathway by BC cells (Manavathi et al., 2013). Different endocrine therapies work by various mechanisms, which can be divided into three different categories: selective estrogen receptor modulators (SERMs), aromatase inhibitors (AI), and CDK4/6 inhibitors. Currently, evidence continues to show that BCSCs are responsible for tumor evolution and play a crucial role in achieving ET resistance (Dey et al., 2019; Rodriguez et al., 2019).

Tamoxifen Resistance

Tamoxifen is one of the most famous selective ER modulators, which can antagonize the effects of estrogen and bind in the ER pathway to some particular target genes (Frasor et al., 2004). Thereby, adjustment of each element or transcription in ER pathway can mediate resistance to endocrine treatment by modulating ER activity or by acting as an escape pathway. Primitively, the increase of BCSCs in advanced BC indicated their potential role in tumorigenesis and tamoxifen resistance (Pece et al., 2010). Further, recent studies demonstrated that tamoxifen resistant MCF-7 (TAM-R) cells contained a higher proportion of BCSCs than non-resistant cells (Wang et al., 2012). Therefore, we speculate that BCSCs may play an important role in endocrine resistance, and accumulating studies have confirmed this.

Recent studies provide more direct evidence on BCSCs participating in tamoxifen resistance through some important pathways. The ER signaling pathway functions as a major mechanism responsible for tamoxifen resistance. The expression of ER splicing variants, such as the estrogen related receptors and the identified short variant ERα36, have also contributed to a poor tamoxifen response (Zhang and Wang, 2013). Although considered ERα negative, BCSCs can still be stimulated by estradiol via paracrine mechanisms. A study also showed that ERα could mediate the rapid estrogen signaling in BCSCs and enhance transcription of genes related to stem cells (Gelsomino et al., 2018). ER could also promote the development of BCSCs via a crosstalk with Sox2 (Zhang Y. et al., 2012). In return, Sox2 could promote the non-genomic estrogen-stimulated activity of ER, thus inducing ER phosphorylation at Ser118 site (Zhang Y. et al., 2012; Vazquez-Martin et al., 2013). In fact, phosphorylation, ubiquitination, and other post-translational modifications of ER and its co-regulators affect the sensitivity to different endocrine therapies (Musgrove and Sutherland, 2009). However, the role of estrogen receptors β (ERβ) in BCSCs is still partly unclear, requiring further experiments to explore its relationship with endocrine resistance and BCSCs.

Another important category of pathways involved in endocrine resistance is the growth factor family. Up-regulation of EGFR, HER2, FGFR, and IGF1 receptors (IGF1R) could activate the downstream signaling pathway, especially PI3K pathways, causing tamoxifen resistance (Chakraborty et al., 2010; Arpino et al., 2008). Lately, using gene expression analysis, it was revealed that the activation of the PI3K/AKT/mTOR pathway and the inactivation of the PTEN tumor suppressor were the major alterations in MCF7 cell-derived BCSCs-enriched cells, compared to non-enriched cells. Down-regulation of PI3K, AKT1 and PI3K/mTOR reduced the self-renewal and survival of BCSCs *in vitro* and their tumor initiation and self-renewal ability *in vivo* (Gargini et al., 2015). In general, these data suggest that some regulators, such as IGF1R and PI3K, may be potential targets to recover the resistance to tamoxifen by restraining BCSCs survival and activity.

Alterations in genes involved in stemness-related pathways, such as Wnt/β-catenin, Notch, and Sonic Hedgehog, have been proven highly effective in acquiring tamoxifen resistance. According to recent studies, activation of Wnt and Notch signaling pathways induced tamoxifen resistance and promoted BCSCs activity in MCF-7 (TAM-R) cells, while inhibition of these pathways abolished the resistance (Magnifico et al., 2009; Loh et al., 2013; Lombardo et al., 2014), supporting the important role of BCSCs in endocrine-independent and TAM-resistant proliferation. Furthermore, clinical data demonstrated that upregulation of the HH signaling was related with a reduction in overall survival and recurrence-free survival in estrogen receptor positive BC patients, even leading to tamoxife resistance (Ramaswamy et al., 2012). By contrast, the stem cell-like population, cell migration, and

invasion declined greatly by the inhibition of the HH signaling, thus preventing the progress of tamoxifen resistance (Ramaswamy et al., 2012). Collectively, accumulating evidence reveals complicated mechanisms with overlapping networks of tamoxifen resistance, which partly results from BCSCs-induced evolution, regulated by Notch, Wnt/β-catenin, HH, and other crucial signaling pathways.

Fulvestrant Resistance

Fulvestrant, a new kind of ER downregulator, can effectively reduce the level of ER in BC cells (Dowsett et al., 2005). Actually, fulvestrant was identified as an effective antagonist to endocrine-sensitive BC after failure of previous tamoxifen or aromatase inhibitor therapies (Howell and Robertson, 1995). Although the detailed mechanisms of fulvestrant resistance remain unclear, some pathways, including EGFR/ErbB2, MEK/ERK, NF-kB, PI3K-AKT, and β-catenin, have been associated with development of fulvestrant resistance (McClelland et al., 2001; Gu et al., 2002; Fan et al., 2006). It is interesting that these proteins and pathways are also correlated with the induction and maintenance of BCSCs (Hardt et al., 2012; Harrison et al., 2013; Luo et al., 2015; Majumder et al., 2016). Therefore, we speculate that BCSCs may mediate fulvestrant resistance through these pathways, but further evidence is needed to prove this.

Studies showed that resistance was associated with G protein-coupled estrogen receptor-1 (GPER) and CDK6 overexpression (Giessrigl et al., 2013; Alves et al., 2016). GPER, mediating estrogen-induced proliferation breast epithelial cells, is also essential for the survival of BCSCs (Chan et al., 2020). Recently, a study showed that microRNA-221 contributed to fulvestrant resistance via activation of β-catenin in BC and promoted the generation of BCSCs, stimulating the production of an invasive phenotype that predicts adverse outcomes (Roscigno et al., 2016). Unfortunately, few studies on fulvestrant resistance have been reported; however, the relationship between fulvestrant resistance and BCSCs may become clearer with future research.

Aromatase Inhibitors Resistance

Aromatase inhibitors (AIs) can inactivate aromatase, block aromatase reaction, inhibit estrogen production, and reduce estrogen levels in the blood, being an ideal ET drugs for ER+ BC in postmenopausal women. Three AIs, such as exemestane, letrozole, and anastrozole, exhibited similar resistance mechanisms in ET (Francis et al., 2015). Besides, AIs could modulate the action of androgen through the androgen receptor (AR) as well, thereby inhibiting estrogen-dependent BC growth (Macedo et al., 2006; Takagi et al., 2010). The application of AIs greatly reduced the risk of BC recurrence among postmenopausal women (Magnani et al., 2013). However, AIs resistance inevitably reduces clinical benefits. Multiple mechanisms contribute to AI resistance, involving either estrogen-independent ER growth or ER-independent activation. Among these, the PI3K pathway is a significant therapeutic target. A previous study revealed that these BCSCs showed low ER expression and the activation of PI3K signaling pathway (Hardt et al., 2012), both of which eventually led to AIs resistance (Marsden et al., 2009). Actually, the alpha-specific PI3K inhibitors, such as buparlisib, alpelisib, and taselisib, were currently utilized as novel drugs for AIs-resistant BC in phase III clinical trials (NCT02437318, NTC01610284, NCT02340221).

Stromal cells, extra-cellular matrix (ECM), and other micro-environment conditions (such as hypoxia and acidity) are also responsible for the generation of BCSCs phenotypes and endocrine (AI and TAM) resistance (Generali et al., 2006; Semenza, 2015). A lot of soluble factors that promote tumor growth and vascularization, such as transforming growth factor-β (TGFβ), which induces epithelial-to-mesenchymal transition (EMT), are secreted by cancer-associated fibroblasts (CAFs). Furthermore, downstream signaling pathways, especially PI3K and MAPK pathways, are activated by EGFR and CXCR4, thus inducing endocrine resistance (Loh et al., 2013; Ma et al., 2015). Additionally, CXCR4 was found to enhance BCSCs self-renewal by the activation of PI3K/AKT and MAPK pathways and promoted tumorigenesis through hydrocarbon receptor (AhR) signaling (Dubrovska et al., 2012). Mesenchymal stem cells (MSCs) protected cancer cells from hormone treatment through direct cell interaction and by secreted proteins (Rhodes et al., 2010). In conclusion, the tumor microenvironment is frequently linked to endocrine resistance, partly due to self-renew and maintenance of BCSCs.

Resistance to Targeted Therapy

HER2 is a receptor tyrosine kinase which is over-expressed or genetically amplified in 15–25% of invasive BCs. As we have seen, anti-HER2 drugs, such as trastuzumab and lapatinib, have obviously improved clinical outcomes in HER2-positive BC patients. Yet the emergence of resistance to anti-HER2 drugs becomes a main barrier during the treatment of HER2-positive BC. In order to improve the prognosis of HER2-positive BC patients, it is essential to study the mechanisms of resistance to anti-HER2 therapy (Chihara et al., 2017). Several observations suggested that the resistance to anti-HER2 drugs may be driven by CSCs (BCSCs) (Martin-Castillo et al., 2013; Seo et al., 2016). Therefore, we would like to find out how BCSCs participate in resistance to anti-HER2 drugs in HER2-positive BC.

Trastuzumab Resistance

Trastuzumab is a molecular targeting drug for HER2 tyrosine kinase receptor. The application of trastuzumab has dramatic therapeutic efficacy in HER2+ BC, but the emergence of drug resistance hinders its clinical benefits. Multiple evidence shows that the mutation of PI3KCA (Berns et al., 2007; Dave et al., 2011) and loss of PTEN (Nagata et al., 2004; Koninki et al., 2010; Gallardo et al., 2012) leads to trastuzumab resistance in BC. Indeed, trastuzumab resistance was also associated with CSCs. CSCs may induce drug resistance via the activation of PI3K/AKT, JAK/STAT3 and NF-kB pathways (Wang et al., 2017). Meanwhile, PTEN loss and PI3KCA mutation could lead to abnormal activation of the downstream PI3K/Akt/mTOR pathway, which in turn, regulates BCSCs pool (Dey et al., 2019). Similarly, PTEN down-regulation increased BCSCs population through Akt activation of Wnt signaling pathway

(Korkaya et al., 2012). We can speculate that the loss of PTEN and the mutation of PI3KCA lead to the activation of downstream PI3K/Akt/mTOR pathway in BCSCs, which results in trastuzumab resistance. Another mechanism of trastuzumab resistance was the activation of IL-6 inflammatory loop mediated BCSCs expansion, resulting in drug resistance of BC to trastuzumab. Meanwhile, IL-6 was found to inhibit PTEN when activating Akt, STAT3, and NF-kB pathways (Korkaya et al., 2012). Interestingly, STAT3 activation led to an increase in stem cell properties, which caused over-expression of HER2 and trastuzumab resistance (Chung et al., 2014). Thus, targeting upstream of JAK/STAT3 pathway, for instance IL-6 receptor antibody, could inhibit trastuzumab resistance and reduce the CSC population. A previous study showed that an excellent functional biomarker for trastuzumab resistance is Mucin1 (MUC1), and its cleaved form is named MUC1* (Sand et al., 2020). Interestingly, anti-MUC1* was found to have a dramatic, stimulatory effect on stem cell growth (Hikita et al., 2008). Fessler demonstrated a significant increase in the number of MUC1* in trastuzumb resistant cell lines (Fessler et al., 2009). In conclusion, MUC1* may be a target for reversing drug resistance of trastuzumab. Among these mechanisms, it is not difficult to find that CSCs are critical in trastuzumab resistance. Thus, the BCSCs-targeted strategy may be worth further research in recovering sensitivity of trastuzumab in BC, and may bring benefits to patients at risk of BC recurrence.

Lapatinib Resistance

Lapatinib is an oral small molecule drug, which targets both epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2). Its resistance involves many factors, such as the pathways of receptor tyrosine kinase, non-receptor tyrosine kinase, CSCs, microRNA, tumor metabolism, among others (Shi et al., 2016). MiR-205-5p is a highly conserved miRNA involved in cell differentiation, migration, and proliferation, which was found to be highly expressed in BCSCs. Moreover, it leads to lapatinib resistance by directly repressing HER2 and indirectly inhibiting EGFR (De Cola et al., 2015; Xiao et al., 2019). It was speculated that the lapatinib resistance caused by miR-205 was via the activation of PI3K/AKT signaling pathway. Therefore, down-regulating the expression of miR-205-5p contributed to inhibit the lapatinib resistance in BCSCs. The other resistance mechanism for lapatinib was associated with CD44+/CD24−, which are surface markers of CSCs (Dey et al., 2019). Knocking down CD24 could not only increase the sensitivity of HER2-positive BC cells to lapatinib, but also inhibit Akt phosphorylation (Hosonaga et al., 2014). For this reason, CD24 may be a target to reverse lapatinib resistance in BC. Actually, the use of lapatinib greatly improves BC prognosis. Nevertheless, clinical evidence suggested that lapatinib resistance led to poor therapeutic efficacy in HER2-positive BC patients. As described in the above mechanisms, CSCs seem to be the key to solve lapatinib resistance. Consequently, further understanding of the regulatory mechanisms of CSCs in lapatinib resistance in BC is essential for developing targeting strategies.

Here, we summarize the resistance mechanism of anti-HER2 drugs. The review suggested that the resistance of anti-HER2 drugs usually occurred by inducing CSC characteristics. TGFβ is a transforming growth cytokine and SMAD is an effector transforming factor in TGFβ signaling pathway. The acquisition of malignant features, such as EMT, cancer cell stemness, and drug resistance in cancer cells was closely related to TGFβ-SMAD3 signaling pathway. Sustained stimulation of TGFβ could induce SMAD3 to phosphorylate intensely and enhance the CSC traits of BC, thereby leading to HER2-positive BC resistance. Therefore, TGFβ-SMAD3 pathway plays a vital role in inducing and maintaining resistance to anti-HER2 drugs (Chihara et al., 2017). BCSCs undoubtedly participate in the process of resistance to HER2-positive BC too. Targeting BCSCs may be a possible way for us to solve the problem of resistance to anti-HER2 drugs.

Therapeutic Strategies for Targeting Breast Cancer Stem Cells to Reverse Resistance

Drug resistance has turned out to be one of major problems in BC therapy, while recent studies found that BCSCs are shown to be the culprit for this phenomenon. Nevertheless, the mechanisms of drug resistance mediated by BCSCs have not been fully understood. Currently, the following vital mechanisms are recognized to be related to treatment resistance, which include overexpression of ATP-binding cassette (ABC) transporter and ALDH1, enhanced DNA repair mechanism, an altered cell cycle, resistance to apoptosis, and all microenvironment influences (Rebucci and Michiels, 2013; Smalley et al., 2013; Cojoc et al., 2015). Therefore, targeting these mechanisms may help us develop new therapies for BCSCs to reverse drug resistance in BC. We will discuss some of the current ways used to target BCSC below. The novel of therapeutic strategies for reversing drug-resistance in BCSCs are displayed in **Table 3** and **Figure 4**.

Targeting Signaling Pathways

Hedgehog (Hh) signaling is a crucial regulator of proliferation, maintenance, and self-renewal of BCSCs. There is a link between the activation of HH signaling and the over expression of MDR1 and ABCG2 in BCSCs. Targeting ABCG2 or MDR1 with cyclosporin A, through inhibition of HH signaling, has shown to regulate and decrease the expression of ABCG2 and ABCG5 (Mao and Unadkat, 2015; Sims-Mourtada et al., 2015). HH signaling showed aberrant activation in Tamoxifen resistant cell lines; instead, knocking down the HH pathway can inhibit growth of tamoxifen resistant cells (Bhateja et al., 2019). Currently, two smoothened (SMO) inhibitors have made their way to clinical trials: GDC-0449 (vismodegib) with paclitaxel, epirubicin, and cyclophosphamide (NCT02694224), and LDE225 (sonidegib) combined with Docetaxel (NCT02027376). Both of the drugs were tested in triple negative BC (Hui et al., 2013; Cazet et al., 2018). It seems that oral HH inhibitors appear to be fairly safe throughout clinical testing.

Confirmatory evidence has recently revealed that the PI3K/Akt/mTOR pathway plays a significant role in regulating BCSC pool. A study observed that Akt signaling altered the subcellular

TABLE 2 | Resistance mechanisms for major drugs in BC therapy.

Drug resistance	Related markers or pathways	Mode of action	*In vitro* or *in vivo* or clinical trial	References
Resistance to chemotherapy				
Paclitaxel	JAK/STAT3-CPT1B-FAO-LPEs	Paclitaxel resistance is regulated by JAK/STAT3-CPT1B-related fatty acid oxidation in BCSCs	*In vitro*	Wang T. et al. (2018)
	MYC/MCL1-(mtOXPHOS) -(ROS) -HIF-1α	paclitaxel resistance is regulated by mitochondrial oxidative phosphorylation (mtOXPHOS) via MYC/MCL1-(mtOXPHOS) - (ROS)-HIF-1αpathway in BCSCs	*In vitro*	(Lee et al., 2017)
	ROS-HIF1/2α-IL-6/IL-8/MDR1	Chemotherapy-induced HIF activity enriched the BCSCs through IL-6 and IL-8 signaling and increased the expression of multidrug resistant proteins (MDR1)	*In vitro*	(Samanta et al., 2014)
	EIF2AK3/EIF2AK4-pEIF2S1-ATF4	Paclitaxel resistance is regulated by redox homoeostasis (ISR) in BCSCs	*In vitro* and *in vivo*	Chen et al. (2019a)
	Jagged2- microRNA-200	Jagged2 promotes the maintenance of BCSCs properties and paclitaxel resistance by regulating the over-expression of microRNA-200	*In vitro* and *in vivo*	Li C. Y. et al. (2018)
	IGF2BP3/CD44-IGF2-Hedgehog signalling	CD44-expressing fibroblasts can inhibit paclitaxel-induced apoptosis, leading to paclitaxel resistance	*In vitro*	Liu Y. et al. (2017)
	ABCB1	Amplification of chromosome region 7q21 coordinated the overexpression of resistance-related proteins and caused cancer cells to develop multidrug resistance.	—	(Genovese et al., 2017)
	ABCB1/ABCG2	Atp binding cassette (ABC) transporter linked to paclitaxel resistance	—	Arnason and Harkness (2015); Robey et al. (2018)
	MTDH/NF-κb signalling	MTDH reduces NF-κB expression and increases p65/p-p65 expression, causing paclitaxel resistance	*In vitro* and *in vivo*	(Yang et al., 2018)
	ERα-activated-DNMT1/DNMT3b	DNMT1 induces DNA methylation and promotes paclitaxel resistance	*In vitro*	(Si et al., 2016)
	MENA/MAPK signalling	MENA subtype expression changes microtubule status after paclitaxel	*In vitro* and *in vivo*	(Oudin et al., 2017)
Anthracyclines	SLC34A2-Bmi1-ABCC5 signalling.	Increases the expression of SLC34A2 in BCSCs induces chemotherapy resistance to Dox through the slc34a2-bmi1-abcc5 signaling pathway.	*In vitro* and *in vivo*	(Ge et al., 2016)
	Glucosylceramide synthase (GCS)	The overexpression of GCS in BC cells is induced by Dox and is related to the pluripotency of BCSCs	*In vitro* and *in vivo*	(Bhinge et al., 2012)
	HIF-2α/BCRP axis	Chemotherapy-mediated HIF-2α directly promotes the expression of BCRP and coordinates the ability of anti-dox in BCSCs.	*In vitro*	(He et al., 2019)
	TOPOII	Mesenchymal stem cells can effectively repair DNA double-strand breaks induced by topoisomerase inhibitors	*In vitro*	(Nicolay et al., 2016)
	ANXA3/NF-κb signalling pathway	ANXA3 overexpression increased the heterogeneity and adriamyclins resistance in BCSCs by the actvation of NF-κB signalling pathway.	*In vitro* and *in vivo*	(Du et al., 2018)
	KLF4 signalling pathway	Adriamyclins chemotherapy increased the expression of CD133, ALDH1A1, ABCG2, and the maintenance of BCSCs characteristics	*In vitro* and *in vivo*	(Li et al., 2017)
Resistance to endocrine Therapy				
Tamoxifen	CD44 + CD24-	High CD44 + /CD24 - ratio is displayed in tamoxifen resistant BC	*In vitro*	(Wang et al., 2012)
	Stem cell markers	Upregulates ALDH, Sox2,Oct4, and CXCR4 in tamoxifen resistant cells	—	(Piva et al., 2014; Gwak et al., 2017; Raffo et al., 2013; Dubrovska et al., 2012; Wang et al., 2012)
	ER signaling pathway	Mutations in the ERα promote the generation of BCSCs markers and induce tamoxifen resistance	*In vitro*	(Gelsomino et al., 2018)
	PI3K/AKT/mTOR signalling	Promotes self-renewal and survival of BCSCs in tamoxifen resistant cells	*In vitro*	(Gargini et al., 2015; Kolev et al., 2015)
	IGFR	Maintains BCSCs surface markers expression and tumorigenesis by the activation of AKT	*In vitro* and *in vivo*	(Chang et al., 2013)
	Wnt/β-catenin pathway	Activation along with the enrichment BCSCs in tamoxifen resistant	*In vitro*	(Loh et al., 2013; Angeloni et al., 2015)
	Notch signalling	Develops tamoxifen resistance via regulating BCSCs	*In vitro*	(Magnifico et al., 2009; Yun et al., 2013)
	IL6/STAT3	Promotes BCSCs and stimulates tamoxifen resistance	*In vitro*	(Wang et al., 2012)
	Hh pathway	Maintains the self-renewal of BCSCs in response to tamoxifen treatment	*In vitro* and *in vivo*	(Ramaswamy et al., 2012)
	TGF-β	Generates the phenotype of BCSCs and induces tamoxifen resistance	*In vitro*	(Liu et al., 2012; Kopp et al., 1995)

(Continued on following page)

TABLE 2 | (*Continued*) Resistance mechanisms for major drugs in BC therapy.

Drug resistance	Related markers or pathways	Mode of action	*In vitro* or *in vivo* or clinical trial	References
Fulvestrant	ER signaling pathway	ERβ as a therapeutic target to in BCSCs to re-sensitizes fulvestrant and tamoxifen resistant cells	*In vitro* and *in vivo*	(Ma et al., 2017)
	Stem cell markers	Up-regulation of ALDH1, NANOG, OCT4 and SOX2 in response to tamoxifen or fulvestrant	*In vitro*	(Lillo et al., 2017)
	NOTCH	Maintains the activity of BCSCs to resistant fulvestrant	*In vitro* and *in vivo*	(Simoes et al., 2015)
AI	CD44/CD24	High CD44 + /CD24 - ratio is demonstrated in AI-resistant cell	*In vitro*	(Wang et al., 2013; Uchiumi et al., 2019)
Letrozole	PI3K/Akt/mTOR signalling pathway	BCBSs-mediated letrozole resistance by regulating PI3K/Akt/mTOR signaling pathway	*In vitro*	Liu Y. et al. (2019)
		Promotes BCSCs enrichment in MCF-7, and inversing by mTOR inhibitors	*In vitro* and *in vivo*	(Liu et al., 2014)
	JNK signaling pathway	Promotes the stemness of BC cells to cause aromatase inhibitors resistance	*In vitro*	(Pelekanou et al., 2018)
	Stem cell markers	Up-regulation of ALDH1, Oct 4, SOX2, and nanog in resistance cells	*In vitro*	(Nasr et al., 2018)
	HER2 signaling	Mediates AI resistance via regulation of stem cell markers, such as breast cancer resistance protein (BCRP)	*In vitro*	(Gilani et al., 2012)
Letrozole or exemestane	HIF-1α	Improves the generation of BCSCs to resistant to letrozole and exemestane	*In vitro*	(Kazi et al., 2014)
exemestane	RTKs pathway	Accumulates stemn-like cancer cells and resistant to exemestane	—	(Farahmand et al., 2018)
palbociclib	PI3K/Akt/mTOR signalling	Increases the ability of stemness and migration in palbociclib-resistant BCSCs	*In vitro*	Chen et al. (2019b)
	IL-6/STAT3 pathway	Promotes BCSCs enrichment	*In vitro* and *in vivo*	(Kettner et al., 2019)
	EMT	Promotes the capacity of migration and invasion via regulating BCSCs in CDK4/6 inhibitor-resistant BC	*In vitro* and *in vivo*	(Kettner et al., 2019; Pandey et al., 2019)
Resistance to Targeted Therapy				
Trastuzumab	PI3K/AKT signalling	Induces trastuzumab resistance via activating PI3K/AKT pathway in BCSCs	*In vitro* and *in vivo*	(Choi et al., 2019)
	JAK/STAT3 signalling	STAT3 activation increases CSCs properties then results in trastuzumab resistance	*In vitro*	(Chung et al., 2014)
	Wnt/β-catenin signalling	Over-activating wnt signalling pathway promotes CSCs then leads to trastuzumab resistance	*In vitro*	(Wu et al., 2012; Choi et al., 2019)
	MUC1	The number of MUC1 increases in trastuzumb resistant cell lines while anti-MUC1 inhibits CSCs proliferation	*In vitro*	(Sand et al., 2020)
	$CD44^+/CD24^-$	Acts as a predictor of poor response to trastuzumab	Clinical trial	(Seo et al., 2016)
Trastuzumab				
Lapatinib	TGFβ- Smad	Enhances the CSCs traits then leads to resistance of targeted therapy	*In vitro*	(Chihara et al., 2017)
Lapatinib	PI3K/AKT signalling	Directly represses HER2 and indirectly inhibits EGFR	*In vitro*	(Iorio et al., 2009; De Cola et al., 2015)
	$CD44^+/CD24^-$	Decreases the sensitivity of HER2+ BC cells to lapatinib	*In vitro*	(Hosonaga et al., 2014)

MTDH, Metadherin; ISR, The integrated stress response; MUC1, Mucin 1.

localization of BCRP, thereby regulating drug efflux activity in CSCs. Inhibitors of PI3K, which can not only be blocked via Akt signaling, resulted in the suppression of cancer cell proliferation, but also enhanced the sensitivity of chemoresistant cells (Hu et al., 2008). Another observation suggested that suppressing Akt that is downstream of HER2 signaling might efficiently target BCSCs in HER2-resistant tumors (Korkaya et al., 2009). Consequently, a series of PI3K and Akt selective inhibitors, which are being clinically investigated, demonstrates promising prospects.

Notch signaling is another pathway associated with treatment resistance. miR-34a regulates Notch-1 pathway in sustaining stem cell properties of BCSC populations, thereby suggesting that the miR-34a/Notch-1 pathway might be a potential therapeutic target for treating BC (Chen et al., 2016). Activation of Notch signaling is regulated by a proteolytic enzyme (γ-secretase), so γ-secretase inhibitor is the most clinically promising candidate in reversing drug resistance (Real and Ferrando, 2009). Psoralidin had been shown to effectively inhibits BCSCs proliferation and self-renewal through downregulating Notch1 signaling (Suman et al., 2013). Besides, vitamin D compounds showed activity against BCSCs by impeding the expression of Notch signaling components, such as Notch1, Notch2, Notch3, JAG1, and JAG2 (Shan et al., 2017). Meanwhile, a study showed that targeting FGFR mitochondrial metabolism-Notch1 axis may be effective to abrogate drug-resistant CSCs in TNBC (Bhola et al., 2016). Hence, Notch signaling pathway plays an important role in drug resistance mediated by BCSCs.

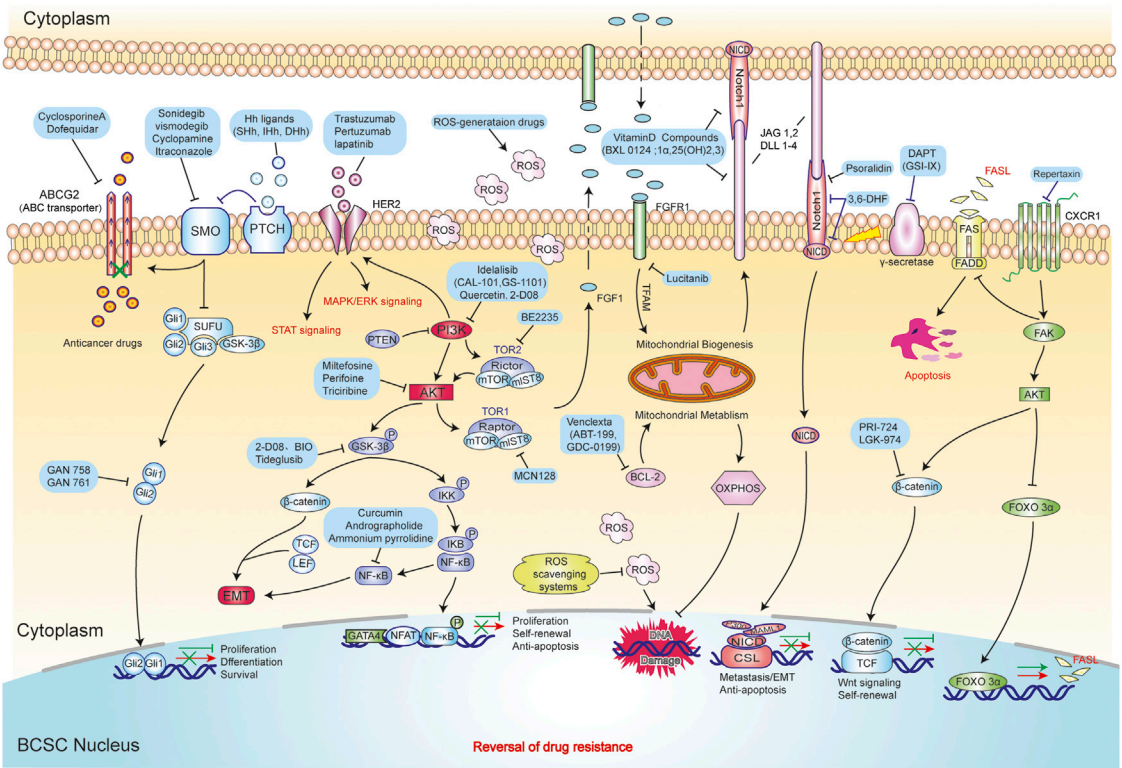

FIGURE 4 | The novel strategies of drug resistance reversal in BCSCs. These strategies mainly include inhibited proliferation ability (Hh, NF-κB signaling pathway) and self-renewal ability (EMT and Wnt/β-catenin signaling pathway), promoted DNA damage (ROS scavenging system and Mitochondrial metabolism) and apoptosis (Notch and FOXO/FASL/FAS signaling pathway).

Targeting Tumor Microenvironment

G-protein coupled receptors (GPCRs) are very important in the survival of BCSCs before and after the chemotherapy process. Chemokine receptors CXCR1 and CXCR2 generally play a role in chemotaxis of neutrophils, macrophages, and endothelial cells in a physiological microenvironment. Antagonizing CXCR1 by CXCR1-neutralizing antibody or by the small molecule inhibitor repertaxin selectively depleted more BCSCs than bulk tumor cells *in vitro*. This was followed by massive apoptosis of bulk tumor cells through FASL/FAS signaling via FAK/AKT/FOXO3A pathway (Ginestier et al., 2010). Repertaxin has already shown satisfactory effects in Phase I trials. Moreover, the chemokine receptor CXCR4 is expressed in BCSCs and forms a target in restraining or removal of BCSCs. Activation of this receptor is thought to facilitate the metastasis of mesenchymal BCSCs. CXCR4 probably stimulated the extracellular signal regulated kinase (ERK) pathway in BCSCs by activating PKA/MAPKAP2 pathway (Yi et al., 2014), thus providing resources for the research of BCSC-targeted cancer therapy through blocking these pathways by inhibiting receptors.

Targeting Breast Cancer Stem Cell Metabolism

The induction of oxidative stress is an important mechanism of action for many anticancer agents. BCSCs possess a highly active DNA repair system, which repairs DNA damages, particularly after chemotherapy treatment. Previous trials suggested that the ability of BCSCs to repair DNA damage is significantly related to reactive oxygen species (ROS), the levels of ROS are markedly lower in BCSCs than in non-CSCs (NCSCs) due to the high expression of free radical scavenging systems in BCSCs, such as superoxide dismutase, catalase, and glutathione peroxidase, which keep them from genotoxic damage of ROS. Thus, reduction of ROS scavengers in BCSCs markedly decreased their clonogenicity and resulted in therapeutic sensitization (Phillips et al., 2006; Diehn et al., 2009). Through H_2O_2-induced BCSC loss of function, ROS-generating drugs may have the therapeutic potential to eradicate drug-resistant BCSCs via induction of premature senescence (Zhong et al., 2019). Moreover, increasing mitochondrial activity is associated with resistance to DNA damage in BC. BCSCs are obviously dependent on glucose and mitochondrial metabolism. BCL-2 protein is a famous regulator of mitochondrial metabolism, inhibition of BCL-2 can result in the inhibition of oxidative phosphorylation (OXPHOS), which will lead to the reduction of BCSCs depending on OXPHOS (Deshmukh et al., 2016).

Besides potentiated ROS scavenging systems, BCSCs can protect themselves from several chemotherapeutic drugs which target the cell cycle process by maintaining a quiescent state in G0

TABLE 3 | Therapeutic strategies to reversing drug-resistance in BCSCs.

Drug/Compound	Target	Mode of action	*In vitro* or *in vivo* or clinical trial	References
Surface markers				
HA-decorated nanoparticles and salinomycin	CD44	Increases efficiency of drug delivery by the system of CD44-HA-Nanoparticles loaded with salinomycin	*In vitro*	(Muntimadugu et al., 2016)
Doxycycline	CD44, ALDH1	Inhibits BCSCs by apoptosis	Clinical trial	(Scatena et al., 2018)
Lentivirus-mediated CD44 shRNA	CD44	Sensitizes BCSCs to doxorubicin	*In vitro*	(Hu et al., 2016)
CD133-targeted polymeric nanoparticles	CD133	Reduces tumor initiating cell by conjugating anti-CD133 monoclonal antibody to nanoparticles	*In vivo*	(Swaminathan et al., 2013)
scFv- PE38KDEL	CD133	Promotes BCSCs apoptosis by inducing cytotoxicity	*In vitro* and *in vivo*	(Ohlfest et al., 2013)
Quercetin	ALDH	Inhibits expression of Sox2, Oct4, nanog, and	*In vitro*	Wang R. et al. (2018)
Withaferin A		Bmi-1	*In vitro*	Kim and Singh (2014)
Benztropine mesylate Deptropine citrate		Inhibits sphere formation and self-renewal of BCSCs	*In vitro* and *in vivo*	(Cui et al., 2017)
Signaling pathway				
Cyclopamine	Hedgehog signaling	Suppresses the activation of the SMO transmembrane receptor protein	*In vivo*	(Kubo et al., 2004)
Monoclonal antibody (5E1)		Inhibits breast cancer growth and metastasis.	*In vivo*	(O'Toole et al., 2011)
Nitidine chloride		Inhibits the stemness of BCSCs by downregulates the marker of CD44	*In vitro*	(Sun et al., 2016)
GANT61 (gli protein inhibitor)		Inhibits expression of glioma-associated oncogene in the Hh signaling pathway	*In vitro*	(Koike et al., 2017)
Vismodegib		Sensitizes BC cells to commonly used chemotherapy drugs by the inactivation of Hedgehog signaling	*In vivo*	(Hui et al., 2013; Palomeras et al., 2018)
Sonidegib		Inhibits the expression of BCSCs markers to sensitize BC cells to docetaxel	*In vitro* and *in vivo*	(Cazet et al., 2018; Palomeras et al., 2018)
NVPBGT226	PI3K signaling	Novel ATP-competitive mTOR kinase inhibitors for advanced breast cancer	*In vitro* and *in vivo*	(Cidado and Park, 2012)
Perifosine		Restores Tamoxifen sensitivity in resistant breast cancer cells	*In vitro*	(Farahmand et al., 2018)
Everolimus (RAD001)		Sensitizes advanced breast cancer to aromatase inhibitor	Clinical trial	(Baselga et al., 2012)
MK2206		Inhibits growth and induces breast cancer cells apoptosis	Clinical trial	(Chien et al., 2019)
PF-03084014 (nirogacestat)	Notch signaling	Sensitizes BCSCs to known chemotherapy drugs by blocking notch signaling	*In vitro* and clinical trial	Zhang C. C. et al. (2012); (Zhang and Grivennikov 2013); Locatelli et al., (2017); Ocana et al., 2010)
MK-0752		Promotes the sensitivity of BCSCs to docetaxel by strong modulation of Notch signaling	*In vitro*, *in vivo* and clinical trial	(Aktas et al., 2009; Schott et al., 2013; Venkatesh et al., 2018)
LY3039478 (crenigacestat)		γ-secretase inhibitor to promote inactivation of notch signaling	Clinical trial	(McCartney et al., 2018)
Capsaicin		Inhibits the entry of NICD to nuclear	*In vitro*	(Shim and Song, 2015)
Psoralidin		Promotes apoptosis and inhibits BCSCs proliferation and repairing	*In vitro*	(Suman et al., 2013)
RO4929097 (RG-4733)		γ-secretase inhibitor to promote inactivation of notch signaling	Clinical trial	(Strosberg et al., 2012; Koury et al., 2017; Venkatesh et al., 2018)
Foxy-5	Wnt/β-catenin	Simulates the effect of Wnt5a to inhibits metastasis	*In vivo*	(Canesin et al., 2017; Palomeras et al., 2018; Goldsberry et al., 2019)
Sulforaphane		Inhibits BCSCs self-renewal by the downregulation of the wnt/β-catenin signaling.	*In vitro*	(Li et al., 2010)
Microenvironment				
AMD3100 (CXCR4 antagonist)	SDF-I/CXCR4	Inhibits BCSC self-renewal and maintenance	*In vitro*	Liu B. Q. et al. (2017)
Reparixin	CXCR signaling	Induces BCSCs apoptosis through FASL/FAS signaling	*In vitro* and clinical trial	(Schott et al., 2013)
Evofosfamide (TH-302)	Hypoxia	Suppresses BC growth by selectively cytotoxic	*In vitro* and *in vivo*	(Liapis et al., 2016)
Echinomycin	Hypoxia response element	Reduces cytotoxic in breast cancer cells	*In vitro*	(Lathan and Von Hoff, 1984)
Tumor metabolism				

(Continued on following page)

TABLE 3 | (*Continued*) Therapeutic strategies to reversing drug-resistance in BCSCs.

Drug/Compound	Target	Mode of action	*In vitro* or *in vivo* or clinical trial	References
VLX600	Mitochondrial OXPHOS	Makes BCSCs death by inhibiting BCL-2	*In vitro*	(Dey et al., 2019)
Etomoxir	Carnitine palmitoyltransferase-1 inhibitor	Activates metabolic by cAMP-induced	*In vitro*	(Manerba et al., 2019)
Salinomycin	Sodium potassium gradient	Selectively eradicates BCSCs selectively via lysosomal iron Targeting.	*In vitro*	(Versini et al., 2020)
XCT790	ERRn-PGC1	Targets FOXM1 and mitochondrial biogenesis to block both the survival and propagation of BCSCs	*In vitro*	(De Luca et al., 2015)
Others				
MS-209	P-glycoprotein	Makes BCSCs more sensitive to docetaxel	*In vitro* and *in vivo*	(Naito et al., 2002)
Glucosamine	STAT 3	Inhibits BCSCs the ability to form mammosphere	*In vitro*	(Hosea et al., 2018)
Apigenin	Hippo	Inhibits BCSCs migration and metastasis by downregulating transcription activity of TAZ and YAP1	*In vitro* and *in vivo*	(Li et al., 2018)
MLN4924	Sox-2	Suppresses stem cell property and makes breast cancer cells more sensitive of tamoxifen	*In vitro*	(Yin et al., 2019)
MRX34	MiR-34a	Contains miR-34a mimic and a lipid vector and inhibits cellular proliferation, invasion and tumor sphere formation.	*In vitro*, *in vivo* and clinical trial	(Adams et al., 2016; Mohammady et al., 2019)
αEPCR-1535	Protein C receptor	Attenuates tumor growth	*In vitro*	(Schaffner et al., 2013)

NICD, Notch intracellular membrane domain.

phase (Yoshida and Saya, 2016). BCSCs can adopt dormancy-associated phenotypes through upregulating autophagic pathways (Vera-Ramirez et al., 2018). Salinomycin is a kind of ionophore antibiotic, which has been shown to be effective in clearing BCSCs through autophagy (Jiang et al., 2018). Recently, studies showed that the mechanistic link between autophagy and metastastic dormancy was associated with Spleen Tyrosine Kinase (SYK) in epithelial-mesenchymal transition (EMT) required for BC metastasis. Fostamatinib, a SYK pharmacologic inhibitior, prevents mesenchymal-epithelial transition (MET), which can inhibit metastatic tumor outgrowth (Shinde et al., 2019). Currently, tyrosine kinase inhibitors are being tested in clinical trials.

Nano-therapeutics Against Breast Cancer Stem Cell

Nanoparticle (NP)-mediated therapy is an effective delivery strategy for cancer therapeutics. It contributes to specific delivery of a chemotherapeutic drug, RNAi, or antibodies to the stem cell population by recognizing antibodies/aptamers against BCSC-specific markers.

CD44 is the first discovered and the most commonly used surface marker of BCSCs, which plays an important role in all aspects of tumor cells, such as growth and proliferation, migration, differentiation, apoptosis, self-renewal, microenvironment, EMT, and drug resistance (Jin et al., 2017). As a cell receptor, CD44 mediates the communication with the microenvironment through interacting with certain extracellular ligands. For the past few years, the development of an antibody against CD44, which could induce BCSCs terminal differentiation, had already been found to be effective and has been gradually accepted (Naor et al., 1997). In aggressive BC, the combination of anti-human CD44 monoclonal antibody with doxorubicin and cyclophosphamide using NPs has been used to prevent tumor recurrence (Fan et al., 2010; Wu et al., 2017).

Micro RNAs (miRs) play a key role in the sustenance and heterogeneity of BCSCs in BC. They can regulate proteins associated with drug resistance in human BC. For instance, miR-21 may facilitate the inhibition of tumor proliferation, growth, and migration (Han et al., 2012); miR-100 inhibits self-renewal of BCSCs and tumorigenesis (Deng et al., 2014); miR-199a can increase stem cell properties in BCSCs (Celia-Terrassa et al., 2017). miR-205-5p is highly expressed in BCSCs and is related to therapy resistance (De Cola et al., 2015). Moreover, research shows that the high expression of STAT3 affects doxorubicin resistance of BCSCs, and miR-124 reverses this resistance of BCSCs through targeting STAT3 to control the HIF-1 signaling pathway (Liu C. et al., 2019). Consequently, targeting miRs and delivering siRNAs to tumors using NPs is an effective strategy to reverse drug resistance and enhance drug efficacy.

Aldehyde dehydrogenase 1 (ALDH1) is a NAD(P)+-dependent enzyme, which is the key enzyme to oxidize intracellular aldehydes to carboxylic acids. ALDH1 is found to be highly active in BCSCs, increasing their proficiency by removing toxic oxygen radicals from the tumor microenvironment (Charafe-Jauffret et al., 2013). By consulting relevant literatures, we also found that the increased levels of ALDH family members were correlated with chemoresistance (Croker et al., 2009; Tanei et al., 2009). ALDHs inhibition sensitizes BCSCs to chemotherapy (Croker and Allan, 2012). NPs containing doxorubicin and chloroquine have been shown to reduce ALDH high population of MDA-MB-231 cells (Li et al., 2015), and several ALDH inhibitors are currently in the preclinical stage.

Other Therapeutic Approaches

CSCs manifest a high number of proteins on their cell surface, such as ABC transporters, ABCB1 (P-gp, MDR1), ABCG2 (BCRP1), ABCC11 (MRP8), and ABCB, which are strongly expressed in CSC's chemo-resistance (Dean, 2009). How do CSCs develop drug resistance through the protein molecule above? In BC, a recent study has indicated that the prominently activated ATP binding cassette (ABC) or drug efflux pump of BCSCs can successfully pump out chemotherapeutic drugs, such as anthracycline or taxanes, which are known as the most essential drugs of BC treatment (Cojoc et al., 2015). Furthermore, other scholars have found that an increased level of ABCG2 in BCSCs enabled rapid expulsion of cytotoxic drugs, conferring cellular resistance to antitumor drugs (Hirschmann-Jax et al., 2004). A recent study has confirmed that SOX2-ABCG2-TWIST1 axis can promote stemness and chemoresistance in TNBC, further indicating that ABC proteins are potential targets for BCSCs eradication (Mukherjee et al., 2017). Dofequidar, an ABC transporter inhibitor, could increase the sensitivity of BCSC to anticancer drugs; it showed promising results in patients with advanced or recurrent BC when combined with other chemotherapeutic agents, such as cyclophosphamide, doxorubicin, and fluorouracil (Saeki et al., 2007). Additionally, SOX2 is a key transcription factor that plays critical roles in maintaining stem cell properties and conferring drug resistance. MLN4924 can repress the expression of SOX2, leading to suppression of stem cell properties and sensitization of BC cells to tamoxifen (Yin et al., 2019).

CONCLUSION

BC remains the most frequent cancer in women, and significant public health issue globally (Zavala et al., 2019). Both of the developing and developed world are suffering from BC incidence and mortality (Global Burden of Disease Cancer Collaboration et al., 2015). Due to limitations of therapeutic strategies, it is urgent to explore novel and effective strategies. The important role of BCSCs in drug resistance, recurrence, and metastasis of BC has attracted more and more attention. Many studies have also enlightened the drug resistance mechanism of BCSCs. Currently, various treatments targeting BCSCs have been in preclinical and clinical trials. Unfortunately, the mechanism of drug resistance that is controlled by BCSC rarely functions individually. In the process of antagonizing anticancer drugs, these mechanisms interact with each other and form a complex functional network of drug resistance. Hence, inhibiting a drug resistant pathway is likely to trigger feedback mechanisms that ultimately allow BCSCs to escape the effects of the drug. Therefore, the therapy based on the combination of multiple targets for BCSCs' functional network is the most promising approach. Furthermore, existing nanobiology technologies should be fully utilized, through finding specific surface markers of targeting BCSCs, to locate and eliminate BCSCs accurately. Recently, biologically and chemically synthesized gold nanoparticles (AuNPs) (Virmani et al., 2019), silver nanoparticles (AgNPs) (Muthupandian et al., 2019) and selenium nanoparticles (SeNPs) (Vahidi et al., 2020) have attracted significant attention for their anticancer effects against cancers such as lung cancer, colorectal Cancer (Barabadi et al., 2020a), cervical cancer (Barabadi et al., 2020b) and prostate cancer (Barabadi et al., 2019a). Fortunately, AuNPs (Barabadi et al., 2019b) and AgNPs (Saravanan et al., 2020) have also been reported to play an important role in the treatment of BC. With the development of cancer nanomedicine, it is expected that biologically and chemically synthesized NPs may emerge as potential BCSCs therapeutic agents alone or in combination with anti-cancer drugs before long of future. In conclusion, these therapies targeting BCSCs will lay the foundation for reversing drug resistance and attaining favorable prognosis in BC.

AUTHOR CONTRIBUTIONS

QZ and MZ conceived and drafted the manuscript. LZ and XM discussed the concepts of the manuscript. QZ drew the figures. LZ and XM approved the version to be submitted.

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REFERENCES

Abad, E Graifer, D, and Lyakhovich, A. (2020). DNA damage response and resistance of cancer stem cells. *Cancer Lett.* 474, 106–117. doi:10.1016/j.canlet.2020.01.008

Adams, B. D., Parsons, C., and Slack, F. J. (2016). The tumor-suppressive and potential therapeutic functions of miR-34a in epithelial carcinomas. *Expert Opin. Ther. Targets* 20, 737–753. doi:10.1517/14728222.2016.1114102

Aktas, B., Tewes, M., Fehm, T., Hauch, S., Kimmig, R., and Kasimir-Bauer, S. (2009). Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. *Breast Cancer Res.* 11, R46. doi:10.1186/bcr2333

Alexe, G., Alexe, S, Axelrod, D. E., Bonates, T. O, Lozina, I. I., Reiss, M., and Hammer, P. L. (2006). Breast cancer prognosis by combinatorial analysis of gene expression data. *Breast Cancer Res.* 8, R41. doi:10.1186/bcr1512

Al-Hajj, M., Wicha, M. S, Benito-Hernandez, A, Morrison, S. J., and Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 100, 3983–3988. doi:10.1073/pnas.0530291100

Alves, C. L., Elias, D., Lyng, M., Bak, M., Kirkegaard, T., Lykkesfeldt, A. E, and Ditzel, H. J. (2016). High CDK6 Protects Cells from Fulvestrant-Mediated Apoptosis and is a Predictor of Resistance to Fulvestrant in Estrogen Receptor-

Positive Metastatic Breast Cancer. *Clin. Cancer Res.* 22, 5514–5526. doi:10.1158/1078-0432.CCR-15-1984

Angeloni, V., Tiberio, P., Appierto, V., and Daidone, M. G. (2015). Implications of stemness-related signaling pathways in breast cancer response to therapy. *Semin. Cancer Biol.* 31, 43–51. doi:10.1016/j.semcancer.2014.08.004

Arnason, T., and Harkness, T. (2015). Development, maintenance, and reversal of multiple drug resistance: at the crossroads of TFPI1, ABC transporters, and HIF1. *Cancers* 7, 2063–2082. doi:10.3390/cancers7040877

Arpino, G., Wiechmann, L., Osborne, C. K., and Schiff, R. (2008). Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocr. Rev.* 29, 217–233. doi:10.1210/er.2006-0045

Baccelli, I., Schneeweiss, A., Riethdorf, S., Stenzinger, A., Schillert, A., Vogel, V., et al. (2013). Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. *Nat. Biotechnol.* 31, 539–544. doi:10.1038/nbt.2576

Bai, X., Ni, J., Beretov, J., Graham, P., and Li, Y. (2018). Cancer stem cell in breast cancer therapeutic resistance, *Cancer Treat Rev*. 69, 152–163. doi:10.1016/j.ctrv.2018.07.004

Baker, A., Wyatt, D., Bocchetta, M., Li, J., Filipovic, A., Green, A., et al. (2018). Notch-1-PTEN-ERK1/2 signaling axis promotes HER2+ breast cancer cell proliferation and stem cell survival. *Oncogene* 37, 4489–4504. doi:10.1038/s41388-018-0251-y

Bane, A., Viloria-Petit, A., Pinnaduwage, D., Mulligan, A. M., O'Malley, F. P., and Andrulis, I. L. (2013). Clinical-pathologic significance of cancer stem cell marker expression in familial breast cancers. *Breast Cancer Res. Treat.* 140, 195–205. doi:10.1007/s10549-013-2591-1

Barabadi, H., Kamali, K. D., Shoushtari, F. J., Tajani, B., and Saravanan, M. (2019a). Emerging theranostic silver and gold nanomaterials to combat prostate cancer: a systematic review. *J. Cluster Sci.* 30 (6), 1375–1382. doi:10.1007/s10876-019-01588-7

Barabadi, H., Mahjoub, M. A., Tajani, B., Ahmadi, A., Junejo, Y., and Saravanan, M. (2019b). Emerging theranostic biogenic silver nanomaterials for breast cancer: a systematic review. *J. Cluster Sci.* 30 (2), 259–279. doi:10.1007/s10876-018-01491-7

Barabadi, H., Vahidi, H., Kamali, K. D., Rashedi, M., and Muthupandian, S. (2020a). Emerging theranostic silver nanomaterials to combat colorectal cancer: a systematic review. *J. Cluster Sci.* 31, 311–321. doi:10.1007/s10876-019-01668-8

Barabadi, H., Vahidi, H., Kamali, K. D., Rashedi, M., and Saravanan, M. (2020b). Antineoplastic biogenic silver nanomaterials to combat cervical cancer: a novel approach in cancer therapeutics. *J. Cluster Sci.* 31 (4), 659–672. doi:10.1007/s10876-019-01697-3

Barker, N., Ridgway, R. A., van Es, J. H., van de Wetering, M., Begthel, H., van den Born, M., et al. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* 457, 608–611. doi:10.1038/nature07602

Baselga, J., Campone, M., Piccart, M., Burris, H. A., Rugo, H. S., Sahmoud, T., et al. (2012). Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *N. Engl. J. Med.* 366, 520–529. doi:10.1056/NEJMoa1109653

Battula, V. L., Shi, Y., Evans, K. W., Wang, R. Y., Spaeth, E. L., Jacamo, R. O., et al. (2012). Ganglioside GD2 identifies breast cancer stem cells and promotes tumorigenesis. *J. Clin. Invest.* 122, 2066–2078. doi:10.1172/JCI59735

Battula, V. L., Nguyen, K., Sun, J., Pitner, M. K., Yuan, B., Bartholomeusz, C., et al. (2017). IKK inhibition by BMS-345541 suppresses breast tumorigenesis and metastases by targeting GD2+ cancer stem cells. *Oncotarget* 8, 36936–36949. doi:10.18632/oncotarget.16294

Berns, K., Horlings, H. M., Hennessy, B. T., Madiredjo, M., Hijmans, E. M., Beelen, K., et al. (2007). A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell* 12, 395–402. doi:10.1016/j.ccr.2007.08.030

Bhateja, P., Cherian, M., Majumder, S., and Ramaswamy, B. (2019). The hedgehog signaling pathway: a viable target in breast cancer?. *Cancers (Basel)* 11 (8), 1126. doi:10.3390/cancers11081126

Bhinge, K. N., Gupta, V., Hosain, S. B., Satyanarayanajois, S. D., Meyer, S. A., Blaylock, B., et al. (2012). The opposite effects of doxorubicin on bone marrow stem cells versus breast cancer stem cells depend on glucosylceramide synthase. *Int. J. Biochem. Cell Biol.* 44, 1770–1778. doi:10.1016/j.biocel.2012.06.010

Bhola, N. E., Balko, J. M., Dugger, T. C., Kuba, M. G., Sanchez, V., Sanders, M., et al. (2013). TGF-β inhibition enhances chemotherapy action against triple-negative breast cancer. *J. Clin. Invest.* 123, 1348–1358. doi:10.1172/JCI65416

Bhola, N. E., Jansen, V. M., Koch, J. P., Li, H., Formisano, L., Williams, J. A., et al. (2016). Treatment of triple-negative breast cancer with TORC1/2 inhibitors sustains a drug-resistant and notch-dependent cancer stem cell population. *Cancer Res.* 76, 440–452. doi:10.1158/0008-5472.CAN-15-1640-T

Bock, C., Kuhn, C., Ditsch, N., Krebold, R., Heublein, S., Mayr, D., et al. (2014). Strong correlation between N-cadherin and CD133 in breast cancer: role of both markers in metastatic events. *J. Cancer Res. Clin. Oncol.* 140, 1873–1881. doi:10.1007/s00432-014-1750-z

Borgna, S., Armellin, M., di Gennaro, A., Maestro, R., and Santarosa, M. (2012). Mesenchymal traits are selected along with stem features in breast cancer cells grown as mammospheres. *Cell Cycle* 11, 4242–4251. doi:10.4161/cc.22543

Bourguignon, L. Y., Singleton, P. A., Zhu, H., and Diedrich, F. (2003). Hyaluronan-mediated CD44 interaction with RhoGEF and Rho kinase promotes Grb2-associated binder-1 phosphorylation and phosphatidylinositol 3-kinase signaling leading to cytokine (macrophage-colony stimulating factor) production and breast tumor progression. *J. Biol. Chem.* 278, 29420–29434. doi:10.1074/jbc.M301885200

Bourguignon, L. Y., Singleton, P. A., Diedrich, F., Stern, R., and Gilad, E. (2004). CD44 interaction with Na+-H+ exchanger (NHE1) creates acidic microenvironments leading to hyaluronidase-2 and cathepsin B activation and breast tumor cell invasion. *J. Biol. Chem.* 279, 26991–27007. doi:10.1074/jbc.M311838200

Bourguignon, L. Y. W. (2019). Matrix hyaluronan-CD44 interaction activates MicroRNA and LncRNA signaling associated with chemoresistance, invasion, and tumor progression. *Front. Oncol* 9, 492. doi:10.3389/fonc.2019.00492

Bourguignon, L. Y., Wong, G., Earle, C., Krueger, K., and Spevak, C. (2010). Hyaluronan-CD44 interaction promotes c-Src-mediated twist signaling, microRNA-10b expression, and RhoA/RhoC up-regulation, leading to Rho-kinase-associated cytoskeleton activation and breast tumor cell invasion. *J. Biol. Chem.*, 285, 36721–36735. doi:10.1074/jbc.M110.162305

Bousquet, G., El Bouchtaoui, M., Sophie, T., Leboeuf, C., de Bazelaire, C., Ratajczak, P., et al. (2017). Targeting autophagic cancer stem-cells to reverse chemoresistance in human triple negative breast cancer. *Oncotarget* 8, 35205–35221. doi: doi:10.18632/oncotarget.16925

Brugnoli, F., Grassilli, S., Lanuti, P., Marchisio, M., Al-Qassab, Y., Vezzali, F., et al. (2017). Up-modulation of PLC-beta2 reduces the number and malignancy of triple-negative breast tumor cells with a CD133(+)/EpCAM(+) phenotype: a promising target for preventing progression of TNBC. *BMC Cancer* 17, 617. doi:10.1186/s12885-017-3592-y

Bumbaca, B., and Li, W. (2018). Taxane resistance in castration-resistant prostate cancer: mechanisms and therapeutic strategies. *Acta Pharm. Sin. B* 8, 518–529. doi:10.1016/j.apsb.2018.04.007

Canesin, G., Evans-Axelsson, S., Hellsten, R., Krzyzanowska, A., Prasad, C. P., Bjartell, A., et al. (2017). Treatment with the WNT5A-mimicking peptide Foxy-5 effectively reduces the metastatic spread of WNT5A-low prostate cancer cells in an orthotopic mouse model. *PLoS One* 12, e0184418. doi:10.1371/journal.pone.0184418

Capeloa, T., Benyahia, Z., Zampieri, L. X., Blackman, M. C. N. M., and Sonveaux, P. (2020). Metabolic and non-metabolic pathways that control cancer resistance to anthracyclines. *Semin. Cell Dev. Biol.* 98, 181–191. doi:10.1016/j.semcdb.2019.05.006

Cazet, A. S., Hui, M. N., Elsworth, B. L., Wu, S. Z., Roden, D., Chan, C. L., et al. (2018). Targeting stromal remodeling and cancer stem cell plasticity overcomes chemoresistance in triple negative breast cancer. *Nat. Commun.* 9, 2897. doi:10.1038/s41467-018-05220-6

Celia-Terrassa, T., Liu, D. D., Choudhury, A., Hang, X., Wei, Y., Zamalloa, J., et al. (2017). Normal and cancerous mammary stem cells evade interferon-induced constraint through the miR-199a-LCOR axis. *Nat. Cell Biol.* 19, 711–723. doi:10.1038/ncb3533

Chakraborty, A. K., Welsh, A., and Digiovanna, M. P. (2010). Co-targeting the insulin-like growth factor I receptor enhances growth-inhibitory and pro-apoptotic effects of anti-estrogens in human breast cancer cell lines. *Breast Cancer Res. Treat.* 120 327–335. doi:10.1007/s10549-009-0382-5

Chan, Y. T., Lai, A. C., Lin, R. J., Wang, Y. H., Wang, Y. T., Chang, W. W., et al. (2020). GPER-induced signaling is essential for the survival of breast cancer stem cells. *Int. J. Cancer* 146. 1674–1685. doi:10.1002/ijc.32588

Chang, W. W., Lin, R. J., Yu, J., Chang, W. Y., Fu, C. H., Lai, A., et al. (2013). The expression and significance of insulin-like growth factor-1 receptor and its pathway on breast cancer stem/progenitors, *Breast Cancer Res.* 15, R39. doi:10.1186/bcr3423

Charafe-Jauffret, E., Ginestier, C., Bertucci, F., Cabaud, O., Wicinski, J., Finetti, P., et al. (2013). ALDH1-positive cancer stem cells predict engraftment of primary breast tumors and are governed by a common stem cell program. *Cancer Res* 73, 7290–7300. doi:10.1158/0008-5472.CAN-12-4704

Chen, I. X., Chauhan, V. P., Posada, J., Ng, M. R., Wu, M. W., Adstamongkonkul, P., et al. (2019). Blocking CXCR4 alleviates desmoplasia, increases T-lymphocyte infiltration, and improves immunotherapy in metastatic breast cancer, *Proc. Natl. Acad. Sci. U.S.A.* 116, 4558–4566. doi:10.1073/pnas.1815515116

Chen, J., Chang, H., Peng, X., Gu, Y., Yi, L., Zhang, Q., et al. (2016). 3,6-dihydroxyflavone suppresses the epithelial-mesenchymal transition in breast cancer cells by inhibiting the Notch signaling pathway. *Sci. Rep.* 6, 28858. doi:10.1038/srep28858

Chen, L., He, J., Zhou, J., Xiao, Z., Ding, N., Duan, Y., et al. (2019a). EIF2A promotes cell survival during paclitaxel treatment *in vitro* and *in vivo*, *J. Cell Mol. Med.* 23, 6060–6071. doi:10.1111/jcmm.14469

Chen, L., Yang, G., and Dong, H. (2019b). Everolimus reverses palbociclib resistance in ER+ human breast cancer cells by inhibiting phosphatidylinositol 3-kinase(PI3K)/Akt/Mammalian target of rapamycin (mTOR) pathway. *Med. Sci. Mon.* 25, 77–86. doi:10.12659/msm.912929

Chihara, Y., Shimoda, M., Hori, A., Ohara, A., Naoi, Y., Ikeda, J. I., et al. (2017). A small-molecule inhibitor of SMAD3 attenuates resistance to anti-HER2 drugs in HER2-positive breast cancer cells. *Breast Cancer Res. Treat.* 166, 55–68. doi:10.1007/s10549-017-4382-6

Cho, Y., Lee, H. W., Kang, H. G., Kim, H. Y., Kim, S. J., and Chun, K. H (2015). Cleaved CD44 intracellular domain supports activation of stemness factors and promotes tumorigenesis of breast cancer. *Oncotarget* 6, 8709–8721. doi:10.18632/oncotarget.3325

Choi, H. J., Jin, S., Cho, H., Won, H. Y., An, H. W., Jeong, G. Y., et al. (2019). CDK12 drives breast tumor initiation and trastuzumab resistance via WNT and IRS1-ErbB-PI3K signaling. *EMBO Rep.* 20, e48058. doi:10.15252/embr.201948058

Chien, A. J., Tripathy, D., Albain, K. S., Symmans, W. F., Rugo, H. S., Melisko, M. E., et al. (2019). MK-2206 and standard neoadjuvant chemotherapy improves response in patients with human epidermal growth factor receptor 2-positive and/or hormone receptor-negative breast cancers in the I-spy 2 trial. *J. Clin. Oncol.* 38, 1059–1069. doi:10.1200/JCO.19.01027

Chung, S. S., Giehl, N., Wu, Y., and Vadgama, J. V. (2014). STAT3 activation in HER2-overexpressing breast cancer promotes epithelial-mesenchymal transition and cancer stem cell traits. *Int. J. Oncol.* 44, 403–411. doi:10.3892/ijo.2013.2195

Cidado, J., and Park, B. H. (2012). Targeting the PI3K/Akt/mTOR pathway for breast cancer therapy. *J. Mammary Gland Biol. Neoplasia* 17, 205–216. doi:10.1007/s10911-012-9264-2

Clarke, M. F., Dick, J. E., Dirks, P. B., Eaves, C. J., Jamieson, C. H., Jones, D. L., et al. (2006). Cancer stem cells–perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res* 66, 9339–9344. doi:10.1158/0008-5472.CAN-06-3126

Cojoc, M., Mabert, K., Muders, M. H., and Dubrovska, A. (2015). A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms. *Semin. Cancer Biol.* 31, 16–27. doi:10.1016/j.semcancer.2014.06.004

Creighton, C. J., Li, X., Landis, M., Dixon, J. M., Neumeister, V. M., Sjolund, A., et al. (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13820–13825. doi:10.1073/pnas.0905718106

Croker, A. K., Goodale, D., Chu, J., Postenka, C., Hedley, B. D., Hess, D. A., et al. (2009). High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. *J. Cell Mol. Med.* 13, 2236–2252. doi:10.1111/j.1582-4934.2008.00455.x

Croker, A. K., and Allan, A. L. (2012). Inhibition of aldehyde dehydrogenase (ALDH) activity reduces chemotherapy and radiation resistance of stem-like ALDHhiCD44$^+$ human breast cancer cells. *Breast Cancer Res. Treat.* 133, 75–87. doi:10.1007/s10549-011-1692-y

Cui, J., Hollmen, M., Li, L., Chen, Y., Proulx, S. T., Reker, D., et al. (2017). New use of an old drug: inhibition of breast cancer stem cells by benztropine mesylate. *Oncotarget* 8, 1007–1022. doi:10.18632/oncotarget.13537

Dave, B., Migliaccio, I., Gutierrez, M. C., Wu, M. F., Chamness, G. C., Wong, H., et al. (2011). Loss of phosphatase and tensin homolog or phosphoinositol-3 kinase activation and response to trastuzumab or lapatinib in human epidermal growth factor receptor 2-overexpressing locally advanced breast cancers. *J. Clin. Oncol.* 29, 166–173. doi:10.1200/JCO.2009.27.7814

Dean, M. (2009). ABC transporters, drug resistance, and cancer stem cells. *J. Mammary Gland Biol. Neoplasia* 14, 3–9. doi:10.1007/s10911-009-9109-9

Deng, L., Shang, L., Bai, S., Chen, J., He, X., Martin-Trevino, R., et al. (2014). MicroRNA100 inhibits self-renewal of breast cancer stem-like cells and breast tumor development. *Cancer Res.* 74, 6648–6660. doi:10.1158/0008-5472.CAN-13-3710

De Cola, A., Volpe, S., Budani, M. C., Ferracin, M., Lattanzio, R., Turdo, A., et al. (2015). miR-205-5p-mediated downregulation of ErbB/HER receptors in breast cancer stem cells results in targeted therapy resistance. *Cell Death Dis.* 6, e1823. doi:10.1038/cddis.2015.192

De Luca, A., Fiorillo, M., Peiris-Pages, M., Ozsvari, B., Smith, D. L., Sanchez-Alvarez, R., et al. (2015). Mitochondrial biogenesis is required for the anchorage-independent survival and propagation of stem-like cancer cells. *Oncotarget* 6, 14777–14795. doi:10.18632/oncotarget.4401

DeSantis, C. E., Ma, J., Gaudet, M. M., Newman, L. A., Miller, K. D., Goding Sauer, A., et al. (2019). Breast cancer statistics, 2019. *CA Cancer J. Clin.* 69, 438–451. doi:10.3322/caac.21583

Deshmukh, A., Deshpande, K., Arfuso, F., Newsholme, P., and Dharmarajan, A. (2016). Cancer stem cell metabolism: a potential target for cancer therapy. *Mol. Cancer* 15, 69. doi:10.1186/s12943-016-0555-x

Dey, P., Rathod, M., and De, A. (2019). Targeting stem cells in the realm of drug-resistant breast cancer. *Breast Cancer (Dove Med Press)* 11, 115–135. doi:10.2147/BCTT.S189224

Diehn, M., Cho, R. W., Lobo, N. A., Kalisky, T., Dorie, M. J., Kulp, A. N., et al. (2009). Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* 458, 780–783. doi:10.1038/nature07733

Dowsett, M., Nicholson, R. I., and Pietras, R. J. (2005). Biological characteristics of the pure antiestrogen fulvestrant: overcoming endocrine resistance. *Breast Cancer Res. Treat.* 93 (Suppl. 1), S11–S18. doi:10.1007/s10549-005-9037-3

Droog, M., Beelen, K., Linn, S., and Zwart, W., Tamoxifen resistance: from bench to bedside, *Eur. J. Pharmacol.*, 717 (2013) 47–57. doi:10.1016/j.ejphar.2012.11.071

Dubrovska, A., Hartung, A., Bouchez, L. C., Walker, J. R., Reddy, V. A., Cho, C. Y., et al. (2012). CXCR4 activation maintains a stem cell population in tamoxifen-resistant breast cancer cells through AhR signalling. *Br. J. Cancer* 107, 43–52. doi:10.1038/bjc.2012.105

Du, R., Liu, B., Zhou, L., Wang, D., He, X., Xu, X., et al. (2018). Downregulation of annexin A3 inhibits tumor metastasis and decreases drug resistance in breast cancer. *Cell Death Dis.* 9, 126. doi:10.1038/s41419-017-0143-z

Eiro, N., Gonzalez, L. O., Fraile, M., Cid, S., Schneider, J., and Vizoso, F. J. (2019). Breast cancer tumor stroma: cellular components, phenotypic heterogeneity, intercellular communication, prognostic implications and therapeutic opportunities. *Cancers* 11, 11. doi:10.3390/cancers11050664

Fan, L., Li, F., Zhang, H., Wang, Y., Cheng, C., Li, X., et al. (2010). Co-delivery of PDTC and doxorubicin by multifunctional micellar nanoparticles to achieve active targeted drug delivery and overcome multidrug resistance. *Biomaterials* 31, 5634–5642. doi:10.1016/j.biomaterials.2010.03.066

Fan, M., Yan, P. S., Hartman-Frey, C., Chen, L., Paik, H., Oyer, S. L., et al. (2006). Diverse gene expression and DNA methylation profiles correlate with differential adaptation of breast cancer cells to the antiestrogens tamoxifen and fulvestrant. *Cancer Res.* 66, 11954–11966. doi:10.1158/0008-5472.CAN-06-1666

Farahmand, L., Mansouri, S., Jafarbeik-Iravani, N., Teymourzadeh, A., and Majidzadeh, A. K. (2018). Stemness phenotype in tamoxifen resistant breast cancer cells may be induced by interactions between receptor tyrosine kinases and ERalpha-66, *Recent Pat. Anti-Cancer Drug Discov.* 13, 302–307. doi:10.2174/1574892813666180305164634

Fedier, A., Schwarz, V. A., Walt, H., Carpini, R. D., Haller, U., and Fink, D. (2001). Resistance to topoisomerase poisons due to loss of DNA mismatch repair. *Int. J. Cancer* 93, 571–576. doi:10.1002/ijc.1356

Fessler, S. P., Wotkowicz, M. T., Mahanta, S. K., and Bamdad, C. (2009). MUC1* is a determinant of trastuzumab (Herceptin) resistance in breast cancer cells. *Breast Cancer Res. Treat.* 118, 113–124. doi:10.1007/s10549-009-0412-3

Francis, P. A., Regan, M. M., Fleming, G. F., Láng, I., Ciruelos, E., Bellet, M., et al. (2015). Adjuvant ovarian suppression in premenopausal breast cancer. *N. Engl. J. Med.* 372, 436–446. doi:10.1056/NEJMoa1412379

Frasor, J., Stossi, F., Danes, J. M., Komm, B., Lyttle, C. R., and Katzenellenbogen, B. S. (2004). Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. *Cancer Res.* 64, 1522–1533. doi:10.1158/0008-5472.can-03-3326

Gaio, E., Conte, C., Esposito, D., Reddi, E., Quaglia, F., and Moret, F. (2020). CD44 targeting mediated by polymeric nanoparticles and combination of chlorine TPCS2a-PDT and docetaxel-chemotherapy for efficient killing of breast differentiated and stem cancer cells in vitro. *Cancers (Basel)* 12, 278. doi:10.3390/cancers12020278

Gallardo, A., Lerma, E., Escuin, D., Tibau, A., Munoz, J., Ojeda, B., et al. (2012). Increased signalling of EGFR and IGF1R, and deregulation of PTEN/PI3K/Akt pathway are related with trastuzumab resistance in HER2 breast carcinomas. *Br. J. Cancer* 106, 1367–1373. doi:10.1038/bjc.2012.85

Gargini, R., Cerliani, J. P., Escoll, M., Anton, I. M., and Wandosell, F. (2015). Cancer stem cell-like phenotype and survival are coordinately regulated by Akt/FoxO/Bim pathway. *Stem Cell.* 33, 646–660. doi:10.1002/stem.1904

Gao, R., Li, D., Xun, J., Zhou, W., Li, J., Wang, J., et al. (2018). CD44ICD promotes breast cancer stemness via PFKFB4-mediated glucose metabolism. *Theranostics* 8, 6248–6262. doi:10.7150/thno.28721

Ge, G., Zhou, C., Ren, Y., Tang, X., Wang, K., Zhang, W., et al. (2016). Enhanced SLC34A2 in breast cancer stem cell-like cells induces chemotherapeutic resistance to doxorubicin via SLC34A2-Bmi1-ABCC5 signaling. *Tumour Biol.* 37, 5049–5062. doi:10.1007/s13277-015-4226-0

Gelsomino, L., Panza, S., Giordano, C., Barone, I., Gu, G., Spina, E., et al. (2018). Mutations in the estrogen receptor alpha hormone binding domain promote stem cell phenotype through notch activation in breast cancer cell lines. *Cancer Lett.* 428, 12–20. doi:10.1016/j.canlet.2018.04.023

Generali, D., Berruti, A., Brizzi, M. P., Campo, L., Bonardi, S., Wigfield, S., et al. (2006). Hypoxia-inducible factor-1alpha expression predicts a poor response to primary chemoendocrine therapy and disease-free survival in primary human breast cancer, *Clin. Cancer Res.* 12, 4562–4568. doi:10.1158/1078-0432.CCR-05-2690

Genovese, I., Ilari, A., Assaraf, Y. G., Fazi, F., and Colotti, G. (2017) Not only P-glycoprotein: amplification of the ABCB1-containing chromosome region 7q21 confers multidrug resistance upon cancer cells by coordinated overexpression of an assortment of resistance-related proteins. *Drug Resist. Updates* 32, 23–46. doi:10.1016/j.drup.2017.10.003

Ghatak, S., Misra, S., and Toole, B. P. (2002). Hyaluronan oligosaccharides inhibit anchorage-independent growth of tumor cells by suppressing the phosphoinositide 3-kinase/Akt cell survival pathway. *J. Biol. Chem.* 277, 38013–38020. doi:10.1074/jbc.M202404200

Giannakakou, P., Sackett, D. L., Kang, Y. K., Zhan, Z., Buters, J. T., Fojo, T., et al. (1997). Paclitaxel-resistant human ovarian cancer cells have mutant beta-tubulins that exhibit impaired paclitaxel-driven polymerization. *J. Biol. Chem.* 272, 17118–17125. doi:10.1074/jbc.272.27.17118

Giessrigl, B., Schmidt, W. M., Kalipciyan, M., Jeitler, M., Bilban, M., Gollinger, M., et al. (2013). Fulvestrant induces resistance by modulating GPER and CDK6 expression: implication of methyltransferases, deacetylases and the hSWI/SNF chromatin remodelling complex. *Br. J. Cancer* 109, 2751–2762. doi:10.1038/bjc.2013.583

Ginestier, C., Hur, M. H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., et al. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 1, 555–567. doi:10.1016/j.stem.2007.08.014

Ginestier, C., Wicinski, J., Cervera, N., Monville, F., Finetti, P., Bertucci, F., et al. (2009). Retinoid signaling regulates breast cancer stem cell differentiation. *Cell Cycle* 8, 3297–3302. doi:10.4161/cc.8.20.9761

Ginestier, C., Liu, S., Diebel, M. E., Korkaya, H., Luo, M., Brown, M., et al. (2010). CXCR1 blockade selectively targets human breast cancer stem cells *in vitro* and in xenografts. *J. Clin. Invest.* 120, 485–497. doi:10.1172/JCI39397

Gil, M., Seshadri, M., Komorowski, M. P., Abrams, S. I., and Kozbor, D. (2013). Targeting CXCL12/CXCR4 signaling with oncolytic virotherapy disrupts tumor vasculature and inhibits breast cancer metastases. *Proc. Natl. Acad. Sci. U.S.A.* 110, E1291–E1300. doi:10.1073/pnas.1220580110

Gilani, R. A., Kazi, A. A., Shah, P., Schech, A. J., Chumsri, S., Sabnis, G., et al. The importance of HER2 signaling in the tumor-initiating cell population in aromatase inhibitor-resistant breast cancer, *Breast Cancer Res. Treat.* 135 (2012) 681–692. doi:10.1007/s10549-012-2148-8

Global Burden of Disease Cancer Collaboration, Fitzmaurice, C., Dicker, D., Pain, A., Hamavid, H., Moradi-Lakeh, M., et al. The global burden of cancer 2013, *JAMA Oncol.* 1 (2015) 505–527. doi:10.1001/jamaoncol.2015.0735

Goldsberry, W. N., Londono, A., Randall, T. D., Norian, L. A., and Arend, R. C. (2019). *A review of the role of Wnt in cancer immunomodulation*. Cancers (Basel), 11.

Gottesman, M. M., Fojo, T., and Bates, S. E. (2002). Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat. Rev. Cancer* 2, 48–58. doi:10.1038/nrc706

Gu, Z., Lee, R. Y., Skaar, T. C., Bouker, K. B., Welch, J. N., Lu, J., et al. (2002). Association of interferon regulatory factor-1, nucleophosmin, nuclear factor-kappaB, and cyclic AMP response element binding with acquired resistance to Faslodex (ICI 182,780). *Cancer Res.* 62, 3428–3437 .

Gwak, J. M., Kim, M., Kim, H. J., Jang, M. H., and Park, S. Y. (2017). Expression of embryonal stem cell transcription factors in breast cancer: Oct4 as an indicator for poor clinical outcome and tamoxifen resistance. *Oncotarget* 8, 36305–36318. doi:10.18632/oncotarget.16750

Gyorffy, B., Serra, V., Jurchott, K., Abdul-Ghani, R., Garber, M., Stein, U., et al. (2005). Prediction of doxorubicin sensitivity in breast tumors based on gene expression profiles of drug-resistant cell lines correlates with patient survival. *Oncogene* 24, 7542–7551. doi:10.1038/sj.onc.1208908

Han, M., Liu, M., Wang, Y., Mo, Z., Bi, X., Liu, Z., et al. (2012). Re-expression of miR-21 contributes to migration and invasion by inducing epithelial-mesenchymal transition consistent with cancer stem cell characteristics in MCF-7 cells. *Mol. Cell. Biochem.* 363, 427–436. doi:10.1007/s11010-011-1195-5

Harbeck, N., and Gnant, M. (2017). Breast cancer. *Lancet* 389, 1134–1150. doi:10.1016/S0140-6736(16)31891-8

Hardt, O., Wild, S., Oerlecke, I., Hofmann, K., Luo, S., Wiencek, Y., et al. (2012). Highly sensitive profiling of CD44+/CD24- breast cancer stem cells by combining global mRNA amplification and next generation sequencing: evidence for a hyperactive PI3K pathway, *Cancer Lett.* 325, 165–174. doi:10.1016/j.canlet.2012.06.010

Harrison, H., Simoes, B. M., Rogerson, L., Howell, S. J., Landberg, G., and Clarke, R. B. (2013). Oestrogen increases the activity of oestrogen receptor negative breast cancer stem cells through paracrine EGFR and Notch signalling. *Breast Cancer Res.* 15, R21. doi:10.1186/bcr3396

Hasanabady, M. H., and Kalalinia, F. (2016). ABCG2 inhibition as a therapeutic approach for overcoming multidrug resistance in cancer. *J. Biosci.* 41, 313–324. doi:10.1007/s12038-016-9601-5

He, M., Fu, Y., Yan, Y., Xiao, Q., Wu, H., Yao, W., et al. (2015). The Hedgehog signalling pathway mediates drug response of MCF-7 mammosphere cells in breast cancer patients. *Clin. Sci.* 129, 809–822. doi:10.1042/CS20140592

He, M., Wu, H., Jiang, Q., Liu, Y., Han, L., Yan, Y., et al. (2019). Hypoxia-inducible factor-2alpha directly promotes BCRP expression and mediates the resistance of ovarian cancer stem cells to adriamycin. *Mol. Oncol.* 13, 403–421. doi:10.1002/1878-0261.12419

Hikita, S. T., Kosik, K. S., Clegg, D. O., and Bamdad, C. (2008). MUC1* mediates the growth of human pluripotent stem cells. *PloS One* 3, e3312. doi:10.1371/journal.pone.0003312

Hirschmann-Jax, C., Foster, A. E., Wulf, G. G., Nuchtern, J. G., Jax, T. W., Gobel, U., et al. (2004). A distinct "side population" of cells with high drug efflux capacity in human tumor cells. *Proc. Natl. Acad. Sci. U.S.A.* 101, 14228–14233. doi:10.1073/pnas.0400067101

Hill, A., McFarlane, S., Mulligan, K., Gillespie, H., Draffin, J. E., Trimble, A., et al. (2006). Cortactin underpins CD44-promoted invasion and adhesion of breast

cancer cells to bone marrow endothelial cells. *Oncogene* 25, 6079–6091. doi:10.1038/sj.onc.1209628

Horwitz, S. B., Lothstein, L., Manfredi, J. J., Mellado, W., Parness, J., Roy, S. N., et al. (1986). Taxol: mechanisms of action and resistance. *Ann. N. Y. Acad. Sci.*, 466, 733–744. doi:10.1111/j.1749-6632.1986.tb38455.x

Hosea, R., Hardiany, N. S., Ohneda, O., and Wanandi, S. I. (2018). Glucosamine decreases the stemness of human ALDH(+) breast cancer stem cells by inactivating STAT3. *Oncol. Lett.* 16, 4737–4744. doi:10.3892/ol.2018.9222

Hosonaga, M., Arima, Y., Sugihara, E., Kohno, N., and Saya, H. (2014). Expression of CD24 is associated with HER2 expression and supports HER2-Akt signaling in HER2-positive breast cancer cells. *Cancer Sci.* 105, 779–787. doi:10.1111/cas.12427

Howell, A. (2008). The endocrine prevention of breast cancer, *Best Pract. Res. Clin. Endocrinol. Metabol.* 22, 615–623. doi:10.1016/j.beem.2008.09.002

Howell, A., and Robertson, J. (1995). Response to a specific antioestrogen (ICI 182780) in tamoxifen-resistant breast cancer. *Lancet* 345, 29–30. doi:10.1016/s0140-6736(95)90739-4

Hu, C., Li, H., Li, J., Zhu, Z., Yin, S., Hao, X., et al. (2008). Analysis of ABCG2 expression and side population identifies intrinsic drug efflux in the HCC cell line MHCC-97L and its modulation by Akt signaling. *Carcinogenesis* 29, 2289–2297. doi:10.1093/carcin/bgn223

Hu, C., Xu, L., Liang, S., Zhang, Z., Zhang, Y., and Zhang, F. (2016), Lentivirus-mediated shRNA targeting Nanog inhibits cell proliferation and attenuates cancer stem cell activities in breast cancer. *J. Drug Target.* 24, 422–432. doi:10.3109/1061186X.2015.1082567

Hui, M., Cazet, A., Nair, R., Watkins, D. N., O'Toole, S. A., and Swarbrick, A., The Hedgehog signalling pathway in breast development, carcinogenesis and cancer therapy, *Breast Cancer Res.* 15 (2013) 203. doi:10.1186/bcr3401

Iorio, M. V., Casalini, P., Piovan, C., Di Leva, G., Merlo, A., Triulzi, T., et al. (2009). microRNA-205 regulates HER3 in human breast cancer. *Cancer Res.* 69, 2195–2200. doi:10.1158/0008-5472.CAN-08-2920

Ishikawa, T., Utoh, M., Sawada, N., Nishida, M., Fukase, Y., Sekiguchi, F., et al. (1998). Tumor selective delivery of 5-fluorouracil by capecitabine, a new oral fluoropyrimidine carbamate, in human cancer xenografts. *Biochem. Pharmacol.* 55, 1091–1097. doi:10.1016/s0006-2952(97)00682-5

Jang, G. B., Hong, I. S., Kim, R. J., Lee, S. Y., Park, S. J., Lee, E. S., et al. (2015). Wnt/β-Catenin small-molecule inhibitor CWP232228 preferentially inhibits the growth of breast cancer stem-like cells. *Cancer Res.* 75, 1691–1702. doi:10.1158/0008-5472.CAN-14-2041

Jia, D., Tan, Y., Liu, H., Ooi, S., Li, L., Wright, K., et al. (2016). Cardamonin reduces chemotherapy-enriched breast cancer stem-like cells *in vitro* and *in vivo*. *Oncotarget* 7, 771–785. doi:10.18632/oncotarget.5819

Jiang, J., Li, H., Qaed, E., Zhang, J., Song, Y., Wu, R., et al. (2018). Salinomycin, as an autophagy modulator-- a new avenue to anticancer: a review. *J. Exp. Clin. Cancer Res.* 37, 26. doi:10.1186/s13046-018-0680-z

Jin, X., Jin, X., and Kim, H. (2017). Cancer stem cells and differentiation therapy. *Tumour Biol.* 39, 1010428317729933. doi:10.1177/1010428317729933

Kazi, A. A., Gilani, R. A., Schech, A. J., Chumsri, S., Sabnis, G., Shah, P., et al. (2014). Nonhypoxic regulation and role of hypoxia-inducible factor 1 in aromatase inhibitor resistant breast cancer. *Breast Cancer Res.* 16, R15. doi:10.1186/bcr3609

Kettner, N. M., Vijayaraghavan, S., Durak, M. G., Bui, T., Kohansal, M., Ha, M. J., et al. (2019). Combined inhibition of STAT3 and DNA repair in palbociclib-resistant ER-positive breast cancer. *Clin. Cancer Res.* 25, 3996–4013. doi:10.1158/1078-0432.CCR-18-3274

Kim, S. H., and Singh, S. V. (2014). Mammary cancer chemoprevention by withaferin A is accompanied by *in vivo* suppression of self-renewal of cancer stem cells. *Cancer Prev. Res.* 7, 738–747. doi:10.1158/1940-6207.CAPR-13-0445

Koninki, K., Barok, M., Tanner, M., Staff, S., Pitkanen, J., Hemmila, P., et al. (2010). Multiple molecular mechanisms underlying trastuzumab and lapatinib resistance in JIMT-1 breast cancer cells. *Cancer Lett.* 294, 211–219. doi:10.1016/j.canlet.2010.02.002

Koike, Y., Ohta, Y., Saitoh, W., Yamashita, T., Kanomata, N., Moriya, T., et al. (2017). Anti-cell growth and anti-cancer stem cell activities of the non-canonical hedgehog inhibitor GANT61 in triple-negative breast cancer cells. *Breast Cancer* 24, 683–693. doi:10.1007/s12282-017-0757-0

Kolev, V. N., Wright, Q. G., Vidal, C. M., Ring, J. E., Shapiro, I. M., Ricono, J., et al. (2015). PI3K/mTOR dual inhibitor VS-5584 preferentially targets cancer stem cells, *Cancer Res.* 75, 446–455. doi:10.1158/0008-5472.CAN-14-1223

Kopp, A., Jonat, W., Schmahl, M., and Knabbe, C. (1995). Transforming growth factor beta 2 (TGF-beta 2) levels in plasma of patients with metastatic breast cancer treated with tamoxifen. *Cancer Res.* 55, 4512–4515.

Korkaya, H., Paulson, A., Charafe-Jauffret, E., Ginestier, C., Brown, M., Dutcher, J., et al. Regulation of mammary stem/progenitor cells by PTEN/Akt/beta-catenin signaling. *PLoS Biol.* 7, e1000121 (2009). doi:10.1371/journal.pbio.1000121

Korkaya, H., Kim, G. I., Davis, A., Malik, F., Henry, N. L., Ithimakin, S., et al. (2012). Activation of an IL6 inflammatory loop mediates trastuzumab resistance in HER2+ breast cancer by expanding the cancer stem cell population. *Mol. Cell* 47, 570–584. doi:10.1016/j.molcel.2012.06.014

Koury, J., Zhong, L., and Hao, J. (2017). Targeting signaling pathways in cancer stem cells for cancer treatment. *Stem Cell. Int.* 2017 2925869. doi:10.1155/2017/2925869

Kubo, M., Nakamura, M., Tasaki, A., Yamanaka, N., Nakashima, H., Nomura, M., et al. (2004). Hedgehog signaling pathway is a new therapeutic target for patients with breast cancer. *Cancer Res.* 64, 6071–6074. doi:10.1158/0008-5472.CAN-04-0416

Lathan, B., and Von Hoff, D. D. (1984). Cytotoxic activity of echinomycin in a human tumor cloning system. *Cancer Drug Deliv.* 1, 191–198. doi:10.1089/cdd.1984.1.191

Latorre, E., Carelli, S., Raimondi, I., D'Agostino, V., Castiglioni, I., Zucal, C., et al. The ribonucleic complex HuR-MALAT1 represses CD133 expression and suppresses epithelial-mesenchymal transition in breast cancer, *Cancer Res.* 76 (2016) 2626–2636. doi:10.1158/0008-5472.CAN-15-2018

Leccia, F., Del Vecchio, L., Mariotti, E., Di Noto, R., Morel, A. P., Puisieux, A., et al. (2014). ABCG2, a novel antigen to sort luminal progenitors of BRCA1- breast cancer cells. *Mol. Cancer* 13, 213. doi:10.1186/1476-4598-13-213

Lee, E., Nichols, P., Spicer, D., Groshen, S., Yu, M. C., and Lee, A. S. (2006). GRP78 as a novel predictor of responsiveness to chemotherapy in breast cancer. *Cancer Res.* 66, 7849–7853. doi:10.1158/0008-5472.CAN-06-1660

Lee, K. M., Giltnane, J. M., Balko, J. M., Schwarz, L. J., Guerrero-Zotano, A. L., Hutchinson, K. E., et al. (2017). MYC and MCL1 cooperatively promote chemotherapy-resistant breast cancer stem cells via regulation of mitochondrial oxidative phosphorylation, *Cell Metabol.* 26, 633–647.e7. doi:10.1016/j.cmet.2017.09.009

Li, Y., Xian, M., Yang, B., Ying, M., and He, Q. (2017). Inhibition of KLF4 by statins reverses adriamycin-induced metastasis and cancer stemness in Osteosarcoma cells, *Stem Cell Rep.* 8, 1617–1629. doi:10.1016/j.stemcr.2017.04.025

Li, Y., Zhang, T., Korkaya, H., Liu, S., Lee, H. F., Newman, B., et al. (2010). Sulforaphane, a dietary component of broccoli/broccoli sprouts, inhibits breast cancer stem cells. *Clin. Cancer Res.* 16, 2580–2590. doi:10.1158/1078-0432.CCR-09-2937

Li, C. Y., Miao, K. L., Chen, Y., Liu, L. Y., Zhao, G. B., Lin, M. H., et al. (2018). Jagged2 promotes cancer stem cell properties of triple negative breast cancer cells and paclitaxel resistance via regulating microRNA-200. *Eur. Rev. Med. Pharmacol. Sci.*, 22, 6008–6014. doi:10.26355/eurrev_201809_15936

Li, Y. W., Xu, J., Zhu, G. Y., Huang, Z. J., Lu, Y., Li, X. Q., et al. (2018). Apigenin suppresses the stem cell-like properties of triple-negative breast cancer cells by inhibiting YAP/TAZ activity. *Cell Death Dis.* 4, 105. doi:10.1038/s41420-018-0124-8

Li, S., Fan, Y., Kumagai, A., Kawakita, E., Kitada, M., Kanasaki, K., et al. (2020). Deficiency in dipeptidyl peptidase-4 promotes chemoresistance through the CXCL12/CXCR4/mTOR/TGFbeta signaling pathway in breast cancer cells. *Int. J. Mol. Sci.* 21, 805. doi:10.3390/ijms21030805

Li, S. Y., Sun, R., Wang, H. X., Shen, S., Liu, Y., Du, X. J., et al. (2015). Combination therapy with epigenetic-targeted and chemotherapeutic drugs delivered by nanoparticles to enhance the chemotherapy response and overcome resistance by breast cancer stem cells. *J. Contr. Release* 205, 7–14. doi:10.1016/j.jconrel.2014.11.011

Liang, Y. J., Wang, C. Y., Wang, I. A., Chen, Y. W., Li, L. T., Lin, C. Y., et al. (2017). Interaction of glycosphingolipids GD3 and GD2 with growth factor receptors maintains breast cancer stem cell phenotype. *Oncotarget*. 8, 47454–47473. doi:10.18632/oncotarget.17665

Liapis, V., Zinonos, I., Labrinidis, A., Hay, S., Ponomarev, V., Panagopoulos, V., et al. (2016). Anticancer efficacy of the hypoxia-activated prodrug evofosfamide

(TH-302) in osteolytic breast cancer murine models. *Cancer Med.* 5, 534–545. doi:10.1002/cam4.599

Lillo, M. A., Nichols, C., Perry, C., Runke, S., Krutilina, R., Seagroves, T. N., et al. (2017). Methylparaben stimulates tumor initiating cells in ER+ breast cancer models, *J. Appl. Toxicol.* 37, 417–425. doi:10.1002/jat.3374

Liu, C., Xing, H., Guo, C., Yang, Z., Wang, Y., and Wang, Y. (2019). MiR-124 reversed the doxorubicin resistance of breast cancer stem cells through STAT3/HIF-1 signaling pathways. *Cell Cycle* 18, 2215–2227. doi:10.1080/15384101.2019.1638182

Liu, J., Ye, Z., Xiang, M., Chang, B., Cui, J., Ji, T., et al. (2019). Functional extracellular vesicles engineered with lipid-grafted hyaluronic acid effectively reverse cancer drug resistance. *Biomaterials* 223, 119475. doi:10.1016/j.biomaterials.2019.119475

Liu, Y., Zhang, X. B., Liu, J. J., Zhang, S., and Zhang, J. (2019). [NVP-BKM120 in combination with letrozole inhibit human breast cancer stem cells via PI3K/mTOR pathway]. *Zhonghua Yixue Zazhi* 99, 1075–1080. doi:10.3760/cma.j.issn.0376-2491.2019.14.008

Liu, L., Yang, M., Kang, R., Wang, Z., Zhao, Y., Yu, Y., et al. (2011). DAMP-mediated autophagy contributes to drug resistance. *Autophagy* 7, 112–114. doi:10.4161/auto.7.1.14005

Liu, T. J., Sun, B. C., Zhao, X. L., Zhao, X. M., Sun, T., Gu, Q., et al. (2013). CD133+ cells with cancer stem cell characteristics associates with vasculogenic mimicry in triple-negative breast cancer. *Oncogene* 32, 544–553. doi:10.1038/onc.2012.85

Liu, Y., Zhang, X., Liu, J., Hou, G., Zhang, S., and Zhang, J. (2014). Everolimus in combination with letrozole inhibit human breast cancer MCF-7/Aro stem cells via PI3K/mTOR pathway: an experimental study, *Tumour Biol.* 35, 1275–1286. doi:10.1007/s13277-013-1170-8

Liu, Y., Yu, C., Wu, Y., Sun, X., Su, Q., You, C., et al. (2017). CD44. *J. Cell Mol. Med.* 21, 1979–1988. doi:10.1111/jcmm.13118

Liu, B. Q., Zhang, S., Li, S., An, M. X., Li, C., Yan, J., et al. (2017). BAG3 promotes stem cell-like phenotype in breast cancer by upregulation of CXCR4 via interaction with its transcript. *Cell Death Dis.* 8, e2933. doi:10.1038/cddis.2017.324

Liu, Z., Bandyopadhyay, A., Nichols, R. W., Wang, L., Hinck, A. P., Wang, S., et al. (2012). Blockade of autocrine TGF-beta signaling inhibits stem cell phenotype, survival, and metastasis of murine breast cancer cells, *J. Stem Cell Res. Ther.* 2, 1–8. doi:10.4172/2157-7633.1000116

Locatelli, M. A., Aftimos, P., Dees, E. C., LoRusso, P. M., Pegram, M. D., Awada, A., et al. (2017). Phase I study of the gamma secretase inhibitor PF-03084014 in combination with docetaxel in patients with advanced triple-negative breast cancer, *Oncotarget*. 8, 2320–2328. doi:10.18632/oncotarget.13727

Loh, Y. N., Hedditch, E. L., Baker, L. A., Jary, E., Ward, R. L., and Ford, C. E. (2013). The Wnt signalling pathway is upregulated in an *in vitro* model of acquired tamoxifen resistant breast cancer. *BMC Cancer* 13, 174. doi:10.1186/1471-2407-13-174

Lombardo, Y., Faronato, M., Filipovic, A., Vircillo, V., Magnani, L., and Coombes, R. C. (2014). Nicastrin and Notch4 drive endocrine therapy resistance and epithelial to mesenchymal transition in MCF7 breast cancer cells. *Breast Cancer Res.* 16, R62. doi:10.1186/bcr3675

Louderbough, J. M., and Schroeder, J. A. (2011). Understanding the dual nature of CD44 in breast cancer progression. *Mol. Cancer Res.* 9, 1573–1586. doi:10.1158/1541-7786.MCR-11-0156

Luker, K. E., Lewin, S. A., Mihalko, L. A., Schmidt, B. T., Winkler, J. S., Coggins, N. L., et al. (2012). Scavenging of CXCL12 by CXCR7 promotes tumor growth and metastasis of CXCR4-positive breast cancer cells. *Oncogene* 31, 4750–4758. doi:10.1038/onc.2011.633

Luo, M. L., Gong, C., Chen, C. H., Hu, H., Huang, P., Zheng, M., et al. (2015). The Rab2A GTPase promotes breast cancer stem cells and tumorigenesis via Erk signaling activation. *Cell Rep.* 11, 111–124. doi:10.1016/j.celrep.2015.03.002

Lv, Y., Xu, C., Zhao, X., Lin, C., Yang, X., Xin, X., et al. (2018a). Nanoplatform assembled from a CD44-targeted prodrug and smart liposomes for dual targeting of tumor microenvironment and cancer cells. *ACS Nano* 12, 1519–1536. doi:10.1021/acsnano.7b08051

Lv, Y., Zhao, X., Zhu, S., Li, S., Xiao, Q., He, W., et al. (2018b). Targeting intracellular MMPs efficiently inhibits tumor metastasis and angiogenesis. *Theranostics* 8, 2830–2845. doi:10.7150/thno.23209

Lytle, N. K., Barber, A. G., and Reya, T. (2018). Stem cell fate in cancer growth, progression and therapy resistance. *Nat. Rev. Cancer* 18, 669–680. doi:10.1038/s41568-018-0056-x

Ma, C. X., Reinert, T., Chmielewska, I., and Ellis, M. J. (2015). Mechanisms of aromatase inhibitor resistance. *Nat. Rev. Cancer* 15, 261–275. doi:10.1038/nrc3920

Ma, R., Karthik, G. M., Lovrot, J., Haglund, F., Rosin, G., Katchy, A., et al. (2017). Estrogen receptor beta as a therapeutic target in breast cancer stem cells. *J. Natl. Cancer Inst.* 109, 1–14. doi:10.1093/jnci/djw236

Macedo, L. F., Guo, Z., Tilghman, S. L., Sabnis, G. J., Qiu, Y., and Brodie, A. (2006). Role of androgens on MCF-7 breast cancer cell growth and on the inhibitory effect of letrozole. *Cancer Res.* 66, 7775–7782. doi:10.1158/0008-5472.CAN-05-3984

Magnani, L., Stoeck, A., Zhang, X., Lanczky, A., Mirabella, A. C., Wang, T. L., et al. (2013). Genome-wide reprogramming of the chromatin landscape underlies endocrine therapy resistance in breast cancer. *Proc. Natl. Acad. Sci. U.S.A.* 110, E1490–E1499. doi:10.1073/pnas.1219992110

Magnani, M., Ortuso, F., Soro, S., Alcaro, S., Tramontano, A., and Botta, M. (2006). The betaI/betaIII-tubulin isoforms and their complexes with antimitotic agents. Docking and molecular dynamics studies. *FEBS J.* 273, 3301–3310. doi:10.1111/j.1742-4658.2006.05340.x

Magnifico, A., Albano, L., Campaner, S., Delia, D., Castiglioni, F., Gasparini, P., et al. (2009). Tumor-initiating cells of HER2-positive carcinoma cell lines express the highest oncoprotein levels and are sensitive to trastuzumab. *Clin. Cancer Res.* 15, 2010–2021. doi:10.1158/1078-0432.CCR-08-1327

Manavathi, B., Dey, O., Gajulapalli, V. N., Bhatia, R. S., Bugide, S., and Kumar, R. (2013). Derailed estrogen signaling and breast cancer: an authentic couple. *Endocr. Rev.* 34, 1–32. doi:10.1210/er.2011-1057

Manerba, M., Govoni, M., Manet, I., Leale, A., Comparone, A., and Di Stefano, G. (2019). Metabolic activation triggered by cAMP in MCF-7 cells generates lethal vulnerability to combined oxamate/etomoxir. *Biochim. Biophys. Acta Gen. Subj.* 1863, 1177–1186. doi:10.1016/j.bbagen.2019.04.008

Majumder, M., Xin, X., Liu, L., Tutunea-Fatan, E., Rodriguez-Torres, M., Vincent, K., et al. (2016). COX-2 induces breast cancer stem cells via EP4/PI3K/AKT/NOTCH/WNT Axis. *Stem Cell.* 34, 2290–2305. doi:10.1002/stem.2426

Mao, Q., and Unadkat, J. D. (2015). Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update. *AAPS J.* 17, 65–82. doi:10.1208/s12248-014-9668-6

Marcato, P., Dean, C. A., Pan, D., Araslanova, R., Gillis, M., Joshi, M., et al. (2011). Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. *Stem Cell.* 29, 32–45. doi:10.1002/stem.563

Marsden, C. G., Wright, M. J., Pochampally, R., and Rowan, B. G. (2009). Breast tumor-initiating cells isolated from patient core biopsies for study of hormone action. *Methods Mol. Biol.* 590, 363–375. doi:10.1007/978-1-60327-378-7_23

Martin-Castillo, B., Oliveras-Ferraros, C., Vazquez-Martin, A., Cufi, S., Moreno, J. M., Corominas-Faja, B., et al. (2013). Basal/HER2 breast carcinomas: integrating molecular taxonomy with cancer stem cell dynamics to predict primary resistance to trastuzumab (Herceptin). *Cell Cycle* 12, 225–245. doi:10.4161/cc.23274

Marotta, L. L., Almendro, V., Marusyk, A., Shipitsin, M., Schemme, J., Walker, S. R., et al. (2011). The JAK2/STAT3 signaling pathway is required for growth of $CD44^+CD24^-$ stem cell-like breast cancer cells in human tumors. *J. Clin. Invest.* 121, 2723–2735. doi:10.1172/JCI44745

McCartney, A., Moretti, E., Sanna, G., Pestrin, M., Risi, E., Malorni, L., et al. (2018). The role of abemaciclib in treatment of advanced breast cancer. *Ther. Adv. Med. Oncol.* 10, 1758835918776925. doi:10.1177/1758835918776925

McClelland, R. A., Barrow, D., Madden, T. A., Dutkowski, C. M., Pamment, J., Knowlden, J. M., et al. (2001). Enhanced epidermal growth factor receptor signaling in MCF7 breast cancer cells after long-term culture in the presence of the pure antiestrogen ICI 182,780 (Faslodex). *Endocrinology* 142, 2776–2788. doi:10.1210/endo.142.7.8259

Meyer, M. J., Fleming, J. M., Lin, A. F., Hussnain, S. A., Ginsburg, E., and Vonderhaar, B. K. (2010). CD44posCD49fhiCD133/2hi defines xenograft-initiating cells in estrogen receptor-negative breast cancer. *Cancer Res.* 70, 4624–4633. doi:10.1158/0008-5472.CAN-09-3619

Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410, 50–56. doi:10.1038/35065016

Mohammady, M., Ghetmiri, S. I., Baharizade, M., Morowvat, M. H., and Torabi, S. (2019). Expanding the biotherapeutics realm via miR-34a: "potent clever little" agent in breast cancer therapy. *Curr. Pharmaceut. Biotechnol.* 20, 665–673. doi:10.2174/1389201020666190617162042

Mukherjee, P., Gupta, A., Chattopadhyay, D., and Chatterji, U. (2017). Modulation of SOX2 expression delineates an end-point for paclitaxel-effectiveness in breast cancer stem cells. *Sci. Rep.* 7, 9170. doi:10.1038/s41598-017-08971-2

Mukherjee, S., Manna, A., Bhattacharjee, P., Mazumdar, M., Saha, S., Chakraborty, S., et al. (2016). Non-migratory tumorigenic intrinsic cancer stem cells ensure breast cancer metastasis by generation of CXCR4(+) migrating cancer stem cells, *Oncogene*, 35, 4937–4948. doi:10.1038/onc.2016.26

Muntimadugu, E., Kumar, R., Saladi, S., Rafeeqi, T. A., and Khan, W. (2016). CD44 targeted chemotherapy for co-eradication of breast cancer stem cells and cancer cells using polymeric nanoparticles of salinomycin and paclitaxel. *Colloids Surf. B Biointerfaces* 143, 532–546. doi:10.1016/j.colsurfb.2016.03.075

Munz, M., Baeuerle, P. A., and Gires, O. (2009). The emerging role of EpCAM in cancer and stem cell signaling, *Cancer Res.* 69, 5627–5629. doi:10.1158/0008-5472.CAN-09-0654

Musgrove, E. A., and Sutherland, R. L. (2009). Biological determinants of endocrine resistance in breast cancer, *Nat. Rev. Cancer*, 9, 631–643. doi:10.1038/nrc2713

Muthupandian, S., Barabadi, H., Ramachandran, B., Venkatraman, G., and Ponmurugan, K. (2019). Emerging plant-based anti-cancer green nanomaterials in present scenario. *Compr. Anal. Chem.* 87, 291–318. doi:10.1016/bs.coac.2019.09.001

Nalla, L. V., Kalia, K., and Khairnar, A. (2019). Self-renewal signaling pathways in breast cancer stem cells. *Int. J. Biochem. Cell Biol.* 107,140–153. doi:10.1016/j.biocel.2018.12.017

Nagata, Y., Lan, K. H., Zhou, X., Tan, M., Esteva, F. J., Sahin, A. A., et al. (2004). PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* 6, 117–127. doi:10.1016/j.ccr.2004.06.022

Naito, M., Matsuba, Y., Sato, S., Hirata, H., and Tsuruo, T. (2002). MS-209, a quinoline-type reversal agent, potentiates antitumor efficacy of docetaxel in multidrug-resistant solid tumor xenograft models. *Clin. Cancer Res.* 8, 582–588

Nasr, M., Farghaly, M., Elsaba, T., El-Mokhtar, M., Radwan, R., Elsabahy, M., et al. (2018). Resistance of primary breast cancer cells with enhanced pluripotency and stem cell activity to sex hormonal stimulation and suppression. *Int. J. Biochem. Cell Biol.* 105, 84–93. doi:10.1016/j.biocel.2018.10.005

Naor, D., Sionov, R. V., and Ish-Shalom, D. (1997). CD44: structure, function, and association with the malignant process. *Adv. Cancer Res.* 71, 241–319. doi:10.1016/s0065-230x(08)60101-3

Nguyen, Y. T., Moon, J. Y., Ediriweera, M. K., and Cho, S. K. (2020). Phenethyl isothiocyanate suppresses stemness in the chemo- and radio-resistant triple-negative breast cancer cell line MDA-MB-231/IR via downregulation of metadheri, *Cancers*, 12, 268. 10.3390/cancers12020268.

Nicolay, N. H., Ruhle, A., Perez, R. L., Trinh, T., Sisombath, S., Weber, K. J., et al. (2016). Mesenchymal stem cells exhibit resistance to topoisomerase inhibition. *Cancer Lett.* 374, 75–84. doi:10.1016/j.canlet.2016.02.007

Nourbakhsh, M., Farzaneh, S., Taghikhani, A., Zarghi, A., and Noori, S. (2020). The effect of a newly synthesized ferrocene derivative AGAINST MCF-7 breast cancer cells and spheroid stem cells through ROS production and inhibition of JAK2/STAT3 signaling pathway. *Anticancer Agents Med. Chem.* 20, 875–886. doi:10.2174/1871520620666200101151743

Ocana, A., Gil-Martin, M., Martin, M., Rojo, F., Antolin, S., Guerrero, A., et al. (2017). A phase I study of the SRC kinase inhibitor dasatinib with trastuzumab and paclitaxel as first line therapy for patients with HER2-overexpressing advanced breast cancer. *Oncotarget* 8, 73144–73153. doi:10.18632/oncotarget.17113

Ohlfest, J. R., Zellmer, D. M., Panyam, J., Swaminathan, S. K., Oh, S., Waldron, N. N., et al. (2013). Immunotoxin targeting CD133(+) breast carcinoma cells, *Drug Deliv. Transl. Res.* 3, 195–204. doi:10.1007/s13346-012-0066-2

Ojo, D., Wei, F., Liu, Y., Wang, E., Zhang, H., Lin, X., et al. (2015). Factors promoting tamoxifen resistance in breast cancer via stimulating breast cancer stem cell expansion. *Curr. Med. Chem.* 22, 2360–2374. doi:10.2174/0929867322666150416095744

Oudin, M. J., Barbier, L., Schafer, C., Kosciuk, T., Miller, M. A., Han, S., et al. (2017). MENA confers resistance to paclitaxel in triple-negative breast cancer. *Mol. Cancer Ther.* 16, 143–155. doi:10.1158/1535-7163.MCT-16-0413

Ouhtit, A., Rizeq, B., Saleh, H. A., Rahman, M. M., and Zayed, H. (2018). Novel CD44-downstream signaling pathways mediating breast tumor invasion. *Int. J. Biol. Sci.* 14, 1782–1790. doi:10.7150/ijbs.23586

Oskarsson, T., Batlle, E., and Massagué, J. (2014). Metastatic stem cells: sources, niches, and vital pathways. *Cell Stem Cell* 14, 306–321. doi:10.1016/j.stem.2014.02.002

O'Toole, S. A., Machalek, D. A., Shearer, R. F., Millar, E. K., Nair, R., Schofield, P., et al. (2011). Hedgehog overexpression is associated with stromal interactions and predicts for poor outcome in breast cancer. *Cancer Res.* 71, 4002–4014. doi:10.1158/0008-5472.CAN-10-3738

Palomeras, S., Ruiz-Martinez, S., and Puig, T. (2018). Targeting breast cancer stem cells to overcome treatment resistance. *Molecules* 23, 2193. doi:10.3390/molecules23092193

Pandey, K., An, H. J., Kim, S. K., Lee, S. A., Kim, S., Lim, S. M., et al. (2019). Molecular mechanisms of resistance to CDK4/6 inhibitors in breast cancer: a review, *Int. J. Cancer* 145, 1179–1188. doi:10.1002/ijc.32020

Pece, S., Tosoni, D., Confalonieri, S., Mazzarol, G., Vecchi, M., Ronzoni, S., et al. (2010). Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. *Cell* 140, 62–73. doi:10.1016/j.cell.2009.12.007

Pelekanou, V., Notas, G., Athanasouli, P., Alexakis, K., Kiagiadaki, F., Peroulis, N., et al. (2018). BCMA (TNFRSF17) induces APRIL and BAFF mediated breast cancer cell stemness *Front. Oncol.* 8, 301. doi:10.3389/fonc.2018.00301

Pena-Solorzano, D., Stark, S. A., Konig, B., Sierra, C. A., and Ochoa-Puentes, C., ABCG2/BCRP: specific and nonspecific modulators. *Med. Res. Rev.*, 37 (2017) 987–1050. doi:10.1002/med.21428

Phillips, T. M., McBride, W. H., and Pajonk, F. (2006). The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. *J. Natl. Cancer Inst.* 98, 1777–1785. doi:10.1093/jnci/djj495

Piva, M., Domenici, G., Iriondo, O., Rabano, M., Simoes, B. M., Comaills, V., et al. (2014). Sox2 promotes tamoxifen resistance in breast cancer cells. *EMBO Mol. Med.* 6, 66–79. doi:10.1002/emmm.201303411

Ponta, H., Sherman, L., and Herrlich, P. A. (2003). CD44: from adhesion molecules to signalling regulators. *Nat. Rev. Mol. Cell Biol.* 4, 33–45. doi:10.1038/nrm1004

Ponti, D., Costa, A., Zaffaroni, N., Pratesi, G., Petrangolini, G., Coradini, D., et al. (2005). Isolation and *in vitro* propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res.* 65, 5506–5511. doi:10.1158/0008-5472.CAN-05-0626

Posada, I. M. D., Lectez, B., Sharma, M., Oetken-Lindholm, C., Yetukuri, L., Zhou, Y., et al. (2017). Rapalogs can promote cancer cell stemness *in vitro* in a Galectin-1 and H-ras-dependent manner, *Oncotarget*, 8, 44550–44566. doi:10.18632/oncotarget.17819

Press, M. F., Sauter, G., Buyse, M., Bernstein, L., Guzman, R., Santiago, A., et al. (2011). Alteration of topoisomerase II-alpha gene in human breast cancer: association with responsiveness to anthracycline-based chemotherapy. *J. Clin. Oncol.* 29, 859–867. doi:10.1200/JCO.2009.27.5644

Raffo, D., Berardi, D. E., Pontiggia, O., Todaro, L., de Kier Joffe, E. B., and Simian, M. (2013). Tamoxifen selects for breast cancer cells with mammosphere forming capacity and increased growth rate. *Breast Cancer Res. Treat.* 142, 537–548. doi:10.1007/s10549-013-2760-2

Ramaswamy, B., Lu, Y., Teng, K. Y., Nuovo, G., Li, X., Shapiro, C. L., et al. (2012). Hedgehog signaling is a novel therapeutic target in tamoxifen-resistant breast cancer aberrantly activated by PI3K/AKT pathway. *Cancer Res.* 72, 5048–5059. doi:10.1158/0008-5472.CAN-12-1248

Real, P. J., and Ferrando, A. A. (2009). NOTCH inhibition and glucocorticoid therapy in T-cell acute lymphoblastic leukemia. *Leukemia* 23, 1374–1377. doi:10.1038/leu.2009.75

Rebucci, M., and Michiels, C. (2013). Molecular aspects of cancer cell resistance to chemotherapy. *Biochem. Pharmacol.* 85, 1219–1226. doi:10.1016/j.bcp.2013.02.017

Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* 414, 105–111. doi:10.1038/35102167

Rhodes, L. V., Muir, S. E., Elliott, S., Guillot, L. M., Antoon, J. W., Penfornis, P., et al. (2010). Adult human mesenchymal stem cells enhance breast tumorigenesis and promote hormone independence. *Breast Cancer Res. Treat.* 121, 293–300. doi:10.1007/s10549-009-0458-2

Robey, R. W., Pluchino, K. M., Hall, M. D., Fojo, A. T., Bates, S. E., and Gottesman, M. M. (2018). Revisiting the role of ABC transporters in multidrug-resistant cancer. *Nat. Rev. Cancer* 18, 452–464. doi:10.1038/s41568-018-0005-8

Rodriguez, D., Ramkairsingh, M., Lin, X., Kapoor, A., Major, P., and Tang, D. (2019). The central contributions of breast cancer stem cells in developing resistance to endocrine therapy in estrogen receptor (ER)-Positive breast cancer. *Cancers (Basel)* 11, 1028. doi:10.3390/cancers11071028

Roscigno, G., Quintavalle, C., Donnarumma, E., Puoti, I., Diaz-Lagares, A., Iaboni, M., et al. (2016). MiR-221 promotes stemness of breast cancer cells by targeting DNMT3b. *Oncotarget* 7, 580–592. doi:10.18632/oncotarget.5979

Saeki, T., Nomizum, T., Toi, M., Ito, Y., Noguchi, S., Kobayashi, T., et al. (2007). Dofequidar fumarate (MS-209) in combination with cyclophosphamide, doxorubicin, and fluorouracil for patients with advanced or recurrent breast cancer. *J. Clin. Oncol.* 25, 411–417. doi:10.1200/JCO.2006.08.1646

Samanta, D., Gilkes, D. M., Chaturvedi, P., Xiang, L., and Semenza, G. L. (2014). Hypoxia-inducible factors are required for chemotherapy resistance of breast cancer stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5429–E5438. doi:10.1073/pnas.1421438111

Sand, A., Piacsek, M., Donohoe, D. L., Duffin, A. T., Riddell, G. T., Sun, C., et al. (2020). WEE1 inhibitor, AZD1775, overcomes trastuzumab resistance by targeting cancer stem-like properties in HER2-positive breast cancer. *Cancer Lett.* 472, 119–131. doi:10.1016/j.canlet.2019.12.023

Sansone, P., Ceccarelli, C., Berishaj, M., Chang, Q., Rajasekhar, V. K., Perna, F., et al. (2016). Self-renewal of CD133(hi) cells by IL6/Notch3 signalling regulates endocrine resistance in metastatic breast cancer. *Nat. Commun.* 7, 10442. doi:10.1038/ncomms10442

Sarkar, A., Chanda, A., Regmi, S. C., Karve, K., Deng, L., Jay, G. D., et al. (2019). Recombinant human PRG4 (rhPRG4) suppresses breast cancer cell invasion by inhibiting TGFbeta-Hyaluronan-CD44 signalling pathway. *PLoS One* 14, e0219697. doi:10.1371/journal.pone.0219697

Saravanan, M., Vahidi, H., Cruz, D. M., Vernet-Crua, A., and Barabadi, H. (2020). Emerging antineoplastic biogenic gold nanomaterials for breast cancer therapeutics: a systematic review. *Int. J. Nanomed.* 15, 3577–3595. doi:10.2147/IJN.S240293

Scatena, C., Roncella, M., Di Paolo, A., Aretini, P., Menicagli, M., Fanelli, G., et al. (2018). Doxycycline, an inhibitor of mitochondrial biogenesis, effectively reduces cancer stem cells (CSCs) in early breast cancer patients: a clinical pilot study. *Front. Oncol.* 8, 452. doi:10.3389/fonc.2018.00452

Schaffner, F., Yokota, N., Carneiro-Lobo, T., Kitano, M., Schaffer, M., Anderson, G. M., et al. (2013). Endothelial protein C receptor function in murine and human breast cancer development. *PLoS One* 8, e61071. doi:10.1371/journal.pone.0061071

Schott, A. F., Landis, M. D., Dontu, G., Griffith, K. A., Layman, R. M., Krop, I., et al. (2013). Preclinical and clinical studies of gamma secretase inhibitors with docetaxel on human breast tumors. *Clin. Cancer Res.* 19, 1512–1524. doi:10.1158/1078-0432.CCR-11-3326

Semenza, G. L. (2015). Regulation of the breast cancer stem cell phenotype by hypoxia-inducible factors. *Clin. Sci.* 129, 1037–1045. doi:10.1042/CS20150451

Sena, L. A., and Chandel, N. S. (2012). Physiological roles of mitochondrial reactive oxygen species. *Mol. Cell* 48, 158–167. doi:10.1016/j.molcel.2012.09.025

Seo, A. N., Lee, H. J., Kim, E. J., Jang, M. H., Kim, Y. J., Kim, J. H., et al. (2016). Expression of breast cancer stem cell markers as predictors of prognosis and response to trastuzumab in HER2-positive breast cancer. *Br. J. Cancer* 114, 1109–1116. doi:10.1038/bjc.2016.101

Shi, H., Zhang, W., Zhi, Q., and Jiang, M. (2016). Lapatinib resistance in HER2+ cancers: latest findings and new concepts on molecular mechanisms. *Tumour Biol.* 37, 15411–15431. doi:10.1007/s13277-016-5467-2

Shan, N. L., Wahler, J., Lee, H. J., Bak, M. J., Gupta, S. D., Maehr, H., et al. (2017). Vitamin D compounds inhibit cancer stem-like cells and induce differentiation in triple negative breast cancer. *J. Steroid Biochem. Mol. Biol.* 173, 122–129. doi:10.1016/j.jsbmb.2016.12.001

Shigdar, S., Qiao, L., Zhou, S. F., Xiang, D., Wang, T., Li, Y., et al. (2013). RNA aptamers targeting cancer stem cell marker CD133. *Cancer Lett.* 330, 84–95. doi:10.1016/j.canlet.2012.11.032

Shim, Y., and Song, J. M. (2015). Quantum dot nanoprobe-based high-content monitoring of notch pathway inhibition of breast cancer stem cell by capsaicin. *Mol. Cell. Probes* 29, 376–381. doi:10.1016/j.mcp.2015.09.004

Shinde, A., Hardy, S. D., Kim, D., Akhand, S. S., Jolly, M. K., Wang, W. H., et al. (2019). Spleen tyrosine kinase-mediated autophagy is required for epithelial-mesenchymal plasticity and metastasis in breast cancer. *Cancer Res.* 79, 1831–1843. doi:10.1158/0008-5472.CAN-18-2636

Si, X., Liu, Y., Lv, J., Ding, H., Zhang, X. A., Shao, L., et al. (2016). ERalpha propelled aberrant global DNA hypermethylation by activating the DNMT1 gene to enhance anticancer drug resistance in human breast cancer cells. *Oncotarget* 7, 20966–20980. doi:10.18632/oncotarget.8038

Siegel, R. L., Miller, K. D., and Jemal, A. (2019). Cancer statistics, 2019. *CA Cancer J. Clin.* 69, 7–34. doi:10.3322/caac.21551

Simoes, B. M., O'Brien, C. S., Eyre, R., Silva, A., Yu, L., Sarmiento-Castro, A., et al. (2015). Anti-estrogen resistance in human breast tumors is driven by JAG1-NOTCH4-dependent cancer stem cell activity. *Cell Rep.* 12, 1968–1977. doi:10.1016/j.celrep.2015.08.050

Sims-Mourtada, J., Opdenaker, L. M., Davis, J., Arnold, K. M., and Flynn, D. (2015). Taxane-induced hedgehog signaling is linked to expansion of breast cancer stem-like populations after chemotherapy. *Mol. Carcinog.* 54, 1480–1493. doi:10.1002/mc.22225

Sledge, G. W., Jr., Loehrer, P. J., Sr., Roth, B. J., and Einhorn, L. H. (1988). Cisplatin as first-line therapy for metastatic breast cancer. *J. Clin. Oncol.* 6, 1811–1814. doi:10.1200/JCO.1988.6.12.1811

Smalley, M., Piggott, L., and Clarkson, R. (2013). Breast cancer stem cells: obstacles to therapy. *Cancer Lett.* 338, 57–62. doi:10.1016/j.canlet.2012.04.023

Solzak, J. P., Atale, R. V., Hancock, B. A., Sinn, A. L., Pollok, K. E., Jones, D. R., et al. (2017). Dual PI3K and Wnt pathway inhibition is a synergistic combination against triple negative breast cancer. *NPJ Breast Cancer* 3, 17. doi:10.1038/s41523-017-0016-8

Sridharan, S., Howard, C. M., Tilley, A. M. C., Subramaniyan, B., Tiwari, A. K., Ruch, R. J., et al. (2019). Novel and alternative targets against breast cancer stemness to combat chemoresistance. *Front. Oncol.* 9, 1003. doi:10.3389/fonc.2019.01003

Strosberg, J. R., Yeatman, T., Weber, J., Coppola, D., Schell, M. J., Han, G., et al. (2012). A phase II study of RO4929097 in metastatic colorectal cancer. *Eur. J. Cancer* 48, 997–1003. doi:10.1016/j.ejca.2012.02.056

Sun, M., Yang, C., Zheng, J., Wang, M., Chen, M., Le, D. Q. S., et al. (2015). Enhanced efficacy of chemotherapy for breast cancer stem cells by simultaneous suppression of multidrug resistance and antiapoptotic cellular defense. *Acta Biomater.* 28, 171–182. doi:10.1016/j.actbio.2015.09.029

Sun, M., Zhang, N., Wang, X., Li, Y., Qi, W., Zhang, H., et al. (2016). Hedgehog pathway is involved in nitidine chloride induced inhibition of epithelial-mesenchymal transition and cancer stem cells-like properties in breast cancer cells. *Cell Biosci.* 6, 44. doi:10.1186/s13578-016-0104-8

Sun, W. L., Chen, J., Wang, Y. P., and Zheng, H. (2011). Autophagy protects breast cancer cells from epirubicin-induced apoptosis and facilitates epirubicin-resistance development. *Autophagy* 7, 1035–1044. doi:10.4161/auto.7.9.16521

Suman, S., Das, T. P., and Damodaran, C. (2013). Silencing NOTCH signaling causes growth arrest in both breast cancer stem cells and breast cancer cells. *Br. J. Cancer* 109, 2587–2596. doi:10.1038/bjc.2013.642

Swaminathan, S. K., Roger, E., Toti, U., Niu, L., Ohlfest, J. R., and Panyam, J. (2013). CD133-targeted paclitaxel delivery inhibits local tumor recurrence in a mouse model of breast cancer. *J. Contr. Release* 171, 280–287. doi:10.1016/j.jconrel.2013.07.014

Takagi, K., Miki, Y., Nagasaki, S., Hirakawa, H., Onodera, Y., Akahira, J., et al. (2010). Increased intratumoral androgens in human breast carcinoma following aromatase inhibitor exemestane treatment. *Endocr. Relat. Cancer* 17, 415–430. doi:10.1677/ERC-09-0257

Tanei, T., Morimoto, K., Shimazu, K., Kim, S. J., Tanji, Y., Taguchi, T., et al. (2009). Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers. *Clin. Cancer Res.* 15, 4234–4241. doi:10.1158/1078-0432.CCR-08-1479

Uchiumi, K., Tsuboi, K., Sato, N., Ito, T., Hirakawa, H., Niwa, T., et al. (2019). Cancer stem-like properties of hormonal therapy-resistant breast cancer cells. *Breast Cancer* 26, 459–470. doi:10.1007/s12282-018-00944-1

Valenti, G., Quinn, H. M., Heynen, G. J. J. E., Lan, L., Holland, J. D., Vogel, R., et al. (2017). Cancer stem cells regulate cancer-associated fibroblasts via activation of hedgehog signaling in mammary gland tumors. *Cancer Res.* 77, 2134–2147. doi:10.1158/0008-5472.CAN-15-3490

van't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., et al. (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415, 530–536. doi:10.1038/415530a

Vahidi, H., Barabadi, H., and Saravanan, M. (2020). Emerging selenium nanoparticles to combat cancer: a systematic review. *J. Cluster Sci.* 31, 301–309. 10.1007/s10876-019-01671-z

Vazquez-Martin, A., Cufi, S., Lopez-Bonet, E., Corominas-Faja, B., Cuyas, E., Vellon, L., et al. (2013). Reprogramming of non-genomic estrogen signaling by

the stemness factor SOX2 enhances the tumor-initiating capacity of breast cancer cells. *Cell Cycle* 12, 3471–3477. doi:10.4161/cc.26692

Venkatesh, V., Nataraj, R., Thangaraj, G. S., Karthikeyan, M., Gnanasekaran, A., Kaginelli, S. B., et al. (2018). Targeting Notch signalling pathway of cancer stem cells. *Stem Cell Invest.* 5, 5. doi:10.21037/sci.2018.02.02

Vera-Ramirez, L., Vodnala, S. K., Nini, R., Hunter, K. W., and Green, J. E. (2018). Autophagy promotes the survival of dormant breast cancer cells and metastatic tumour recurrence. *Nat. Commun.* 9, 1944. doi:10.1038/s41467-018-04070-6

Versini, A., Colombeau, L., Hienzsch, A., Gaillet, C., Retailleau, P., Debieu, S., et al. (2020). Salinomycin derivatives kill breast cancer stem cells via lysosomal iron targeting.*Chemistry* 26, 7416–7424. doi:10.1002/chem.202000335

Virmani, I., Sasi, C., Priyadarshini, E., Kumar, R., and Meena, R. (2019). Comparative anticancer potential of biologically and chemically synthesized gold nanoparticles. *J. Cluster Sci.* 31, 1–10. doi:10.1007/s10876-019-01695-5

Wang, A., Qu, L., and Wang, L. (2017). At the crossroads of cancer stem cells and targeted therapy resistance. *Cancer Lett.* 385, 87–96. doi:10.1016/j.canlet.2016.10.039

Wang, D., Xu, J., Liu, B., He, X., Zhou, L., Hu, X., et al. (2018). IL6 blockade potentiates the anti-tumor effects of γ-secretase inhibitors in Notch3-expressing breast cancer. *Cell Death Differ.* 25, 330–339. doi:10.1038/cdd.2017.162

Wang, R., Yang, L., Li, S., Ye, D., Yang, L., Liu, Q., et al. (2018). Quercetin inhibits breast cancer stem cells via downregulation of aldehyde dehydrogenase 1A1 (ALDH1A1), chemokine receptor type 4 (CXCR4), Mucin 1 (MUC1), and epithelial cell adhesion molecule (EpCAM). *Med. Sci. Mon. Int. Med. J. Exp. Clin. Res.* 24, 412–420. doi:10.12659/msm.908022

Wang, T., Fahrmann, J. F., Lee, H., Li, Y. J., Tripathi, S. C., Yue, C., et al. (2018). JAK/STAT3-Regulated fatty acid β-oxidation is critical for breast cancer stem cell self-renewal and chemoresistance. *Cell Metabol.* 27, 136–150.e5. .doi:10.1016/j.cmet.2017.11.001

Wang, J., Gildea, J. J., and Yue, W. (2013). Aromatase overexpression induces malignant changes in estrogen receptor alpha negative MCF-10A cells. *Oncogene* 32, 5233–5240. doi:10.1038/onc.2012.558

Wang, L., Tian, H., Yuan, J., Wu, H., Wu, J., and Zhu, X. (2015). CONSORT: Sam68 is directly regulated by MiR-204 and promotes the self-renewal potential of breast cancer cells by activating the wnt/beta-catenin signaling pathway. *Medicine* 94, e2228. doi:10.1097/MD.0000000000002228

Wang, T., Gantier, M. P., Xiang, D., Bean, A. G., Bruce, M., Zhou, S. F., et al. (2015). EpCAM aptamer-mediated Survivin silencing sensitized cancer stem cells to doxorubicin in a breast cancer model. *Theranostics* 5, 1456–1472. doi:10.7150/thno.11692

Wang, X., Wang, G., Zhao, Y., Liu, X., Ding, Q., Shi, J., et al. (2012). STAT3 mediates resistance of CD44(+)CD24(-/low) breast cancer stem cells to tamoxifen *in vitro*. *J. Biomed. Res.* 26, 325–335. doi:10.7555/JBR.26.20110050

Wijdeven, R. H., Pang, B., van der Zanden, S. Y., Qiao, X., Blomen, V., Hoogstraat, M., et al. (2015). Genome-Wide identification and characterization of novel factors conferring resistance to topoisomerase II poisons in cancer, *Cancer Res.* 75, 4176–4187. doi:10.1158/0008-5472.CAN-15-0380

Wright, M. H., Calcagno, A. M., Salcido, C. D., Carlson, M. D., Ambudkar, S. V., and Varticovski, L. (2008). Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics. *Breast Cancer Res.* 10, R10. doi:10.1186/bcr1855

Wu, D., Si, M., Xue, H. Y., and Wong, H. L. (2017). Nanomedicine applications in the treatment of breast cancer: current state of the art. *Int. J. Nanomed.* 12, 5879–5892. doi:10.2147/IJN.S123437

Wu, Y., Ginther, C., Kim, J., Mosher, N., Chung, S., Slamon, D., et al. (2012). Expression of Wnt3 activates Wnt/beta-catenin pathway and promotes EMT-like phenotype in trastuzumab-resistant HER2-overexpressing breast cancer cells. *Mol. Cancer Res.* 10, 1597–1606. doi:10.1158/1541-7786.MCR-12-0155-T

Xiao, Y., Humphries, B., Yang, C., and Wang, Z. (2019). MiR-205 dysregulations in breast cancer: the complexity and opportunities. *Noncoding RNA* 5, 53. doi:10.3390/ncrna5040053

Xu, H., Zhou, Y., Li, W., Zhang, B., Zhang, H., Zhao, S., et al. (2018). Tumor-derived mesenchymal-stem-cell-secreted IL-6 enhances resistance to cisplatin via the STAT3 pathway in breast cancer. *Oncol. Lett.* 15, 9142–9150. doi:10.3892/ol.2018.8463

Yang, L., Tian, Y., Leong, W. S., Song, H., Yang, W., Wang, M., et al. (2018). Efficient and tumor-specific knockdown of MTDH gene attenuates paclitaxel resistance of breast cancer cells both *in vivo* and *in vitro*. *Breast Cancer Res.* 20, 113. doi:10.1186/s13058-018-1042-7

Yang, Y., Hao, E., Pan, X., Tan, D., Du, Z., Xie, J., et al. (2019). Gomisin M2 from Baizuan suppresses breast cancer stem cell proliferation in a zebrafish xenograft model. *Aging* 11, 8347–8361. 10.18632/aging.102323.

Yang, Z., Guo, F., Albers, A. E., Sehouli, J., and Kaufmann, A. M. (2019). Disulfiram modulates ROS accumulation and overcomes synergistically cisplatin resistance in breast cancer cell lines. *Biomed. Pharmacother.* 113, 108727. doi:10.1016/j.biopha.2019.108727

Yi, T., Zhai, B., Yu, Y., Kiyotsugu, Y., Raschle, T., Etzkorn, M., et al. (2014). Quantitative phosphoproteomic analysis reveals system-wide signaling pathways downstream of SDF-1/CXCR4 in breast cancer stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 111, E2182–E2190. doi:10.1073/pnas.1404943111

Yin, Y., Xie, C. M., Li, H., Tan, M., Chen, G., Schiff, R., et al. (2019). The FBXW2-MSX2-SOX2 axis regulates stem cell property and drug resistance of cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 116, 20528–20538. doi:10.1073/pnas.1905973116

Yoshida, G. J., and Saya, H. (2016). Therapeutic strategies targeting cancer stem cells. *Cancer Sci.* 107, 5–11. doi:10.1111/cas.12817

Yousaf, M., and Ali, M. (2020). Modulation of ABCG2 surface expression by Rab5 and Rab21 to overcome multidrug resistance in cancer cells. *Xenobiotica* 50, 1–9. doi:10.1080/00498254.2020.1716107

Yun, J., Pannuti, A., Espinoza, I., Zhu, H., Hicks, C., Zhu, X., et al. (2013). Crosstalk between PKCalpha and Notch-4 in endocrine-resistant breast cancer cells. *Oncogenesis* 2, e60. doi:10.1038/oncsis.2013.26

Zavala, V. A., Serrano-Gomez, S. J., Dutil, J., and Fejerman, L. (2019). Genetic epidemiology of breast cancer in Latin America. *Genes* 10, 153. doi:10.3390/genes10020153

Zhang, C. C., Pavlicek, A., Zhang, Q., Lira, M. E., Painter, C. L., Yan, Z., et al. (2012). Biomarker and pharmacologic evaluation of the gamma-secretase inhibitor PF-03084014 in breast cancer models, *Clin. Cancer Res.* 18, 5008–5019. doi:10.1158/1078-0432.CCR-12-1379

Zhang, Y., Eades, G., Yao, Y., Li, Q., and Zhou, Q. (2012). Estrogen receptor α signaling regulates breast tumor-initiating cells by down-regulating miR-140 which targets the transcription factor SOX2. *J. Biol. Chem.* 287, 41514–41522. doi:10.1074/jbc.M112.404871

Zhang, W., and Grivennikov, S. I. (2013). Top Notch cancer stem cells by paracrine NF-kappaB signaling in breast cancer, *Breast Cancer Res.* 15, 316. doi:10.1186/bcr3565

Zhang, X., and Wang, Z. Y. (2013). Estrogen receptor-α variant, ER-α36, is involved in tamoxifen resistance and estrogen hypersensitivity, *Endocrinology* 154, 1990–1998. doi:10.1210/en.2013-1116

Zhao, D., Mo, Y., Li, M. T., Zou, S. W., Cheng, Z. L., Sun, Y. P., et al. (2014). NOTCH-induced aldehyde dehydrogenase 1A1 deacetylation promotes breast cancer stem cells. *J. Clin. Invest.* 124, 5453–5465. doi:10.1172/JCI76611

Zhong, G., Qin, S., Townsend, D., Schulte, B. A., Tew, K. D., and Wang, G. Y. (2019). Oxidative stress induces senescence in breast cancer stem cells. *Biochem. Biophys. Res. Commun.* 514, 1204–1209. doi:10.1016/j.bbrc.2019.05.098

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REVIEW
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Advances in Rodent Models for Breast Cancer Formation, Progression, and Therapeutic Testing

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Breast cancer is a highly complicated disease. Advancement in the treatment and prevention of breast cancer lies in elucidation of the mechanism of carcinogenesis and progression. Rodent models of breast cancer have developed into premier tools for investigating the mechanisms and genetic pathways in breast cancer progression and metastasis and for developing and evaluating clinical therapeutics. Every rodent model has advantages and disadvantages, and the selection of appropriate rodent models with which to investigate breast cancer is a key decision in research. Design of a suitable rodent model for a specific research purpose is based on the integration of the advantages and disadvantages of different models. Our purpose in writing this review is to elaborate on various rodent models for breast cancer formation, progression, and therapeutic testing.

Keywords: mouse model, mouse intraductal model, transgenic mouse model, breast cancer, rodent model

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INTRODUCTION

Breast cancer is the most commonly diagnosed cancer and one of the most common cause of cancer death in women worldwide. Advancement in treatment and prevention of breast cancer lies in elucidation of the mechanism of carcinogenesis and progression. Rodent models of breast cancer have developed into premier tools for breast cancer research, and they have generated important insights into the mechanisms that underpin development of the disease and interventional therapies. This review summarizes various rodent models for breast cancer formation, progression, and therapeutic testing.

A BRIEF HISTORY OF RODENT CANCER MODELS

In the past century, rodent models have proved to be powerful tools in improving knowledge of the underlying mechanisms and genetic pathways of breast cancer and have also created approaches for modeling clinical tumor subtypes and developing innovative cancer therapies. Certain mouse lines can naturally develop breast cancer, or they can be transplanted with breast cancer. Tumors can also be induced by manipulating the mouse genome or by delivery of a viral infection or carcinogen. The relatively low cost of mice and their high reproductive cycle of only 9 weeks make them excellent models for cancer research. In 1911, the first transplantable mouse mammary tumor line and the

epithelial origin of a spontaneous mammary tumor were described by Haaland (1). Jacksons Laboratories showed that a retrovirus caused a high incidence of mammary tumors in mice in 1936 (2). The first xenograft breast cancer model was reported in 1962 *via* the heterotransplantation of human breast cancer into an immune-deficient mouse (3). The development of genetically engineered animal models offered a great leap in understanding the genetic basis of breast cancer. The first report of a transgenic mouse model of breast cancer, Oncomouse. In 1984, The Philip Leder research group generated transgenic mice using mouse mammary tumor virus (MMTV)/c-myc fusion gene expression. The mice developed mammary adenocarcinomas spontaneously (4), and 3 years later, they produced transgenic mice with coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes, which resulted in a dramatic and synergistic acceleration of tumor formation (5). These milestones established an entirely new research tool with which to explore the genetic processes of breast cancer. The first transgenic mouse model (GEMM) of HER2-positive breast cancer, reported in 1988, represented another milestone in breast cancer research (6). In 1999, Chuxia Deng and colleagues succeeded in developing a mouse model that ablated BRCA1 specifically in mammary epithelial cells, resulting in mammary tumors (7). This mouse model offered a notably large amount of information that greatly facilitated our understanding of the gender- and tissue-specific tumor suppressor functions of BRCA1 and enriched insights into applying these preclinical models of disease to breast cancer research. However, the GEMM requires time-consuming and expensive work, and another main drawback is that it is highly difficult to control the spatial and temporal expression of a gene of interest. Actually, most human breast cancers are not due to hereditary mutations, rather they arise from normal cells that later suffered spontaneous mutations. The technique of virus-mediated gene transfer into selected mammary cells (such as stem cells and specific progenitor cells) at selected times can overcome many of the shortcomings of the existing mouse models and more closely mimics human breast cancer formation in which only one or a few cells mutate to initiate cancer development (8).

TRANSPLANTABLE MOUSE MODELS FOR BREAST CANCER

Human cancer cells can be grown as transplants in mice. These transplantable models are simple but have been proven to be highly useful for assessment of breast cancer features, biological behaviors, metastatic potential, and response to therapy.

Cancer Cell Line Transplantation Mouse Model

Cell-derived xenograft (CDX) of human breast cancer is performed from aggressive cancer cell lines. The CDX model from different tumor cell lines has unique characteristics, including relatively homogenous histological features, molecular subtype, and metastatic potential, among other features. The CDX model makes it possible for different mammary cancer cell lines to be transferred to the mouse in a short time, allowing validation of the target genes of interest and the possibility of research on metastasis and therapy response. It represents a relatively homogenous mass but cannot mimic the heterogeneity or the tumor microenvironment of human breast cancer (9). This technique is usually performed in nude mice (deficient in T-cell function) or other immunocompromised mice but cannot mimic the immune system reaction. If the cancer cells are derived from mouse, they can also be grafted into mice with an intact immune system (10, 11). And the long-term growth *in vitro* can alter some features from its origin cell. Triple-negative breast cancer cell lines such as MDA-MB-231, MDA-MB-435, and SUM1315 can be used to generate stable orthotopic or spontaneous metastasis models of breast cancer *via* orthotopic injection in the mammary fat pad (12). The metastatic MDA-MB-231 and SUM149 CDX models can also be generated by injection into the mouse tail vein (13). Not all breast cancer cell lines from human can be used to successfully establish a CDX mouse model (14). ER-positive luminal A cell lines such as T47D or MCF-7 can only form a tumor mass in immunodeficient mice supplemented with subcutaneous estradiol pellets (15), which produces 18–40 times the physiological levels of estrogen in mice (16, 17). HER2 subtype cell lines such as SKBR3 and MDA-MB-453 cells have poor tumorigenic potential (18).

Patient-Derived Tumor Xenograft Mouse Model

Due to the limitation of CDX models, the patient-derived xenograft (PDX) is generated by xenografting fresh human tumor biopsies that recapitulate the diversity of breast cancer into host mice. This model reflects the tumor original behavior, histopathology, drug response, and metastatic potential of the original tumor (19). In brief, human tumor fragments or tumor cell suspensions are implanted into the immunocompromised mice and subsequently passaged through several generations in mice. The more heavily immunocompromised mice are usually used to generate PDX, such as NOD-SCID mice (deficient in T-cell and B-cell functions) and NSG mice (deficient in T-cells, B-cells and NK cells).

The PDX modes are relatively stable after the third passage in mice (20) and have relatively stable biological behaviors, such as gene expression profiles, intrinsic phenotypes, and genomic alteration, that are similar to the source of human breast cancer (21–24). PDX also has selected structural variation or mutation differences with the original primary tumor, perhaps due to the adaption to transplantation into the new microenvironment (25). PDX models appear to retain the heterogeneity of the parental tumor of origin and experience a "bottlenecking" clonal selection during transplantation and serial passaging (26). Ding et al. reported comprehensive genomic sequence analysis of DNA samples from an African-American patient with basal-like breast cancer for peripheral blood, the

primary tumor, a brain metastasis, and a xenograft derived from the primary tumor (25). That group found that the PDX derived from the primary tumor contained all of the primary tumor mutations and displayed a mutation enrichment pattern that resembled the metastasis. The metastatic subclone was present within the primary tumor, the aggressive subclone with clonal selection in PDX. The PDX drug screening test can mimic and predict drug efficacy, especially in triple negative breast cancer (27), and is a pivotal preclinical tool for evaluating drug response and study of the clonal evolution of tumors and new biomarkers (15). TNBC tumors and to a lesser degree the HER2+ tumors, readily adapt to growth in mice, whereas only 2.5% of ER+ tumors successfully formed stable patient-derived breast cancer xenografts (28). PDX models can't mimic the immune system and tumor-host interaction because it must also be grown in immunocompromised mice.

Mouse Intraductal Model (MIND) for Studying Cancer Progression and Immunotherapy

The breast ductal system is a complex series of branching tubules extending from intralobular ductules to the major lactiferous ducts that terminate in the nipple. The mouse intraductal model is based on the special structure of the mammary mouse gland. Human cancer cells can be introduced directly into the mouse mammary ducts in immunodeficient mice to mimic the natural progression of cancer cells in the mammary microenvironment. Behbod et al. established the ductal carcinoma *in situ* (DCIS) model by injecting the human DCIS cell lines (MCF-10 and SUM-255) into mouse mammary ducts *via* up-the-teat injection (29). This approach mimicked breast tumor carcinogenesis and progression from *in situ* to invasive disease and spontaneous metastasis to the relevant sites. In contrast to fat-pad-grafted ER+ tumor cell lines that require estrogen supplement, the MIND of MCF-7 achieved a high engraftment rate without hormone supplements and recapitulated the histopathology and kinetics of human ER-positive tumors (16, 17). These MIND models also often developed bone, lung, and brain metastases, whereas fat pad injection xenografts developed few brain and no bone metastases. The Ki67 indices of MIND MCF-7 tumors were 23%, highly similar to MCF-7 cell lines, and the gene expression signatures are highly similar to the luminal B subtype of clinical samples, as shown by Affymetric microarray and PAM50 (30). For the triple-negative breast cancer mouse model, a fully immunocompetent 4T1-based intraductal model can mimic breast cancer advancement and metastasis to the lungs *via* lymphatic or hematogenous dissemination within 4 weeks (31–33), and it can also disseminate to the liver, brain and kidney (34). 4T1 is a mouse breast cancer line derived from a spontaneously arising mammary tumor in BALB/cfC3H mice (35). The 4T1 MIND models overcome immunosuppression and allow effective immunotherapy research for TNBC (33, 36). This model is predictive, retransplantable, and genomically stable and is an economical and practical mouse model for translational research and study of physiologically relevant hormone action in breast carcinogenesis.

CARCINOGEN-INDUCED RODENT MODELS

Chemical compounds can induce breast cancer. For example, the carcinogen 7,12-dimethylbenzathracene (DMBA), delivered intragastrically by gavage, can induce mammary adenocarcinomas with several morphological types in mice (37). The induced tumors were luminal-like and mostly ER/PR+ (38, 39). Previous research indicated that estrogen exposure was closely related to elevated breast cancer risk in women (40, 41). The 17β-estradiol-induced mammary cancers highly express ER, PR, and GATA binding protein 3 (42–45), and others such as N-nitroso-N-methylurea (NMU) can induce mouse breast cancer similar to that of low-grade estrogen-receptor positive human breast cancer (46–48). Spontaneous chemically induced mouse models are helpful for investigation of the pathogenesis and therapeutics of breast cancer (49).

GENETICALLY ENGINEERED MOUSE MODELS OF BREAST CANCER

Genetically engineered mouse models (GEMM) of breast cancer have supplied a wealth of knowledge for both basic cancer research and translational oncology by introducing DNA into the mouse genome. GEMMs reflect some of the diversity of genetic lesions in human breast cancer. These models include three broad groups: transgenic mouse model, knockout mouse model, and genetic models of breast cancer based on intraductal injection of virus to modify the genes of the mammary cells.

Transgenic Mouse Model

Transgenic mouse models refer to those which have exogenous DNA integrated in their germline as a consequence of experimental DNA transfer application. The integrated DNA may or may not be derived from the same species as the host genome, it may or may not be targeted to an intended site of incorporation in the genome, and it may or may not encode for a functional gene.

The MMTV-PyMT transgenic mouse is a model that carries the polyoma virus middle T-antigen under the control of the mouse mammary tumor virus (MMTV) promoter. The PyMT is involved in multiple oncogenic pathways that lead to an aggressive tumor phenotype such as Src, Ras, and PI3K (50–54). MMTV-PyMT females develop multifocal, poorly differentiated, highly invasive ductal carcinoma as early as 4 weeks of age, reaching the maximum tumor burden at 12 weeks of age, and they also exhibit lung metastasis near 10 weeks of age (55–59). This model is used in breast cancer research to analyze the mechanism of carcinogenesis and alter the tumor microenvironment. Maglione also reported that atypical lesions had levels of detectable ER expression, and the mammary intraepithelial neoplasia and tumor cells had variable sporadic ER-positive nuclei staining (58). Previous research indicated that the PyVT mammary tumors were shown to be ER+, PR+, P53+, and HER-2+ *via* immunohistochemistry at the early stage of

tumor formation, progressing to the triple-negative subtype (57, 58). This model has drug resistance to cisplatin and paclitaxel, but tamoxifen is effective in the prestage and early stage of tumor formation (57).

The Wnt-1 (int-1) proto-oncogene was originally cloned following activation by MMTV insertion in mouse mammary tumors (60, 61). The MMTV-wnt-1 mouse model was established with the MMTV-LTR upstream of wnt1 insertion in the opposite transcription orientation (62, 63). This model is characterized by grossly ductal hyperplasia with extensive fibrosis, and these mice can develop breast cancer at an onset of 24 weeks (64). Females cannot deliver milk to their young because of extensive ductal hyperplasia (64). The tumors in MMTV-wnt-1 transgenic mice are composed of myoepithelial (basal-like) and luminal epithelial cells. β-catenin is an integral player in the Wnt signal transduction pathway, and β-catenin transgenic (MMTV-βcatΔN) mice exhibit mammary gland hyperplasia and mammary adenocarcinoma, which are highly similar to the corresponding lesions in MMTV-wnt-1 mice (65). Wnt10b is a ligand that activates the canonical Wnt/β-catenin pathway, and MMTV-Wnt-10b transgenic mice showed hyperplastic mammary development involving highly branched mammary ducts and gynecomastia (66). LRP6 is a Wnt signaling coreceptor, and MMTV-LRP6 mice exhibit significant hyperplasia with upregulated expression of Axin2, Cyclin D1, and c-Myc (67). MMTV-c-Myc and MMTV-int2 mice also develop pronounced mammary hyperplasia and adenocarcinoma in proportion (65, 68). Indeed, the wnt-associated mouse model has made a great contribution to elaboration of the wnt pathway in breast carcinogenesis.

The first MMTV-ErbB2 transgenic mouse model expressed an activated Erbb2 under promoter of MMTV-LTR, and these mice are viable and fertile (6). There is no phenotypic effect in males. This transgene is expressed at low levels in the normal mammary epithelium, salivary gland, and lung (69, 70), and higher expression is detected in tumor tissue. This model produces multifocal and stochastic mammary tumor formation near 15 weeks of age (69, 71) and lung metastasis with long latency (approximately 32 weeks or longer) (72) and had positive cyclin D1 and CDK4 expression and a high Ki-67 proliferative index. In contrast to the MMTV-ErbB2 mouse line, Muller et al. later established transgenic mice carrying unactivated neu under the MMTV promoter (73). The mice began to develop focal mammary adenocarcinoma surrounded with hyperplastic mammary epithelium at 16 weeks of age, with decreased neu intrinsic tyrosine kinase activity. Many of these tumor-bearing transgenic mice with unactivated neu also developed metastatic tumors in the lung (73). Li et al. found that 37% of tumors in the MMTV-ErbB2 mouse had mis-sense mutations in p53 (74), and thus, they established bitransgenic mice carrying MMTV-neu and a 172Arg-to-His p53 mutant (p53-172H). The bitransgenic mice developed anaplastic and aneuploidy tumors that exhibited increased apoptosis, distinct from the tumors with diminished apoptosis arising in p53-null mice (74).

The C3(1)/SV 40/t-antigen (C3(1)/Tag) mouse model contained a recombinant gene expressing the simian virus 40 early-region transforming sequence under rat prostatic steroid binding protein [C3(1)]. Female hemizygous mice generally developed mammary hyperplasia at 9 weeks of age and mammary intraepithelial neoplasia with similarities to DCIS at 12 weeks, with subsequent development of mammary adenocarcinoma at an onset of 24 weeks in 100% of the animals and 15% incidence of lung metastasis (75–81). This model develops invasive carcinoma independently of hormones or pregnancy (72). All mammary adenocarcinomas were diffuse immunopositive for CK14, CK18, and p53 and negative for αSMA, ERα, PR, and C-erbB-2 (81). Previous study indicated that human basal-like breast cancer exhibits a high frequency of p53 mutation of deletion. It is a suitable mouse model for research on basal-like breast cancer because of the gene expression and DNA somatic alteration levels.

Cyclin D1 is essential in breast carcinogenesis induced by c-neu and v-Ha-ras and not induced by c-myc or Wnt-1 (82). The MMTV-cyclin D1 mouse can develop mammary adenocarcinomas quite late stochastically (83, 84). Cyclin E is a cancer marker that is the limiting factor for the transition from G1 to the S-phase of the cell cycle, which determines ignition of DNA duplication. Previous research indicated that the 27% low-molecular-weight isoform of cyclin E transgenic mice can induce metastatic mammary carcinoma (85).

Knockout Mouse Models of Breast Cancer

Knockout animals are mice with targeted disruption of selected endogenous gene sequences. These models are used to reveal valuable clues related to the biological and molecular function of a gene in the setting of a developing or developed tumor. The constitutive knockout model refers to the whole-body knockout model, i.e., the target gene is knocked out in all tissues at all times. Many tumor suppressors often result in lethality during embryonic development or at developmental stages prior to tumor formation. This obstacle has been effectively overcome by applying the conditional knockout model (86) in which the gene knockout can be spatially and even temporally regulated. With a conditional KO, gene inactivation can occur in a certain tissue type, made possible by Cre-LoxP and Flp-Frt recombinase system. Today, the development of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technique has made conditional knockouts even more popular and widely used. This new technology is more efficient and easier than the Cre-LoxP or Flp-Frt recombinase technology. Therefore, we summarize the tumor phenotype of the popular conditional knockout strains reported in the literature.

BRCA1 inherited mutations predispose carriers to female breast and ovarian cancers. Constitutive knockout of mouse BRCA1 causes recessive mouse embryonic lethality (87), and therefore, the BRCA1 conditional mutant mouse model was used to overcome this obstacle (88). Exon 11 is a large central exon of 3426 bp that represents 60% of the coding sequence in BRCA1 (89). In 1999, Xu established a BRCA1flox11 mutant mouse, which was achieved by deleting only exon 11 of the full-length BRCA1 gene and leaving expression of the short BRCA1 transcript with loxP sites (BRCA1flox11) (7). The 25% BRCA1flox11 mutant mouse develops mammary tumors after a long latency (7). The 94% BRCA1flox11

mouse develops mammary tumors with a long latency (T50 = 17 months), and the tumors exhibit an atypical medullary phenotype strongly reminiscent of basal-like breast tumors (90). Xu et al. found that the BRCA1flox11 mutation mouse often had had spontaneous p53 mutation, and thus they introduced heterozygous deletion of p53 in the BRCA1flox11 mouse, which accelerated tumor formation (91). Weaver et al. also revealed that certain of the tumors had structural abnormalities on the map location of c-myc gene, Rb1, and p53, similar to BRCA1-associated breast cancer in patients (92).

Other conditional BRCA1 alleles are reported to cause functionally null BRCA1 alleles by flanking exon 2 ($BRCA^{f2}$) (90), exons 5–6 ($BRCA1^{f5-6}$) (93), exons 5–13 ($BRCA1^{f5-13}$) (94), or exons 22–24 ($BRCA1^{f22-24}$) (95). The $BRCA1^{f5-13}$ mouse had intermediate to high grade tumors with high mitotic count, expansive growth, moderate to high nuclear grade also displayed ER-negative immunohistochemistry staining with pushing borders, and increased expression of basal epithelial markers, similar to human basal-like breast cancer (94). The 64% mouse with $BRCA1^{f22-24}$ mutation combined with heterozygosity for a p53 mutation developed tumors with basal-like markers in all cases before 22 months of age. This model had high histological grade, central necrotic areas, and presence of homologous metaplastic elements and is a suitable model for metaplastic basal-like breast cancers (95).

Germline mutations of BRCA2 are associated with one-third of hereditary breast cancer. Jonker et al. generated a mouse model with conditional mutants of $BRCA2^{f11}$ (flanking exon 11 of the gene with loxP sites) and found that no $BRCA2^{f11}$ mice developed tumors. The mammary glands and skin frequently developed tumors in females carrying conditional $BRCA2^{f11}$ and p53 knockout alleles (96). The vast majority of the mammary tumors were carcinomas with myoepithelial or basal cell types. Most tumors arising in the conditional $BRCA2^{f11}$ and p53 knockout mice had high-grade invasive ductal carcinoma, with a solid growth pattern, a large CK8-positive and ER-negative cell type with high mitotic count, high-grade nuclei and with pushing borders (96). The tumors often harbor the undifferentiated basal cell type. Based on the results from cross-species comparison by unsupervised clustering, these tumors are closely similar to human BRCA1-mutated breast cancers with basal-like phenotypes. Ludwig generated mice with $BRCA2^{f3-4}$ (flanking exons 3 and 4 of the gene with loxP sites) mutation, which had a high incidence (77%) of breast tumors that developed in one or more glands after a long latency (time for median tumor-free survival of approximately 1.4 years; total of 40 tumors in 20 animals) (97). In addition, Cheung generated a mouse model with $BRCA2^{f9-11}$ (flanking exon 9–10 of the gene with loxP sites), which had mammary adenocarcinomas after a long latency (average, 1.6 years). A subset of these tumors also showed downregulated p53 expression (98).

As mentioned previously, the p53 mutation is linked tightly with breast cancer. The conditional knockout $p53^{\Delta 2-10}$ (deletion of exon 2–10 of the gene with Cre recombinase) model generated by Jonker et al. develops lymphomas and sarcoma rather than epithelial tumors (96), and therefore, those researchers crossed $p53^{\Delta 2-10}$ mice with K14-cre transgenic mice (Cre recombinase expression is restricted to skin and mammary gland epithelium and other epithelial tissues). The resulting K14-cre $p53^{\Delta 2-10}$ mice developed mammary tumors with a median latency (T50) of 288 days. Interestingly, 38% of the mammary tumors were pure epithelial tumors (intermediate to high-grade), 48% were poorly differentiated biphasic carcinoma, and 14% were well differentiated biphasic carcinoma. The molecular signatures of these tumors showed a significant association with human sporadic ER-negative tumors (94). These tumors closely mimic human sporadic basal-like breast cancer. Lin et al. generated a mouse breast cancer model with inactivated p53 (deletion of exon 2–10 of the gene with Cre/loxp) in mammary epithelial cells (99). The tumors are heterogeneous, including adenocarcinoma, myoepithelial adenocarcinoma, spindle cell tumor, and adenosquamous carcinoma, and most were poorly differentiated invasive adenocarcinomas, which share the most histopathological similarity with human breast cancer. A total of 35% had c-myc amplification, and 66% had erbB2 overexpression. The tumors were initially ERα-positive but progressed to ERα-positive and -negative tumors (99), similar to human breast cancer. Multistep histopathological changes and alterations in the ERα expression pattern are observed during progression of mammary carcinogenesis in these models.

PTEN is a tumor suppressor that is frequently mutated in breast cancers. Germline PTEN mutations cause inherited syndromes that lead to an increased risk of breast cancer. Wu Hong and colleague generated $PTEN^{\Delta 5}$ allele (flanking exon 5 of PTEN with loxP sites) and established mammary-specific PTEN deletion mice (100, 101). PTEN null mammary epithelial cells were hyperproliferative and showed decreased apoptosis. Mutant females developed mammary tumors with upregulated populations of cells expressing cytokeratin 5 and 6 within 400 days (101). When a PTEN conditional allele was mated with MMTV-NIC mice, which coupled expression of Cre and activated ErbB2 from the bicistronic transgenic transcript, all female mice developed multifocal mammary tumors and high lung metastases, which displayed histopathological and molecular characteristics of the luminal subtype of primary human breast cancer (102).

Genetic Models of Breast Cancer Based on Intraductal Injection of Virus for Delivery of Oncogenic Mutations to Mimic Human Cancer Formation

Based on molecular biology, breast cancer is highly complicated. Most human cancers are not due to hereditary mutations, and instead, they arise from normal cells that later suffer spontaneous mutations. It is notably difficult to manipulate the spatial and temporal expression of genes in mouse. Genetic models of breast cancer based on intraductal injection of a virus can circumvent selected disadvantages of the typical transgenic or knockout mouse models. Currently, in clinical and basic research, compound techniques of mouse models have more practical applications. The avian leukosis-sarcoma virus (ALSV) and its specific receptor tumor virus A (TVA) play a vital role in this model. Mammalian cells lack the TVA gene sequence, and the

transfer of the TVA gene to specific cells in mouse renders them uniquely susceptible to infection by ALSV-based RCAS virus (103). RCAS viruses can be delivered into mice by injection of virus-producing cells or by injection of concentrated virus (103, 104). Harold Varmus and colleagues constructed the RCAS-TVA avian retroviral system, which can carry oncogenes (e.g., K-ras, c-myc), marker genes (e.g., green fluorescent protein, alkaline phosphatase), dominant negative tumor suppressors (e.g., mutant p53), or recombinases (e.g., Cre) (105). This method offers a precise way to manipulate the temporal and spatial expression of genes in the mammary epithelium. A single TVA mouse stain can be used to evaluate the effects of multiple genes, individually or in combination, instead of generating a mouse line for each gene of interest. Yi Li modeled breast cancer in a mouse with the RCAS-TVA system by mammary gland intraductal injection (106) (**Figure 1**). Precancerous lesions can be detected by 7 days following RCAS-PyMT injection (107). The PyMT oncogene delivered by RCAS-TVA caused multifocal mammary tumors after a notably short median latency of only 12.5 days. The tumors were composed of myoepithelial (basal-like) and luminal epithelial cells and were relatively well differentiated, consisting of many acini and heterogeneous cell types with ER positive expression (8, 108). In mice injected with RCAS-erbB2, precancerous lesions can be detected 14 days after injection (109). The mice developed high grade, poorly differentiated, stroma-rich, and ER-negative mammary tumors (109–111).

CHALLENGES IN MODELING ER+ BREAST CANCER

Majority GEMMs are ER negative and most xenograft mouse models are based on few ER+ cancer cell lines (112). And there are no reliable mouse models of ER+ breast cancer that are also estrogen-dependent (113, 114). For example, STAT1−/− mice express abundant amounts of ER and PR (115), but tumor development is not hormone-dependent (116). A K-Ras mutant has been reported to induce ER+ tumors in mice (114), but the resulting tumors have not been thoroughly tested for estrogen dependency. As we previous indicated that the RACS-TVA approach can especially introduce genetic alterations into only a small number of the mammary cells (103). Lentiviral PyMT produces both luminal and basal-like tumors (55). TVA-PyMT mice and TVA-erbB2 mice had ER expression in greater than 10% of mammary tumor cells (117).

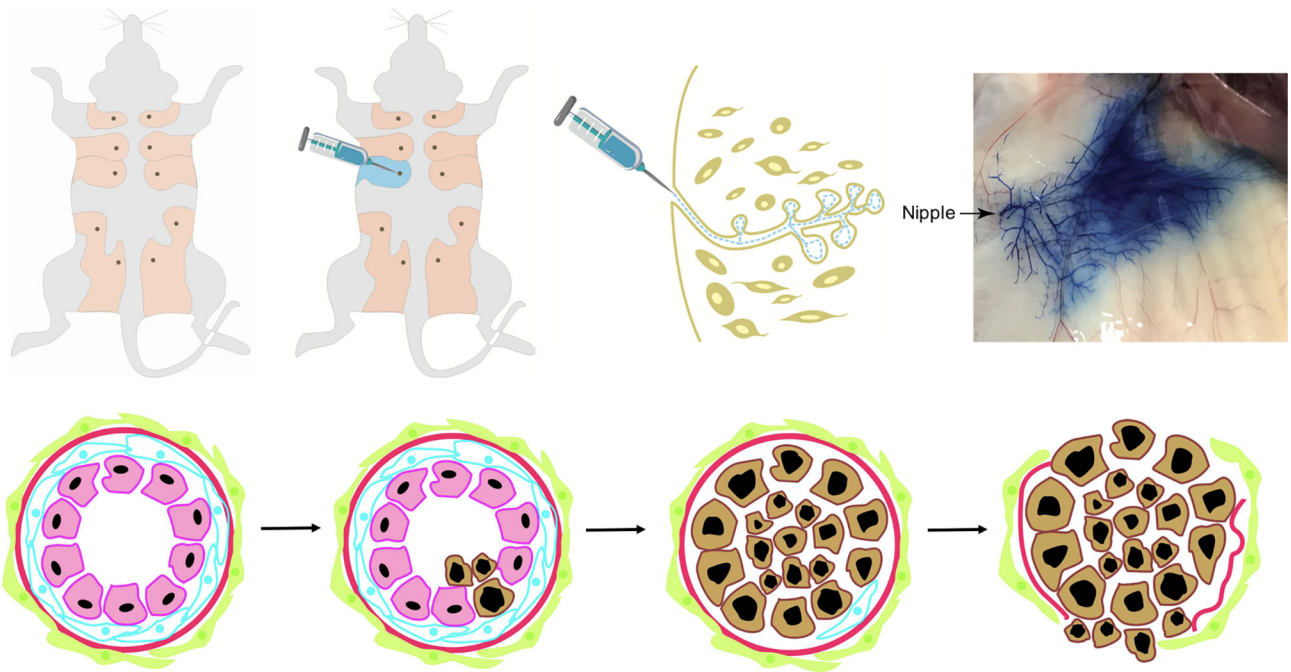

FIGURE 1 | The intraductal injection of retrovirus into mammary glands *in vivo* with virus vector initiates and promotes carcinogenesis in mouse models. It is a novel mouse mammary gland cancer model which mimics human breast cancer non-invasive-to-invasive progression with virus vector. Most human breast cancers are not due to hereditary mutations, rather they arise from normal cells that later suffered spontaneous mutations. This mouse model was established by intraductal injection of retrovirus carrying the oncogenes with blue dye into one of the fifth mouse mammary glands. The technique of virus-mediated gene transfers into selected mammary cells (such as stem cells and specific progenitor cells) at selected times can overcome many of the shortcomings of the existing mouse models and more closely mimics human breast cancer formation in which only one or a few cells mutate to initiate cancer development. It allows temporal analysis of many processes involved in early breast cancer invasive progression including intraductal cancer cell growth, the cell interactions with the surrounding normal epithelial and myoepithelial cells, and their escape into the surrounding stroma. Photo of nipple injection – courtesy of Wen Bu (Baylor College of Medicine).

And PyMT-induced tumors exhibited a two-fold increased ER-positivity *versus* erbB2-induced tumors. Compare with mice mammary glands, rats are more similar to the human breast, rats mammary glands had a ductal tree terminates in TDLUs with connective tissues and organized fibroblasts as sheath around and shows extensive alveolar development (118). Oral DMBA or intravenous or subcutaneous of NMU induced ER+ and PR+ tumors in rats (119), and many of these tumors harbor Ras mutations (49, 120). Ras signaling is frequently activated in human breast cancer, usually not by mutations in a Ras gene per se, but by mutations and overexpression of upstream signaling components such as receptor tyrosine kinases and NF1 mutations (121). Wang et al. found that intraductal injection of retrovirus expressing activated versions of Ras or erbB2 into Sprague/Dawley rats led to ER+ tumors (122). This intraductal model has a defined genetic mutation and is more relevant to human breast cancer etiology than DMBA models. NF1 mutations are enriched in ER+ breast cancers of patients. Crispr-mediated germline knockout of NF1 has been reported to induce ER+ tumors that are estrogen dependent (123). The CRISPRs technology is already widely used to edit somatic cells, and CAS9 rats are already commercially available. Therefore, intraductal injection of a virus carrying gRNA could be used to mutate NF1 and other genes associated with human ER+ cancer to generate somatic models of ER+ cancer in rats. Wen and Yi also described the intraductal injection of lentiviral vector FUCGW carrying the mutated oncogene HrasQ61L to Sprague/Dawley rats led to mammary tumors with high positive expression of both ER and PR (124). This technique is an efficient tool for modeling formation, prevention, and treatment of human breast cancer, especially ER+ breast cancer.

TRANSLATIONAL APPLICATION OF RODENT MODELS FOR BREAST CANCER TREATMENT

Actually, to mimic human breast cancer accurately is very difficult, especially in breast cancer therapy. CDX or PDX models are widely used because of its easy application, large and rapid tumor cohort generation, and simple preclinical data assessment. They can't recapitulate tumorigenicity and treatment response in immunocompromized or immune-competent host system. In clinic, cyclin dependent kinase 4/6 (CDK4/6) inhibitors PD0332991 (palbociclib) has shown great efficacy in the treatment of hormone receptor-positive breast cancer, has received conditional approval from the FDA for metastatic breast cancer. Roberts et al. indicated that palbociclib is effective in a HER2-positive mouse model of breast cancer (MMTV-c-neu) but had no effect in the basal-like breast model C3-TAg (125, 126). The combination of carboplatin plus PD0332991 decreased antitumor activity compared with carboplatin alone in MMTV-c-neu with hematopoietic stem cell dormancy (125). It mimicked the therapy response of palbociclib in different subtype breast cancer. Usary et al. examined a range of therapeutics focused on MEK, mTOR, and PIK3CA/mTOR inhibitors in basal-like (C3-TAg), luminal B (MMTV-c-neu), and claudin-low (T11/TP53$^{-/-}$ OST) GEMM (127). They found variable responses in different GEMM. The MMTV-c-neu and basal-like breast model C3-Tag was the most responsive in general and claudin-low T11/TP53−/− model was the most resistant with only small responses. GEMMs recapitulated characteristics of human breast cancer have become a promising tool in cancer research to predict clinical outcome. A successful GEMM is very slow and laborious so that it has not been widely used. And there are still a lot of deficiencies with GEMM in preclinical research. The "co-clinical" trials which are validated *in vivo* models to pursue high-throughput drug screening and rapid translation of effective anticancer drugs into the clinical setting (128, 129). The co-clinical trials are underway in breast cancer, and we are looking forward to better rodent models for therapeutic testing of breast cancer.

SUMMARY

The selection of appropriate rodent models for investigation of breast cancer is an important experimental decision. Every mouse model has advantages and disadvantages, and thus it is highly important to design a suitable mouse model for each research purpose based on integration of the advantages and disadvantages of different models, and compound techniques of mouse models have more practical application. The rodent models may help to improve the knowledge of breast carcinogenesis mechanism and genetic pathways, as well as creating therapy for modeling clinical breast cancer subtypes and develop innovative cancer therapy.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to articles reviewed in this manuscript, were involved in the drafting and revision, and approved the final version of this manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Cardiff RD, Kenney N. A compendium of the mouse mammary tumor biologist: from the initial observations in the house mouse to the development of genetically engineered mice. *Cold Spring Harb Perspect Biol* (2011) 3(6):a003111. doi: 10.1101/cshperspect.a003111
2. Bittner JJ. Some Possible Effects of Nursing on the Mammary Gland Tumor Incidence In Mice. *Science (New York NY)* (1936) 84(2172):162. doi: 10.1126/science.84.2172.162
3. Kim JB, O'Hare MJ, Stein R. Models of breast cancer: is merging human and animal models the future? *Breast Cancer Res* (2004) 6(1):22–30. doi: 10.1186/bcr645
4. Stewart TA, Pattengale PK, Leder P. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. *Cell* (1984) 38(3):627–37. doi: 10.1016/0092-8674(84)90257-5
5. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. *Cell* (1987) 49(4):465–75. doi: 10.1016/0092-8674(87)90449-1
6. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* (1988) 54(1):105–15. doi: 10.1016/0092-8674(88)90184-5
7. Xu X, Wagner KU, Larson D, Weaver Z, Li C, Ried T, et al. Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nat Genet* (1999) 22(1):37–43. doi: 10.1038/8743
8. Du Z, Podsypanina K, Huang S, McGrath A, Toneff MJ, Bogoslovskaia E, et al. Introduction of oncogenes into mammary glands in vivo with an avian retroviral vector initiates and promotes carcinogenesis in mouse models. *Proc Natl Acad Sci U S A* (2006) 103(46):17396–401. doi: 10.1073/pnas.0608607103
9. Silva JG, Corrales-Medina FF, Maher OM, Tannir N, Huh WW, Rytting ME, et al. Clinical next generation sequencing of pediatric-type malignancies in adult patients identifies novel somatic aberrations. *Oncoscience* (2015) 2(2):187–92. doi: 10.18632/oncoscience.131
10. Tao K, Fang M, Alroy J, Sahagian GG. Imagable 4T1 model for the study of late stage breast cancer. *BMC Cancer* (2008) 8:228. doi: 10.1186/1471-2407-8-228
11. Paschall AV, Liu K. An Orthotopic Mouse Model of Spontaneous Breast Cancer Metastasis. *J Vis Exp* (2016) 114:54040. doi: 10.3791/54040
12. Zhang C, Yan Z, Arango ME, Painter CL, Anderes K. Advancing bioluminescence imaging technology for the evaluation of anticancer agents in the MDA-MB-435-HAL-Luc mammary fat pad and subrenal capsule tumor models. *Clin Cancer Res* (2009) 15(1):238–46. doi: 10.1158/1078-0432.Ccr-08-0897
13. Kuperwasser C, Dessain S, Bierbaum BE, Garnet D, Sperandio K, Gauvin GP, et al. A mouse model of human breast cancer metastasis to human bone. *Cancer Res* (2005) 65(14):6130–8. doi: 10.1158/0008-5472.Can-04-1408
14. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. *Breast Cancer Res* (2011) 13(4):215. doi: 10.1186/bcr2889
15. Whittle JR, Lewis MT, Lindeman GJ, Visvader JE. Patient-derived xenograft models of breast cancer and their predictive power. *Breast Cancer Res* (2015) 17(1):17. doi: 10.1186/s13058-015-0523-1
16. Sikora MJ, Cooper KL, Bahreini A, Luthra S, Wang G, Chandran UR, et al. Invasive lobular carcinoma cell lines are characterized by unique estrogen-mediated gene expression patterns and altered tamoxifen response. *Cancer Res* (2014) 74(5):1463–74. doi: 10.1158/0008-5472.Can-13-2779
17. Sflomos G, Dormoy V, Metsalu T, Jeitziner R, Battista L, Scabia V, et al. A Preclinical Model for ERα-Positive Breast Cancer Points to the Epithelial Microenvironment as Determinant of Luminal Phenotype and Hormone Response. *Cancer Cell* (2016) 29(3):407–22. doi: 10.1016/j.ccell.2016.02.002
18. Holen I, Speirs V, Morrissey B, Blyth K. In vivo models in breast cancer research: progress, challenges and future directions. *Dis Models Mech* (2017) 10(4):359–71. doi: 10.1242/dmm.028274
19. Murayama T, Gotoh N. Patient-Derived Xenograft Models of Breast Cancer and Their Application. *Cells* (2019) 8(6):621. doi: 10.3390/cells8060621
20. Kawaguchi T, Foster BA, Young J, Takabe K. Current Update of Patient-Derived Xenograft Model for Translational Breast Cancer Research. *J Mammary Gland Biol Neoplasia* (2017) 22(2):131–9. doi: 10.1007/s10911-017-9378-7
21. Li S, Shen D, Shao J, Crowder R, Liu W, Prat A, et al. Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. *Cell Rep* (2013) 4(6):1116–30. doi: 10.1016/j.celrep.2013.08.022
22. DeRose YS, Wang G, Lin YC, Bernard PS, Buys SS, Ebbert MT, et al. Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes. *Nat Med* (2011) 17(11):1514–20. doi: 10.1038/nm.2454
23. Reyal F, Guyader C, Decraene C, Lucchesi C, Auger N, Assayag F, et al. Molecular profiling of patient-derived breast cancer xenografts. *Breast Cancer Res* (2012) 14(1):R11. doi: 10.1186/bcr3095
24. Zhang X, Claerhout S, Prat A, Dobrolecki LE, Petrovic I, Lai Q, et al. A renewable tissue resource of phenotypically stable, biologically and ethnically diverse, patient-derived human breast cancer xenograft models. *Cancer Res* (2013) 73(15):4885–97. doi: 10.1158/0008-5472.Can-12-4081
25. Ding L, Ellis MJ, Li S, Larson DE, Chen K, Wallis JW, et al. Genome remodelling in a basal-like breast cancer metastasis and xenograft. *Nature* (2010) 464(7291):999–1005. doi: 10.1038/nature08989
26. Eirew P, Steif A, Khattra J, Ha G, Yap D, Farahani H, et al. Dynamics of genomic clones in breast cancer patient xenografts at single-cell resolution. *Nature* (2015) 518(7539):422–6. doi: 10.1038/nature13952
27. Engebraaten O, Vollan HKM, Børresen-Dale AL. Triple-negative breast cancer and the need for new therapeutic targets. *Am J Pathol* (2013) 183(4):1064–74. doi: 10.1016/j.ajpath.2013.05.033
28. Kabos P, Finlay-Schultz J, Li C, Kline E, Finlayson C, Wisell J, et al. Patient-derived luminal breast cancer xenografts retain hormone receptor heterogeneity and help define unique estrogen-dependent gene signatures. *Breast Cancer Res Treat* (2012) 135(2):415–32. doi: 10.1007/s10549-012-2164-8
29. Behbod F, Kittrell FS, LaMarca H, Edwards D, Kerbawy S, Heestand JC, et al. An intraductal human-in-mouse transplantation model mimics the subtypes of ductal carcinoma in situ. *Breast Cancer Res* (2009) 11(5):R66. doi: 10.1186/bcr2358
30. Haricharan S, Lei J, Ellis M. Mammary Ductal Environment Is Necessary for Faithful Maintenance of Estrogen Signaling in ER^+ Breast Cancer. *Cancer Cell* (2016) 29(3):249–50. doi: 10.1016/j.ccell.2016.02.017
31. Ghosh A, Sarkar S, Banerjee S, Behbod F, Tawfik O, McGregor D, et al. MIND model for triple-negative breast cancer in syngeneic mice for quick and sequential progression analysis of lung metastasis. *PloS One* (2018) 13(5):e0198143. doi: 10.1371/journal.pone.0198143
32. Atiya HI, Dvorkin-Gheva A, Hassell J, Patel S, Parker RL, Hartstone-Rose A, et al. Intraductal Adaptation of the 4T1 Mouse Model of Breast Cancer Reveals Effects of the Epithelial Microenvironment on Tumor Progression and Metastasis. *Anticancer Res* (2019) 39(5):2277–87. doi: 10.21873/anticanres.13344
33. Steenbrugge J, Breyne K, Demeyere K, De Wever O, Sanders NN, Van Den Broeck W, et al. Anti-inflammatory signaling by mammary tumor cells mediates prometastatic macrophage polarization in an innovative intraductal mouse model for triple-negative breast cancer. *J Exp Clin Cancer Res* (2018) 37(1):191. doi: 10.1186/s13046-018-0860-x
34. Luo XL, Lin L, Hu H, Hu FL, Lin Y, Luo ML, et al. Development and characterization of mammary intraductal (MIND) spontaneous metastasis models for triple-negative breast cancer in syngeneic mice. *Sci Rep* (2020) 10(1):4681. doi: 10.1038/s41598-020-61679-8
35. Dexter DL, Kowalski HM, Blazar BA, Fligiel Z, Vogel R, Heppner GH. Heterogeneity of tumor cells from a single mouse mammary tumor. *Cancer Res* (1978) 38(10):3174–81.
36. Steenbrugge J, Vander Elst N, Demeyere K, De Wever O, Sanders NN, Van Den Broeck W, et al. Comparative Profiling of Metastatic 4T1- vs. Non-metastatic Py230-Based Mammary Tumors in an Intraductal Model for Triple-Negative Breast Cancer. *Front Immunol* (2019) 10:2928:2928. doi: 10.3389/fimmu.2019.02928
37. Barros AC, Muranaka EN, Mori LJ, Pelizon CH, Iriya K, Giocondo G, et al. Induction of experimental mammary carcinogenesis in rats with 7,12-dimethylbenz(a)anthracene. *Rev Hosp Clin* (2004) 59(5):257–61. doi: 10.1590/s0041-87812004000500006

38. Danforth DNJr, Tamarkin L, Lippman ME. Melatonin increases oestrogen receptor binding activity of human breast cancer cells. *Nature* (1983) 305 (5932):323–5. doi: 10.1038/305323a0
39. Sutherland RL, Murphy LC, San Foo M, Green MD, Whybourne AM, Krozowski ZS. High-affinity anti-oestrogen binding site distinct from the oestrogen receptor. *Nature* (1980) 288(5788):273–5. doi: 10.1038/288273a0
40. Travis RC, Key TJ. Oestrogen exposure and breast cancer risk. *Breast Cancer Res* (2003) 5(5):239–47. doi: 10.1186/bcr628
41. Seymour L, Bezwoda WR, Meyer K, Behr C. Detection of P24 protein in human breast cancer: influence of receptor status and oestrogen exposure. *Br J Cancer* (1990) 61(6):886–90. doi: 10.1038/bjc.1990.198
42. Mense SM, Remotti F, Bhan A, Singh B, El-Tamer M, Hei TK, et al. Estrogen-induced breast cancer: alterations in breast morphology and oxidative stress as a function of estrogen exposure. *Toxicol Appl Pharmacol* (2008) 232(1):78–85. doi: 10.1016/j.taap.2008.06.007
43. Harvell DM, Strecker TE, Tochacek M, Xie B, Pennington KL, McComb RD, et al. Rat strain-specific actions of 17beta-estradiol in the mammary gland: correlation between estrogen-induced lobuloalveolar hyperplasia and susceptibility to estrogen-induced mammary cancers. *Proc Natl Acad Sci U S A* (2000) 97(6):2779–84. doi: 10.1073/pnas.050569097
44. Jiang S, Katayama H, Wang J, Li SA, Hong Y, Radvanyi L, et al. Estrogen-induced aurora kinase-A (AURKA) gene expression is activated by GATA-3 in estrogen receptor-positive breast cancer cells. *Horm Cancer* (2010) 1 (1):11–20. doi: 10.1007/s12672-010-0006-x
45. Ruhlen RL, Willbrand DM, Besch-Williford CL, Ma L, Shull JD, Sauter ER. Tamoxifen induces regression of estradiol-induced mammary cancer in the ACI.COP-Ept2 rat model. *Breast Cancer Res Treat* (2009) 117(3):517–24. doi: 10.1007/s10549-008-0169-0
46. Kassayová M, Bobrov N, Strojný L, Orendáš P, Demečková V, Jendželovský R, et al. Anticancer and Immunomodulatory Effects of Lactobacillus plantarum LS/07, Inulin and Melatonin in NMU-induced Rat Model of Breast Cancer. *Anticancer Res* (2016) 36(6):2719–28.
47. Chan MM, Lu X, Merchant FM, Iglehart JD, Miron PL. Serial transplantation of NMU-induced rat mammary tumors: a model of human breast cancer progression. *Int J Cancer* (2007) 121(3):474–85. doi: 10.1002/ijc.22684
48. Martinez VG, Crown J, Porter RK, O'Driscoll L. Neuromedin U alters bioenergetics and expands the cancer stem cell phenotype in HER2-positive breast cancer. *Int J Cancer* (2017) 140(12):2771–84. doi: 10.1002/ijc.30705
49. Zeng L, Li W, Chen CS. Breast cancer animal models and applications. *Zool Res* (2020) 41(5):477–94. doi: 10.24272/j.issn.2095-8137.2020.095
50. Raptis L, Brownell HL, Corbley MJ, Wood KW, Wang D , Haliotis T. Cellular ras gene activity is required for full neoplastic transformation by the large tumor antigen of SV40. *Cell Growth Differ* (1997) 8(8):891–901.
51. Raptis L, Marcellus R, Corbley MJ, Krook A, Whitfield J, Anderson SK, et al. Cellular ras gene activity is required for full neoplastic transformation by polyomavirus. *J Virol* (1991) 65(10):5203–10. doi: 10.1128/JVI.65.10.5203-5210.1991
52. Wood LD, Parsons DW, Jones S, Lin J, Sjöblom T, Leary RJ, et al. The genomic landscapes of human breast and colorectal cancers. *Science (New York NY)* (2007) 318(5853):1108–13. doi: 10.1126/science.1145720
53. Fromowitz FB, Viola MV, Chao S, Oravez S, Mishriki Y, Finkel G, et al. ras p21 expression in the progression of breast cancer. *Hum Pathol* (1987) 18 (12):1268–75. doi: 10.1016/s0046-8177(87)80412-4
54. Garcia R, Yu CL, Hudnall A, Catlett R, Nelson KL, Smithgall T, et al. Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. *Cell Growth Differ* (1997) 8 (12):1267–76.
55. Smith BA, Shelton DN, Kieffer C, Milash B, Usary J, Perou CM, et al. Targeting the PyMT Oncogene to Diverse Mammary Cell Populations Enhances Tumor Heterogeneity and Generates Rare Breast Cancer Subtypes. *Genes Cancer* (2012) 3(9-10):550–63. doi: 10.1177/1947601913475359
56. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol* (1992) 12(3):954–61. doi: 10.1128/mcb.12.3.954
57. Shishido SN, Delahaye A, Beck A, Nguyen TA. The anticancer effect of PQ1 in the MMTV-PyVT mouse model. *Int J Cancer* (2014) 134(6):1474–83. doi: 10.1002/ijc.28461
58. Maglione JE, Moghanaki D, Young LJ, Manner CK, Ellies LG, Joseph SO, et al. Transgenic Polyoma middle-T mice model premalignant mammary disease. *Cancer Res* (2001) 61(22):8298–305.
59. Maglione JE, McGoldrick ET, Young LJ, Namba R, Gregg JP, Liu L, et al. Polyomavirus middle T-induced mammary intraepithelial neoplasia outgrowths: single origin, divergent evolution, and multiple outcomes. *Mol Cancer Ther* (2004) 3(8):941–53.
60. Nusse R, Brown A, Papkoff J, Scambler P, Shackleford G, McMahon A, et al. A new nomenclature for int-1 and related genes: the Wnt gene family. *Cell* (1991) 64(2):231. doi: 10.1016/0092-8674(91)90633-a
61. McMahon AP, Bradley A. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* (1990) 62(6):1073–85. doi: 10.1016/0092-8674(90)90385-r
62. Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T, Varmus HE. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* (1988) 55(4):619–25. doi: 10.1016/0092-8674(88)90220-6
63. Foo AL, Sly PD. Pulmonary function in a hospital population of asthmatic children. *J Asthma* (1991) 28(4):273–80. doi: 10.3109/02770909109073384
64. Li Y, Hively WP, Varmus HE. Use of MMTV-Wnt-1 transgenic mice for studying the genetic basis of breast cancer. *Oncogene* (2000) 19(8):1002–9. doi: 10.1038/sj.onc.1203273
65. Michaelson JS, Leder P. beta-catenin is a downstream effector of Wnt-mediated tumorigenesis in the mammary gland. *Oncogene* (2001) 20 (37):5093–9. doi: 10.1038/sj.onc.1204586
66. Lane TF, Leder P. Wnt-10b directs hypermorphic development and transformation in mammary glands of male and female mice. *Oncogene* (1997) 15(18):2133–44. doi: 10.1038/sj.onc.1201593
67. Zhang J, Li Y, Liu Q, Lu W, Bu G. Wnt signaling activation and mammary gland hyperplasia in MMTV-LRP6 transgenic mice: implication for breast cancer tumorigenesis. *Oncogene* (2010) 29(4):539–49. doi: 10.1038/onc.2009.339
68. Muller WJ, Lee FS, Dickson C, Peters G, Pattengale P, Leder P. The int-2 gene product acts as an epithelial growth factor in transgenic mice. *EMBO J* (1990) 9(3):907–13. doi: 10.1002/j.1460-2075.1990.tb08188.x
69. Cardiff RD, Sinn E, Muller W, Leder P. Transgenic oncogene mice. Tumor phenotype predicts genotype. *Am J Pathol* (1991) 139(3):495–501.
70. Ray D, Terao Y, Nimbalkar D, Hirai H, Osmundson EC, Zou X, et al. Hemizygous disruption of Cdc25A inhibits cellular transformation and mammary tumorigenesis in mice. *Cancer Res* (2007) 67(14):6605–11. doi: 10.1158/0008-5472.Can-06-4815
71. Hodgson MC, Vanostran G, Alghamdi S, Poppiti RJ, Agoulnik AI, Agoulnik IU. Reduced androgen receptor expression accelerates the onset of ERBB2 induced breast tumors in female mice. *PloS One* (2013) 8(4):e60455. doi: 10.1371/journal.pone.0060455
72. Fantozzi A, Christofori G. Mouse models of breast cancer metastasis. *Breast Cancer Res* (2006) 8(4):212. doi: 10.1186/bcr1530
73. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci USA* (1992) 89(22):10578–82. doi: 10.1073/pnas.89.22.10578
74. Li B, Rosen JM, McMenamin-Balano J, Muller WJ, Perkins AS. neu/ERBB2 cooperates with p53-172H during mammary tumorigenesis in transgenic mice. *Mol Cell Biol* (1997) 17(6):3155–63. doi: 10.1128/mcb.17.6.3155
75. Maroulakou IG, Anver M, Garrett L, Green JE. Prostate and mammary adenocarcinoma in transgenic mice carrying a rat C3(1) simian virus 40 large tumor antigen fusion gene. *Proc Natl Acad Sci U S A* (1994) 91 (23):11236–40. doi: 10.1073/pnas.91.23.11236
76. Pathania R, Ramachandran S, Elangovan S, Padia R, Yang P, Cinghu S, et al. DNMT1 is essential for mammary and cancer stem cell maintenance and tumorigenesis. *Nat Commun* (2015) 6:6910. doi: 10.1038/ncomms7910
77. Shibata MA, Jorcyk CL, Liu ML, Yoshidome K, Gold LG, Green JE. The C3 (1)/SV40 T antigen transgenic mouse model of prostate and mammary cancer. *Toxicol Pathol* (1998) 26(1):177–82. doi: 10.1177/019262339802600121

78. Wild R, Yokoyama Y, Dings RP, Ramakrishnan S. VEGF-DT385 toxin conjugate inhibits mammary adenocarcinoma development in a transgenic mouse model of spontaneous tumorigenesis. *Breast Cancer Res Treat* (2004) 85(2):161–71. doi: 10.1023/B:BREA.0000025407.02896.ec
79. Song W, Hwang Y, Youngblood VM, Cook RS, Balko JM, Chen J, et al. Targeting EphA2 impairs cell cycle progression and growth of basal-like/triple-negative breast cancers. *Oncogene* (2017) 36(40):5620–30. doi: 10.1038/onc.2017.170
80. Liu R, Varghese S, Rabkin SD. Oncolytic herpes simplex virus vector therapy of breast cancer in C3(1)/SV40 T-antigen transgenic mice. *Cancer Res* (2005) 65(4):1532–40. doi: 10.1158/0008-5472.Can-04-3353
81. Erisman JW, Domburg N, de Vries W, Kros H, de Haan B , Sanders K. The Dutch N-cascade in the European perspective. *Sci China C Life Sci* (2005) 48 (Suppl 2):827–42. doi: 10.1007/bf03187122
82. Sutherland RL, Musgrove EA. Cyclin D1 and mammary carcinoma: new insights from transgenic mouse models. *Breast Cancer Res* (2002) 4(1):14–7. doi: 10.1186/bcr411
83. Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, Schmidt EV. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* (1994) 369(6482):669–71. doi: 10.1038/369669a0
84. van Rossum AG, van Bragt MP, Schuuring-Scholtes E, van der Ploeg JC, van Krieken JH, Kluin PM, et al. Transgenic mice with mammary gland targeted expression of human cortactin do not develop (pre-malignant) breast tumors: studies in MMTV-cortactin and MMTV-cortactin/-cyclin D1 bitransgenic mice. *BMC Cancer* (2006) 6:58. doi: 10.1186/1471-2407-6-58
85. Garcia Y, Kahn O, Rabardel L, Chansou B, Salmon L, Tuchagues JP. Two-Step Spin Conversion for the Three-Dimensional Compound Tris(4,4'-bis-1,2,4-triazole)iron(II) Diperchlorate. *Inorg Chem* (1999) 38(21):4663–70. doi: 10.1021/ic990511q
86. Deng CX. Conditional knockout mouse models of cancer. *Cold Spring Harb Protoc* (2014) 2014(12):1217–33. doi: 10.1101/pdb.top074393
87. Hakem R, de la Pompa JL, Sirard C, Mo R, Woo M, Hakem A, et al. The tumor suppressor gene Brca1 is required for embryonic cellular proliferation in the mouse. *Cell* (1996) 85(7):1009–23. doi: 10.1016/s0092-8674(00)81302-1
88. Deng CX. Tumor formation in Brca1 conditional mutant mice. *Environ Mol Mutagen* (2002) 39(2-3):171–7. doi: 10.1002/em.10069
89. Raval C, Saeed K. Anaesthetic management of a patient of Brugada syndrome for an emergency appendicectomy. *Anesth Essays Res* (2012) 6 (1):101–4. doi: 10.4103/0259-1162.103390
90. Shakya R, Szabolcs M, McCarthy E, Ospina E, Basso K, Nandula S, et al. The basal-like mammary carcinomas induced by Brca1 or Bard1 inactivation implicate the BRCA1/BARD1 heterodimer in tumor suppression. *Proc Natl Acad Sci U S A* (2008) 105(19):7040–5. doi: 10.1073/pnas.0711032105
91. Xu X, Qiao W, Linke SP, Cao L, Li WM, Furth PA, et al. Genetic interactions between tumor suppressors Brca1 and p53 in apoptosis, cell cycle and tumorigenesis. *Nat Genet* (2001) 28(3):266–71. doi: 10.1038/90108
92. Weaver Z, Montagna C, Xu X, Howard T, Gadina M, Brodie SG, et al. Mammary tumors in mice conditionally mutant for Brca1 exhibit gross genomic instability and centrosome amplification yet display a recurring distribution of genomic imbalances that is similar to human breast cancer. *Oncogene* (2002) 21(33):5097–107. doi: 10.1038/sj.onc.1205636
93. McPherson JP, Lemmers B, Hirao A, Hakem A, Abraham J, Migon E, et al. Collaboration of Brca1 and Chk2 in tumorigenesis. *Genes Dev* (2004) 18 (10):1144–53. doi: 10.1101/gad.1192704
94. Liu X, Holstege H, van der Gulden H, Treur-Mulder M, Zevenhoven J, Velds A, et al. Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. *Proc Natl Acad Sci U S A* (2007) 104(29):12111–6. doi: 10.1073/pnas.0702969104
95. McCarthy A, Savage K, Gabriel A, Naceur C, Reis-Filho JS, Ashworth A. A mouse model of basal-like breast carcinoma with metaplastic elements. *J Pathol* (2007) 211(4):389–98. doi: 10.1002/path.2124
96. Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. *Nat Genet* (2001) 29(4):418–25. doi: 10.1038/ng747
97. Ludwig T, Fisher P, Murty V, Efstratiadis A. Development of mammary adenocarcinomas by tissue-specific knockout of Brca2 in mice. *Oncogene* (2001) 20(30):3937–48. doi: 10.1038/sj.onc.1204512
98. Cheung AM, Elia A, Tsao MS, Done S, Wagner KU, Hennighausen L, et al. Brca2 deficiency does not impair mammary epithelium development but promotes mammary adenocarcinoma formation in p53(+/-) mutant mice. *Cancer Res* (2004) 64(6):1959–65. doi: 10.1158/0008-5472.can-03-2270
99. Lin SC, Lee KF, Nikitin AY, Hilsenbeck SG, Cardiff RD, Li A, et al. Somatic mutation of p53 leads to estrogen receptor alpha-positive and -negative mouse mammary tumors with high frequency of metastasis. *Cancer Res* (2004) 64(10):3525–32. doi: 10.1158/0008-5472.Can-03-3524
100. Lesche R, Groszer M, Gao J, Wang Y, Messing A, Sun H, et al. Cre/loxP-mediated inactivation of the murine Pten tumor suppressor gene. *Genesis (New York NY 2000)* (2002) 32(2):148–9. doi: 10.1002/gene.10036
101. Li G, Robinson GW, Lesche R, Martinez-Diaz H, Jiang Z, Rozengurt N, et al. Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland. *Development (Cambridge England)* (2002) 129(17):4159–70.
102. Schade B, Rao T, Dourdin N, Lesurf R, Hallett M, Cardiff RD, et al. PTEN deficiency in a luminal ErbB-2 mouse model results in dramatic acceleration of mammary tumorigenesis and metastasis. *J Biol Chem* (2009) 284 (28):19018–26. doi: 10.1074/jbc.M109.018937
103. Ahronian LG, Lewis BC. Using the RCAS-TVA system to model human cancer in mice. *Cold Spring Harb Protoc* (2014) 2014(11):1128–35. doi: 10.1101/pdb.top069831
104. Ahronian LG, Lewis BC. In vivo delivery of RCAS virus to mice. *Cold Spring Harb Protoc* (2014) 2014(11):1167–9. doi: 10.1101/pdb.prot077982
105. Fisher GH, Orsulic S, Holland E, Hively WP, Li Y, Lewis BC, et al. Development of a flexible and specific gene delivery system for production of murine tumor models. *Oncogene* (1999) 18(38):5253–60. doi: 10.1038/sj.onc.1203087
106. Reddy JP, Li Y. The RCAS-TVA system for introduction of oncogenes into selected somatic mammary epithelial cells in vivo. *J Mammary Gland Biol Neoplasia* (2009) 14(4):405–9. doi: 10.1007/s10911-009-9157-1
107. Sun H, Zhu Y, He QF, Shu LY, Zhang W, Chai YM. Reinforcement strategy for lateral rafting plate fixation in posterolateral column fractures of the tibial plateau: The magic screw technique. *Injury* (2017) 48(12):2814–26. doi: 10.1016/j.injury.2017.10.033
108. Siwko SK, Bu W, Gutierrez C, Lewis B, Jechlinger M, Schaffhausen B, et al. Lentivirus-mediated oncogene introduction into mammary cells in vivo induces tumors. *Neoplasia (New York NY)* (2008) 10(7):653–662, 651 p following 662. doi: 10.1593/neo.08266
109. Haricharan S, Hein SM, Dong J, Toneff MJ, Aina OH, Rao PH, et al. Contribution of an alveolar cell of origin to the high-grade malignant phenotype of pregnancy-associated breast cancer. *Oncogene* (2014) 33 (50):5729–39. doi: 10.1038/onc.2013.521
110. Haricharan S, Dong J, Hein S, Reddy JP, Du Z, Toneff M, et al. Mechanism and preclinical prevention of increased breast cancer risk caused by pregnancy. *eLife* (2013) 2:e00996. doi: 10.7554/eLife.00996
111. Sinha VC, Qin L, Li Y. A p53/ARF-dependent anticancer barrier activates senescence and blocks tumorigenesis without impacting apoptosis. *Mol Cancer Res* (2015) 13(2):231–8. doi: 10.1158/1541-7786.Mcr-14-0481-t
112. Hakamy A, Bolton CE, Gibson JE, McKeever TM. Risk of fall in patients with COPD. *Thorax* (2018) 73(11):1079–80. doi: 10.1136/thoraxjnl-2017-211008
113. Champagne E, Scarpellino L, Lane P, Acha-Orbea H. CD28/CTLA4-B7 interaction is dispensable for T cell stimulation by mouse mammary tumor virus superantigen but not for B cell differentiation and virus dissemination. *Eur J Immunol* (1996) 26(7):1595–602. doi: 10.1002/eji.1830260728
114. Andò S, Malivindi R, Catalano S, Rizza P, Barone I, Panza S, et al. Conditional expression of Ki-Ras(G12V) in the mammary epithelium of transgenic mice induces estrogen receptor alpha (ERα)-positive adenocarcinoma. *Oncogene* (2017) 36(46):6420–31. doi: 10.1038/onc.2017.252
115. Chan SR, Vermi W, Luo J, Lucini L, Rickert C, Fowler AM, et al. STAT1-deficient mice spontaneously develop estrogen receptor α-positive luminal mammary carcinomas. *Breast Cancer Res* (2012) 14(1):R16. doi: 10.1186/bcr3100
116. Chan SR, Rickert CG, Vermi W, Sheehan KC, Arthur C, Allen JA, et al. Dysregulated STAT1-SOCS1 control of JAK2 promotes mammary luminal progenitor cell survival and drives ERα(+) tumorigenesis. *Cell Death Differ* (2014) 21(2):234–46. doi: 10.1038/cdd.2013.116
117. Holloway KR, Sinha VC, Bu W, Toneff M, Dong J, Peng Y, et al. Targeting Oncogenes into a Defined Subset of Mammary Cells Demonstrates That the Initiating Oncogenic Mutation Defines the Resulting Tumor Phenotype. *Int J Biol Sci* (2016) 12(4):381–8. doi: 10.7150/ijbs.12947

118. Masso-Welch PA, Darcy KM, Stangle-Castor NC, Ip MM. A developmental atlas of rat mammary gland histology. *J Mammary Gland Biol Neoplasia* (2000) 5(2):165–85. doi: 10.1023/a:1026491221687
119. Russo J, Russo IH. Atlas and histologic classification of tumors of the rat mammary gland. *J Mammary Gland Biol Neoplasia* (2000) 5(2):187–200. doi: 10.1023/a:1026443305758
120. Shirai K, Uemura Y, Fukumoto M, Tsukamoto T, Pascual R, Nandi S, et al. Synergistic effect of MNU and DMBA in mammary carcinogenesis and H-ras activation in female Sprague-Dawley rats. *Cancer Lett* (1997) 120(1):87–93. doi: 10.1016/s0304-3835(97)00293-0
121. Griffith OL, Spies NC, Anurag M, Griffith M, Luo J, Tu D, et al. The prognostic effects of somatic mutations in ER-positive breast cancer. *Nat Commun* (2018) 9(1):3476. doi: 10.1038/s41467-018-05914-x
122. Wang B, Kennan WS, Yasukawa-Barnes J, Lindstrom MJ, Gould MN. Difference in the response of neu and ras oncogene-induced rat mammary carcinomas to early and late ovariectomy. *Cancer Res* (1992) 52(15):4102–5.
123. Dischinger PS, Tovar EA, Essenburg CJ, Madaj ZB, Gardner EE, Callaghan ME, et al. NF1 deficiency correlates with estrogen receptor signaling and diminished survival in breast cancer. *NPJ Breast Cancer* (2018) 4:29. doi: 10.1038/s41523-018-0080-8
124. Bu W, Li Y. Intraductal Injection of Lentivirus Vectors for Stably Introducing Genes into Rat Mammary Epithelial Cells in Vivo. *J Mammary Gland Biol Neoplasia* (2020). doi: 10.1007/s10911-020-09469-w
125. Roberts PJ, Bisi JE, Strum JC, Combest AJ, Darr DB, Usary JE, et al. Multiple roles of cyclin-dependent kinase 4/6 inhibitors in cancer therapy. *J Natl Cancer Inst* (2012) 104(6):476–87. doi: 10.1093/jnci/djs002
126. Roberts PJ, Usary JE, Darr DB, Dillon PM, Pfefferle AD, Whittle MC, et al. Combined PI3K/mTOR and MEK inhibition provides broad antitumor activity in faithful murine cancer models. *Clin Cancer Res* (2012) 18 (19):5290–303. doi: 10.1158/1078-0432.Ccr-12-0563
127. Usary J, Zhao W, Darr D, Roberts PJ, Liu M, Balletta L, et al. Predicting drug responsiveness in human cancers using genetically engineered mice. *Clin Cancer Res* (2013) 19(17):4889–99. doi: 10.1158/1078-0432.Ccr-13-0522
128. Lunardi A, Ala U, Epping MT, Salmena L, Clohessy JG, Webster KA, et al. A co-clinical approach identifies mechanisms and potential therapies for androgen deprivation resistance in prostate cancer. *Nat Genet* (2013) 45 (7):747–55. doi: 10.1038/ng.2650
129. Federico L, Chong Z, Zhang D, McGrail DJ, Zhao W, Jeong KJ, et al. A murine preclinical syngeneic transplantation model for breast cancer precision medicine. *Sci Adv* (2017) 3(4):e1600957. doi: 10.1126/sciadv.1600957

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Dezocine, An Opioid Analgesic, Exerts Antitumor Effects in Triple-Negative Breast Cancer by Targeting Nicotinamide Phosphoribosyltransferase

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Opioids are a potential adjuvant treatment for certain cancers; while they are primarily used to relieve chronic pain, these drugs may also affect cancer progression and recurrence. Dezocine is one opioid commonly used in China, but its effects on cancer cells are unknown. Here, we demonstrated the inhibitory effect of dezocine on triple-negative breast cancer (TNBC) cells, and determined the underlying molecular mechanism. We found that dezocine suppressed cell proliferation, migration and invasion, and induced apoptosis in TNBC cells. Xenograft models demonstrated the inhibitory effects of dezocine treatment on TNBC tumor growth *in vivo*. The anticancer effects of dezocine were independent of opioid receptors, which are not highly expressed by normal breast or breast cancer tissues. A pull-down assay and LC-MS/MS analysis indicated that dezocine directly targets NAMPT: computer modeling verified that the free energy of dezocine kinetically bound into the pocket of NAMPT was −17.4 kcal/mol. Consequently, dezocine treatment inhibited NAMPT enzyme activity, resulting in cellular NAD abolishment. We confirmed the dezocine-induced inhibition of cell proliferation by both NAMPT knockdown and upon treatment with the inhibitor FK866. Our results suggest that both dezocine and NAMPT might represent novel therapeutic targets for TNBC.

Keywords: triple-negative breast cancer, dezocine, opioid, NAMPT, proliferation, metastasis

INTRODUCTION

Breast cancer is the most common malignancy suffered by women, accounting for 30% of all diagnosed cases (Siegel et al., 2019). Advances in disease treatment and management have improved survival rates, but breast cancer patients still experience the second highest mortality rate of all female cancer patients. However, mortality rate differs depending on breast cancer subtype, with some being more aggressive and difficult to treat than others. One example is triple negative breast cancer (TNBC), which accounts for 15% of all diagnosed breast carcinomas and is characterized by a lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression (Waks and Winer, 2019). This means that traditional treatments that target these

biomarkers are largely ineffective, and patients with TNBC typically experience an unfavorable prognosis. At present, TNBC is treated with standard chemotherapy combined with PARP inhibitors or DNA-targeting platinum drugs, such as carboplatin (Aggarwal et al., 2019). However, the heterogenous nature of the disease means that even these aggressive, combination treatment regimens are often ineffective, and novel treatment approaches are urgently required to improve prognosis for patients with TNBC.

For this reason, our group sought to investigate the potential of opioid drugs as an adjuvant treatment for TNBC. Opioids are widely used for relieving moderate to severe pain in the clinic, including chronic cancer pain, but previous studies have suggested that regional anesthesia and analgesia may also impact cancer progression and recurrence (Tedore, 2015; Forget et al., 2019; Wall et al., 2019). Furthermore, both agonistic and antagonistic opioid ligands have been found to affect cancer growth and development (Boland et al., 2014; Szczepaniak et al., 2020). For example, the mu opioid receptor (MOR) serves an important role in cancer progression by regulating angiogenesis, EMT, mTOR, Src and other signaling pathways (Singleton et al., 2015). MOR is highly expressed in human non-small cell lung cancer (NSCLC) tumor tissues, and the MOR agonist morphine increases Lewis lung carcinoma (LLC) cell proliferation, while MOR knockout mice or the opioid antagonist MNTX infusion attenuates LLC tumor growth and reduces lung metastasis (Mathew et al., 2011). In addition, increased MOR expression is associated with shorter progression-free survival (PFS) and overall survival (OS) in patients with metastatic prostate cancer (Zylla et al., 2013). Recent studies have also linked MOR overexpression to hepatocellular carcinoma (HCC) progression and poor prognosis in HCC patients, while MOR silencing is found to reduce HCC-associated tumorigenesis (Chen et al., 2019; Li et al., 2019). Morphine promotes triple negative breast cancer (TNBC) progression, angiogenesis and metastasis in xenograft mouse models and *in vitro* studies (Bimonte et al., 2015; Liu et al., 2020), but the effect of morphine in published literature remains controversial (Bimonte et al., 2013). Another opioid analgesic, dezocine, is a MOR and kappa receptor (KOR) mixed agonist-antagonist, with a stronger affinity to MOR (Wang et al., 2018). It also acts as a norepinephrine and serotonin reuptake inhibitor via the norepinephrine transporter (NET) and serotonin transporter (SERT) (Liu et al., 2014). It is widely used in China, particularly for the relief of chronic cancer pain. The link to MOR suggests that dezocine treatment may also affect cancer progression and metastasis; however, the effect of dezocine on cancer cells remains unknown.

In the present study, we found that dezocine inhibited cell growth, induced apoptosis, and suppressed metastasis in TNBC cell lines. Furthermore, we determined that dezocine exerted these anticancer effects by directly targeting nicotinamide phosphoribosyltransferase (NAMPT), which triggered the downregulation of NAMPT enzyme activity and NAD levels. Importantly, Xenograft models indicated the inhibitory effects of dezocine *in vivo*. These findings suggest that dezocine may have potential as a novel adjuvant treatment to inhibit TNBC progression.

MATERIALS AND METHODS

Cell Lines and Reagents

SH-SY5Y and U937 cell lines were obtained from the Beijing Stem Cell Bank, Chinese Academy of Sciences (Beijing, China). All other cell lines were purchased from the American Type Culture Collection (Manassas, VA, United States). MDA-MB-231, BT549, MDA-MB-468 and MCF7 cells were cultured in DMEM (HyClone; GE Healthcare, Chicago, IL, United States) containing 10% fetal bovine serum (FBS; PAN-Seratech GmbH, Aidenbach, Germany). HCC1937 and U937 cells were cultured in RPMI medium (HyClone; GE Healthcare) containing 10% FBS. MCF10A cells were cultured using the MEGM Bullet kit (Lonza Group, Basel, Switzerland) and 10 ng/ml cholera toxin. SH-SY5Y cells were cultured in DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, United States) containing 10% FBS. All cell lines were propagated at 37°C with 95% humidity in a 5% CO_2 incubator.

Dezocine was obtained from Yangzi River Pharmaceuticals Group (Taizhou, Jiangsu, China). FK866 and NMN were purchased from Selleck Chemicals LLC, Houston, TX, United States). Morphine was obtained from Northeast Pharmaceutical Group Shenyang No.1 Pharmaceutical Co. Ltd (Shenyang, Liaoning, China). Morphine, dezocine and NMN were dissolved in ddH_2O. FK866 was dissolved in ethanol. All compounds were diluted in appropriate media for cell culture studies.

Cell Viability Assay

Cell viability was measured using Cell Counting Kit-8 (CCK-8; Dalian Meliun Biotech Co., Ltd., Dalian, Liaoning, China) according to the manufacturer's protocol. Cells were seeded in 96-well plates at a density of 5000 cells/well, and were incubated in a final volume of 100 μL culture medium per well. After 24 h, the cells were treated with dezocine or FK866 for 48 h and cell viability was tested via the addition of CCK-8 reagent. To determine the half maximal inhibitory concentration (IC50), cells were treated with different concentrations of dezocine (0–320 μg/ml) for 48 h, and cell viability was measured.

Colony Formation and Proliferation Assays

Cells were seeded in 6-well plates at a density of 1000 cells/well and were incubated in a final volume of 2 ml/well culture medium containing different concentrations of dezocine (0, 10, 20 μg/ml). After 8–10 days incubation, the plates were washed with PBS and the cells were fixed with 100% methanol at 4°C for 10 min. Then, cells were stained with 0.05% crystal violet for 10 min at room temperature, and washed twice with ddH_2O.

DNA synthesis was detected in MDA-MB-231 and BT549 cells using the Cell-Light EdU Apollo488 *In Vitro* kit (Guangzhou RiboBio Co., Ltd., Guangzhou, Guangdong, China), according to the manufacturer's protocol. Briefly, cells were seeded in a 96-well plate at a density of 5000 cells/well, and were incubated in a final volume of 200 ml/well culture medium containing different concentrations of dezocine (10–40 μg/ml) for 48 h. The cells were then stained with Apollo488 and Hoechst33342. Images in 5 fields for each sample were randomly captured, and EdU-positive and Hoechst 33,342-positive cells were counted using

Image-Pro Plus 6.0 software (Media Cybernetics, lnc., Rockville, MD, United States)

Cell Apoptosis Analysis

MDA-MB-231 and BT549 cells were seeded into 6-well plates. After 24 h, the cells were treated with dezocine at 0, 10, 20, 40 μg/ml for 48 h. The cells were then labeled with Annexin V and PI using the Annexin V-FITC Apoptosis Detection kit (BioVision, Inc., Milpitas, CA, United States) and were analyzed using FACSCalibur platform (BD Biosciences, Franklin Lakes, NJ, United States).

Wound Healing Assays

Cells were seeded into 6-well plates, cultured until they reached 90% confluence, then starved in serum-free medium overnight. Following this, the cell monolayer was scratched using a sterile 200 μL tip and the plates were washed with PBS 2–3 times to remove floating cells. Then, DMEM containing 10% FBS and dezocine at 0, 10, 20, 40 μg/ml was added. Wound healing was monitored using an Olympus IX73 inverted microscope (Olympus Corporation, Tokyo, Japan) to capture photos of the migrating cells at 0, 24 and 48 h.

Transwell Assays

A total of 5×10^5 cells suspended in 200 μL serum-free medium were seeded in the upper chamber of 24-well Transwell inserts (8-μm pores, Corning; Thermo Fisher Scientific, Inc.) coated with or without Matrigel (BD Biosciences) for invasion and migration assays, respectively. The lower chamber contained 600 μL DMEM with 10% FBS. After incubation for 18 h, non-migrating cells on the upper side of the membrane were removed with a cotton swab, and the remaining cells were fixed and stained with 0.5% crystal violet. Images of 5 random fields were then captured using an Olympus IX73 inverted microscope (Olympus Corporation).

Western Blot

MDA-MB-231 and BT549 cells cultured in 60 mm dishes were treated with dezocine at 0, 10, 20, 40 μg/ml for 48 h. The cells were collected with sample buffer containing protease inhibitors, and protein concentration was determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Protein samples were separated by SDS-PAGE, and then transferred onto polyvinylidene fluoride membranes. Then, the membranes were labeled with corresponding primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Protein expression was then detected using the Pierce ECL western blot substrate (Thermo Fisher Scientific, Inc.). The antibodies used are listed in **Supplementary Table S1**.

Total Ribonucleic Acid Isolation and RT-qPCR

Total RNA was extracted from the cultured cells and the concentration was measured with a Nanodrop spectrometer N1000 (Thermo Fisher Scientific, Inc.). The extracted RNA was then reverse-transcribed to cDNA using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). The cDNA was then diluted 10-fold and prepared according to the SYBR Green Reagent specification (Takara Bio, Inc., Kusatsu, Japan). The protocol was as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 30 s. mRNA expression of the target genes was analyzed using the $2^{-\Delta\Delta Cq}$ method. Each real-time PCR reaction was repeated three times and normalized to the internal reference *GAPDH*. The primers used are listed in Table 2.

In Vitro Dezocine Pull Down Assay

2.5 mg Dezocine was incubated with Sepharose 4B beads (200 ml/g; GE Healthcare Life Sciences) in binding buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 4 μg/ml bovine serum albumin, 0.02 mM PMSF, 1X protease inhibitor mixture) with gentle rocking overnight at 4°C to form dezocine-Sepharose 4B. The dezocine-Sepharose 4B beads were then washed three times with washing buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 0.02 mM PMSF). MDA-MB-231 cellular supernatant fraction (1 mg) was then incubated with 200 μL dezocine-Sepharose 4B or Sepharose 4B (as a negative control) in binding buffer. After incubation overnight at 4°C with gentle rocking, the beads were washed five times with washing buffer, and the proteins bound to the beads were analyzed by LC-MS/MS and western blotting.

Protein In-Gel Digestion

The proteins from dezocine pull down assay was separated and then Coomassie-stained bands on the polyacrylamide gel were excised and transferred into a 1.5 ml microcentrifuge tube, where they were rinsed twice with Mill-Q water. The spots were then de-stained with 100 μL 50 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) and incubated with occasional vortexing for 30 min, depending on the intensity of the staining. Then, the de-staining solution was discarded and the tubes were washed twice with 200 μL of Mill-Q water. Next, 400 μL 100% acetonitrile was added to dry the gel spots in a SpeedVac for 10 min. Finally, the gel spots were rehydrated in a minimal volume of sequencing-grade porcine trypsin or chrymotrypsin solution (Promega Corporation, Madison, WI, United States; 20 μg/ml in 25 mM NH_4HCO_3) and incubated at 37°C overnight. Supernatant was transferred into a 200 μL microcentrifuge tube, and the gels were extracted with extraction buffer (67% acetonitrile containing 1% trifluoroacetic acid). The peptide extract and gel spot supernatant were combined and then completely dried in a SpeedVac.

Mass Spectrometry and nanoLC-Electrospray Ionization-Mass Spectrometry Analysis

The lyophilized peptide was re-suspended in 2% acetonitrile containing 0.1% formic acid and then 4 μL aliquots was loaded into a ChromXP C18 (3 μm, 120 Å) trap column. Online chromatography separation was performed on the Ekspert nanoLC 415 system (SCIEX, Concord, ON, Canada). Trapping and desalting were performed at a flow rate of 4 μL/min for 5 min, with 100% solvent A (water/acetonitrile/formic acid (98/2/0.1%;

B, 2/98/0.1%)). Then, an elution gradient of 8–38% solvent B was used on an analytical column (75 μm × 15 cm C18–3 μm 120 Å; ChromXP, Eksigent) for 30 min. Information-dependent acquisition (IDA) MS techniques were used to acquire tandem MS data on a Triple TOF 6600 tandem mass spectrometer (SCIEX), fitted with a Nanospray III ion source. Data were acquired using an ion spray voltage of 2.4 kV, curtain gas of 35 PSI, nebulizer gas of 12 PSI, and an interface heater temperature of 150°C. The MS was performed with TOF-MS scans. For IDA, survey scans were acquired in 250 ms and up to 40 product ion scans (50 ms) were collected if a threshold of 260 cps with a charge state of 2-4 was exceeded. A rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for 16s.

The MS/MS data were analyzed for protein identication and quantication using ProteinPilot Software v.5.0 (SCIEX). The local false discovery rate was estimated with the integrated PSPEP tool in the ProteinPilot Software as 1.0%, after searching against a decoy concatenated uniprot human protein database (20,191 entries). The following settings were then selected: sample type, identification; cystine alkylation, iodoacetamide; digestion, trypsin; instrument, TripleTOF 6600; species, none; search effort, thorough ID. For each identified peptide confidence should be >95%, and the proteins should have at least 2 unique peptides.

Computer Modeling Analysis

The initial protein structure was built based on its X-ray crystal structure (PDB code: 2GVJ) (Khan et al., 2006). Computational docking was performed using the program Autodock Vina (Trott and Olson, 2010). The search space for docking was large enough to include the default pocket of the protein and for the ligand to rotate in. Potential binding configurations were then selected based on their binding affinity energy. Molecular dynamic simulations were performed from the selected docking conformation with Amber18 software using FF14SB force filed for the protein, GaFF2 forcefield for the ligand, and the TIP3P water model (Jorgensen et al., 1983; Maier et al., 2015). The protein was solvated in a rhombic octahedral box with periodic boundary conditions and a distance of 10 Å between the boundary and the nearest protein atoms. Sodium and chloride ions were added to neutralize the simulated system. The system was minimized for 10,000 steps using a steepest descent algorithm, followed by a 1 ns heating process to increase the temperature from 10 to 310 K, and 1 ns of NPT simulation with weak restraints on heavy atoms. The 20 ns of NPT MD production simulation was performed at 310 K, and snapshots from the last 10 ns were used for MM/GBSA calculations.

In Vitro Nicotinamide Phosphoribosyltransferase Inhibition Assays

Dezocine (40 μg/ml), 10 nM FK866 and 20 μM NMN in ddH_2O were prepared, and *in vitro* NAMPT enzyme inhibitory activity assays were performed using the NAMPT Colorimetric Assay kit (Abcam, Cambridge, United Kingdom), according to manufacturers' protocol.

Determination of Intracellular NAD^+ Levels

MDA-MB-231 and BT549 cells, cultured in 6-well plates, were treated with dezocine at 0, 10, 20, 40 ug/ml or 10 nM FK866 for 48 h. Cells were then collected using a NAD^+/NADH extraction buffer. Intracellular NAD^+ content was determined with a NAD^+/NADH Assay kit with WST-8 (Beyotime Institute of Biotechnology, Beijing, China), according to the manufacturer's protocol. The resultant value was normalized to total cell number.

Knockdown of Nicotinamide Phosphoribosyltransferase

NAMPT-knockdown cells were generated through lentiviral-mediated delivery of NAMPT small hairpin (sh)RNA. The shRNA oligos were synthesized by GENEWIZ (South Plainfield, NJ, United States) and cloned into the pLKO.1 expression construct (using pLKO.1-scramble shRNA as control). The shRNA sequences used were provided in **Supplementary Table S3**. The resultant pLKO.1-shRNA plasmids were co-transfected into HEK293T cells with the pCMV-VSV-G packaging plasmids and envelope pCMV-delta-8.2 envelope plasmids for production of the shRNA lentivirus, as described below. After 48 h, the supernatant fractions from the cell cultures were collected and filtered through a 0.45 μm filter. Cells were then infected with the viral supernatant fractions and supplemented with polybrene. The culture medium was replaced with fresh growth medium 16 h post-infection, with 2 μg/ml puromycin for 48 h-selection. The cells were cultured in this medium until the control cells died. Knockdown efficiency was then evaluated by qPCR and western blot analysis. The shRNA sequences used are listed in **Supplementary Table S3**.

Transient Transfection

The MOR and KOR expression plasmids, OPRM1-Tango and OPRK1-Tango, were obtained from Addgene (Watertown, MA, United States). A total of 5 × 105 MDA-MB-231 or BT549 cells were inoculated in a 100-mm dish. When the cells reached 70% confluence, they were transfected with the target plasmids and Lipofectamine 3000™ (Life Technologies; Thermo Fisher Scientific, Inc.) and configured in Opti-MEM for 10 min at room temperature. The transfection mixture was incubated with TNBC cell lines for 48 h, and protein and gene expression were detected by western blot or qPCR, respectively.

Xenografts in Nude Mice

All animal research procedures were performed according to the protocols of the Animal Care and Use Ethics Committee of Shenzhen University Health Science Center and all animals were treated in strict accordance with protocols approved by the Institutional Animal Use Committee of the Health Science Center, Shenzhen University. Female NU/NU mice (Charles River. Beijing, China, ~4–6 weeks old; n = 10) were subcutaneously injected with $10X10^6$ MDA-MB-231 cells in 200 uL PBS supplied with 25% of Matrigel (Corning, NY, United States) on the right side of the back. 3 weeks after inoculation, these mice were then randomly allocated into control group (n = 5) and experimental groups (n = 5) for treatment. PBS (control group) or dezocine (30 mg/kg;

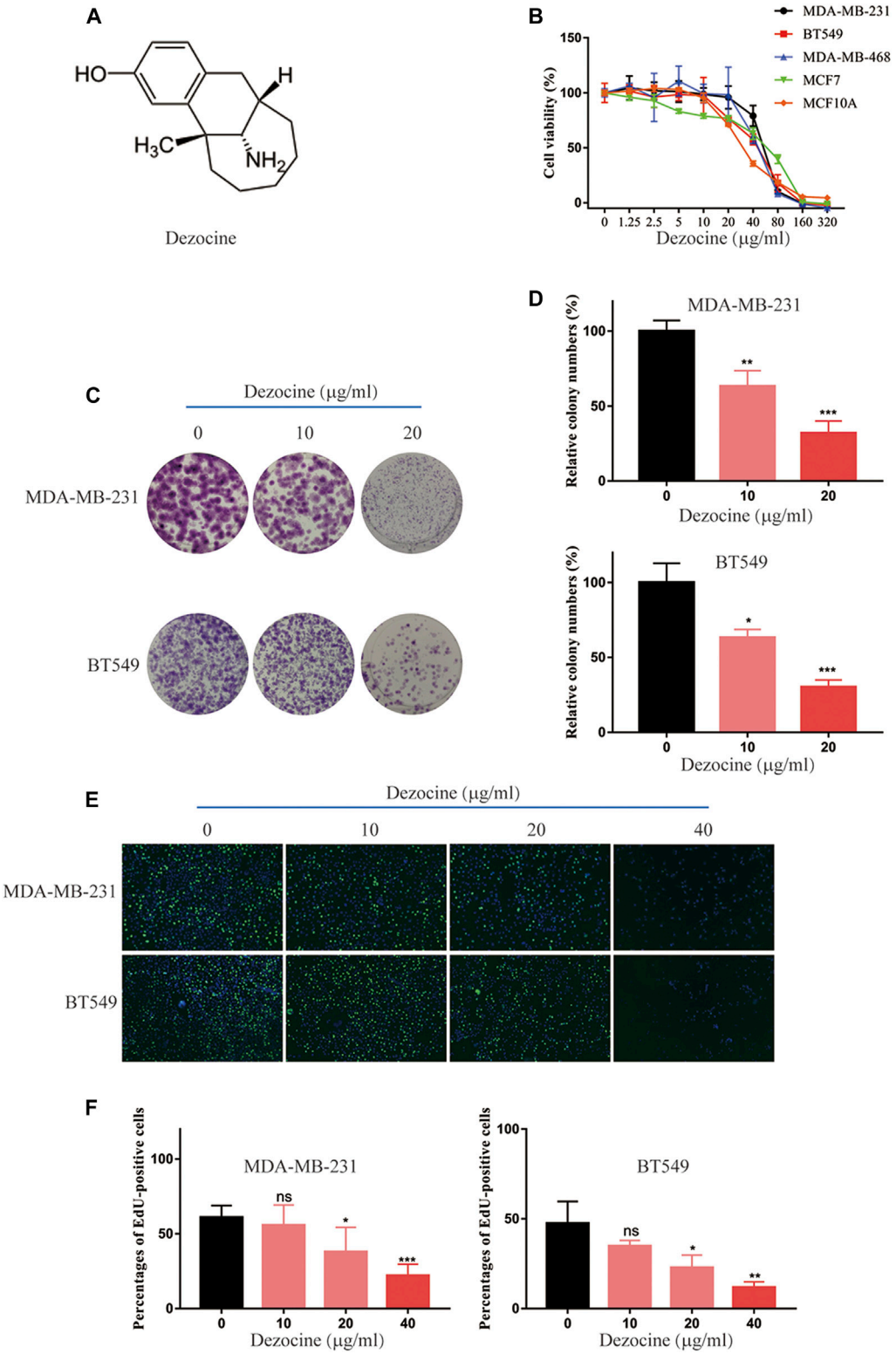

FIGURE 1 | Dezocine inhibits breast cancer cell viability, colony formation and DNA synthesis. **(A)** The molecular structure of dezocine. **(B)** MDA-MB-231, BT549, MDA-MB-468, and MCF7 cells were treated with the indicated concentrations of dezocine for 48 h, and cell viability assays were performed. **(C)** Representative images of colony formation assays performed with dezocine-treated MDA-MB-231 and BT549 cells, with **(D)** quantification. **(E)** DNA synthesis was measured in dezocine-treated MDA-MB-231 and BT549 cells using EdU incorporation assays, with **(F)** quantification. Percentages of EdU-positive cells out of total cells are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. negative control.

experimental group) was daily intraperitoneally injected for 4 weeks. The tumor growth was monitored by measurement of tumor diameter twice a week, and the tumor volume was calculated with the formula: volume = (length x width^2)/2. At the end of treatment, the mice were sacrificed and tumor xenografts were excised and weighted.

Statistical Analysis

All experiments were performed at least in triplicate. Data were analyzed using GraphPad Prism 7 statistical software (GraphPad Software, Inc., La Jolla, CA, United States). Data are presented as the mean ± standard deviation. Two-tailed, unpaired Student's t-tests were used to compare the difference between two groups with similar variance. For all tests, $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Dezocine Exerts Anti-Tumor Effects on Triple-Negative Breast Cancer Cancer Cells

Dezocine (**Figure 1A**) is an opioid analgesic used to relieve pain in cancer patients, but its direct influence on cancer cells remains to be determined. In the present study, we initially tested the effect of dezocine treatment on cell viability in a range of cell lines, which included TNBC, ER-positive breast cancer and mammary epithelial cell lines. The viability of all the cell lines was inhibited in a dose-dependent manner by treatment with dezocine for 48 h (**Figure 1B**). The IC50 values for all cell lines was in the 30–50 μg/ml range, suggesting that dezocine does not exhibit cell type specificity. Immortalized MCF10A mammary epithelial cells were also sensitive to dezocine (IC50 = 32.12 μg/ml; **Table 1**). Colony formation assays further confirmed the dose-dependent, dezocine-induced inhibition of MDA-MB-231 and BT549 TNBC cell proliferation, with fewer cells visible on the plates as the dose increased from 10 μg/ml to 20 μg/ml (**Figures 1C,D**). Furthermore, EdU assays were conducted to analyze the impact of dezocine treatment on DNA synthesis in MDA-MB-231 and BT549 cells. Dezocine significantly decreased DNA synthesis in both MDA-MB-231 and BT549 cells in a dose-dependent manner (**Figures 1E,F**); consistent with the results of cell viability and clonogenic assays.

Dezocine Induces Apoptosis in MDA-MB-231 and BT549 Cells

Flow cytometry revealed that dezocine treatment (10–40 μg/ml; 48 h) significantly increased the proportion of Annexin V-positive apoptotic cells, and this effect was dose-dependent (**Figures 2A,B**). Furthermore, the protein expression of apoptotic markers was upregulated after dezocine treatment (**Figures 2C,D**), which was consistent with the flow cytometry results.

Dezocine Inhibits Cell Migration and Invasion in MDA-MB-231 and BT549 Cells

The results of the wound healing assay revealed that both MDA-MB-231 and BT549 cell migration was suppressed by dezocine treatment for 24 or 48 h (**Figures 3A–D**). This effect was once again dose-dependent. Transwell migration assays confirmed this result, as well as its dose-dependent nature (**Figures 3E–G**). Similar results were also obtained from Transwell assays using Matrigel, suggesting that MDA-MB-231 and BT549 cell invasion was also inhibited by 10 or 20 ug/ml dezocine treatment (**Figures 3H–J**). Furthermore, protein expression of the mesenchymal markers N-cadherin, vimentin, TCF8 and beta-catenin was downregulated by dezocine treatment for 48 h (**Figures 3K,L**). Taken together, these results clearly demonstrate the inhibitory effect of dezocine on the migration and invasion of TNBC cell lines *in vitro*.

Dezocine Suppresses the Progression of Triple-Negative Breast Cancer in An Opioid Receptor Independent Manner

Dezocine functions as an analgesic via opioid receptors, but whether the mechanism underlying the dezocine-induced inhibition TNBC occurs via these receptors remains unknown. We first detected the mRNA expression of opioid receptors (MOR, KOR and DOR) with RT-qPCR in breast cancer cell lines and normal MCF10A breast epithelial cells, using the SH-SY5Y neuroblastoma cell line and U937 monocyte cell line as controls. The mRNA expression levels of opioid receptors were comparatively low in both normal breast and breast cancer cell lines, with MOR expression levels being particularly reduced (**Figure 4A**). Furthermore, we tested the effect of opioid receptor overexpression in TNBC cells, and found that MOR and KOR overexpression inhibited MDA-MB-231 and BT549 cell proliferation (**Figure 4B**). To further investigate whether dezocine works as an opioid receptor agonist here, MDA-MB-231 and BT549 cells were incubated with 40 μg/ml dezocine for 48 h after pretreatment for 15 min with 10 uM NAL/NTX, which are opioid receptor antagonists. However, neither NAL nor NTX rescued MDA-MB-231 and BT549 cell viability following dezocine treatment (**Figures 4C,D**). Considering dezocine also acts as an antagonist of opioid receptors, TNBC cells were treated with 40 μg/ml dezocine for 48 h after pretreatment for 15 min with 20 ug/ml morphine, which acts as an agonist of opioid receptors. The inhibitory effect of dezocine on cell viability was not reversed by morphine (**Figure 4E**), and these data together implied that dezocine exerts its anti-tumor effect on TNBC cells in an opioid receptor-independent manner.

Dezocine Inhibits Nicotinamide Phosphoribosyltransferase Enzyme Activity and Reduces Cellular NAD Content Through Targeting Nicotinamide Phosphoribosyltransferase

To reveal the direct target and molecular mechanisms underlying the effects of dezocine, a pull-down assay and HPLC-MS/MS were performed. The proteins pulled down from MDA-MB-231 lysate by dezocine-sepharose 4B beads were separated by SDS-

PAGE, and LC-MS/MS was performed to compare them with those pulled down by sepharose 4B beads (**Supplementary Figure 1A**). Among these candidate targets from LC-MS/MS (**Supplementary Table S3**), NAMPT was confirmed to be captured by dezocine-conjugated beads in both MDA-MB-231and BT549 cells through western blot analysis, suggesting that dezocine targeted NAMPT directly (**Figure 5A**). Furthermore, computer modeling analysis indicated that dezocine binds in the NAMPT pocket, and the free energy of dezocine kinetically bound into this pocket was −17.4 kcal/mol (**Figure 5B**). This data further confirmed that NAMPT is the direct target of dezocine.

Next, NAMPT mRNA expression was confirmed in breast cancer cell lines by RT-qPCR, and higher NAMPT expression levels were observed in MDA-MB-231 and BT549 cell lines (**Supplementary Figure 2A**). However, following dezocine treatment for 48 h, NAMPT protein expression did not change in MDA-MB-231 and BT549 cells (**Figure 5C**). This indicated that dezocine did not affect NAMPT protein stability.

NAMPT is a rate-limiting enzyme involved in the nicotinamide adenine dinucleotide salvage pathway. To determine the activity of the enzyme with dezocine binding, an NAMPT activity assay was performed and the NAMPT inhibitor FK866 was used as a control. Both dezocine and FK866 inhibited NAMPT enzyme activity, while NMN rescued NAMPT activity *in vitro* (**Figure 5D**). Furthermore, as demonstrated with an NAD/NADH assay, dezocine reduced NAD production in a dose-dependent manner in both MDA-MB-231and BT549 cells (**Figures 5E,F**). Together, these results suggested that dezocine bound directly to NAMPT and inhibited the activity of the enzyme.

FK866T is an NAMPT inhibitor that reduces cell proliferation by decreasing intracellular NAD levels. In TNBC cells, the inhibitory effects of FK866 were confirmed by cell viability assays, while dezocine showed a similar inhibitory effect (**Supplementary Figures 2B,C**). The suppression of cell growth was further confirmed by knockdown of NAMPT (knockdown efficiency was detected, **Supplementary Figure 2D**) in MDA-MB-231and BT549 cells (**Supplementary Figures 2E,F**).

Dezocine Inhibits Triple-Negative Breast Cancer Tumor Growth *In Vivo*

Having shown the effects of dezocine *in vitro*, we subsequently wanted to determine the inhibitory effects of dezocine *in vivo*. We subcutaneously injected MDA-MB-231 cells (10×10^6) into the right flanks of nude mice. 3 weeks after inoculation, the mice were exposed to PBS or dezocine (30 mg/kg) treatment for 4 weeks (**Figure 6A**). Xenografts taken from mice in the dezocine-treated group were significantly smaller than those taken from the PBS-treated group (**Figures 6B,C**). Indeed, we measured the tumor volume twice a week after injection of MDA-MB-231 cells, and found that it was significantly reduced in the dezocine-treated group compared to the PBS-treated group (**Figure 6D**). However, there was no significant difference in the body weights between the two groups of mice (**Supplementary Figure S3**), suggesting the dosage of dezocine we used was systemic safety.

TABLE 1 | IC50 of dezocine in TNBC, ERα$^+$ breast cancer and mammary epithelial cell lines.

Types	Cell line	IC50 (μg/ml)
ERα$^+$	MCF7	44.06
TNBC	MDA-MB-231	52
	MDA-MB-468	43.22
	BT549	40
Normal	MCF10A	32.12

TNBC, triple negative breast cancer; ER, estrogen receptor.

DISCUSSION

Pain remains a prevalent concern for patients with cancer, and has a significant impact on clinical outcome. Opioids are widely used as clinical analgesic agents for cancer patients. Dezocine is a synthetized opioid analgesic and is broadly prescribed for pain relieving in China, occupying over 45% of the domestic market of opioid analgesics. Thus, it is very important to understand the role of dezocine in cancer treatment. However, the effect of dezocine on cancer cells remains unknown. In the present study, we demonstrated that dezocine inhibited DNA synthesis, cell proliferation, cell migration and invasion in TNBC cell lines *in vitro*. Dezocine treatment also induced apoptosis in TNBC cells, confirmed by the upregulation of pro-apoptotic proteins, such as cleaved PARP and cleaved caspase 3. Importantly, our xenograft models demonstrated the inhibitory effects of dezocine treatment on TNBC tumor growth *in vivo*. These results clearly demonstrate the anticancer effects of dezocine in TNBC.

Dezocine is a MOR and KOR mixed agonist-antagonist, with a higher affinity for MOR (Liu et al., 2014; Wang et al., 2018). MOR overexpression has been observed in human NSCLC, prostate cancer and HCC, and is regarded as a molecular marker for poor prognosis. For example, MOR silencing and MOR antagonists treatment have previously been demonstrated to suppress carcinoma progression (Mathew et al., 2011; Zylla et al., 2013; Chen et al., 2019; Li et al., 2019). However, the results of our study indicated that opioid receptor expression in breast cancer cell lines and mammary epithelial cell lines is relatively low compared with SH-SY5Y and U937 cells. This suggested that the expression pattern of opioid receptors in breast cancer is completely different from that in lung cancer and HCC. When MOR and KOR were overexpressed in TNBC cells, cell proliferation was suppressed; but this is not consistent with the results seen in lung cancer and HCC. Furthermore, neither NAL/NTX, which acts as an antagonist, nor the agonist morphine, managed to suppress the inhibitory effects of dezocine. This indicated that dezocine acts in an opioid receptor independent manner, suggesting that opioid receptor function depends on cancer type and tissue specificity.

Our pull-down assay followed by LC-MS/MS found that dezocine targeted NAMPT directly; and computer modeling analysis confirmed that dezocine bound to NAMPT. NAMPT, also known as pre-B-cell colony-enhancing factor 1 (PBEF1) or visfatin, acts as a rate-limiting enzyme in the nicotinamide adenine dinucleotide (NAD^+) salvage pathway. It catalyzes the condensation of nicotinamide and 5-phosphoribosyl-1-pyrophosphate to nicotinamide mononucleotide during NAD^+ biosynthesis, and extracellular NAMPT exerts

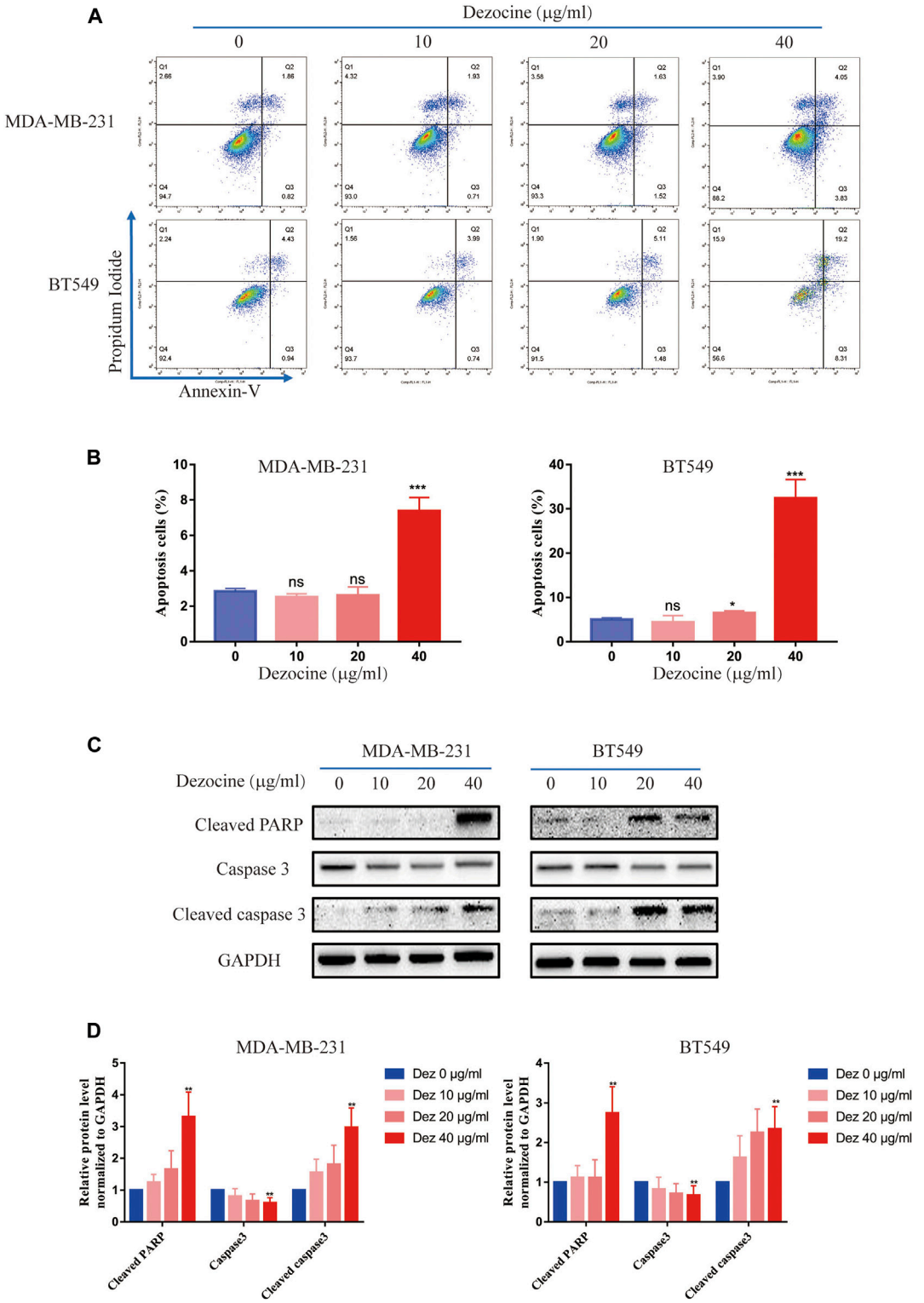

FIGURE 2 | Dezocine induces apoptosis in MDA-MB-231 and BT549 cells. **(A)** Apoptosis was measured with Annexin V and PI staining in MDA-MB-231 and BT549 cells treated with dezocine for 48 h, followed by flow cytometry. **(B)** Quantification of flow cytometry data. **(C)** Apoptosis-related protein expression was detected by western blot. **(D)** Quantification of western blot data. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. control.

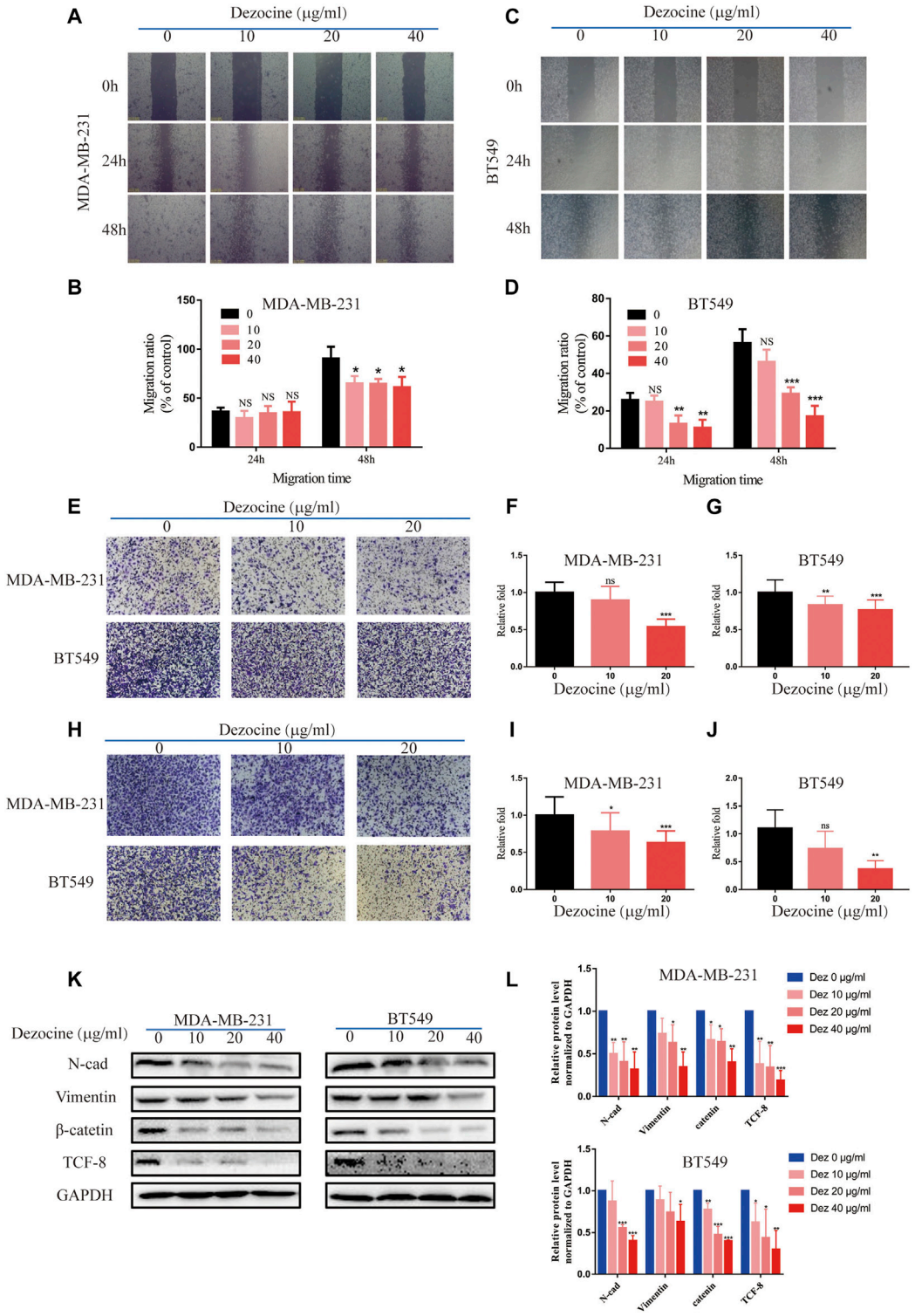

FIGURE 3 | Dezocine inhibits MDA-MB-231 and BT549 cell migration and invasion. **(A)** Wound healing assays revealed that dezocine inhibited MDA-MB-231 cell migration, with **(B)** quantification; similar results were found for **(C)** BT459 cells, with **(D)** quantification. **(E)** Transwell assays were performed to detect cell migration following dezocine treatment, with quantification of **(F)** MDA-MB-231 cells and **(G)** BT549 cells. **(H)** Transwell assays with Matrigel were performed to detect cell invasion following dezocine treatment, with quantification in **(I)** MDA-MB-231 and **(J)** BT549 cells. **(K)** MDA-MB-231 and BT549 cells were treated with dezocine for 48 h, and the expression levels of epithelial and mesenchymal markers were detected by western blot. **(L)** Quantification of western blot data. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

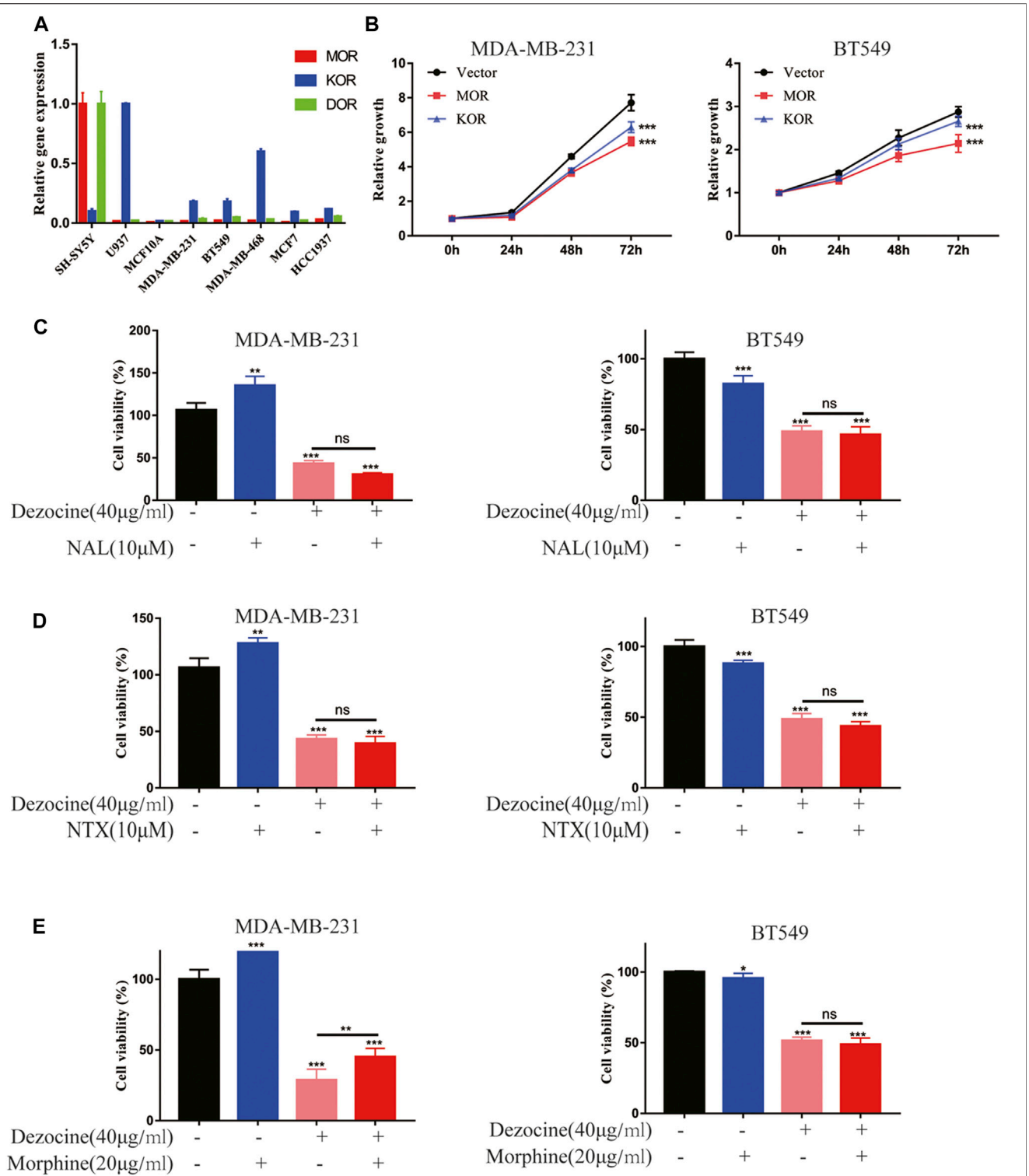

FIGURE 4 | Dezocine suppresses TNBC progression in an opioid receptor-independent manner. **(A)** mRNA expression levels of the opioid receptors MOR, KOR and DOR were determined in the indicated cell lines, including breast cancer cells and MCF10A breast epithelial cells, with SH-SY5Y and U937 as controls. **(B)** MDA-MB-231 and BT549 cells were transiently transfected with MOR or KOR plasmids, and cell proliferation was determined. MDA-MB-231 and BT549 cells were pre-treated with **(C)** NAL or **(D)** NTX or **(E)** morphine for 15 min prior to treatment with dezocine for 48 h, and cell viability was measured with a CCK8 assay. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

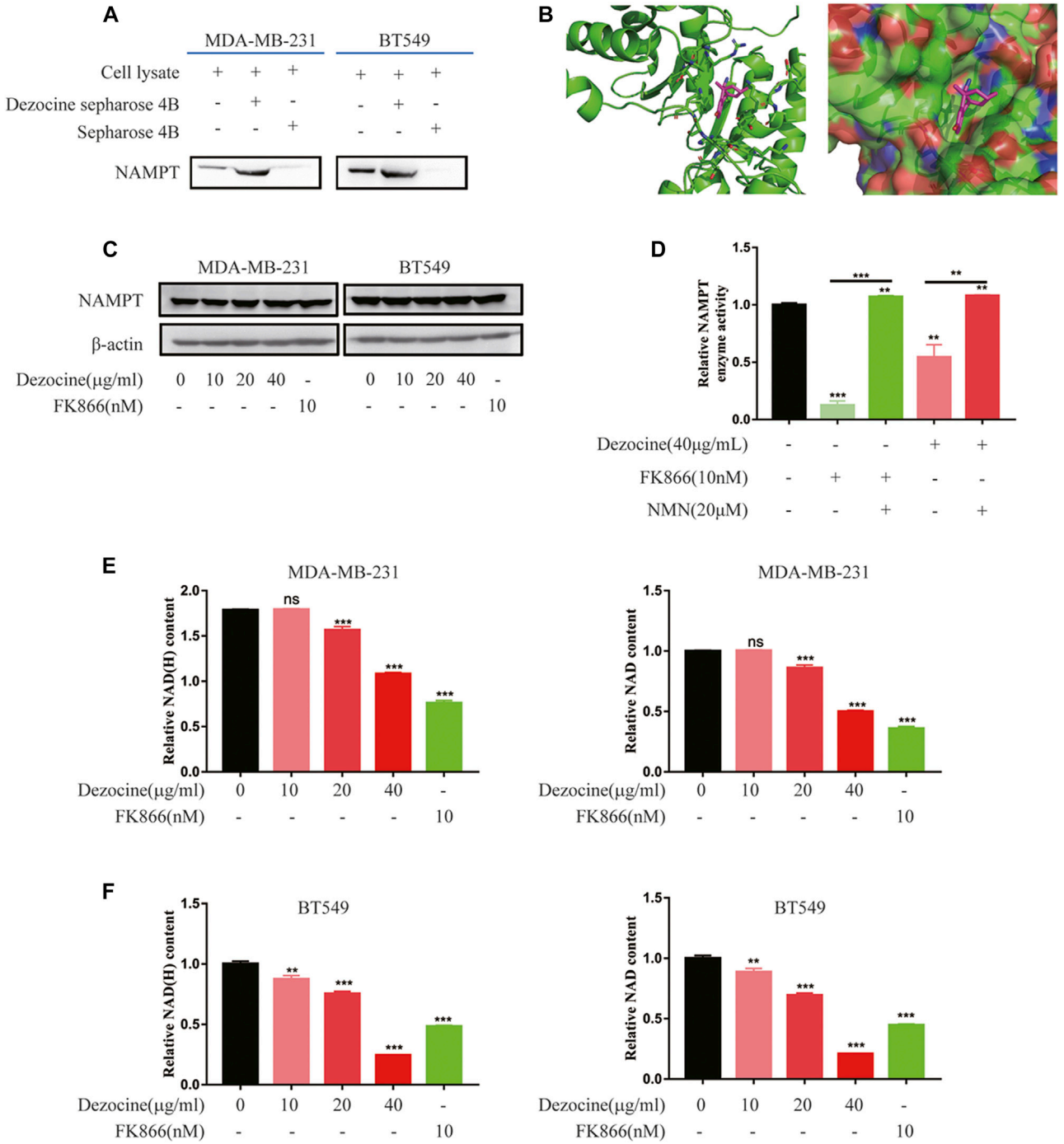

FIGURE 5 | Dezocine inhibits NAMPT enzyme activity, which reduces cellular NAD content. **(A)** A pull-down assay with dezocine-sepharose 4B beads was performed in MDA-MB-231 and BT549 cells, and NAMPT protein expression was detected with western blot analysis. **(B)** Computer modeling analysis revealed that dezocine binds to NAMPT. NAMPT is presented as a graphic, with the residues nearby to dezocine represented as sticks in the left. NAMPT is presented as the surface in the right. **(C)** MDA-MB-231 and BT549 cells were treated with dezocine for 48 h, and NAMPT expression levels were detected. FK866 was used as the control. **(D)** NAMPT enzyme activity was examined *in vitro*. NAD content was then detected in **(E)** MDA-MB-231 and **(F)** BT549 cells. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control, with additional comparisons indicated by lines.

additional cytokine-like activity (Garten et al., 2015). NAD is a co-enzyme that participates in a number of cell metabolic pathways, including glycolysis, with increased NAD levels enhancing glycolysis. This can provide cancer cells with energy and promote tumor progression. Furthermore, NAD is a substrate of NAD-dependent enzymes such as poly (ADP-

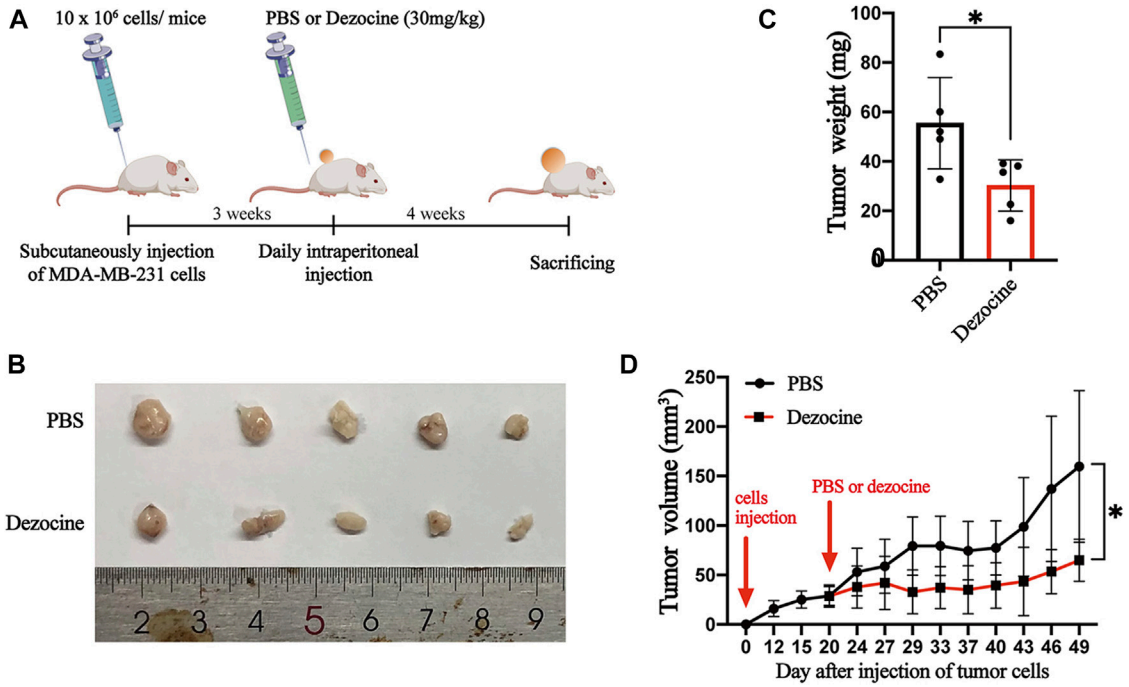

FIGURE 6 | Dezocine exhibits anti-tumor effects on xenografts *in vivo*. **(A)** MDA-MB-231 cells (10×10^6 cells) was subcutaneously injected into the flank of mice. 3 weeks after tumor cells inoculation, a total of 30 mg/kg dezocine in PBS solution was daily injected intraperitoneally for 4 weeks. **(B)** The photographs of tumors from mice treated with PBS or dezocine. **(C)** The mean of tumor weights was measured after PBS or dezocine treatment ($p = 0.03$). **(D)** The tumor volumes from mice were measured twice a week post-injection ($p = 0.02$). *$P < 0.05$ vs. control.

ribose)polymerase (PARP), sirtuins, and NAD glycohydrolase, which regulate DNA repair, gene expression and stress responses, all of which have implications for cancer (Yaku et al., 2018). Thus, through its control of NAD biosynthesis, NAMPT has a crucial role in cancer cell metabolism. NAMPT overexpression has been observed in multiple malignant tumors, including breast, ovarian, thyroid, gastric, prostate and colorectal cancers, gliomas, and malignant lymphomas (Tan et al., 2015). NAMPT promotes the proliferation and survival of rapidly dividing cancer cells by elevating NAD levels and enhancing glycolysis (Tan et al., 2013; Yamamoto et al., 2017). As a result, NAMPT is considered a promising novel therapeutic target for cancer treatment. NAMPT inhibitors such as FK866, GMX1777, and GMX1778, have been developed as a candidate novel therapeutic strategy for cancer (Yaku et al., 2018). In our study, NAMPT expression was verified in breast cancer cell lines and mammary epithelial cell lines, and the results were in accordance with those previously obtained in a range of solid tumors, including breast cancer (Shackelford et al., 2013). A study including 176 breast cancer patients also demonstrated that higher NAMPT levels are correlated with poorer survival, with high-grade tumors having significantly higher NAMPT/p73 mRNA ratios (Sharif et al., 2016). Furthermore, NAMPT has been found to induce breast cancer cell proliferation through the AKT/PI3K and ERK/MAPK signaling pathways, and to protect against apoptosis (Gholinejad et al., 2017). In the present study, MDA-MB-231and BT549 cell proliferation was suppressed by NAMPT knockdown, which are similar to those results found previously.

Furthermore, we found that dezocine treatment led to the inhibition of NAMPT activity and the reduction of cellular NAD, and this effect was dose-dependent. As a result, TNBC cell proliferation was suppressed. Similar results were observed following FK866 treatment in our study. FK866 was the first inhibitor of NAMPT to be developed, and is regarded as a candidate novel therapeutic drug through blocking NAMPT activity. FK866 induced apoptosis-mediated cell death in chronic lymphocytic leukemia cells and HepG2 liver carcinoma cells (Hasmann and Schemainda, 2003; Gehrke et al., 2014), inhibited the epithelial-mesenchymal transition in hepatocarcinoma MHCC97-H cells (Zhang et al., 2018). However, NAMPT has also been reported to induce the epithelial-to-mesenchymal transition independently of its enzymatic activity (Soncini et al., 2014). Furthermore, NAMPT knockdown has been found to increase the aggressiveness of human breast cancer metastasis through the regulation of integrins (Santidrian et al., 2014). The results of the present study suggested that dezocine suppresses cell migration and invasion in TNBC cells, however, the underlying molecular mechanisms require to be further elucidated.

In summary, the present study is the first to report the efficacy of dezocine against TNBC *in vitro* and *in vivo*. Furthermore, we demonstrated that dezocine binds directly to NAMPT, inhibiting its enzyme activity and downregulating NAD in TNBC cells. The lack of effective treatments for TNBC is a global health concern, and the development novel treatment strategies is urgently required. Drug repurposing has emerged as a novel strategy

for cancer therapy. Dezocine represents a potential candidate treatment for TNBC and perhaps other cancers, and, furthermore, NAMPT may represent a candidate therapeutic target in TNBC. Notably, previous study has shown that the combination of NAMPT inhibitor FK866 with olaparib inhibited TNBC growth *in vivo* than either single agent alone (Bajrami et al., 2012), supporting the potential use of dezocine alongside Olaparib or other therapeutic agents, to increase overall efficacy in TNBC. The further investigation is warranted in future studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: dezocine pull down LC-MSMS in MDA-MB-231 CELLS has been deposited in the ProteomeXchange repository, accession number: PXD022583.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Use Committee of the Health Science Center, Shenzhen University.

AUTHOR CONTRIBUTIONS

DZ and AY conceived and designed the experiments. CX, WC, CC, SL, YZ, TX, GS, and YZ performed the experiments and MS/MS analysis. KC performed the computer modeling analysis. CX, CC carried out statistical analysis. CX and DZ wrote/reviewed the paper. DZ, AY, and YZ oversaw the research project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.600296/full#supplementary-material.

REFERENCES

Aggarwal, S., Verma, S. S., Aggarwal, S., and Gupta, S. C. (2019). Drug repurposing for breast cancer therapy: old weapon for new battle. *Semin. Cancer Biol.* 68. 8-20. doi:10.1158/1538-7445.sabcs18-p2-14-14

Bajrami, I., Kigozi, A., Van Weverwijk, A., Brough, R., Frankum, J., Lord, C. J., et al. (2012). Synthetic lethality of PARP and NAMPT inhibition in triple-negative breast cancer cells. *EMBO Mol. Med.* 4, 1087–1096. doi:10.1002/emmm.201201250

Bimonte, S., Barbieri, A., Palma, G., and Arra, C. (2013). The role of morphine in animal models of human cancer: does morphine promote or inhibit the tumor growth?. *Biomed. Res. Int.* 2013, 258141. doi:10.1155/2013/258141

Bimonte, S., Barbieri, A., Rea, D., Palma, G., Luciano, A., Cuomo, A., et al. (2015). Morphine promotes tumor angiogenesis and increases breast cancer progression. *Biomed. Res. Int.* 2015, 161508. doi:10.1155/2015/161508

Boland, J. W., McWilliams, K., Ahmedzai, S. H., and Pockley, A. G. (2014). Effects of opioids on immunologic parameters that are relevant to anti-tumour immune potential in patients with cancer: a systematic literature review. *Br. J. Cancer* 111, 866–873. doi:10.1038/bjc.2014.384

Chen, D. T., Pan, J. H., Chen, Y. H., Xing, W., Yan, Y., Yuan, Y. F., et al. (2019). The mu-opioid receptor is a molecular marker for poor prognosis in hepatocellular carcinoma and represents a potential therapeutic target. *Br. J. Anaesth.* 122, e157–e167. doi:10.1016/j.bja.2018.09.030

Forget, P., Aguirre, J. A., Bencic, I., Borgeat, A., Cama, A., Condron, C., et al. (2019). How anesthetic, analgesic and other non-surgical techniques during cancer surgery might affect postoperative oncologic outcomes: a summary of current state of evidence. *Cancers (Basel)* 11, 592. doi:10.3390/cancers11050592

Garten, A., Schuster, S., Penke, M., Gorski, T., De Giorgis, T., and Kiess, W. (2015). Physiological and pathophysiological roles of NAMPT and NAD metabolism. *Nat. Rev. Endocrinol.* 11, 535–546. doi:10.1038/nrendo.2015.117

Gehrke, I., Bouchard, E. D. J., Beiggi, S., Poeppl, A. G., Johnston, J. B., Gibson, S. B., et al. (2014). On-target effect of FK866, a nicotinamide phosphoribosyl transferase inhibitor, by apoptosis-mediated death in chronic lymphocytic leukemia cells. *Clin. Cancer Res.* 20, 4861–4872. doi:10.1158/1078-0432.ccr-14-0624

Gholinejad, Z., Kheiripour, N., Nourbakhsh, M., Ilbeigi, D., Behroozfar, K., Hesari, Z., et al. (2017). Extracellular NAMPT/Visfatin induces proliferation through ERK1/2 and AKT and inhibits apoptosis in breast cancer cells. *Peptides* 92, 9–15. doi:10.1016/j.peptides.2017.04.007

Hasmann, M., and Schemainda, I. (2003). FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis. *Cancer Res.* 63, 7436–7442.

Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Klein, M. L. (1983). Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* 79, 926–935. doi:10.1063/1.445869

Khan, J. A., Tao, X., and Tong, L. (2006). Molecular basis for the inhibition of human NMPRTase, a novel target for anticancer agents. *Nat. Struct. Mol. Biol.* 13, 582–588. doi:10.1038/nsmb1105

Li, Y., Li, G., Tao, T., Kang, X., Liu, C., Zhang, X., et al. (2019). The μ-opioid receptor (MOR) promotes tumor initiation in hepatocellular carcinoma. *Cancer Lett.* 453, 1–9. doi:10.1016/j.canlet.2019.03.038

Liu, R., Huang, X.-P., Yeliseev, A., Xi, J., and Roth, B. L. (2014). Novel molecular targets of dezocine and their clinical implications. *Anesthesiology* 120, 714–723. doi:10.1097/aln.0000000000000076

Liu, Z., Cheng, S., Fu, G., Ji, F., Wang, C., and Cao, M. (2020). Postoperative administration of ketorolac averts morphine-induced angiogenesis and metastasis in triple-negative breast cancer. *Life Sci.* 251, 117604. doi:10.1016/j.lfs.2020.117604

Maier, J. A., Martinez, C., Kasavajhala, K., Wickstrom, L., Hauser, K. E., and Simmerling, C. (2015). ff14SB: improving the accuracy of protein side chain and backbone parameters from ff99SB. *J. Chem. Theor. Comput.* 11, 3696–3713. doi:10.1021/acs.jctc.5b00255

Mathew, B., Lennon, F. E., Siegler, J., Mirzapoiazova, T., Mambetsariev, N., Sammani, S., et al. (2011). The novel role of the mu opioid receptor in lung cancer progression. *Anesth. Analgesia* 112, 558–567. doi:10.1213/ane.0b013e31820568af

Santidrian, A. F., Leboeuf, S. E., Wold, E. D., Ritland, M., Forsyth, J. S., and Felding, B. H. (2014). Nicotinamide phosphoribosyltransferase can affect metastatic activity and cell adhesive functions by regulating integrins in breast cancer. *DNA Repair* 23, 79–87. doi:10.1016/j.dnarep.2014.08.006

Shackelford, R. E., Mayhall, K., Maxwell, N. M., Kandil, E., and Coppola, D. (2013). Nicotinamide phosphoribosyltransferase in malignancy: a review. *Genes & Cancer* 4, 447–456. doi:10.1177/1947601913507576

Sharif, T., Ahn, D.-G., Liu, R.-Z., Pringle, E., Martell, E., Dai, C., et al. (2016). The NAD+ salvage pathway modulates cancer cell viability via p73. *Cell Death Differ* 23, 669–680. doi:10.1038/cdd.2015.134

Siegel, R. L., Miller, K. D., and Jemal, A. (2019). *Cancer* statistics, 2019. *CA A. Cancer J. Clin.* 69, 7–34. doi:10.3322/caac.21551

Singleton, P. A., Moss, J., Karp, D. D., Atkins, J. T., and Janku, F. (2015). The mu opioid receptor: a new target for cancer therapy?. *Cancer* 121, 2681–2688. doi:10.1002/cncr.29460

Soncini, D., Caffa, I., Zoppoli, G., Cea, M., Cagnetta, A., Passalacqua, M., et al. (2014). Nicotinamide phosphoribosyltransferase promotes epithelial-to-mesenchymal transition as a soluble factor independent of its enzymatic activity. *J. Biol. Chem.* 289, 34189–34204. doi:10.1074/jbc.m114.594721

Szczepaniak, A., Fichna, J., and Zielinska, M. (2020). Opioids in cancer development, progression and metastasis: focus on colorectal cancer. *Curr. Treat. Options. Oncol.* 21, 6. doi:10.1007/s11864-019-0699-1

Tan, B., Dong, S., Shepard, R. L., Kays, L., Roth, K. D., Geeganage, S., et al. (2015). Inhibition of nicotinamide phosphoribosyltransferase (NAMPT), an enzyme essential for NAD+ biosynthesis, leads to altered carbohydrate metabolism in cancer cells. *J. Biol. Chem.* 290, 15812–15824. doi:10.1074/jbc.m114.632141

Tan, B., Young, D. A., Lu, Z.-H., Wang, T., Meier, T. I., Shepard, R. L., et al. (2013). Pharmacological inhibition of nicotinamide phosphoribosyltransferase (NAMPT), an enzyme essential for NAD+ biosynthesis, in human cancer cells. *J. Biol. Chem.* 288, 3500–3511. doi:10.1074/jbc.m112.394510

Tedore, T. (2015). Regional anaesthesia and analgesia: relationship to cancer recurrence and survival. *Br. J. Anaesth.* 115 (Suppl. 2), ii34–ii45. doi:10.1093/bja/aev375

Trott, O., and Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31, 455–461. doi:10.1002/jcc.21334

Waks, A. G., and Winer, E. P. (2019). Breast cancer treatment. *JAMA* 321, 288–300. doi:10.1001/jama.2018.19323

Wall, T., Sherwin, A., Ma, D., and Buggy, D. J. (2019). Influence of perioperative anaesthetic and analgesic interventions on oncological outcomes: a narrative review. *Br. J. Anaesth.* 123, 135–150. doi:10.1016/j.bja.2019.04.062

Wang, Y. H., Chai, J. R., Xu, X. J., Ye, R. F., Zan, G. Y., Liu, G. Y., et al. (2018). Pharmacological characterization of dezocine, a potent analgesic acting as a kappa partial agonist and mu partial agonist. *Sci. Rep.* 8, 14087. doi:10.1038/s41598-018-32568-y

Yaku, K., Okabe, K., Hikosaka, K., and Nakagawa, T. (2018). NAD metabolism in cancer therapeutics. *Front. Oncol.* 8, 622. doi:10.3389/fonc.2018.00622

Yamamoto, M., Inohara, H., and Nakagawa, T. (2017). Targeting metabolic pathways for head and neck cancers therapeutics. *Cancer Metastasis Rev.* 36, 503–514. doi:10.1007/s10555-017-9691-z

Zhang, B., Shi, D., Zhang, X., Liang, G., Liu, W., and Qiao, S. (2018). FK866 inhibits the epithelial-mesenchymal transition of hepatocarcinoma MHCC97-H cells. *Oncol. Lett.* 16, 7231–7238. doi:10.3892/ol.2018.9541

Zylla, D., Gourley, B. L., Vang, D., Jackson, S., Boatman, S., Lindgren, B., et al. (2013). Opioid requirement, opioid receptor expression, and clinical outcomes in patients with advanced prostate cancer. *Cancer* 119, 4103–4110. doi:10.1002/cncr.28345

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The handling Editor declared a shared affiliation, though no other collaboration, with one of the authors KC.

frontiers in Oncology

REVIEW
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Breast Cancer Resistance to Cyclin-Dependent Kinases 4/6 Inhibitors: Intricacy of the Molecular Mechanisms

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Breast cancer is a common malignant tumor in women, with a highest incidence and mortality among all of the female malignant tumors. Notably, targeted therapy has achieved impressive success in the treatment of breast cancer. As one class of the anti-tumor targeted therapeutics, Cyclin-Dependent Kinases 4/6CDK4/6inhibitors have shown good clinical activity in treating breast cancer. Nevertheless, despite the promising clinical outcomes, intrinsic or acquired resistance to CDK4/6 inhibitors has limited the benefits of this novel target therapy. In the present review, we provide an overview of the currently known molecular mechanisms of resistance to CDK4/6 inhibitors, and discuss the potential strategies to overcoming drug resistance improving the outcomes for breast cancer patients treated with CDK4/6 inhibitors.

Keywords: breast cancer, CDK4/6 inhibitors, drug resistance, molecular mechanisms, combination administration

INTRODUCTION

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that play key roles in regulating cell cycle (1). CDK 4 and 6, two critical kinases among CDKs mediate the cellular transition from G0/G1 phase to S phase during cell cycle: dysregulation of CDK 4/6, result in uncontrolled cell division. The main effect of CDK4/6 inhibitor is to bind with cyclin D specifically, block cell cycle transformation, and stop cell cycle in G1 phase, thereby inhibiting tumor cell proliferation (2). Importantly, CDK4/6 inhibitors have showed great efficacy in treatment of breast cancer. Based on the PALOMA-1trail, FDA approved palbociclib, the first CDK 4/6 inhibitor, in combination with letrozole as first-line treatment for patients with ER-positive, HER2-negative advanced breast cancer (ABC) or metastatic breast cancer (MBC) (3). At present, three selective CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) have been approved by FDA (4, 5). These three CDK4/6 inhibitors are used in combination with endocrine therapies or fulvestrant for patients with ER+ Her− metastatic breast cancer. Clinical trials PALOMA-2, MONALEESA-2, and MONARCH-3 have showed that when combined with aromatase inhibitors, CDK4/6 inhibitors could significantly prolong the progression-free survival in postmenopausal women with HR-positive metastatic breast cancer (6–8). Nevertheless, despite promising clinical outcomes, acquired or intrinsic resistance to CDK4/6 inhibitors often occurs, and this constitutes a major hindrance to successful treatment and limits the

therapeutic benefits of those targeted therapeutics for patients with this disease. Therefore, understanding the molecular mechanisms and pathways involved in resistance to CDK4/6 inhibitors may help develop effective strategies to circumventing drug resistance and selecting patient populations who can benefit from this targeted therapy. Here, we review and discuss the known molecular mechanisms and pathways that modulate the cellular sensitivity or resistance to CDK4/6 inhibitors, and provide our outlook on this subject (6–8).

POTENTIAL RESISTANCE MECHANISMS

Breast cancer cells can be intrinsically resistant to CDK4/6 or develop acquired resistance to those agents. CDK4/6 can phosphorylate retinoblastoma protein (Rb1), and the phosphorylation leads to Rb1 functional inactivation, then Rb1 uncoupling from E2Fs transcription factors and release E2Fs. CDK4/6 inhibitors exert their effects through breaking the CDK4/6-Rb-E2F pathway (9, 10). The tumor cells with loss of Rb1 and lack of the major targets, intrinsic resistance to CDK4/6 inhibitors may occur (11, 12). The major obstacle to successful treatment with CDK4/6 inhibitors is the acquired resistance that frequently occurs in the patients who have received this therapy. Tumor cells can acquire the ability to escape CDK4/6 action (13). Understanding potential mechanisms of acquired resistance to CDK4/6 inhibitors may help find effective ways to preventing or overcoming drug resistance to this class of therapeutics (**Figure 1**).

DIRECT CELL CYCLE MECHANISMS

Loss of Drug Target Genes

RB1

The tumor suppressor Rb1 is a key checkpoint in the cell cycle and a major target of CDK4/6 inhibitors. In both of preclinical and clinical settings, Rb1 mutations were found (14). In the tumor cell line with acquired resistance to palbociclib, it was

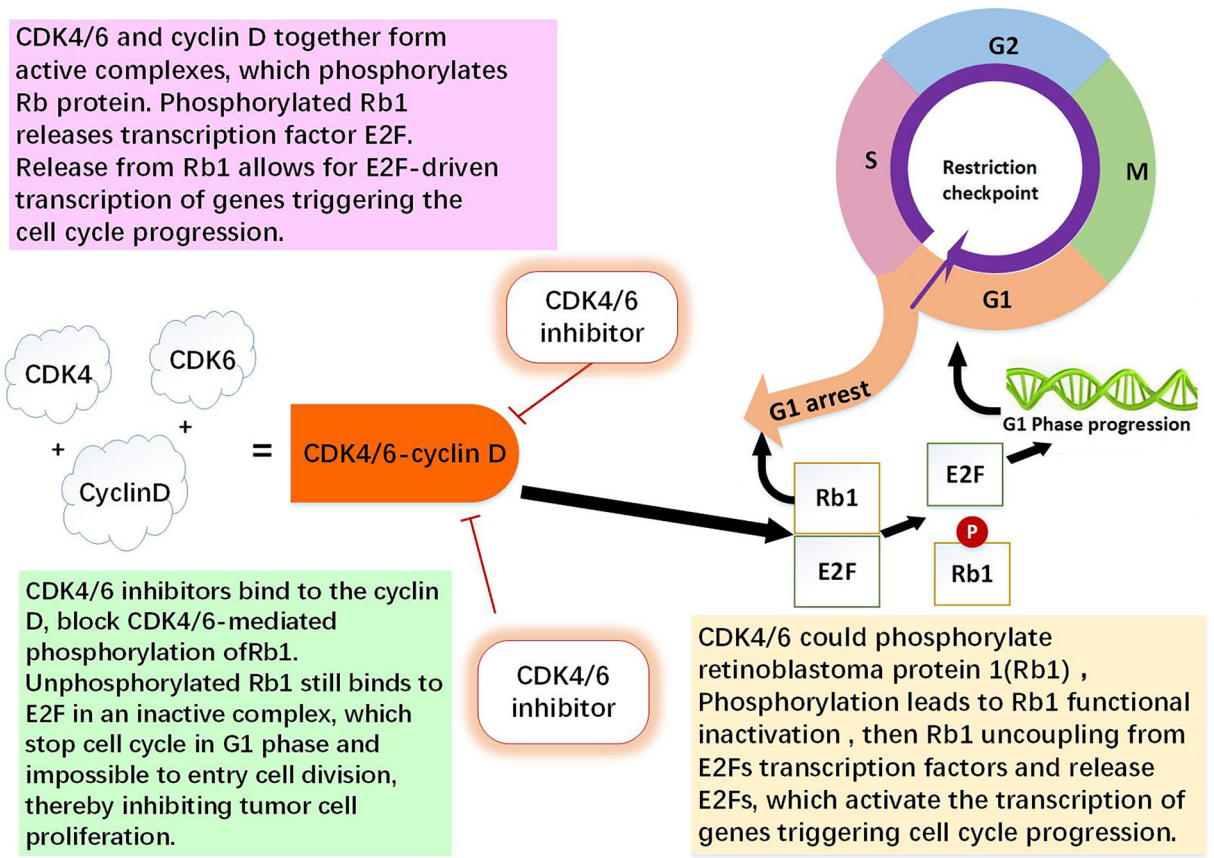

FIGURE 1 | The role of CDK4/6-cyclin D and CDK4/6 inhibitor. CDK4 and CDK6 play key roles in cell proliferation. Cyclin D is regulator of the CDK4 and CDK6 kinases. CDK4/6 and cyclin D together form active complexes, which phosphorylates Rb1 protein. Rb1 is an onco-suppressor which repress the transcription of genes required for the cell cycle, limit the expression of transcription factor E2F target genes which are involved in cell cycle progression. Phosphorylated Rb1 releases E2F. Release from Rb1 allows for E2F-driven genes triggering the cell cycle progression. CDK4/6 inhibitors bind to the cyclin D specifically, thereby block CDK4/6-mediated phosphorylation of Rb1. Non-phosphorylated Rb1 still binds to E2F in an inactive complex, which leading to cell cycle arrest in G0/G1 phase and impossible to entry cell division, thereby inhibiting tumor cell proliferation (1, 9, 10, 13).

demonstrated that resistance to CDK4/6 inhibitors was mediated through Rb1 loss, and restoration of Rb1 expression rendered tumor cells sensitivity to the CDK4/6 inhibitor (15). Chronic loss of Rb1 was found to be a cause of resistance to CDK4/6 inhibitors in breast cancer (16, 17). Using the breast cancer cell lines sensitive or resistant to palbociclib, it was showed that the complex change of Rb1 pathway was related to resistance to CDK4/6 inhibitor, Rb1 deficient in function is an important factor that contributes to palbociclib and abemaciclib resistance in breast cancer patients (18, 19). In clinical settings, researchers sequenced the somatic genomic mutations of three HR+ breast cancer samples before and after drug resistance to CDK4/6 inhibitors occurred and found that Rb1 mutation, allele substitution or exon deletion only existed in the blood samples after but not before drug resistance (20). Many researches showed that Rb1 loss could activate bypass of cyclin D1-CDK4/6-dependent pathway, leading to acquired resistance to CDK4/6 inhibition (14). These observations suggest that despite loss of Rb1, progression of the cell cycle continues *via* the activation of other cell cycle machinery, and inhibition of the bypass axis in combination with the CDK4/6 inhibitors may be effective in overcoming resistance to these targeted therapies. However, in the PALOMA-3 randomized phase III trial, the circulating tumor DNA sequencing from patients showed that Rb1 mutations occurred only in 6 of 127 (4.7%) patients (21). Thus, further clinical evidence is needed to analyze the frequency of Rb1 mutation in breast cancer patients receiving CDK 4/6 treatment (**Figure 2**, **Table 1**).

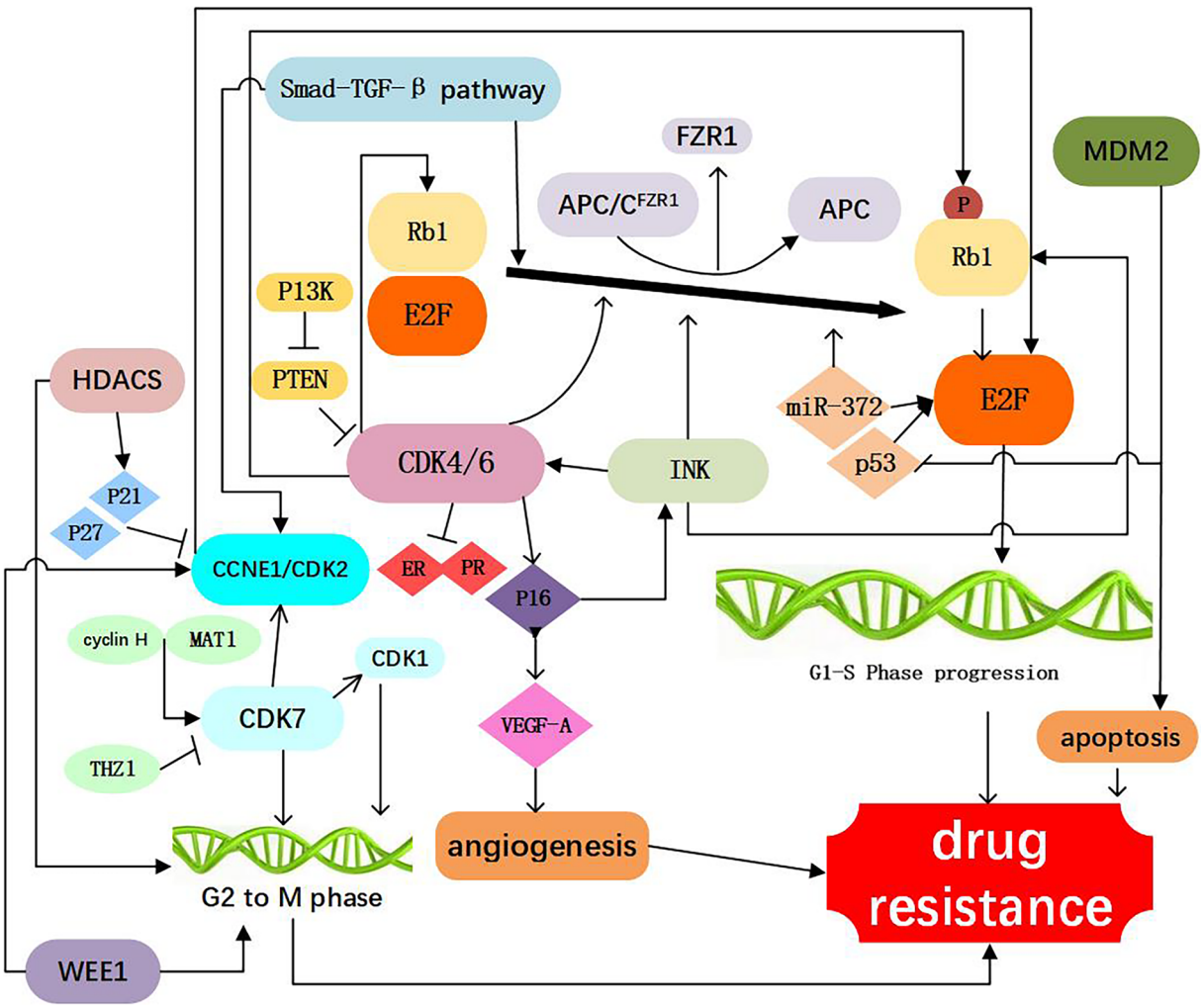

FIGURE 2 | Resistance to CDK4/6 inhibitors: Direct Cell Cycle Mechanism: 1. Loss of drug target genes: APC/CFZR1 promote the phosphorylation of Rb1 and regulate cell transition from G1 to S. knockdown of Rb1 and FZR1 synergistically bypassed cell division arrest induced by the CDK4/6 inhibitor (14–23); 2. Increased activity of the CDK4 and CDK6: amplification of CDK4/6 account for a decreased CDK4/6 targeted phosphorylation of Rb1 and a decreased sensitivity of breast cancer cells to CDK4/6 inhibitor (2, 24–34); 3. Abnormal regulations of upstream and Downstream kinases: CCNE1/CDK2, CDK7, E2F, INK, PTEN, Smad-TGF-β pathway which are involved in the progression of cell cycle, as shown in **Figure 1**, are responsible for resistance to CDK4/6 inhibitors (12, 15, 17, 26, 35–60); 4. Activation of alternate genes like HDACS, WEE1, MDM2, partly help the cancer cell escape from the drugs work (61–67).

TABLE 1 | Mechanisms of acquired resistance to CDK4/6 inhibitors: Direct cell cycle mechanisms.

Resistance classify	Resistance mechanism	Detection	Overcome
Loss of drug target genes (14–23)	Loss of Rb1	1. Cell biology experiments 2. Proteomics 3. Clinical trial	1. Restore Rb1 expression 2. Bypass way
	Loss of APC/C^{FZR1}	1. Cell biology experiments	1. Restore FZR1 expression
Increased activity of the target genes (2, 24–34)	CDK4 amplification	1. Cell biology experiments 2. Proteomics 3. Immunohistochemistry 4. Clinical trial	1. Knockdown of CDK4 2. Bypass way
	CDK6 amplification	1. Cell biology experiments 2. Proteomics 3. Immunohistochemistry 4. Clinical trial	1. Knockdown of CDK6 2. Bypass way
Abnormal regulations of upstream and downstream kinases (12, 15, 17, 26, 35–60)	Increased expression of CCNE1/CDK2	1. Cell biology experiments 2. Proteomics 3. Immunohistochemistry 4. Chip-seq analysis	1.CDK2 inhibitor 2. Bypass way
	CDK7 overexpression	1. Cell biology experiments 2. Proteomics 3. Immunohistochemistry	CDK7 inhibitor
	E2F overexpression	1. Cell biology experiments 2. Proteomics 3. Biopsies mRNA gene expression	1. E2F inhibitor 2. Inhibition regulate gene or protein downstream of E2F
	p16INK4A (p16) overexpression	1. Cell biology experiments 2. Proteomics	1. Restore p16 expression 2. p16 methylation
	Loss of PTEN	1. Cell biology experiments 2. Proteomics 3. Biopsy	1. Restore PTEN expression
	Smad-TGF-β pathway dysregulation	1. Cell biology experiments 2. Proteomics	1. Activate smad3 2. TGF-β inhibitor 3. Inhibition of EMT
Activation of alternate genes (61–67)	WEE1 overexpression	1. Cell biology experiments 2. Proteomics	WEE1 inhibitor
	MDM2 overexpression	1. Cell biology experiments 2. Proteomics	MDM2 inhibitors

CDK, Cyclin-dependent kinases; Rb1, Retinoblastoma protein1; APC/C, anaphase promoting complex/cyclosome; PTEN, Phosphatase and tensin homolog; TGF-β, transforming growth factor β; WEE1, serine/threonine kinases gene; MDM2, Mouse double minute 2 homolog.

APC/C^{FZR1}

Similar to Rb1, the ubiquitin ligase anaphase promoting complex/cyclosome (APC/C) play an important role in cell cycle regulation. APC/C and pRb interact *via* the co-activator of APC/C^{FZR1}, providing an alternative pathway to regulate transition from G1 to S by pRb through a post-translational mechanism (22). FZR1 is a candidate CDK4/6-cyclin D substrate and as an important determinant in response to CDK4/6 inhibitors. It was found that the loss of FZR1 resulted in uncontrolled cell cycle progression from G1 to S phase. In human breast cancer cell lines, simultaneous knockdown of Rb and FZR1 synergistically bypassed cell division arrest induced by the CDK4/6 inhibitor PD-0332991 (23). The precise mechanism of resistance to CDK4/6 inhibitors associated with the loss of FZR1 remains unclear. It is likely that loss of FZR1 corresponds with the loss of Rb; however, this possibility remains to be further investigated (**Figure 2**, **Table 1**).

Increased Activity of the Target Genes

CDK4

CDK4 is an important component of the cyclind-CDK4/6-Rb1 pathway, and was observed in 25% luminal B and 14% Luminal A breast cancers (24). In addition, aberrant expression of CDK4 activates the cyclind-CDK4/6-Rb1 pathway and results in drug resistance (25). It has been demonstrated that CDK4 was elevated in palbociclib resistant cell lines (26). Also, amplification of CDK4 has been reported in melanoma, glioma, rhabdomyosarcoma, and lung cancer and confers resistance to CDK4/6 inhibitors in these malignancies (27–30). The researchers found that increasing phosphorylation of p27 could inhibit CDK4 and regulate the cyclin D/CDK4/p27 complex activity, which could make breast cancer cells more resistant to palbociclib (2, 31), above study suggesting a potential strategy to prevent adaptation to CDK4/6 inhibitors (**Figure 2**, **Table 1**).

CDK6

The functions of CDK6 are both kinase-dependent and non-kinase-dependent (32). After a prolonged exposure to CDK4/6 inhibitor LY2835219, a significant amplification of CDK6 was found in several breast cancer cell lines, and this may account for a decreased CDK4/6 targeted phosphorylation of Rb1 and a decreased sensitivity of breast cancer cells to CDK4/6 inhibitor (32). Further experiments confirmed that forced overexpression of CDK6 indeed mediated drug resistance. Overexpression of CDK6 not only mediates resistance to CDK4/6 inhibitors, but also leads to decreased expression of estrogen and progesterone

receptors. These studies also suggest that the efficacy of CDK4/6 inhibitors in breast cancer cells is modulated by ER. Therefore, CDK6 amplification can decrease the tumor cell sensitivity to both ER antagonists and CDK4/6 inhibitors. Knockdown of CDK6 can restore sensitivity, while enforced overexpression of CDK6 can confer resistance to CDK4/6 inhibitors

A decrease in ER/PR expression was observed in the tumor specimens from patients receiving treatment of CDK4/6 inhibitor and showing insensitivity to CDK4/6 inhibitors (33). The non-kinase dependent function of CD6 lies in its transcriptional regulation function. In the STAT3 and Cyclin D pathways, CDK6 could up-regulate the transcription of P16 and the expression of VEGF-A that can promote angiogenesis, contributing to the progression and drug resistance of breast cancer (32, 34) (**Figure 2**, **Table 1**).

Abnormal Regulations of Upstream and Downstream Kinases

CCNE1/CDK2

The cyclin E (encoded by CCNE1 gene)-CDK2 complexes play a key role in the cell cycle from G1 to S phase. Cyclin E-CDK2 can phosphorylate Rb1, release E2F, and promote entry into the S phase (35, 36). In an analysis of global gene expressions, increased expression of CDK2 was found in the palbociclib-resistant breast cancer cell lines. Also it was suggested that loss of p21 and p27, which has an inhibitory effect function on CDK2, may represent a mechanism leading to bypass of palbociclib (17). It has been reported that when combined CDK2 and CDK4 inhibitors, resistance to palbociclib was no longer obvious, suggesting that cyclin E-CDK2 complexes protein might mediate resistance to CDK4/6 inhibitors (37). Hopefully, next generation CDK inhibitors can target CDK2 to prevent or conquer drug resistance (**Figure 2**, **Table 1**).

CDK7

CDK7, one of the major cell cycle regulators, acts as a CDK-activating kinase (CAK) by maintaining CDK1 and CDK2 activity. CDK7 promotes the cell transition from G2 phase to M phase (38). It has been demonstrated that CDK7 overexpression occurred in the estrogen receptor-positive, palbociclib-resistant breast cancer cells (26), suggesting that CDK7 is involved in cellular resistance to CDK4/6 inhibitors. The CDK7 selective inhibitor, THZ1, can significantly inhibit the proliferation of triple negative breast cancer cells at the nmol/L concentration (39, 40). Also, the sensitivity of breast cancer cells to CDK7 inhibitors appears to be associated with the loss of ER and Rb1 CN expression (26). Thus, CDK7 inhibitors may play an important role in both of the targeted therapy and cellular resistance to CDK4/6 inhibitors (**Figure 2**, **Table 1**).

E2F

The CDK-Rb-E2F pathway plays a critical role in the control of cell cycle in breast cancer. At the early stage of G1, E2F binds to Rb1 protein and forms a functional complex. Phosphorylation of Rb1 protein by CDK activates E2F. Activation of E2F can promote the transition of cells from G1 phase to S phase. It has been reported that in the CDK4/6 inhibitor-resistant cell lines, the CDK-Rb-E2F pathway reactivate (41). Researchers found that in tumor biopsies resistant to palbociclib, CCND3, CCNE1, and CDKN2D are persistently elevated before palbociclib used, all three genes are known E2F1 transcription targets, suggesting persistent E2F activity in resistant tumors (42). It was also revealed that E2F1 was up-regulated in patients with tumor lymph node metastasis and advanced stage (43) and patients with increased E2F expression was associated with lower overall survival (OS), relapse-free survival (RFS), distant metastasis-free survival (DMFS) (44). Therefore, E2F might be exploited as a therapeutic target both for suppressing drug resistance to CDK4/6 inhibitors and biomarkers and therapeutic targets for breast cancer in breast cancer.

INK

CDK4/6 activity is regulated by the INK4 family proteins (p16INK4A, p15INK4B, p18INK4C, and p19INK4D), can inhibit the expression of CDK4 and lead to cell cycle arrest in the G1/S phase, thus considered as a natural tumor inhibitor (45). The P16 (p16INK4A) protein, encoded by the $CDKN2A^{ink4a}$ gene, play an important role of the INK4 family. It has been reported that CDK4/6 inhibitors can inhibit cancer cell cycle progression because of P16 gene deletion (46). Cancer cells with P16 methylation are more sensitive to palbociclib than those control (47, 48). It has been found that overexpression of p16 and loss of Rb1 often occur simultaneously. When p16 overexpression is accompanied by Rb1 deficiency, CDK4/6 inhibitors are inactive due to the Rb1 deficiency. With the presence of Rb1, overexpression of p16 (be consistent) leads to a decrease of CDK4 and resistance to CDK4/6 inhibitors (12). Further studies are needed to delineate the precise mechanistic association between Rb1 loss and P16 overexpression, which may help design novel therapeutic strategies to overcoming the acquired resistance to CDK4/6 inhibitors (**Figure 2**, **Table 1**).

PTEN

PTEN a tumor suppressor gene, is one of the frequently mutated genes in human cancers (49). The increased expression of PTEN leads to the inactivation of CDK, which enables the Rb1 keep dephosphorylating, while binding to transcription factor E2F, which ultimately inhibits cell proliferation. these ways may influence the effect of CDK4/6 inhibitors (49). Researchers analyzed serial biopsies from breast cancer patients treated with the combination of ribociclib and letrozole and found that ablation of PTEN was sufficient to promote resistance to CDK4/6 inhibition (50). The increased AKT expression could reduce PTEN expression and render breast cancer cells resistant to CDK4/6 inhibitors (51). In breast cancer cells, loss of PTEN also conferred resistance to alpelisib. Moreover, loss of PTEN expression can cause dual resistance to CDK4/6 inhibitors and PI3K inhibitors (52) (**Figure 2**, **Table 1**).

Smad-TGF-β Pathway

Smad–transforming growth factor β (TGF-β) pathway contributes to G1 arrest in breast cancer cells (53). TGF-β

signaling is transduced through Smad2 and Smad3 and forms a complex with Smad4 to regulate target gene expression relevant to cell growth and differentiation (54, 55). Smad3, which has antiproliferative effects, has a key role in TGF-β signaling cascade. Smad3 can regulate cell cycle arrest, and has been shown to be correlated with resistance to CDK4/6 inhibitors (53). Mechanistically, cyclin E-CDK2 and cyclin D1-CDK4/6 complexes can suppress Smad3 through its phosphorylation, and the suppression of Smad3 releases the Rb1-E2F blockade and restore cell cycle arrest in breast cancer cells (53, 56). TGF-β can phosphorylate and activate Smad2 and Smad3 and form a complex with Smad4, and this contributes to the induction and progression of EMT. EMT can promote invasion and metastasis of cancer cells and increase drug resistance (57). Consistently, inhibition of the CDK2-mediated phosphorylation of Smad3 reduces TNBC cell migration and invasion through changes in EMT-related signaling factors (58). According to these findings, resistance of tumor cells to CDK4/6 inhibitors may result from suppression of Smad3 that is associated with the activated cyclin E-CDK2 axis and EMT (15, 36, 59, 60). Thus, the Smad-TGF-β pathway might be considered as a potential therapeutic target for overcome drug resistance to CDK4/6 inhibitors (**Figure 2**, **Table 1**).

Activation of Alternate Genes Are Involved in the Progression of Cell Cycle

WEE1

WEE1 is a protein tyrosine kinase that phosphorylates CDK1 and CDK2 and causes their inhibition (61). WEE1 inhibits CDK1 to maintain the cell in an inactive state and prevent mitosis. WEE1 also inhibits CDK2 to delay the replication process and allow time for DNA repair. Both of these events occur in breast cancer cells (61, 62). Inhibiting the expression of WEE1 can sensitize the drug resistant cancer cells to CDK4/6 inhibitors, probably because that inhibiting WEE1 can increase the expression of CD4 (63). In the ribociclib-resistant cancer cells, a down-regulation of the G2/M checkpoint was observed (64). Drug resistant cancer cells exhibited collateral sensitivity to the Wee-1 inhibitor, adavosertib (AZD1775). Combined treatment with ribociclib and adavosertib can elicit significantly stronger antiproliferative effect on drug resistant tumor cells cells than ribociclib alone (64) (**Figure 2**, **Table 1**).

MDM2

Mouse double minute 2 homolog (MDM2) is a negative regulatory protein of tumor suppressor p53 and can inhibit cellular senescence. MDM2 binds to p53 protein and inhibits the function of this tumor suppressor (65). Overexpression of MDM2 drives breast oncogenesis and blocks apoptosis of breast cancer cells, resulting in resistance of tumor cells to CDK4/6 inhibitors. Therefore, the use of MDM2 inhibitors may reverse cellular resistance to CDK4/6 inhibitors, and this has been in human liposarcoma (66). Indeed, the MDM2 inhibitor, CGM097, in combination with a CDK4/6 inhibitor palbociclib and fulvestrant has shown promising therapeutic benefits in reversing the tumor resistance to CDK4/6 inhibitors and to endocrine therapy (67) (**Figure 2**, **Table 1**).

INDIRECT CELL CYCLE MECHANISMS

Bypass Pathways of the Cell Cycle

mTOR Pathway

Abnormal activation of mammalian target of rapamycin (mTOR) pathway is an important target for development of anti-cancer drug, the most common mechanism of mTOR activation in breast cancer is *via* phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling, PI3K/AKT/mTOR pathway is closely associated with cellular resistance to CDK4/6 inhibitors (15, 68–70). It was reported that mTOR signaling is dysregulated in breast cancer patients following abemaciclib treatment (70) and PI3K/mTOR pathway has been shown to be upregulated in response to chronic exposure to CDK4/6 inhibitors (71). Also, pre-treatment with mTOR inhibitors was shown to prevent or delay the resistance to CDK4/6 inhibitors (72). In a kinome-wide siRNA screen, it was found that the AKT pathway is highly activated in the ribociclib resistant breast cancer cells (73). Combination of PI3K and CDK 4/6 inhibitors could reduce cell viability and overcome intrinsic and adaptive resistance leading to tumor regressions (74). Further studies demonstrated that ribociclib in combination with an AKT inhibitor or PI3K inhibitor has a significantly stronger inhibitory effect on the growth of transplanted tumor in mouse models, as compared with ribociclib alone, supporting the role of PI3K signaling pathway in mediating resistance to the CDK4/6 inhibitor (73). Thus, coinhibition of the PI3K/mTOR and CDK4/6 pathways may prevent induction of drug resistance. Furthermore, it has been showed in a preclinical model that a PI3K inhibitor combined with a CDK4/6 inhibitor has a significant stronger inhibitory effect on proliferation of breast cancer cells than the single drug (41). Taken together, the PI3K/AKT/mTOR inhibitors may represent a class of sensitizers in CDK4/6-targeted therapy (**Figure 3**, **Table 2**).

AP-1

High expression of AP-1 can lead to resistance to CDK4/6 inhibitors. AP-1 family consists of C-FOS, C-Jun, ATF, and MAF, and is involved in the regulation of a variety of genes, including cyclinD (75). The high expression of C-Jun is common in breast cancer and affects the expression of ER (76). It was found in breast cancer cells that are resistant to palbociclib which the transcriptions of AP-1 and C-FOS were increased, and AP-1 blockade in combination with palbociclib could effectively inhibit cell proliferation and reduce pRb and CDK2 levels as compared to single agent treatment (77). These observations suggest that co-treatment with Ap-1 specific inhibitors and CDK4/6 inhibitors may elicit anti-tumor synergistic effects. AP-1 and c-FOS inhibitors have entered Phase II clinical trial (T-5224) (78) (**Figure 3**, **Table 2**).

FGFR

The fibroblast growth factor receptor (FGFR) is growth factor receptor tyrosine kinases (79). Development of normal mammary gland requires active transcription of FGFR mediated proto-protein kinase and FGFR is closely associated with the development and progression of breast cancer (80, 81).

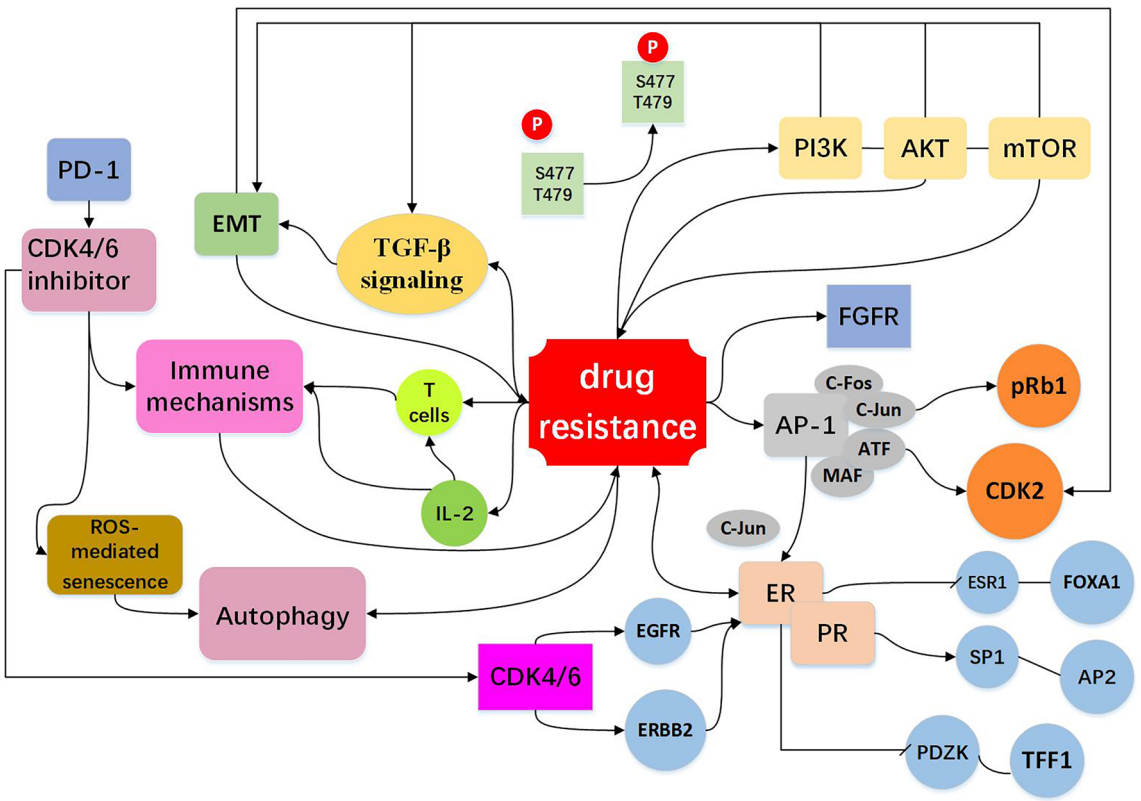

FIGURE 3 | Resistance to CDK4/6 inhibitors: Indirect Cell Cycle Mechanism Bypass pathways of the cell cycle: mTOR activation is *via* phosphoinositide PI3K/AKT signaling. The PI3K/AKT/mTOR pathway regulate cell signal transduction, have extensive links with other bypasses, for example EMT and TGF-β pathway (15, 41, 68–74). High expression of AP-1 (75–78), FGFR amplification (79–82), loss of ER or PR (13, 16, 26, 72) expression drives cells to escape CDK4/6 inhibition and act as bypass pathways for the progression of the cell. Other mechanisms include EMT (10, 83–87), immune mechanisms (88–91) and autophagy directly or indirectly influence drug resistance shown in the figure (10, 92–96).

Based on the combination of letrozole with ribociclib, the clinical trial MONALESA-2 observed that FGFR1 amplification was related to a lower PFS (79). It was also demonstrated that FGFR1 expression was increased in breast cancer MCF-7 cells treated with fulvestrant and palbociclib (82), and lucitanib, an anti-FGFR drug, can decrease drug resistance. As FGFR1 can stimulate the proliferation capacity of cancer cells, inhibiting both FGFR/FGF and the CDK4/6 pathways might be an effective approach to preventing or circumventing resistance to a single agent (**Figure 3**, **Table 2**).

ER and PR

ER and PR are the major factors that mediate cyclinD-CDK4/6 activity in estrogen receptor-positive (ER+) and progesterone receptor-positive (PR+) breast cancer cells (13). Effect of ER on resistance to CDK4/6 inhibitors involves both cell cycle and non-cell cycle mechanisms. In a preliminary clinical study, it was found that the expressions of ER/PR were lost in the palbociclib resistant tumor samples and down-regulated in the palbociclib resistant breast cancer cells (16, 26). Chip-seq analysis uncovered that ER was deficient in binding to ESR1 and FOXA1, but enriched in binding to SP1 and AP2, and these were accompanied by decreased expression of regulatory genes such as PDZK1 and TFF1.These data indicate that drug-resistant cells are genetically altered by chromosome remodeling. In other pathways discussed above, high expression of AP-1 leads to overexpression of C-Jun, which inhibits ER activity and modulates the efficacy of CDK4/6 inhibitors (76). Similarly, CDK4/6 blockade can lead to up-regulation of EGFR/ERBB and down-regulation of ER signaling pathway, and this negative feedback regulation can impact the efficacy of CDK4/6 inhibitors (26) (**Figure 3**, **Table 2**).

Other Mechanisms

EMT

Epithelial-mesenchymal transformation (EMT) is a biological process in which epithelial cells lose their polarity obtain the ability to invade and migrate. EMT has important roles in tumor cell metastasis, tumor stem cell formation, drug resistance, and other malignant phenotypes. A number of EMT-related signaling pathways are involved in drug resistance in cancer cells (83–85). The gene set enrichment analysis (GSEA) revealed enrichment of pathways that regulate EMT and cancer stem cells (IL-6/Stat3, IL-2/STAT-5, Notch, Wnt) in the cells resistant to

TABLE 2 | Mechanisms of acquired resistance to CDK4/6 inhibitors: Indirect cell cycle mechanisms.

Resistance classify	Resistance mechanism	Detection	Overcome
Bypass pathways of the cell cycle (13, 15, 16, 26, 41, 68–82)	mTOR pathway	1. Clinical trial 2. Cell biology experiments 3. Immunohistochemistry 4. Animal model	1. mTOR inhibitor 2. AKT inhibitor 3. PI3K inhibitor
	High expression of AP-1	1. Clinical trial 2. Cell biology experiments 3. Immunohistochemistry	1. AP-1 inhibitor
	FGFR amplification	1. Clinical trial 2. Cell biology experiments 3. Immunohistochemistry	1. Anti-FGFR drug
	Loss of ER or PR expression.	1. Preliminary clinical study 2. Chip-seq analysis 3. Cell biology experiments	1. ER regulator/blocker 2. Bypass way
Other mechanisms (10, 83–96)	EMT	1. Gene set enrichment analysis (GSEA) 2. Proteomics 3. Immunohistochemistry 4. Cell biology experiments	1. Inhibition of EMT 2. Bypass way
	Immune mechanisms	1. Proteomics 2. Experimental animal models 3. Cell biology experiments	1. Immune checkpoint inhibitors 2. Immunotherapy
	Autophagy	1. Proteomics 2. Immunohistochemistry	1. Autophagy inhibitor 2. Autophagy proteins

PI3K, phosphatidylinositide 3-kinases; AKT, protein kinase B;mTOR, mammalian target of rapamycin; AP-1, Activator protein 1; ER, estrogen receptor; PR, progesterone receptor; FGFR, fibroblast growth factor; EMT, Epithelial-mesenchymal transformation; receptor.

palbociclib (10). Indeed, anti-CDK4/6 therapy can induce EMT and enhance cell invasion through activating TGF-β signaling (60, 86). It was suggested that EMT is an important determinant of success/failure of targeted therapies by interfering with the compensatory changes such as deregulation of CDK2 activity (87). Low cyclin D1 (CCND1) expression displays increased expression of EMT markers, increased migration of breast cancer cells and drug resistance (86) (**Figure 3**, **Table 2**).

Immune Mechanisms

CDK4/6 inhibitors not only induce tumor cell cycle arrest, but also promote anti-tumor immunity (88–90). In murine models of breast carcinoma, it was found that CDK4/6 inhibitors can activate tumor expression of endogenous retroviral elements that enhance tumor antigen presentation. CDK4/6 inhibitors also suppress the proliferation of suppressive regulatory T cells (Tregs) and enhance the cytotoxic T cell-mediated killing of tumor cells. It was also found that CDK4/6 inhibitors could promote anti-tumor immunity by phosphorylating NFAT4, a transcription factor of T cells, thereby increasing IL-2 levels (91). CDK4/6 inhibitors reduced the proliferation of T cells, but increased tumor infiltration and activation of effector T cells. In addition, CDK4/6 inhibition can augment the response to PD-1 blockade in multiple *in vivo* murine syngeneic tumor models (91). These studies provide a rationale for combining CDK4/6 inhibitors with immunotherapy to more effectively killing tumor cells and preventing drug resistance (**Figure 3**, **Table 2**).

Autophagy

Autophagy is a cellular process that eliminates the damaged or aged cells and is the key machinery for bulk degradation of superfluous or aberrant cytoplasmic components. Autophagy is a double-edged sword in drug sensitivity/drug resistance (92–94). Autophagy could elevate the maintenance of cancer stem cells which may enhance drug resistance, while autophagy may help tumor cells to clear the drug-induced damage which decreasing the impact of chemotherapy and enhances therapeutic response (95, 96). It was demonstrated that CDK4/6 inhibition induces ROS mediated senescence and autophagy, blockade of autophagy significantly improves the efficacy of CDK4/6 inhibition (10). It was reported that high expression of autophagy proteins like LC3B can be utilized to combat resistance to cell-cycle-targeted therapies, such as CDK4/6 inhibitors (94). More research is needed to clarify the relationship between the CDK4/6 inhibitor and autophagy, this will provide a better prospect for the clinical application (**Figure 3**, **Table 2**).

Summary and Perspectives

CDK4/6 inhibitors are an effective therapeutic option for patients. A number of clinical trials have demonstrated the effectiveness and benefits of CDK4/6 inhibitors in improving the progression-free survival (PFS) of patients with ER-positive, HER2-negative advanced breast cancer (ABC) or metastatic breast cancer (MBC) when combined with endocrine therapy. The approval of palbociclib was based on the results from the PALOMA-1/TRIO-18, PALOMA-2, and PALOMA-3 trials. In the PALOMA-1 trail, combined therapy of letrozole with palbociclib significantly improved PFS as compared with single-agent letrozole. The PALOMA-2 trial confirmed the clinical activity of combination of palbociclib with letrozole. In PALOMA-3 trial, combined treatment of palbociclib with fulvestrant has shown benefits in patients with HR-positive, HER2-negative ABC or MBC. Thus, FDA approved the combined use of palbociclib with fulvestran based on this trial (3, 6, 21, 97). Abemaciclib was approved based

on the results of MONARCH 1, MONARCH2, and MONARCH3, and combination of abemaciclib with fulvestrant has been approved for treatment of patients with HR-positive, HER2-negative ABC or MBC. MONARCH 3 trial showed that abemaciclib plus anastrozole or letrozole produced a significantly longer median PFS than the placebo plus anastrozole or letrozole. FDA has approved the combined therapy of abemaciclib in with an aromatase inhibitor as first-line treatment for postmenopausal women with HR-positive, HER2-negative ABC (8, 98, 99). In addition, ribociclib in combination with letrozole was approved as the first-line treatment for postmenopausal women with HR-positive and HER2-negative ABC or MBC, and the combination of ribociclib with fulvestrant was approved for the treatment of postmenopausal women with HR-positive and HER2-negative ABC, based on the outcomes from clinical trials. MONALEESA-7 trial compared patience received ovarian function suppression and endocrine therapy plus ribociclib or not, in the ribociclib group, the PFS and overall survival (OS) was significantly long than placebo group (100–102). The recent study SOLAR-1, indicated that when alpelisib was combined with fulvestrant to treat the patients with PIK3CA-mutated, HR+, HER2- ABC patients, the PFS was increased from 5.7 to 11.2 months, a statistically significant prolongation (103). In China, the CDK4/6 inhibitors have been introduced into the first-line treatment for patients with advanced estrogenic receptor positive breast cancer. While this new targeted therapy has benefited numerous patients with advanced breast cancer, drug resistance to CDK4/6 inhibitors remain to be a major impediment to successful treatment of the disease. Novel approaches to preventing or overcoming the resistance to CDK4/6 inhibitors would certainly increase the value and benefits of these agents to breast cancer patients. However, to reach this goal, we need to have a better understanding of the multiplicity and complexity of the molecular mechanisms involved in resistance to CDK4/6 inhibitors. Also, despite enormous advances in this targeted therapy in treating breast cancer, its clinical efficacy and benefits are limited by the patient populations that do not benefit from this remedy, and this might be associated with a variety of factors such as tumor heterogeneity and target alterations. Identification and development of predictive and reliable biomarkers for the response to CD4/6 inhibitors shall significantly improve the outcome and value of the CD4/6-targeted therapy through better selecting appropriate patients for specific therapeutic regimens, thus are urgently needed. With a better understanding of the molecular mechanism behind resistance to CDK4/6 inhibitors, we could anticipate that patients can better benefit from novel therapeutic strategies that prevent and circumvent drug resistance and reinforce the efficacy of this targeted therapy.

AUTHOR CONTRIBUTIONS

HJ contributed to the conception of the study. RL finished the first manuscript preparation. BW revised the manuscript. SW, XL, JR, JL, KB, YW helped perform the analysis with constructive discussions. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Balter M, Vogel G. NOBEL PRIZE IN PHYSIOLOGY OR MEDICINE: Cycling Toward Stockholm. *Science* (2001) 294(5542):502–3. doi: 10.1126/science.294.5542.502
2. Guarducci C, Bonechi M, Boccalini G, Benelli M, Risi E, Di Leo A, et al. Mechanisms of Resistance to CDK4/6 Inhibitors in Breast Cancer and Potential Biomarkers of Response. *Breast Care (Basel Switzerland)* (2017) 12(5):304–8. doi: 10.1159/000484167
3. Finn RS, Crown JP, Lang I, Boer K, Bondarenko IM, Kulyk SO, et al. The Cyclin-Dependent Kinase 4/6 Inhibitor Palbociclib in Combination with Letrozole Versus Letrozole Alone as First-Line Treatment of Oestrogen Receptor-Positive, HER2-Negative, Advanced Breast Cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. *Lancet Oncol* (2015) 16(1):25–35. doi: 10.1016/s1470-2045(14)71159-3
4. Corona SP, Generali D. Abemaciclib: A CDK4/6 Inhibitor for the Treatment of HR+/HER2- Advanced Breast Cancer. *Drug Des Devel Ther* (2018) 12:321–30. doi: 10.2147/DDDT.S137783
5. Spring LM, Wander SA, Andre F, Moy B, Turner NC, Bardia A. Cyclin-Dependent Kinase 4 and 6 Inhibitors for Hormone Receptor-Positive Breast Cancer: Past, Present, and Future. *Lancet* (2020) 395(10226):817–27. doi: 10.1016/s0140-6736(20)30165-3
6. Diéras V, Harbeck N, Joy AA, Gelmon K, Ettl J, Verma S, et al. Palbociclib with Letrozole in Postmenopausal Women with ER+/HER2–Advanced Breast Cancer: Hematologic Safety Analysis of the Randomized PALOMA-2 Trial. *Oncologist* (2019) 24(12):1514–25. doi: 10.1634/theoncologist.2019-0019
7. Duso B, Trapani D, Viale G, Criscitiello C, D'Amico P, Belli C, et al. Clinical Efficacy of Ribociclib as a First-Line Therapy for HR-Positive, Advanced Breast Cancer. *Expert Opin Pharmacotherapy* (2018) 19(3):299–305. doi: 10.1080/14656566.2018.1429407
8. Johnston S, Martin M, Di Leo A, Im S-A, Awada A, Forrester T, et al. MONARCH 3 Final PFS: A Randomized Study of Abemaciclib as Initial Therapy For Advanced Breast Cancer. *NPJ Breast Cancer* (2019) 5(1):244–59. doi: 10.1038/s41523-018-0097-z
9. C G. RB and Cell Cycle Progression. *Oncogene* (2006) 38(25):5220–7. doi: 10.1038/sj.onc.1209615
10. Vijayaraghavan S, Karakas C, Doostan I, Chen X, Bui T, Yi M, et al. CDK4/6 and Autophagy Inhibitors Synergistically Induce Senescence in Rb Positive Cytoplasmic Cyclin E Negative Cancers. *Nat Commun* (2017) 8(27):15916. doi: 10.1038/ncomms15916
11. Jeffry LD. Therapeutic Response to CDK4/6 Inhibition in Breast Cancer Defined by Ex Vivo Analyses of Human Tumors. *Cell Cycle (Georgetown Tex)* (2012) 14(11):2756–61. doi: 10.4161/cc.21195
12. Dean JL, McClendon AK, Hickey TE, Butler LM, Tilley WD, Witkiewicz AK, et al. Therapeutic Response to CDK4/6 Inhibition in Breast Cancer Defined by Ex Vivo Analyses of Human Tumors. *Cell Cycle (Georgetown Tex)* (2012) 11(14):2756–61. doi: 10.4161/cc.21195
13. Finn RS, Aleshin A, Slamon DJ. Targeting the Cyclin-Dependent Kinases (CDK) 4/6 in Estrogen Receptor-Positive Breast Cancers. *Breast Cancer Res* (2016) 18(1):17. doi: 10.1186/s13058-015-0661-5
14. Xi J, Ma CX. Sequencing Endocrine Therapy for Metastatic Breast Cancer: What Do We Do After Disease Progression on a CDK4/6 Inhibitor? *Curr Oncol Rep* (2020) 22(6):57. doi: 10.1007/s11912-020-00917-8
15. Herrera-Abreu MT, Palafox M, Asghar U, Rivas MA, Cutts RJ, Garcia-Murillas I, et al. Early Adaptation and Acquired Resistance to CDK4/6 Inhibition in Estrogen Receptor-Positive Breast Cancer. *Cancer Res* (2016) 76(8):2301–13. doi: 10.1158/0008-5472.CAN-15-0728

16. Yang C, Li Z, Bhatt T, Dickler M, Giri D, Scaltriti M, et al. Acquired CDK6 Amplification Promotes Breast Cancer Resistance to CDK4/6 Inhibitors and Loss of ER Signaling and Dependence. *Oncogene* (2017) 36(16):2255–64. doi: 10.1038/onc.2016.379
17. Dean JL, Thangavel C, McClendon AK, Reed CA, Knudsen ES. Therapeutic CDK4/6 Inhibition in Breast Cancer: Key Mechanisms of Response and Failure. *Oncogene* (2010) 29(28):4018–32. doi: 10.1038/onc.2010.154
18. Malorni L, Piazza S, Ciani Y, Guarducci C, Bonechi M, Biagioni C, et al. A Gene Expression Signature of Retinoblastoma Loss-of-Function is a Predictive Biomarker of Resistance to Palbociclib in Breast Cancer Cell Lines and is Prognostic in Patients with ER Positive Early Breast Cancer. *Oncotarget* (2016) 7(42):68012–22. doi: 10.18632/oncotarget.12010
19. Gong X, Litchfield LM, Webster Y, Chio LC, Wong SS, Stewart TR, et al. Genomic Aberrations that Activate D-type Cyclins Are Associated with Enhanced Sensitivity to the CDK4 and CDK6 Inhibitor Abemaciclib. *Cancer Cell* (2017) 32(6):761–76.e766. doi: 10.1016/j.ccell.2017.11.006
20. Condorelli R, Spring L, O'Shaughnessy J, Lacroix L, Bailleux C, Scott V, et al. Polyclonal RB1 Mutations and Acquired Resistance to CDK 4/6 Inhibitors in Patients With Metastatic Breast Cancer. *Ann Oncol* (2018) 29(3):640–5. doi: 10.1093/annonc/mdx784
21. O'Leary B, Cutts RJ, Liu Y, Hrebien S, Huang X, Fenwick K, et al. The Genetic Landscape and Clonal Evolution of Breast Cancer Resistance to Palbociclib plus Fulvestrant in the PALOMA-3 Trial. *Cancer Discov* (2018) 8 (11):1390–403. doi: 10.1158/2159-8290.Cd-18-0264
22. Ramanujan A, Tiwari S. APC/C and Retinoblastoma Interaction: Cross-Talk of Retinoblastoma Protein With the Ubiquitin Proteasome Pathway. *Biosci Rep* (2016) 36(5):e00377–7. doi: 10.1042/bsr20160152
23. The I, Ruijtenberg S, Bouchet BP, Cristobal A, Prinsen MBW, van Mourik T, et al. Rb and FZR1/Cdh1 Determine CDK4/6-Cyclin D Requirement in C. elegans and Human Cancer Cells. *Nat Commun* (2015) 6(1):5906. doi: 10.1038/ncomms6906
24. Koboldt DC, Fulton RS, McLellan MD, Schmidt H, Kalicki-Veizer J, McMichael JF, et al. Comprehensive Molecular Portraits of Human Breast Tumours. *Nature* (2012) 490(7418):61–70. doi: 10.1038/nature11412
25. Hamilton E, Infante JR. Targeting CDK4/6 in Patients With Cancer. *Cancer Treat Rev* (2016) 45:129–38. doi: 10.1016/j.ctrv.2016.03.002
26. Pancholi S, Ribas R, Simigdala N, Schuster E, Nikitorowicz-Buniak J, Ressa A, et al. Tumour Kinome Re-Wiring Governs Resistance to Palbociclib in Oestrogen Receptor Positive Breast Cancers, Highlighting New Therapeutic Modalities. *Oncogene* (2020) 39(25):4781–97. doi: 10.1038/s41388-020-1284-6
27. Eran H. A Landscape of Driver Mutations in Melanoma. *Cell* (2012) 2 (150):251–63. doi: 10.1016/j.cell.2012.06.024
28. Ling C. p16-Cdk4-Rb Axis Controls Sensitivity to a Cyclin-Dependent Kinase Inhibitor PD0332991 in Glioblastoma Xenograft Cells. *Neuro-oncology* (2012) 7(14):810–81. doi: 10.1093/neuonc/nos114
29. Olanich ME, Sun W, Hewitt SM, Abdullaev Z, Pack SD, Barr FG. CDK4 Amplification Reduces Sensitivity to CDK4/6 Inhibition in Fusion-Positive Rhabdomyosarcoma. *Clin Cancer Res* (2015) 21(21):4947–59. doi: 10.1158/1078-0432.Ccr-14-2955
30. Jiang H, Luo N, Zhang X, Song C, Zang J. EGFR L861Q and CDK4 Amplification Responding to Afatinib Combined With Palbociclib Treatment in a Patient With Advanced Lung Squamous Cell Carcinoma. *Lung Cancer* (2020) 145:216–218. doi: 10.1016/j.lungcan.2020.04.001
31. Patel P, Asbach B, Shteyn E, Gomez C, Coltoff A, Bhuyan S, et al. Brk/Protein Tyrosine Kinase 6 Phosphorylates p27KIP1, Regulating the Activity of Cyclin D-Cyclin-Dependent Kinase 4. *Mol Cell Biol* (2015) 35(9):1506–22. doi: 10.1128/mcb.01206-14
32. Kollmann K, Heller G, Schneckenleithner C, Warsch W, Scheicher R, et al. A Kinase-Independent Function of CDK6 Links the Cell Cycle to Tumor Angiogenesis. *Cancer Cell* (2016) 30(2):359–360. doi: 10.1016/j.ccell.2016.07.003
33. Finn RS, Aleshin A, Slamon DJ. Targeting the Cyclin-Dependent Kinases (CDK) 4/6 in Estrogen Receptor-Positive Breast Cancers. *Breast Cancer Res BCR* (2016) 18(1):17. doi: 10.1186/s13058-015-0661-5
34. Gacche RN, Assaraf YG. Redundant Angiogenic Signaling and Tumor Drug Resistance. *Drug Resist Update* (2018) 36:47–76. doi: 10.1016/j.drup.2018.01.002
35. Pandey K, An HJ, Kim SK, Lee SA, Kim S, Lim SM, et al. Molecular Mechanisms of Resistance to CDK4/6 Inhibitors in Breast Cancer: A Review. *Int J Cancer* (2019) 145(5):1179–88. doi: 10.1002/ijc.32020
36. GA B, Alan DJ. Cell Cycle Progression Without Cyclin E/CDK2: Breaking Down the Walls of Dogma. *Cancer Cell* (2003) 4(3):160–2. doi: 10.1016/S1535-6108(03)00217-4
37. Caldon CE, Sergio CM, Kang J, Muthukaruppan A, Boersma MN, Stone A, et al. Cyclin E2 Overexpression is Associated With Endocrine Resistance But Not Insensitivity to CDK2 Inhibition in Human Breast Cancer Cells. *Mol Cancer Ther* (2012) 11(7):1488–99. doi: 10.1158/1535-7163.Mct-11-0963
38. Schachter MM, Merrick KA, Larochelle S, Hirschi A, Zhang C, Shokat KM, et al. A Cdk7-Cdk4 T-Loop Phosphorylation Cascade Promotes G1 Progression. *Mol Cell* (2013) 50(2):250–60. doi: 10.1016/j.molcel.2013.04.003
39. Wang Y, Zhang T, Kwiatkowski N, Abraham BJ, Lee TI, Xie S. CDK7-Dependent Transcriptional Addiction in Triple-Negative Breast Cancer. *Cell* (2015) 163(1):174–86. doi: 10.1016/j.cell.2015.08.063
40. Zhenghui Z, Wei L, Liyan. Yangshune Correlation Analysis of Cyclin D1, Aurora Kinase-A, Fibroblast Growth Factor Receptor2 Gene Single Nucleotide Polymorphism With Pathological Indicators and Prognosis of Breast Cancer. *Chin J Exp Surgery|Chin J Exp Surg* (2020) 7(37):1208–11. doi: 10.3760/cma.j.cn421213-20200709-00496
41. Michaloglou C, Crafter C, Siersbæk R, Delpuech O, Curwen J, Carnevalli L, et al. Combined Inhibition of mTOR and CDK4/6 Is Required for Optimal Blockade of E2F Function and Long-term Growth Inhibition in Estrogen Receptor-Positive Breast Cancer. *Mol Cancer Ther* (2018) 17: molcanther.0537.2017. doi: 10.1158/1535-7163.MCT-17-0537
42. Ma CX, Gao F, Luo J, Northfelt DW, Goetz M, Forero A, et al. NeoPalAna: Neoadjuvant Palbociclib, a Cyclin-Dependent Kinase 4/6 Inhibitor, and Anastrozole for Clinical Stage 2 or 3 Estrogen Receptor-Positive Breast Cancer. *Clin Cancer Res* (2017) 23(15):4055–65. doi: 10.1158/1078-0432.Ccr-16-3206
43. Yan Y, Xu H, Wang J, Wu X, Wen W, Liang Y, et al. Inhibition of Breast Cancer Cells by Targeting E2F-1 Gene and Expressing IL15 Oncolytic Adenovirus. *Biosci Rep* (2019) 39(7):BSR20190384. doi: 10.1042/bsr20190384
44. Li Y, Huang J, Yang D, Xiang S, Sun J, Li H, et al. Expression Patterns of E2F Transcription Factors and Their Potential Prognostic Roles in Breast Cancer. *Oncol Lett* (2018) 15(6):9216–30. doi: 10.3892/ol.2018.8514
45. Sun F, Li N, Tong X, Zeng J, He S, Gai T, et al. Ara-c Induces Cell Cycle G1/S Arrest by Inducing Upregulation of the INK4 Family Gene or Directly Inhibiting the Formation of the Cell Cycle-Dependent Complex CDK4/Cyclin D1. *Cell Cycle* (2019) 18(18):2293–306. doi: 10.1080/15384101.2019.1644913
46. Kong Y, Sheng X, Wu X, Yan J, Ma M, Yu J, et al. Frequent Genetic Aberrations in the CDK4 Pathway in Acral Melanoma Indicate the Potential for CDK4/6 Inhibitors in Targeted Therapy. *Clin Cancer Res* (2017) 23 (22):6946–57. doi: 10.1158/1078-0432.Ccr-17-0070
47. Li P, Zhang X, Gu L, Zhou J, Deng D. P16 Methylation Increases the Sensitivity of Cancer Cells to the CDK4/6 Inhibitor Palbociclib. *PloS One* (2019) 14(10):e0223084. doi: 10.1371/journal.pone.0223084
48. Medema RH, Herrera RE, Lam F, Weinberg RA. Growth Suppression by p16ink4 Requires Functional Retinoblastoma Protein. *Proc Natl Acad Sci U S A* (1995) 92(14):6289–93. doi: 10.1073/pnas.92.14.6289
49. Dosil MA, Mirantes C, Eritja N, Felip I, Navaridas R, Gatius S, et al. Palbociclib has Antitumour Effects on Pten-Deficient Endometrial Neoplasias. *J Pathol* (2017) 242(2):152–64. doi: 10.1002/path.4896
50. Wang Y, Ly A, Hosono Y, Murchie E, Walmsley CS. PTEN Loss Mediates Clinical Cross-Resistance to CDK4/6 and PI3Kα Inhibitors in Breast Cancer. *Cancer Discov* (2020) 10(1):72–85. doi: 10.1158/2159-8290.CD-18-0830.
51. Juric D, Castel P, Griffith M, Griffith OL, Won HH, Ellis H. Convergent Loss of PTEN Leads to Clinical Resistance to a PI(3)Kα Inhibitor. *Nature* (2015) 518(7538):240–4. doi: 10.1038/nature13948
52. Razavi P, Dickler MN, Shah PD, Toy W, Brown DN, Won HH, et al. Alterations in PTEN and ESR1 Promote Clinical Resistance to Alpelisib Plus Aromatase Inhibitors. *Nat Cancer* (2020) 1(4):382–93. doi: 10.1038/s43018-020-0047-1

53. Zelivianski S, Cooley A, Kall R, Jeruss JS. Cyclin-Dependent Kinase 4-Mediated Phosphorylation Inhibits Smad3 Activity in Cyclin D-over expressing breast cancer cells. *Mol Cancer Res* (2010) 8(10):1375–87. doi: 10.1158/1541-7786.Mcr-09-0537
54. Kretschmer A, Moepert K, Dames S, Sternberger M, Kaufmann J, Klippel A. Differential Regulation of TGF-Beta Signaling Through Smad2, Smad3 and Smad4. *Oncogene* (2003) 22(43):6748–63. doi: 10.1038/sj.onc.1206791
55. Meirson T, Gil-Henn H. Targeting Invadopodia for Blocking Breast Cancer Metastasis. *Drug Resist Updat* (2018) 39:1–17. doi: 10.1016/j.drup.2018.05.002
56. Yang J, Song K, Krebs TL, Jackson MW. Danielpour D Rb/E2F4 and Smad2/3 link Survivin to TGF-Beta-Induced Apoptosis and Tumor Progression. *Oncogene* (2008) 27(40):5326–38. doi: 10.1038/onc.2008.165
57. Lamouille S, Xu J, Derynck R. Molecular Mechanisms of Epithelial–Mesenchymal Transition. *Nat Rev Mol Cell Biol* (2014) 15(3):178–96. doi: 10.1038/nrm3758
58. Thomas AL, Lind H, Hong A, Dokic D, Oppat K, Rosenthal E, et al. Inhibition of CDK-Mediated Smad3 Phosphorylation Reduces the Pin1-Smad3 Interaction and Aggressiveness of trIple Negative Breast Cancer Cells. *Cell Cycle* (2017) 16(15):1453–64. doi: 10.1080/15384101.2017.1338988
59. Franco J, Witkiewicz AK, Knudsen ES. CDK4/6 Inhibitors Have Potent Activity in combination With Pathway Selective Therapeutic Agents in Models of Pancreatic Cancer. *Oncotarget* (2014) 5(15):6512–25. doi: 10.18632/oncotarget.2270
60. Liu F, Korc M. Cdk4/6 Inhibition Induces Epithelial-Mesenchymal Transition and Enhances Invasiveness in Pancreatic Cancer Cells. *Mol Cancer Ther* (2012) 10(11):2138–48. doi: 10.1158/1535-7163.MCT-12-0562
61. O'Connor MJ. Targeting the DNA Damage Response in Cancer. *Mol Cell* (2015) 60(4):547–60. doi: 10.1016/j.molcel.2015.10.040
62. Geenen JJJ, Schellens JHM. Molecular Pathways: Targeting the Protein Kinase Wee1 in Cancer. *Clin Cancer Res* (2017) 23(16):4540–44. doi: 10.1158/1078-0432.CCR-17-0520
63. Martin L-A, Pancholi S, Ribas R, Gao NS Q, Nikitorowicz-Buniak J, Johnston S, et al. Resistance to Palbociclib Depends on Multiple Targetable Mechanisms Highlighting the Potential of Drug Holidays and Drug Switching To Improve Therapeutic Outcome, Abstracts: 2016 San Antonio Breast Cancer Symposium. *Cancer Res Cancer Res* (2017) 77(4 Suppl):P3-03-09-P3-03-09. doi: 10.1158/1538-7445.
64. Grolmusz VK, Chen J, Emond R, Cosgrove PA, Pflieger L, Nath A, et al. Exploiting collateral sensitivity controls growth of mixed culture of sensitive and resistant cells and decreases selection for resistant cells in a cell line model. *Cancer Cell Int* (2020) 20:253. doi: 10.1186/s12935-020-01337-1
65. Kundu N, Brekman A, Kim JY, Xiao G, Gao C, Bargonetti J. Estrogen-Activated MDM2 Disrupts Mammary Tissue Architecture Through a p53-Independent Pathway. *Oncotarget* (2017) 8(29):47916–30. doi: 10.18632/oncotarget.18147
66. Laroche-Clary A, Chaire V, Algeo M-P, Derieppe M-A, Loarer FL, Italiano A. Combined Targeting of MDM2 and CDK4 is Synergistic in Dedifferentiated Liposarcomas. *J Hematol Oncol* (2017) 10(1):123. doi: 10.1186/s13045-017-0482-3
67. Portman N, Milioli HH, Alexandrou S, Coulson R, Yong A, Fernandez KJ, et al. MDM2 Inhibition in Combination With Endocrine Therapy and CDK4/6 Inhibition For the Treatment of ER-Positive Breast Cancer. *Breast Cancer Res* (2020) 22(1):87. doi: 10.1186/s13058-020-01318-2
68. Michaloglou C, Crafter C, Siersbaek R, Delpuech O, Curwen JO, Carnevalli LS, et al. Combined Inhibition of mTOR and CDK4/6 Is Required for Optimal Blockade of E2F Function and Long-term Growth Inhibition in Estrogen Receptor-positive Breast Cancer. *Mol Cancer Ther* (2018) 17(5):908–20. doi: 10.1158/1535-7163.Mct-17-0537
69. Dey N, De P, Leyland-Jones B. PI3K-AKT-mTOR Inhibitors in Breast Cancers: From Tumor Cell Signaling to Clinical Trials. *Pharmacol Ther* (2017) 175:91–106. S0163725817300517. doi: 10.1016/j.pharmthera.2017.02.037
70. Litchfield LM, Boehnke K, Brahmachary M, Mur C, Bi C, Stephens JR, et al. Combined Inhibition of PIM and CDK4/6 Suppresses Both mTOR Signaling and Rb Phosphorylation and Potentiates PI3K Inhibition in Cancer Cells. *Oncotarget* (2020) 11(17):1478–92. doi: 10.18632/oncotarget.27539
71. McCartney A, Migliaccio I, Bonechi M, Biagioni C, Romagnoli D, De Luca F, et al. Mechanisms of Resistance to CDK4/6 Inhibitors: Potential Implications and Biomarkers for Clinical Practice. *Front Oncol* (2019) 9:666. doi: 10.3389/fonc.2019.00666
72. Occhipinti G, Romagnoli E, Santoni M, Cimadamore A, Sorgentoni G, Cecati M, et al. Sequential or Concomitant Inhibition of Cyclin-Dependent Kinase 4/6 Before mTOR Pathway in Hormone-Positive HER2 Negative Breast Cancer: Biological Insights and Clinical Implications. *Front Genet* (2020) 15(11):349. doi: 10.3389/fgene.2020.00349
73. Jansen VM, Bhola NE, Bauer JA, Formisano L, Lee K-M, Hutchinson KE, et al. Kinome-Wide RNA Interference Screen Reveals a Role for PDK1 in Acquired Resistance to CDK4/6 Inhibition in ER-Positive Breast Cancer. *Cancer Res* (2017) 77(9):2488–99. doi: 10.1158/0008-5472.CAN-16-2653
74. Vora SR, Juric D, Kim N, Mino-Kenudson M, Huynh T, Costa C, et al. CDK 4/6 Inhibitors Sensitize PIK3CA Mutant Breast Cancer to PI3K Inhibitors. *Cancer Cell* (2014) 26(1):136–49. doi: 10.1016/j.ccr.2014.05.020
75. Shaulian E, Karin M. AP-1 in Cell Proliferation and Survival. *Oncogene* (2001) 20(19):2390–400. doi: 10.1038/sj.onc.1204383
76. Shen Q, Uray IP, Li Y, Zhang Y, Hill J, Xu XC, et al. Targeting the Activator Protein 1 Transcription Factor for the Prevention of Estrogen Receptor-Negative Mammary Tumors. *Cancer Prev Res (Phila)* (2008) 1(1):45–55. doi: 10.1158/1940-6207.Capr-08-0034
77. Angelis C, Nardone A, Cataldo ML, Veeraraghavan J, Fu X, Giuliano M, et al. AP-1 as a Potential Mediator of Resistance to the Cyclin-Dependent Kinase (CDK) 4/6-Inhibitor Palbociclib in ER-Positive Endocrine-Resistant Breast Cancer. *Cancer Res* (2018) 78:P4–03. doi: 10.1158/1538-7445.SABCS17-P4-03-05
78. Ye N, Ding Y, Wild C, Shen Q, Zhou J. Small Molecule Inhibitors Targeting Activator Protein 1 (AP-1). *J Med Chem* (2014) 16(57):6930–48. doi: 10.1021/jm5004733
79. Sobhani N, Fan C, Flores-Villanueva PO, Generali D, Li Y. The Fibroblast Growth Factor Receptors in Breast Cancer: from Oncogenesis to Better Treatments. *Int J Mol Sci* (2020) 21(6):2021. doi: 10.3390/ijms21062011
80. Perez-Garcia J, Muñoz-Couselo E, Soberino J, Racca F, Cortes J. Targeting FGFR Pathway in Breast Cancer. *Breast* (2018) 37:126–33. doi: 10.1016/j.breast.2017.10.014
81. Sahores A, May M, Sequeira GR, Fuentes C, Jacobsen B, Lanari C, et al. Targeting FGFR with BGJ398 in Breast Cancer: Effect on Tumor Growth and Metastasis. *Curr Cancer Drug Targets* (2018) 18(10):979–87. doi: 10.2174/1568009618666171214114706
82. Formisano L, Lu Y, Servetto A, Hanker AB, Jansen VM, Bauer JA, et al. Aberrant FGFR Signaling Mediates Resistance to CDK4/6 Inhibitors in ER+ Breast Cancer. *Nat Commun* (2019) 10(1):1373. doi: 10.1038/s41467-019-09068-2
83. Pradella D, Naro C, Sette C, Ghigna C. EMT And Stemness: Flexible Processes Tuned by Alternative Splicing in Development and Cancer Progression. *Mol Cancer* (2017) 16(1):8. doi: 10.1186/s12943-016-0579-2
84. Kim DH, Xing T, Yang Z, Dudek R, Lu Q, Chen Y-H. Epithelial Mesenchymal Transition in Embryonic Development, Tissue Repair and Cancer: A Comprehensive Overview. *J Clin Med* (2017) 1. doi: 10.3390/jcm7010001
85. Du B, Shim JS. Targeting Epithelial-Mesenchymal Transition (EMT) to Overcome Drug Resistance in Cancer. *Molecules* (2016) 21(7):965. doi: 10.3390/molecules21070965
86. Tobin NP, Sims AH, Lundgren KL, Lehn S, Landberg G. Cyclin D1, Id1 and EMT in Breast Cancer. *BMC Cancer* (2011) 11:417. doi: 10.1186/1471-2407-11-417
87. Yaswen P. Reinforcing Targeted Therapeutics With Phenotypic Stability Factors. *Cell Cycle* (2014) 13(24):3818–22. doi: 10.4161/15384101.2014.985071
88. Ernst B, Anderson KS. Immunotherapy for the Treatment of Breast Cancer. *Curr Oncol Rep* (2015) 2(17):5. doi: 10.1007/s11912-014-0426-9
89. Jia H, Truica CI, Wang B, Wang Y, Ren X, Harvey HA, et al. Immunotherapy For Triple-Negative Breast Cancer: Existing Challenges and Exciting Prospects. *Drug Resist Update* (2017) 32:1–15. doi: 10.1016/j.drup.2017.07.002

90. Goel S, DeCristo MJ, Watt AC, BrinJones H, Sceneay J, Li BB. CDK4/6 Inhibition Triggers Anti-Tumour Immunity. *Nature* (2017) 548(7668):471–5. doi: 10.1038/nature23465
91. Deng J, Wang ES, Jenkins RW, Li S, Dries R, Yates K, et al. CDK4/6 Inhibition Augments Antitumor Immunity by Enhancing T-cell Activation. *Cancer Discov* (2018) 8(2):216–33. doi: 10.1158/2159-8290.CD-17-0915
92. Sisinni L, Pietrafesa M, Lepore S, Maddalena F, Condelli V, Esposito F, et al. Endoplasmic Reticulum Stress and Unfolded Protein Response in Breast Cancer: The Balance Between Apoptosis and Autophagy and Its Role in Drug Resistance. *Int J Mol Sci* (2019) 20(4):10.3390/ijms20040857. doi: 10.3390/ijms20040857
93. Chen S, Jiang Y-Z, Huang L, Zhou R-J, Yu K-D, Liu Y, et al. The Residual Tumor Autophagy Marker LC3B Serves as a Prognostic Marker in Local Advanced Breast Cancer after Neoadjuvant Chemotherapy. *Clin Cancer Res* (2013) 19(24):6853–62. doi: 10.1158/1078-0432.CCR-13-1617
94. Lefort S, Joffre C, Kieffer Y, Givel A-M, Bourachot B, Zago G, et al. Inhibition of Autophagy as a New Means of Improving Chemotherapy Efficiency in High-LC3B Triple-Negative Breast Cancers. *Autophagy* (2014) 10(12):2122–42. doi: 10.4161/15548627.2014.981788
95. Rubinstein AD. Kimchi A Life in the Balance - A Mechanistic View of the Crosstalk Between Autophagy and Apoptosis. *J Cell Sci* (2012) 125(Pt 22):5259–68. doi: 10.1242/jcs.115865
96. Gangemi R, Paleari L, Orengo AM, Cesario A, Chessa L, Ferrini S, et al. Cancer Stem Cells: A New Paradigm for Understanding Tumor Growth and Progression and Drug Resistance. *Curr Med Chem* (2009) 16(14):1688–703. doi: 10.2174/092986709788186147
97. Verma S, Bartlett CH, Schnell P, DeMichele AM, Loi S, Ro J, et al. Palbociclib in Combination With Fulvestrant in Women With Hormone Receptor-Positive/HER2-Negative Advanced Metastatic Breast Cancer: Detailed Safety Analysis From a Multicenter, Randomized, Placebo-Controlled, Phase III Study (PALOMA-3). *Oncologist* (2016) 21(10):1165–75. doi: 10.1634/theoncologist.2016-0097
98. Goetz MP, Toi M, Campone M, Sohn J, Paluch-Shimon S, Huober J, et al. MONARCH 3: Abemaciclib As Initial Therapy for Advanced Breast Cancer. *J Clin Oncol* (2017) 35(32):3638–46. doi: 10.1200/JCO.2017.75.6155
99. Dickler MN, Tolaney SM, Rugo HS, Cortés J, Diéras V, Patt D, et al. MONARCH 1, A Phase II Study of Abemaciclib, a CDK4 and CDK6 Inhibitor, as a Single Agent, in Patients with Refractory HR(+)/HER2(-) Metastatic Breast Cancer. *Clin Cancer Res* (2017) 23(17):5218–24. doi: 10.1158/1078-0432.Ccr-17-0754
100. Hortobagyi GN, Stemmer SM, Burris HA, Yap YS, Sonke GS, Paluch-Shimon S, et al. Updated Results from MONALEESA-2, a Phase III Trial of First-Line Ribociclib Plus Letrozole Versus Placebo Plus Letrozole in Hormone Receptor-Positive, HER2-Negative Advanced Breast Cancer. *Ann Oncol* (2018) 29(7):1541–7. doi: 10.1093/annonc/mdy155
101. Tripathy D, Im SA, Colleoni M, Franke F, Bardia A, Harbeck N, et al. Ribociclib Plus Endocrine Therapy for Premenopausal Women With Hormone-Receptor-Positive, Advanced Breast Cancer (MONALEESA-7): A Randomised Phase 3 Trial. *Lancet Oncol* (2018) 19(7):904–15. doi: 10.1016/s1470-2045(18)30292-4
102. Slamon DJ, Neven P, Chia S, Fasching PA, De Laurentiis M, Im SA, et al. Phase III Randomized Study of Ribociclib and Fulvestrant in Hormone Receptor-Positive, Human Epidermal Growth Factor Receptor 2-Negative Advanced Breast Cancer: MONALEESA-3. *J Clin Oncol* (2018) 36(24):2465–72. doi: 10.1200/jco.2018.78.9909
103. André F, Ciruelos EM, Juric D, Loibl S, Campone M, Mayer IA, et al. Alpelisib Plus Fulvestrant for PIK3CA-Mutated, Hormone Receptor-Positive, Human Epidermal Growth Factor Receptor-2-Negative Advanced Breast Cancer: Final Overall Survival Results From SOLAR-1. *Ann Oncol* (2021) 32(2):208–17. doi: 10.1016/j.annonc.2020.11.011

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Pim1 Kinase Inhibitors Exert Anti-Cancer Activity Against HER2-Positive Breast Cancer Cells Through Downregulation of HER2

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The proviral integration site for moloney murine leukemia virus 1 (Pim1) is a serine/threonine kinase and able to promote cell proliferation, survival and drug resistance. Overexpression of Pim1 has been observed in many cancer types and is associated with the poor prognosis of breast cancer. However, it remains unclear whether Pim1 kinase is a potential therapeutic target for breast cancer patients. In this study, we found that Pim1 expression was strongly associated with HER2 expression and that HER2-overexpressing breast cancer cells were more sensitive to Pim1 inhibitor-induced inhibitions of cell viability and metastatic ability. Mechanistically, Pim1 inhibitor suppressed the expression of HER2 at least in part through transcriptional level. More importantly, Pim1 inhibitor overcame the resistance of breast cancer cells to HER2 tyrosine kinase inhibitor lapatinib. In summary, downregulation of HER2 by targeting Pim1 may be a promising and effective therapeutic approach not only for anti-cancer growth but also for circumventing lapatinib resistance in HER2-positive breast cancer patients.

Keywords: HER2, lapatinib, drug resistance, breast cancer, PIM1

INTRODUCTION

Breast cancer is the most common cancer type and ranks second among causes for cancer death in women (Fahad Ullah, 2019). According to the expression pattern of biomarkers, including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2, also known as Neu, ErbB2, EGFR2), breast cancer can be classified into several subtypes (Raica et al., 2009). Among these biomarkers, HER2 overexpression is correlated with poor prognosis prior to the advent of anti-HER2 therapies (Barros et al., 2010; Santa-Maria et al., 2016).

HER2 is a member of human epidermal growth factor receptor (HER/EGFR) tyrosine kinase family, which is frequently overexpressed in many cancer types (Wang, 2017). HER family includes

EGFR, HER2, HER3, and HER4. The overexpressed HER2 form either homo-dimer or hetero-dimer with other members of EGFR family. Thereafter, HER2 is activated through autophosphorylation and transduces the downstream signaling pathways, leading to cycle progression, cell proliferation, survival and cancer stemness for tumor progression (Hsu and Hung, 2016; Nami and Wang, 2017). Therefore, targeted therapy against HER2 tyrosine kinase activity has been developed and approved for HER2-positive breast cancer (Riese and Stern, 1998; Iqbal and Iqbal, 2014). There are two types of HER2-targeted therapy, including HER2 antibody trastuzumab and HER2 tyrosine kinase inhibitor (TKI) lapatinib (Ahmed et al., 2015). Furthermore, lapatinib may act as a surrogate treatment for HER2-overexpressing metastatic breast cancer patients who failed to respond to trastuzumab treatment (Brandes et al., 2010; Hicks et al., 2015). Although these drugs indeed show clinical benefits to HER2-positive breast cancer patients, acquired resistance is still developed eventually and remains a hurdle to be overcome (Nahta et al., 2009; Rexer and Arteaga, 2012; Pernas and Tolaney, 2019). However, the mechanisms underlying resistance remain not fully clarified.

The proviral integration site for moloney murine leukemia virus 1 (Pim1) is a serine/threonine kinase. There are three members in human Pim family, including Pim1, Pim2, and Pim3, which are encoded in chromosome 6, X chromosome, and chromosome 22, respectively. Aberrant elevation of Pim1 has been observed in many cancer types and reported to play a crucial role in tumorigenesis due to the interactions with numerous proteins participating in various signaling pathways involved in cell proliferation, survival, and drug resistance (Narlik-Grassow et al., 2014; Warfel and Kraft, 2015). The oncogenic potential of Pim1 was most extensively investigated in prostate cancer (Holder and Abdulkadir, 2014; Ouhtit et al., 2015; Luszczak et al., 2020). It has reported that AKT inhibitor GSK690693 promotes the transcriptional induction of Pim1 kinase, which increased the protein expressions of receptor tyrosine kinase (RTK), including EGFR, HER2, and HER3, and subsequently resulted in the resistance of prostate cancer cells to AKT inhibition (Cen et al., 2013). Furthermore, Pim kinase inhibitor M-110 was shown to reduce the expression of EGFR, leading to the reduction of extracellular signal-regulated kinase (ERK) pathway activity in prostate cancer (Siu et al., 2011). Although overactivation of HER family was observed in many cancers, especially in breast cancer, ovarian cancer, and non-small cell lung cancer, and correlates with poor prognosis and drug resistance (Wang, 2017), it remains unclear whether Pim1 plays a role in the regulations of HER family expression and TKI resistance and functions a potential therapeutic target in breast cancer. In this study, our data showed that Pim1 positively regulates the expressions of HER2 at the transcriptional level and that targeting Pim1 may be a promising and effective therapeutic approach not only for anti-cancer growth but also for circumventing lapatinib resistance in HER2-positive breast cancer patients.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Human HER2-positive (SkBr3, BT474) and HER2-negative (MDA-MB-231, MCF7, and T47D) breast cancer cell lines were obtained from the American Type Culture Collection. HBL-100 cells and HER2-overexpressing clone (HER18) of MCF-7 cells were 5kind gift from Prof. Mien-Chie Hung. Lapatinib-resistant clones (Sk/LR6 and Sk/LR9) were selected from SkBr3 cells by culturing the cells in increasing concentrations of lapatinib (by 2 μM every 2–3 weeks, up to a maintenance concentration of 10 μM for 3 months). All cell lines were maintained in Dulbecco's Modified Eagle's Medium/F12 containing 10% fetal bovine serum (GeneDireX), 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Lapatinib-resistant clones were maintained in the presence of 1 μM lapatinib.

Preparation of Cell Extracts

Cells were washed with 1X phosphate buffered saline (PBS) once and harvested with RIPA buffer (50 mM Tris (pH7.5), 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM PMSF, 10 μg/ml Aprotinin) plus protease inhibitors, followed by homogenization with sonication and centrifugation at 21,500 × g for 15 min. Whole cell lysates were stored at -20°C until used for the experiments (Lee et al., 2019a; Lee et al., 2020).

Western Blot and Antibodies

As described previously (Lee et al., 2019b), the concentration of total proteins was determined by Bradford protein assay (Bio-Rad), and protein levels were examined by western blot analysis with specific antibodies. Antibody against p-Pim1 Tyr309 was purchased from Assay biotech. Antibodies against AKT, p-ERK Thr202/Tyr 204 and ERK were purchased from Cell Signaling. Antibodies against Pim1 (12H8), EGFR, HER3 (C-17), and HER4 (C-18) were purchased from Santa Cruz. Antibodies against α-Tubulin, Flag®M2 and β-Actin were purchased from SIGMA. Antibody against HER2 was purchased from EMD Millipore. Relative protein expressions were quantified by using ImageJ software (Wayne Rasband, National Institute of Health, United States). The quantification was shown as the relative amounts of each protein normalization with the loading control, and data were represented for three independent experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

Cells were seeded at a density of 3–4 × 10^3 cells/well in a 96-well plate. The next day, cells were cultured with serum-free medium and treated with Pim inhibitors SMI-4a and SGI-1776 at the concentrations of 0, 0.5, 1, 2, 5, 10, 20 μM for 2 days in three independent experiments. Then, the culture medium was refreshed with 100 μl serum-free medium with 5 mg/ml MTT

solution (Sigma) for 3 h followed by wash with PBS 3 times. The formazan in the cells was solubilized in 100 µl DMSO per well, followed by the measurement of absorbance at 570 nm.

Lentivirus Infection of shRNA

Cells were seeded at a density of 2×10^5 cells/well in a 6-well plate overnight. Cells were infected with lentivirus shRNA using a multiplicity of infection of 125 for 24 h. Then, cells were refreshed with the medium containing 2 µg/ml puromycin for 24 h followed by subsequent experiments.

Plasmid DNA Transfection

Cells were seeded at a density of 4×10^5 cells/well in a 6-well plate. The next day, the cells were transfected with 1 µg plasmid DNA per well for 2 days using TransIT-2020 transfection reagent according to the manufacturer's instruction as described previously (Huang et al., 2013), followed by subsequent experiments.

Cell Migration and Invasion Assays

Cell migration and invasion abilities were examined by *in vitro* transwell assay as described previously (Huang et al., 2016). For migration assay, cells at a density of 2×10^5/well were seeded on the non-coated membrane of the upper chamber. For invasion assay, the membrane of the upper chamber was coated with 1–2 mg/ml Matrigel (BD Biosciences), followed by cell seeding at a density of 2×10^5/well with treatment of SMI-4a at the indicated concentration. After 48 h incubation, cells were washed with 1X PBS once, followed by fixation with 4% formaldehyde for 30 min. Cells were washed with 1X PBS once again, followed by 1% crystal violet staining for 15–30 min at room temperature. Cells remaining on the upper chamber were removed using cotton swab. The number of migrating or invading cells was shown and quantified by counting for five fields/field of view at ×200 magnification.

Clonogenic Assay

HER2-negative and -positive breast cancer cells were seeded at a density of 1×10^3/well in a 24-well plate. The next day, cells were treated with SMI-4a for 14 days. The cells were refreshed with a medium containing SMI-4a every 7 days. 2 weeks later, the cell viability was determined by 1% crystal violet staining (buffered with 30% ethanol).

Reverse-Transcription-Quantitative Polymerase Chain Reaction

Total RNA extraction was performed using Trizol™ reagent (Roche). 1 µg RNA was subjected to reverse transcription using M-MLV reverse transcriptase according to manufacturer's instruction (Sigma). The qPCR analysis was performed on Illumina EcoTM system (Bio-genesis Technologies Inc.) using VeriQuest Fast SYBR Green qPCR Master Mix.

Determination of the Half-Maximal Inhibitory Concentration

IC50 of Pim inhibitors was determined by the following equation: lgIC50 = Xm-I (P- (3-Pm-Pn)/4). Xm: lg maximum dose; I: lg (maximum dose/relative dose); *p*: the sum of the positive reaction rate; Pm: the maximum positive reaction rate; Pn: the minimum positive reaction rate.

Statistical Analysis

Pearson correlation was used to study the correlation between IC50, Pim1, or HER family expression in breast cancer cell lines. All data were displayed as mean ± S.E.M for three independent experiments. The significance of the difference between the experimental and control groups was assessed by Student's t-test. The difference is significant if *p*-value is *< 0.05, **<0.01, ***<0.001.

RESULTS

Human epidermal growth factor receptor 2 Expression Was Strongly Associated With the Expression and Inhibitor Sensitivity of Pim1

It is known that induction of Pim1 was accompanied by increases in EGFR expression (Siu et al., 2011; Cen et al., 2013). To address whether Pim1 regulates HER family expression in breast cancer, we first examined the association between Pim1 and HER family protein expressions using a panel of breast cancer cell lines by western blot (**Figure 1A**). The correlation analysis based on R^2 score revealed that Pim1 protein expression significantly and positively correlated with HER2 and HER3, but not HER4, protein expressions and that the correlation between Pim1 and EGFR expressions approaches marginal significance (**Figure 1B**).

To further address whether the correlation between Pim1 and HER family expressions relies on Pim1 kinase activity, Pim1 kinase inhibitors SMI-4a and SGI-1776 were employed. First, we determined the sensitivity of various breast cancer cell lines to these Pim1 inhibitors in MTT assays and analyzed the correlation of Pim1 protein expression with the IC50 of these two inhibitors. The IC50 of these Pim1 inhibitors in various breast cancer cell lines were listed in **Figure 2A**. Alteration of protein level is one of the factors contributing to oncogenic function and may determine the sensitivity of cancer cells to their inhibitors, and the target-independent cell-killing effect of SGI-1776 has been reported (Lin et al., 2019). Therefore, we first analyzed the correlation of Pim1 protein expression with the IC50 of these two inhibitors. As shown in **Figure 2B**, the IC50 of SMI-4a but not SGI-1776 was inversely associated with Pim1 protein expression, indicating that the specific inhibition of Pim1 by SGI-1776 is not the sole mechanism for its anti-cancer activities. We next analyzed the correlation between HER family protein levels and the IC50 of these two inhibitors. We found that the IC50 of SMI-4a significantly and inversely correlated with EGFR, HER2, and HER3 protein levels while the IC50 of SGI-1776 only significantly and negatively correlated with EGFR and HER2 protein level (**Figure 2C**). Taken together, these results suggest that EGFR and HER2 expressions are strongly associated with Pim1 expression and the sensitivity to Pim1 inhibitors in breast cancer cells.

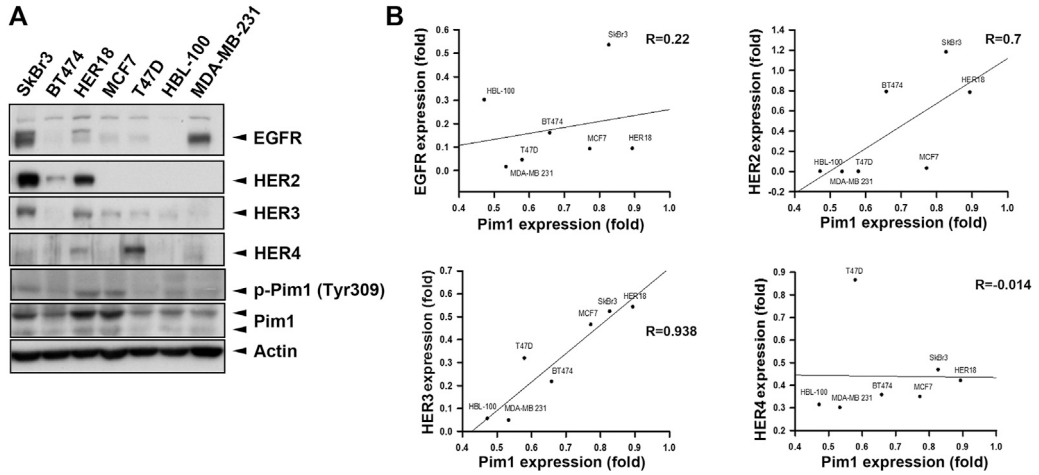

FIGURE 1 | HER2 expression was strongly associated with the Pim1 expression in breast cancer cells. **(A)** Whole cell lysates of breast cancer cells, including SkBr3, BT474, HER18, MCF7, T47D, HBL-100, and MDA-MB-231 cells, were subjected to protein expression analysis in western blot using the indicated antibodies. **(B)** The correlations of Pim1 expression with EGFR, HER2, HER3, and HER4 expression were analyzed by regression analysis based on the results shown in **(A)**.

Human epidermal growth factor receptor 2 -Expressing Breast Cancer Cells Were More Sensitive to Pim1 Inhibitor-Induced Cell Death

Since we observed that HER2 expression was associated with Pim1 inhibitor sensitivity, we further examine whether HER2-positive breast cancers are more sensitive to Pim1 inhibitors. As shown in **Figure 3A**, IC50 values of SMI-4a and SGI-1776 were lower in HER2-positive than in HER2-negative breast cancer cell lines. In order to investigate whether HER2 acts as a determinant for the sensitivity to Pim1 inhibitors, HER2 was overexpressed in different breast cancer cells followed by measuring their sensitivity to SMI-4a. The viabilities of HER2-addicted SkBr3 and BT474 breast cancer cells were suppressed by SMI-4a, and the inhibitory effect was rescued by further increasing HER2 expression in these cell lines (**Figure 3B**). On the other hand, HER2-negative and Pim1 inhibitor-insensitive MCF7 and T47D cells became sensitive to SMI-4a while these cells were transformed to HER2-positive and addicted (HER18 and T47D-HER2) cells in MTT assay (**Figure 3C**). Similar results were also observed in clonogenic assays (**Figure 3D**, **Supplementary Figure S1**). In addition to cell viability, we also examined the effect of the Pim1 inhibitor on cell migration and invasion. As shown in **Figure 4A**, SMI-4a reduced the migration and invasion abilities of HER2-positive SkBr3 breast cancer cells in a dose-dependent manner. The quantitative results of migrated and invaded cell numbers were shown in **Figure 4B**. Collectively, these findings support that HER2 acts as one of the Pim1 downstream effectors and is a critical determinant for the sensitivity of HER2-positive cells to Pim-1 inhibitor. However, the possibility that other downstream effectors of Pim1 mediate the anti-cancer activity of Pim1 inhibitor in HER2-positive breast cancer cells can not be excluded.

Pim1 Inhibitors Suppressed Human epidermal growth factor receptor Family Expression in Human epidermal growth factor receptor 2 -Expressing Breast Cancer Cells

We next investigated the mechanism underlying Pim1 inhibitor-mediated anti-cancer activity in HER2-expressing breast cancer cells. As shown in **Figure 5A**, **Supplementary Figure S2A**, SMI-4a decreased HER2 and p-4E-BP1 protein expression in a dose-dependent manner in SkBr3 cells. The activity of HER2-downstream signaling ERK was also inhibited by SMI-4a. In addition to HER2, EGFR and HER3 protein expressions were attenuated by SMI-4a. Similar results were also observed in another HER2-positive BT474 breast cancer cell line (**Figure 5B**, **Supplementary Figure S2B**). In the RT-qPCR analysis, we found that SMI-4a reduced the mRNA levels of all members of HER family in a dose-dependent manner in both BT474 (**Figure 6A**) and SkBr3 cells (**Figure 6B**). Silence of Pim1 expression with two individual shRNAs also decreased the mRNA expression of HER2 in BT474 cells (**Figure 6C**). Conversely, overexpression of Pim1 also increased HER2 and HER3 expressions in MCF7 cells (**Figure 6D**). These results suggest that Pim1 inhibitors suppressed HER2 expression in HER2-expressing breast cancer cells through the transcriptional level.

Pim1 Inhibitors Overcome Lapatinib Resistance Through Downregulation of HER Family Expression

Lapatinib is a HER2 TKI approved for metastatic HER2-positive breast cancer patients. Development of acquired resistance within one year of treatment limited the clinical benefits of this drug

A

Cell Lines	IC50 of SMI-4a (μM)
SkBr3	14.53
HER18	18.81
BT474	36.04
MCF7	78.01
HBL-100	100.01
T47D	238.21
MDA-MB 231	618.59

Cell Lines	IC50 of SGI-1776 (μM)
SkBr3	3.07
MDA-MB 231	3.49
BT474	5.71
HBL-100	6.65
HER18	7.41
T47D	7.86
MCF7	10.60

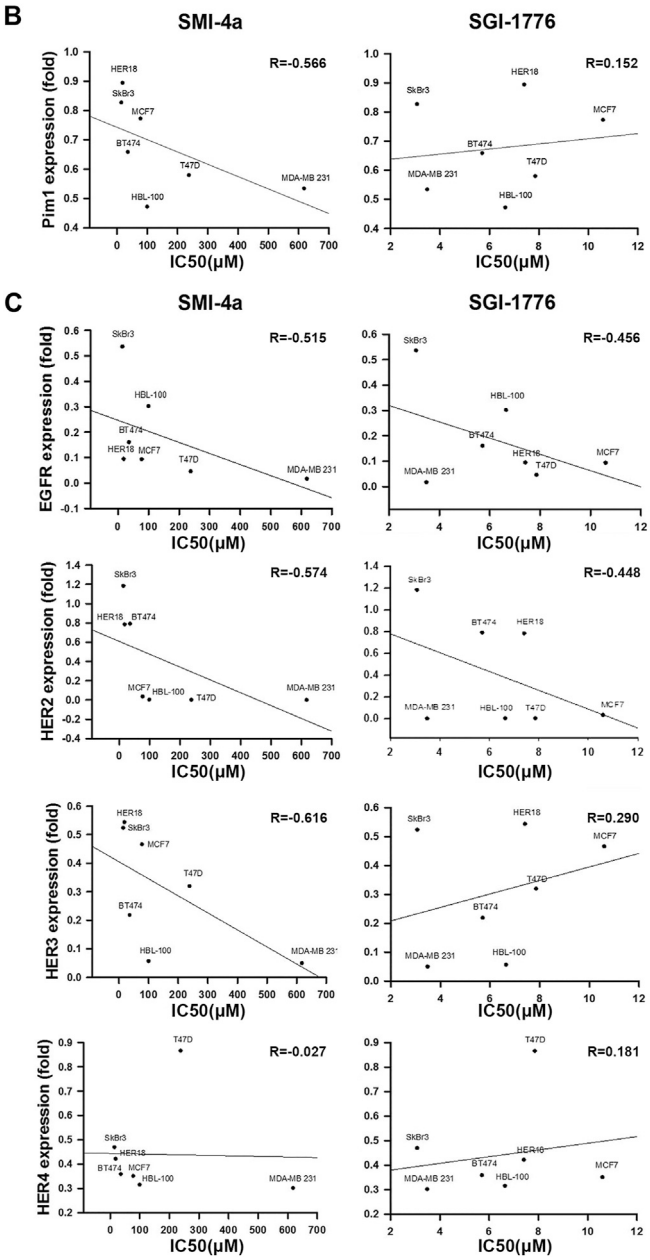

FIGURE 2 | HER2 expression was strongly associated with the sensitivity of breast cancer cells to Pim1 inhibitors**. (A)** The summary table of IC50 of Pim1 inhibitors in various breast cancer cell lines. **(B,C)** The half-maximal inhibitory concentration (IC50) of Pim1 inhibitors, including SMI-4a and SGI-1776 in SkBr3, BT474, HER18, MCF7, T47D, HBL-100, and MDA-MB-231 cells, was determined by MTT assays. The correlations of IC50 of SMI-4a and SGI-1776 with Pim1 **(B)**, EGFR **(C)**, HER2 **(C)**, HER3 **(C)** and HER4 **(C)** expressions were determined by regression analysis.

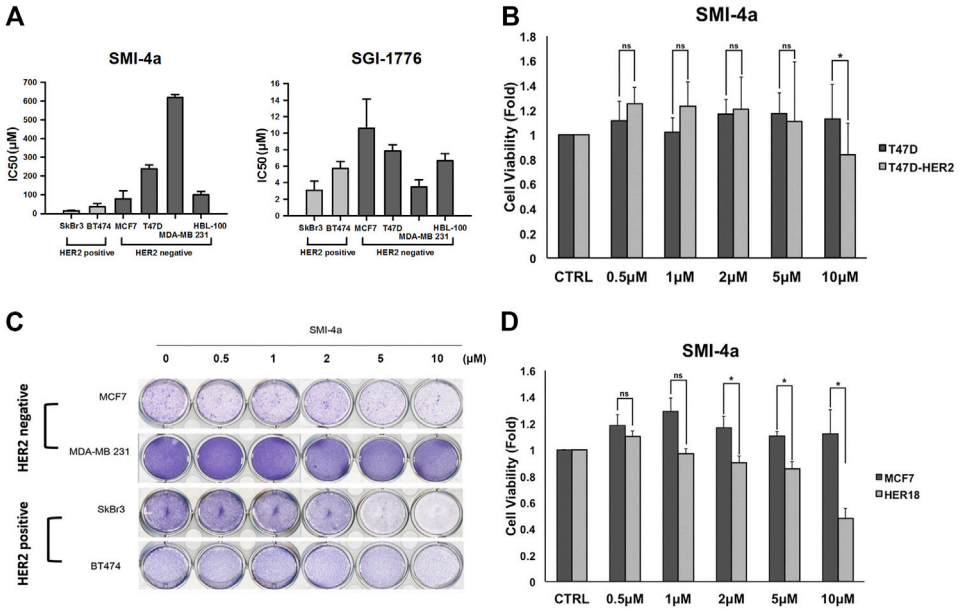

FIGURE 3 | HER2-expressing breast cancer cells were more sensitive to Pim1 inhibitor-induced inhibition of cell viability. **(A)** The IC50 of SMI-4a and SGI-1776 in SkBr3, BT474, MCF7, T47D, HBL-100, and MDA-MB-231 cells was determined by MTT assay. **(B)** HER2 expression vector or empty vector was transiently transfected into HER2-positive SkBr3 or BT474 cells for 2 days, followed by the determination of cell viability in response to SMI-4a in MTT assay. **(C)** The effects of SMI-4a on the cell viabilities of MCF7, HER18, and HER2-transfected T47D cells were determined in MTT assay. Results were expressed as mean ± S.E.M. of three independent experiments. *: $p < 0.05$; **: $p < 0.01$ as compared with control group. **(D)** MCF7, HER18, T47D and T47D-HER2 cells were treated with SMI-4a at 10 μM and subjected to clonogenic assay for 14 days. Cell viability was determined by crystal violet staining.

(D'amato et al., 2015; Shi et al., 2016). HER2 protein, even without tyrosine kinase activity in the presence of lapatinib, still contributes to the viability of lapatinib-resistant cells in a heregulin (HRG) and HER3-dependent manner (Sato et al., 2013). The tumoral Pim1 mRNA expression was higher in lapatinib-treated patients with HER2-positive breast cancers than in the patients without lapatinib treatment in a published gene set (GSE130788) (**Figure 7A**). Since Pim1 upregulates HER family expression, inhibition of HER family expression by Pim1 inhibitor may overcome lapatinib resistance. Interestingly, Sk/LR6 and Sk/LR9 cells, two acquired lapatinib-resistant clones of SkBr3 cells, exhibited higher Pim1 kinase activity as evidenced by the induction of Pim1 phosphorylation at Tyr309 than their parental SkBr3 cells (**Figure 7B**). When Sk/LR6 cells were treated with Pim1 inhibitor SMI-4a, the protein expressions of EGFR, HER2, HER3 as well as p-4E-BP1 were downregulated by Pim-1 inhibition in a dose-dependent manner (**Figure 7C** and **Supplementary Figure S2C, D**). We next examined whether SMI-4a overcomes lapatinib resistance in Sk/LR6 and Sk/LR9 cells. As shown in **Figure 7D**, treatment of SMI-4a, but not lapatinib, obviously inhibited cell viability of Sk/LR6 and Sk/LR9 cells rather than their parental cells. Meanwhile, corresponding blots showed that HER2 expression was suppressed by SMI-4a but not lapatinib in both resistant clones (**Figure 7E**). These results suggest that Pim1 inhibitor suppresses cell viability of lapatinib-resistant cells through reduction of HER2 expression.

DISCUSSION

The members of the HER family are well-known oncogenic driver genes in various cancer types. Although targeting the kinase activity by small molecular inhibitors has shown promising clinical benefits, kinase-independent functions have been proposed to contribute to the development of acquired resistance to these drugs (Zhang et al., 2009; Bhullar et al., 2018). Suppression of the protein expression of these RTKs has been proposed as a potential strategy to overcome the drug resistance (Bonanno et al., 2011; Alexander et al., 2017). In this study, we demonstrated that Pim1 may function as a therapeutic target to downregulate HER2 expression and thereby overcome lapatinib resistance.

Pim1 is a serine/threonine kinase and promotes cell proliferation, survival, and drug resistance. Overexpression of Pim1 has been observed in many cancer types and reported to play a crucial role in tumorigenesis (Narlik-Grassow et al., 2014; Warfel and Kraft, 2015). In previous studies, AKT inhibitor GSK690693 was reported to promote transcriptional induction of Pim1 kinase. Subsequently, Pim1 increased the RTK protein expression, including EGFR, HER2, and HER3 through Cap-independent translation, resulting in the resistance of prostate cancer cells to AKT inhibition (Cen et al., 2013). Moreover, Pim kinase inhibitor M-110 has been shown to reduce the expression of EGFR, leading to lower extracellular signal-regulated kinase (ERK) pathway activity in prostate cancer (Siu et al., 2011). Our results also demonstrated that Pim1 regulates protein expression

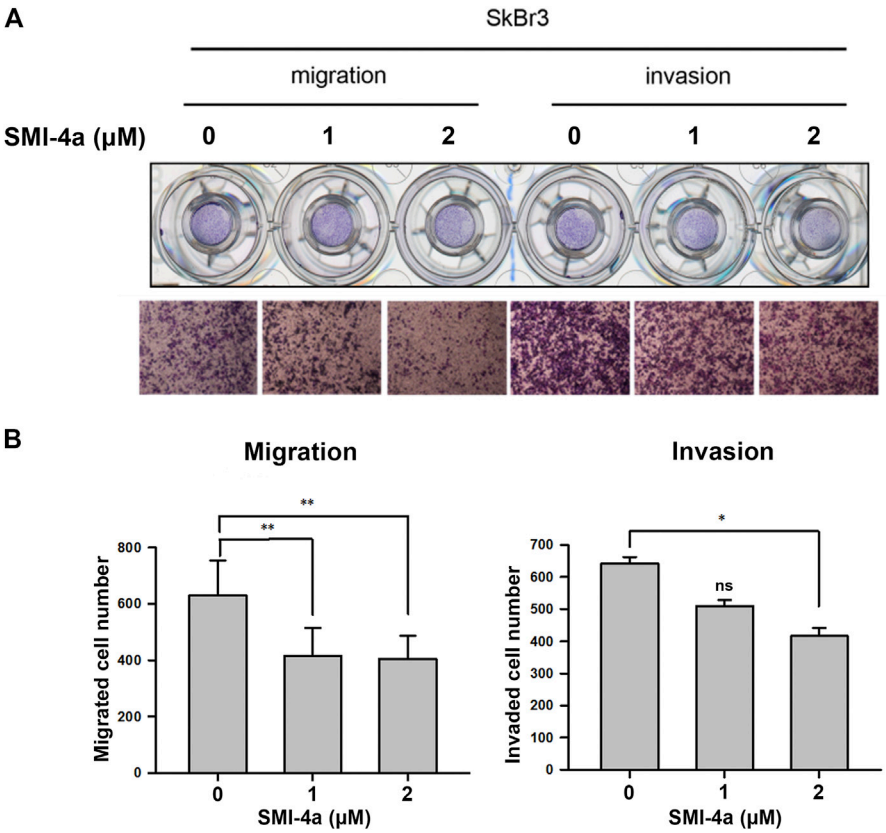

FIGURE 4 | Pim1 inhibitor attenuated cell migration and invasion abilities in HER2-positive SkBr3 cells. **(A,B)** SkBr3 cells were treated with SMI-4a at 0, 1, 2 μM for 2 days and subjected to *in vitro* transwell assay. Cell migration and invasion were observed under microscope and by crystal violet staining **(A)**. The numbers of migrated and invaded cells were calculated and quantified **(B)**. Results were expressed as mean ± S.E.M. of three independent experiments. *: $p < 0.05$; **: $p < 0.01$ as compared with control group.

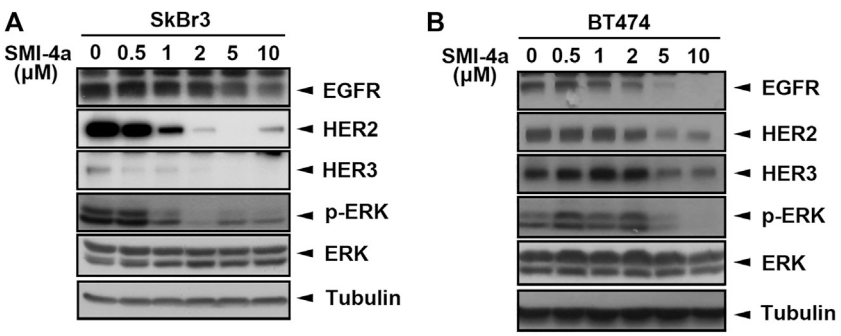

FIGURE 5 | Pim1 inhibitors suppressed HER family expression in HER2-expressing breast cancer cells. **(A,B)** SkBr3 **(A)** and BT474 **(B)** cells were treated with SMI-4a at 0, 0.5, 1, 2, 5, 10 μM, and whole-cell lysates were harvested. Protein expressions were examined by western blot using the indicated antibodies.

of the HER family in breast cancer cells (**Figures 5**, **6**), indicating that a common upregulation of the HER family by Pim-1 in various cancer types. Different to the findings in the previous studies, our data showed that Pim1 regulates the expression of HER family, in particular HER2, at the transcriptional level (**Figure 6**). It is known that Pim1 influences the activity of a number of transcriptional regulators, such as NFATc1, RelA/p65, and c-Myb (Rainio et al., 2002; Winn et al., 2003; Kim et al., 2010). Our previous study indicates that RelA/p65 activation mediates hepatitis B virus X protein-induced HER3 transcription (Chen et al., 2016). Furthermore, transcription factor activator protein-2 (AP-2) was reported to promote EGFR, HER2, and

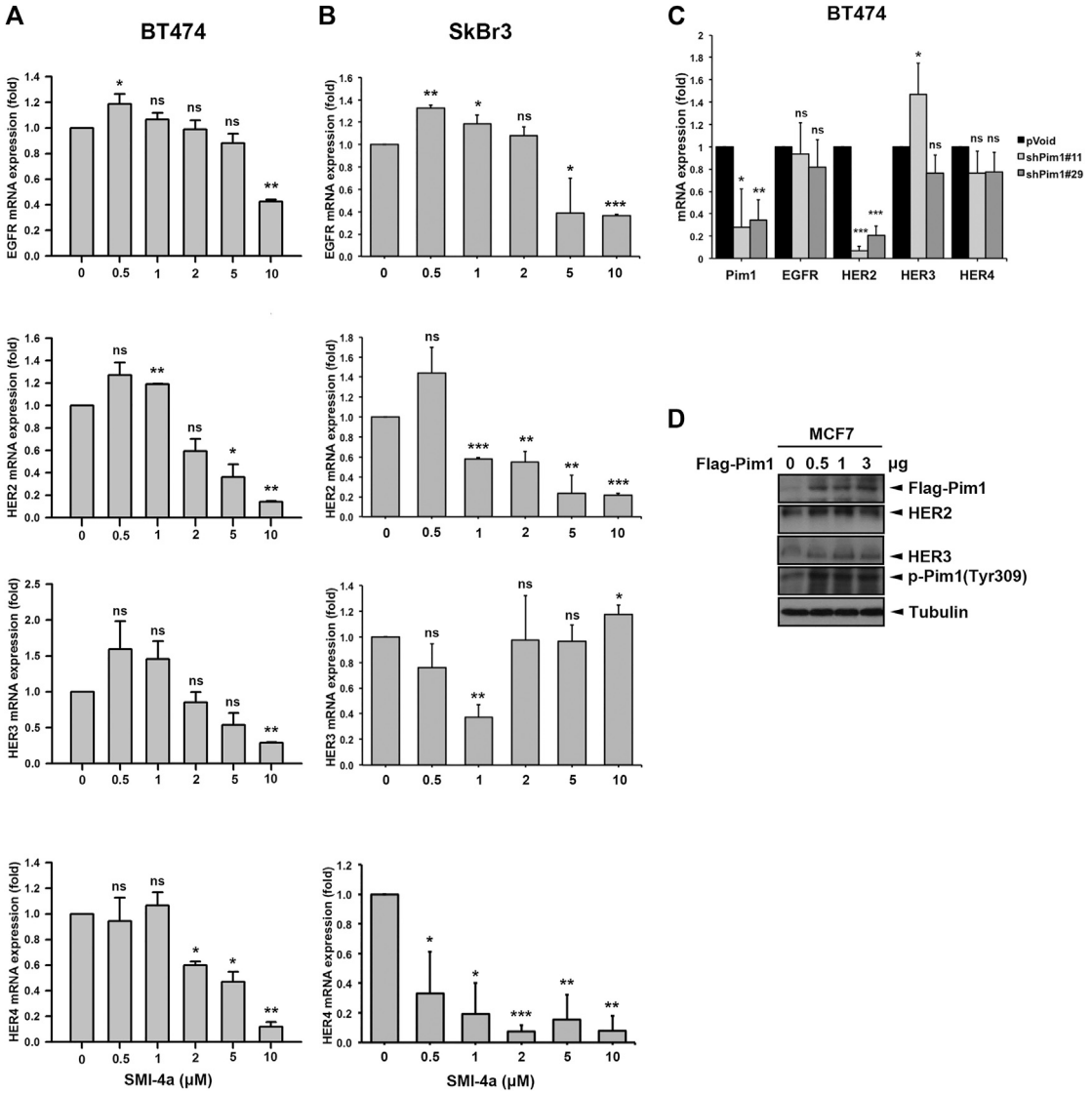

FIGURE 6 | Pim1 transcriptionally upregulated HER2 expression in breast cancer cells. **(A,B)** BT474 **(A)** and SkBr3 **(B)** cells were treated with SMI-4a at 0, 0.5, 1, 2, 5, 10 μM and total RNAs were extracted. The mRNA expressions of the HER family were examined by RT-qPCR followed by normalization with *actin* expression. **(C)** BT474 cells were infected with shPim1#11 or shPim1#18 lentivirus for 2 days followed by total RNA extraction. The mRNA expressions of Pim1 and HER family were examined by RT-qPCR followed by normalization with *actin* expression. **(D)** MCF7 cells were transiently transfected with the indicated concentration of Flag-Pim1 expression vector for 3 days followed by preparation of total lysates. Protein expressions were examined by western blot using the indicated antibodies. Results were expressed as mean ± S.E.M. of three independent experiments. *: $p < 0.05$; **: $p < 0.01$ as compared with control group.

HER3 transcription (Bosher et al., 1995; Johnson, 1996; Bates and Hurst, 1997). Whether RelA/p65 or AP-2 is involved in Pim1-upregulated HER family expression awaits further investigations. In addition to transcriptional control, Pim1 was reported to promote cell cycle progression through induction of p27 phosphorylation and proteasomal degradation (Morishita et al., 2008). Therefore, the potential mechanisms other than transcriptional regulation for Pim1-mediated HER family expression cannot be excluded.

In breast cancer, 20–30% of cases belong to the subgroup of HER2 overexpression, which makes the tumor more aggressive. Therefore, targeted therapy against HER2 activity has been developed and approved for HER2-positive breast cancer (Iqbal and Iqbal, 2014). Although these drugs indeed showed clinical benefits to HER2-positive breast cancer patients, acquired resistance is developed eventually and becomes a hurdle to be overcome (Nahta et al., 2009; Rexer and Arteaga, 2012; D'amato et al., 2015; Pernas and Tolaney, 2019). To date, several mechanisms are proposed for lapatinib resistance. Upregulation of HRG has been observed in lapatinib-resistant cells to confer lapatinib resistance through HER3 and AKT activation, which depends on residual HER2 expression (Sato et al., 2013). In addition to its ligand upregulation, protein expression and phosphorylation of HER3 are induced by

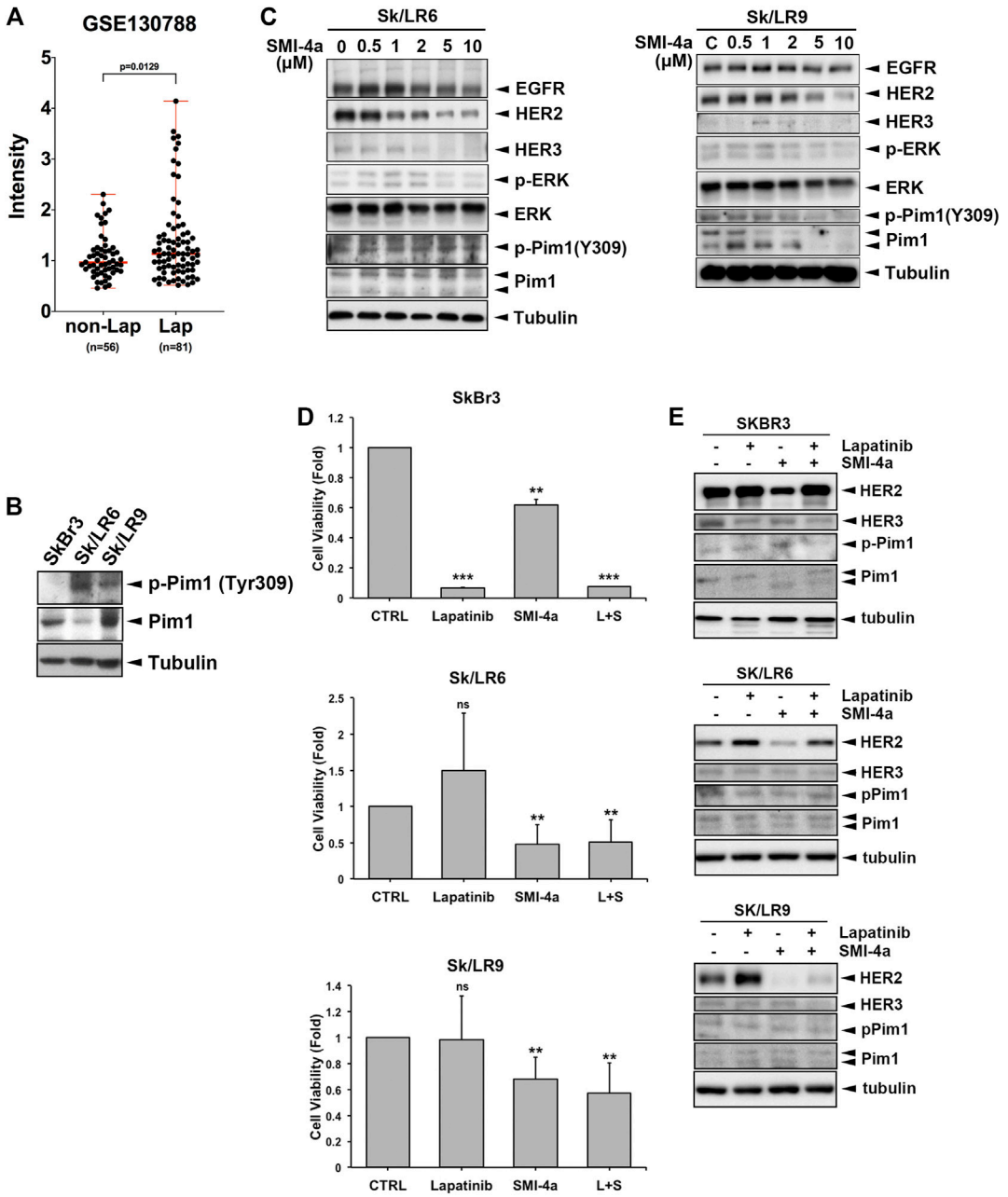

FIGURE 7 | Pim1 inhibitor suppressed HER family expression and cell viability in lapatinib-resistant breast cancer cells. **(A)** The level of Pim1 mRNA in HER2-positive breast cancer patients who were treated with or without lapatinib in GEO database (GSE130788). **(B)** Total lysates of SkBr3 and its derived lapatinib-resistant Sk/LR6 and Sk/LR9 cells were harvested. Protein expressions were examined by western blot using indicated antibodies. **(C)** Sk/LR6 and Sk/LR9 cells were treated with SMI-4a at 0, 0.5, 1, 2, 5, 10 μM and whole-cell lysates were harvested. Protein expressions were examined by western blot using indicated antibodies. **(D)** SkBr3, Sk/LR6 and Sk/LR9 cells were treated with lapatinib (1 μM), SMI-4a (10 μM), the combination of lapatinib and SMI-4a, respectively for 3 days. Cell viability was examined by MTT assay. L + S: the combination of lapatinib and SMI-4a. Results were expressed as mean ± S.E.M. of three independent experiments. **: $p < 0.01$; ***: $p < 0.001$ as compared with control group. **(E)** SkBr3, Sk/LR6, and Sk/LR9 cells were treated with lapatinib (1 μM), SMI-4a (10 μM), the combination of lapatinib and SMI-4a, respectively for 3 days and whole-cell lysates were harvested. Protein expressions were examined by western blot using indicated antibodies.

lapatinib. Phosphorylated HER3 is able to interact with the p85 subunit of PI3K to activate AKT signaling. Upregulated HER3 interacts with other RTK, such as MET, to maintain survival signaling (Sergina et al., 2007; Garrett et al., 2011; Chen et al., 2012). These events limit the therapeutic efficacy of lapatinib. Furthermore, HER2 T798I and EGFR T790M mutations have also been proposed to mediate lapatinib resistance (Trowe et al., 2008). On the other hand, accumulated evidence has revealed that

EGFR promotes cancer cell survival through tyrosine kinase activity-independent mechanisms (Weihua et al., 2008; Tan et al., 2015; Tsuchihashi et al., 2016). Even its kinase activity is inhibited by lapatinib, EGFR still can confer survival signal in cancer cells. Therefore, targeting protein expression of the HER family rather than only its kinase activity may be an effective way for HER2-positive breast cancer cells. Indeed, our results showed that Pim1 inhibitors overcome lapatinib resistance by suppressing protein levels of the HER family (**Figure 7**). In addition, long-term treatment with lapatinib may switch oncogene addiction to the Pim1-regulated pathway, resulting in a stronger viability inhibition by SMI-4a in lapatinib-resistant clones. Moreover, these findings imply the existence of non-tyrosine phosphorylation-dependent functions of HER2, which may cause the drug resistance to lapatinib and need to be explored in further studies. In conclusion, our study indicates that downregulation of HER2 by targeting Pim1 may be a promising and effective therapeutic approach for HER2-positive breast cancer cells and for circumventing lapatinib resistance.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

Study concepts: WH, YJC, and YC; Study design: WH, CH and LL; Data acquisition: BW, FC, YW, YL, YFW and CW; Quality control of data and algorithms: CH, LL and YL; Data analysis and interpretation: BW, FC, YW, YL, YFW, YJC and YC; Statistical analysis: BW, YW, and CW; Manuscript preparation: WH, BW, CH, and LL; Manuscript editing: CW and YJC; Manuscript review: YC and WH. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.614673/full#supplementary-material

REFERENCES

Ahmed, S., Sami, A., and Xiang, J. (2015). HER2-directed Therapy: Current Treatment Options for HER2-Positive Breast Cancer. *Breast Cancer* 22, 101–116. doi:10.1007/s12282-015-0587-x

Alexander, P. B., Chen, R., Gong, C., Yuan, L., Jasper, J. S., Ding, Y., et al. (2017). Distinct Receptor Tyrosine Kinase Subsets Mediate Anti-HER2 Drug Resistance in Breast Cancer. *J. Biol. Chem.* 292, 748–759. doi:10.1074/jbc.m116.754960

Barros, F. F. T., Powe, D. G., Ellis, I. O., and Green, A. R. (2010). Understanding the HER Family in Breast Cancer: Interaction with Ligands, Dimerization and Treatments. *Histopathology* 56, 560–572. doi:10.1111/j.1365-2559.2010.03494.x

Bates, N. P., and Hurst, H. C. (1997). Transcriptional Regulation of Type I Receptor Tyrosine Kinases in the Mammary Gland. *J. Mammary Gland Biol. Neoplasia* 2, 153–163. doi:10.1023/a:1026303814855

Bhullar, K. S., Lagaron, N. O., Mcgowan, E. M., Parmar, I., Jha, A., Hubbard, B. P., et al. (2018). Kinase-targeted Cancer Therapies: Progress, Challenges and Future Directions. *Mol. Cancer* 17, 48. doi:10.1186/s12943-018-0804-2

Bonanno, L., Jirillo, A., and Favaretto, A. (2011). Mechanisms of Acquired Resistance to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors and New Therapeutic Perspectives in Non Small Cell Lung Cancer. *Cdt* 12, 922–933. doi:10.2174/138945011795528958

Bosher, J. M., Williams, T., and Hurst, H. C. (1995). The Developmentally Regulated Transcription Factor AP-2 Is Involved in C-erbB-2 Overexpression in Human Mammary Carcinoma. *Proc. Natl. Acad. Sci.* 92, 744–747. doi:10.1073/pnas.92.3.744

Brandes, A. A., Franceschi, E., Tosoni, A., and Degli Esposti, R. (2010). Trastuzumab and Lapatinib beyond Trastuzumab Progression for Metastatic Breast Cancer: Strategies and Pitfalls. *Expert Rev. Anticancer Ther.* 10, 179–184. doi:10.1586/era.09.156

Cen, B., Mahajan, S., Wang, W., and Kraft, A. S. (2013). Elevation of Receptor Tyrosine Kinases by Small Molecule AKT Inhibitors in Prostate Cancer Is Mediated by Pim-1. *Cancer Res.* 73, 3402–3411. doi:10.1158/0008-5472.can-12-4619

Chen, C.-T., Kim, H., Liska, D., Gao, S., Christensen, J. G., and Weiser, M. R. (2012). MET Activation Mediates Resistance to Lapatinib Inhibition of HER2-Amplified Gastric Cancer Cells. *Mol. Cancer Ther.* 11, 660–669. doi:10.1158/1535-7163.mct-11-0754

Chen, J.-Y., Chen, Y.-J., Yen, C.-J., Chen, W.-S., and Huang, W.-C. (2016). HBx Sensitizes Hepatocellular Carcinoma Cells to Lapatinib by Up-Regulating ErbB3. *Oncotarget* 7, 473–489. doi:10.18632/oncotarget.6337

D'amato, V., Raimondo, L., Formisano, L., Giuliano, M., De Placido, S., Rosa, R., et al. (2015). Mechanisms of Lapatinib Resistance in HER2-Driven Breast Cancer. *Cancer Treat. Rev.* 41, 877–883. doi:10.1016/j.ctrv.2015.08.001

Fahad Ullah, M. (2019). Breast Cancer: Current Perspectives on the Disease Status. *Adv. Exp. Med. Biol.* 1152, 51–64. doi:10.1007/978-3-030-20301-6_4

Garrett, J. T., Olivares, M. G., Rinehart, C., Granja-Ingram, N. D., Sanchez, V., Chakrabarty, A., et al. (2011). Transcriptional and Posttranslational Up-Regulation of HER3 (ErbB3) Compensates for Inhibition of the HER2 Tyrosine Kinase. *Proc. Natl. Acad. Sci.* 108, 5021–5026. doi:10.1073/pnas.1016140108

Hicks, M., Macrae, E. R., Abdel-Rasoul, M., Layman, R., Friedman, S., Querry, J., et al. (2015). Neoadjuvant Dual HER2-Targeted Therapy with Lapatinib and Trastuzumab Improves Pathologic Complete Response in Patients with Early Stage HER2-Positive Breast Cancer: A Meta-Analysis of Randomized Prospective Clinical Trials. *The Oncologist* 20, 337–343. doi:10.1634/theoncologist.2014-0334

Holder, S., and Abdulkadir, S. (2014). PIM1 Kinase as a Target in Prostate Cancer: Roles in Tumorigenesis, Castration Resistance, and Docetaxel Resistance. *Ccdt* 14, 105–114. doi:10.2174/1568009613666131126113854

Hsu, J. L., and Hung, M.-C. (2016). The Role of HER2, EGFR, and Other Receptor Tyrosine Kinases in Breast Cancer. *Cancer Metastasis Rev.* 35, 575–588. doi:10.1007/s10555-016-9649-6

Huang, W.-C., Hung, C.-M., Wei, C.-T., Chen, T.-M., Chien, P.-H., Pan, H.-L., et al. (2016). Interleukin-6 Expression Contributes to Lapatinib Resistance through Maintenance of Stemness Property in HER2-Positive Breast Cancer Cells. *Oncotarget* 7, 62352–62363. doi:10.18632/oncotarget.11471

Huang, W. C., Hsieh, Y. L., Hung, C. M., Chien, P. H., Chien, Y. F., Chen, L. C., et al. (2013). BCRP/ABCG2 Inhibition Sensitizes Hepatocellular Carcinoma Cells to Sorafenib. *PLoS One* 8, e83627. doi:10.1371/journal.pone.0083627

Iqbal, N., and Iqbal, N. (2014). Human Epidermal Growth Factor Receptor 2 (HER2) in Cancers: Overexpression and Therapeutic Implications. *Mol. Biol. Int.* 2014, 852748. doi:10.1155/2014/852748

Johnson, A. C. (1996). Activation of Epidermal Growth Factor Receptor Gene Transcription by Phorbol 12-myristate 13-acetate Is Mediated by Activator Protein 2. *J. Biol. Chem.* 271, 3033–3038. doi:10.1016/s0021-9258(18)97974-3

Kim, K., Kim, J. H., Youn, B. U., Jin, H. M., and Kim, N. (2010). Pim-1 Regulates RANKL-Induced Osteoclastogenesis via NF-Kb Activation and NFATc1 Induction. *J.I.* 185, 7460–7466. doi:10.4049/jimmunol.1000885

Lee, H.-P., Chen, P.-C., Wang, S.-W., Fong, Y.-C., Tsai, C.-H., Tsai, F.-J., et al. (2019a). Plumbagin Suppresses Endothelial Progenitor Cell-Related Angiogenesis *In Vitro* and *In Vivo*. *J. Funct. Foods* 52, 537–544. doi:10.1016/j.jff.2018.11.040

Lee, H.-P., Wang, S.-W., Wu, Y.-C., Lin, L.-W., Tsai, F.-J., Yang, J.-S., et al. (2020). Soya-cerebroside Inhibits VEGF-Facilitated Angiogenesis in Endothelial Progenitor Cells. *Food Agric. Immunol.* 31, 193–204. doi:10.1080/09540105.2020.1713055

Lee, H.-P., Wang, S.-W., Wu, Y.-C., Tsai, C.-H., Tsai, F.-J., Chung, J.-G., et al. (2019b). Glucocerebroside Reduces Endothelial Progenitor Cell-Induced Angiogenesis. *Food Agric. Immunol.* 30, 1033–1045. doi:10.1080/09540105.2019.1660623

Lin, A., Giuliano, C. J., Palladino, A., John, K. M., Abramowicz, C., Yuan, M. L., et al. (2019). Off-target Toxicity Is a Common Mechanism of Action of Cancer Drugs Undergoing Clinical Trials. *Sci. Transl Med.* 11, eaaw8412. doi:10.1126/scitranslmed.aaw8412

Luszczak, S., Kumar, C., Sathyadevan, V. K., Simpson, B. S., Gately, K. A., Whitaker, H. C., et al. (2020). PIM Kinase Inhibition: Co-targeted Therapeutic Approaches in Prostate Cancer. *Signal. Transduct Target. Ther.* 5, 7. doi:10.1038/s41392-020-0109-y

Morishita, D., Katayama, R., Sekimizu, K., Tsuruo, T., and Fujita, N. (2008). Pim Kinases Promote Cell Cycle Progression by Phosphorylating and Down-Regulating p27Kip1 at the Transcriptional and Posttranscriptional Levels. *Cancer Res.* 68, 5076–5085. doi:10.1158/0008-5472.can-08-0634

Nahta, R., Shabaya, S., Ozbay, T., and Rowe, D. (2009). Personalizing HER2-Targeted Therapy in Metastatic Breast Cancer beyond HER2 Status: what We Have Learned from Clinical Specimens. *Cppm* 7, 263–274. doi:10.2174/187569209790112337

Nami, B., and Wang, Z. (2017). HER2 in Breast Cancer Stemness: A Negative Feedback Loop towards Trastuzumab Resistance. *Cancers (Basel)* 9, 40. doi:10.3390/cancers9050040

Narlik-Grassow, M., Blanco-Aparicio, C., and Carnero, A. (2014). The PIM Family of Serine/threonine Kinases in Cancer. *Med. Res. Rev.* 34, 136–159. doi:10.1002/med.21284

Ouhtit, A., Muzumdar, S., Gupta, I., Shanmuganathan, S., and Tamimi, Y. (2015). Understanding the Functional Discrepancy of Pim-1 in Cancer. *Front. Biosci. (Elite Ed.* 7, 208–214. doi:10.2741/728

Pernas, S., and Tolaney, S. M. (2019). HER2-positive Breast Cancer: New Therapeutic Frontiers and Overcoming Resistance. *Ther. Adv. Med. Oncol.* 11, 1758835919833519. doi:10.1177/1758835919833519

Raica, M., Jung, I., Cimpean, A. M., Suciu, C., and Muresan, A. M. (2009). From Conventional Pathologic Diagnosis to the Molecular Classification of Breast Carcinoma: Are We Ready for the Change? *Rom. J. Morphol. Embryol.* 50, 5–13.

Rainio, E.-M., Sandholm, J., and Koskinen, P. J. (2002). Cutting Edge: Transcriptional Activity of NFATc1 Is Enhanced by the Pim-1 Kinase. *J. Immunol.* 168, 1524–1527. doi:10.4049/jimmunol.168.4.1524

Rexer, B. N., and Arteaga, C. L. (2012). Intrinsic and Acquired Resistance to HER2-Targeted Therapies in HER2 Gene-Amplified Breast Cancer: Mechanisms and Clinical Implications. *Crit. Rev. Oncog* 17, 1–16. doi:10.1615/critrevoncog.v17.i1.20

Riese, D. J., 2nd, and Stern, D. F. (1998). Specificity within the EGF family/ErbB Receptor Family Signaling Network. *Bioessays* 20, 41–48. doi:10.1002/(sici)1521-1878(199801)20:1<41::aid-bies7>3.0.co;2-v

Santa-Maria, C. A., Nye, L., Mutonga, M. B., Jain, S., and Gradishar, W. J. (2016). Management of Metastatic HER2-Positive Breast Cancer: Where Are We and where Do We Go from Here? *Oncology (Williston Park)* 30, 148–155.

Sato, Y., Yashiro, M., and Takakura, N. (2013). Heregulin Induces Resistance to Lapatinib-Mediated Growth Inhibition of HER2-Amplified Cancer Cells. *Cancer Sci.* 104, 1618–1625. doi:10.1111/cas.12290

Sergina, N. V., Rausch, M., Wang, D., Blair, J., Hann, B., Shokat, K. M., et al. (2007). Escape from HER-Family Tyrosine Kinase Inhibitor Therapy by the Kinase-Inactive HER3. *Nature* 445, 437–441. doi:10.1038/nature05474

Shi, H., Zhang, W., Zhi, Q., and Jiang, M. (2016). Lapatinib Resistance in HER2+ Cancers: Latest Findings and New Concepts on Molecular Mechanisms. *Tumour Biol.* 37, 15411–15431. doi:10.1007/s13277-016-5467-2

Siu, A., Virtanen, C., and Jongstra, J. (2011). PIM Kinase Isoform Specific Regulation of MIG6 Expression and EGFR Signaling in Prostate Cancer Cells. *Oncotarget* 2, 1134–1144. doi:10.18632/oncotarget.386

Tan, X., Thapa, N., Sun, Y., and Anderson, R. A. (2015). A Kinase-independent Role for EGF Receptor in Autophagy Initiation. *Cell* 160, 145–160. doi:10.1016/j.cell.2014.12.006

Trowe, T., Boukouvala, S., Calkins, K., Cutler, R. E., Jr., Fong, R., Funke, R., et al. (2008). EXEL-7647 Inhibits Mutant Forms of ErbB2 Associated with Lapatinib Resistance and Neoplastic Transformation. *Clin. Cancer Res.* 14, 2465–2475. doi:10.1158/1078-0432.ccr-07-4367

Tsuchihashi, K., Okazaki, S., Ohmura, M., Ishikawa, M., Sampetrean, O., Onishi, N., et al. (2016). The EGF Receptor Promotes the Malignant Potential of Glioma by Regulating Amino Acid Transport System Xc(-). *Cancer Res.* 76, 2954–2963. doi:10.1158/0008-5472.can-15-2121

Wang, Z. (2017). ErbB Receptors and Cancer. *Methods Mol. Biol.* 1652, 3–35. doi:10.1007/978-1-4939-7219-7_1

Warfel, N. A., and Kraft, A. S. (2015). PIM Kinase (And Akt) Biology and Signaling in Tumors. *Pharmacol. Ther.* 151, 41–49. doi:10.1016/j.pharmthera.2015.03.001

Weihua, Z., Tsan, R., Huang, W.-C., Wu, Q., Chiu, C.-H., Fidler, I. J., et al. (2008). Survival of Cancer Cells Is Maintained by EGFR Independent of its Kinase Activity. *Cancer Cell* 13, 385–393. doi:10.1016/j.ccr.2008.03.015

Winn, L. M., Lei, W., and Ness, S. A. (2003). Pim-1 Phosphorylates the DNA Binding Domain of C-Myb. *Cell Cycle* 2, 258–262. doi:10.4161/cc.2.3.383

Zhang, J., Yang, P. L., and Gray, N. S. (2009). Targeting Cancer with Small Molecule Kinase Inhibitors. *Nat. Rev. Cancer* 9, 28–39. doi:10.1038/nrc2559

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