

Elevated estrogen receptor β expression in triple negative breast cancer cells is associated with sensitivity to doxorubicin by inhibiting the PI3K/AKT/mTOR signaling pathway

SHANSHAN LEI^{1,2*}, PEIZHI FAN^{2*}, MENGCHUAN WANG¹, CHAOJIE ZHANG²,
YU JIANG³, SHULIN HUANG², MENG FANG², ZILI HE⁴ and AIGUO WU¹

¹Department of General Surgery, Zhujiang Hospital (The Second School of Clinical Medicine),

Southern Medical University, Guangzhou, Guangdong 510282; ²Department of Breast and Thyroid Surgery;

³Hunan Provincial Key Laboratory of Emergency and Critical Care Metabonomics, Institute of Emergency Medicine;

⁴Laboratory of Hepatobiliary Molecular Oncology, Department of Hepatobiliary Surgery, Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University), Changsha, Hunan 410005, P.R. China

Received June 24, 2019; Accepted March 19, 2020

DOI: 10.3892/etm.2020.8809

Abstract. Based on its pathological characteristics, breast cancer is a highly heterogeneous disease. Triple negative breast cancer (TNBC) is an aggressive subtype, and due to a lack of effective therapeutic targets, patients with TNBC do not significantly benefit from endocrine or anti-HER2 therapy. Conventional chemotherapy has been regarded as the only systemic therapy option for TNBC, but its therapeutic efficacy remains limited. Estrogen receptor β (ER β) has been identified as a tumor suppressor in TNBC. Therefore, the aim of the present study was to identify the role of ER β in regulating the response to chemotherapy, and to investigate its underlying mechanism in TNBC. MDA-MB-231 and BT549 cells were treated with doxorubicin (DOX), liquiritigenin [Liq, (Chengdu Biopurify Phytochemicals, Ltd.); a specific ER β agonist], or a combination of DOX and Liq *in vitro*. The effects of various treatments on cell viability and proliferation were measured using the Cell Counting Kit-8 and colony-formation assays, respectively. MDA-MB-231 and ER β knockdown (ER β -KD) MDA-MB-231 cells were selected for the establishment of ER α -/ER β + and ER α -/ER β - cell models, respectively. The two cell models were treated with DOX, Liq or a combination of DOX and Liq. The effects of the treatment on the PI3K/AKT/mTOR signaling pathway were evaluated by

assessing the protein expression levels of AKT and mTOR using western blot analysis. Low Liq concentrations increased the sensitivity of MDA-MB-231 and BT549 cells to DOX. Moreover, the synergistic effect of Liq and DOX treatment was associated with the inhibition of the PI3K/AKT/mTOR signaling pathway in MDA-MB-231 cells, and the effect was ER β -dependent. The results suggested that elevated ER β expression was associated with sensitivity to doxorubicin by inhibiting the PI3K/AKT/mTOR signaling pathway; therefore, the combined use of conventional chemotherapeutic drugs with ER β agonists may serve as an effective therapy for TNBC.

Introduction

Breast cancer is the most common malignant primary tumor in women worldwide, and the incidence is continually on the increase (1). Triple negative breast cancer (TNBC), which accounts for 10-20% of newly diagnosed cases of breast cancer (1,2), is defined by the absence of estrogen receptor (ER) α , progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression. Based on the pathological features, TNBC is an aggressive subtype with a poor prognosis, due to a high rate of early recurrence and distant metastasis (3). The poor prognosis is due to the lack of efficacy of the current systemic therapies, including endocrine-based and HER2-targeted therapies (4). Conventional chemotherapy is the standard strategy for the systemic treatment of advanced TNBC; however, the therapeutic efficacy in TNBC is not satisfactory. It has been reported that 34% of patients with newly diagnosed TNBC will undergo recurrence within five years, following adjuvant or neoadjuvant chemotherapy (5). Therefore, combination therapies that enhance the sensitivity and improve the tolerance of chemotherapy are required for the effective treatment of TNBC.

ER α is a major determinant in classifying the various subtypes of breast cancer, and is also an indicator of endocrine therapy. The role of ER α in breast cancer has been clearly demonstrated (6). By contrast, ER β , another estrogen receptor

Correspondence to: Dr Aiguo Wu, Department of General Surgery, Zhujiang Hospital (The Second School of Clinical Medicine), Southern Medical University, 253 Middle Industrial Avenue, Guangzhou, Guangdong 510282, P.R. China
E-mail: wagtyz@sina.com

*Contributed equally

Key words: estrogen receptor β , liquiritigenin, triple negative breast cancer, PI3K/AKT/mTOR

subtype, is not well characterized. It has been reported that ER β , which is expressed in 30% of TNBC cases (7), is a key regulator of signal transduction and tumor suppression in breast cancer (8). Furthermore, ER β displays an antiproliferative role in TNBC (9). Patients with ER β -positive TNBC displayed an improved 5-year survival rate compared with patients with ER β -negative TNBC (10). However, research into the role of ER β during TNBC has primarily focused on endocrine therapy, and little has been reported regarding the role and therapeutic value of ER β in chemotherapy. A large-scale retrospective study reported that the upregulation of ER β 1 (the fully functional isoform of ER β) predicted an improved prognosis for patients with TNBC. In the study, 508 out of 571 (89.0%) patients with TNBC were successfully treated with adjuvant chemotherapy (11). However, whether the status of ER β in TNBC is associated with the response to chemotherapy requires further investigation. Therefore, it is important to identify the role of ER β in regulating the response to chemotherapy and its underlying mechanisms in TNBC.

In the present study, the inhibitory effects of doxorubicin and a combination therapy [doxorubicin and liquiritigenin (Liq)] on ER β -positive TNBC MDA-MB-231 and BT549 cell lines were investigated *in vitro*. The results suggested that upregulated ER β expression in TNBC cells was associated with improved sensitivity to doxorubicin by inhibiting the PI3K/AKT/mTOR signaling pathway.

Materials and methods

Cells and reagents. TNBC MDA-MB-231 and BT549 cell lines were purchased from the American Type Culture Collection. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Sigma-Aldrich; Merck KGaA). Liq, an ER β agonist, was purchased from Chengdu Biopurify Phytochemicals, Ltd.

Cell viability assay. To analyze the effects of different treatments on the viability of MDA-MB-231 and BT549 cells, a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.) was performed according to the manufacturer's protocol. Cells (5×10^3 cells/well) were plated in 96-well plates at 37°C for 24 h. Subsequently, various agents, Liq (0-160 μ g/ml, Chengdu Biopurify Phytochemicals, Ltd.) alone, doxorubicin (DOX; 0-4 ng/ml, Sigma-Aldrich; Merck KGaA) alone or a combination of DOX (0-4 ng/ml) and Liq (40 μ g/ml) were added to each well, whilst the control cells were treated with DMSO (Sigma-Aldrich; Merck KGaA). Following a 48-h incubation at 37°C, 10 μ l CCK-8 reagent was added to each well and incubated for a further 2 h at 37°C. The absorbance of each well was measured at a wavelength of 450 nm using a multi-mode microplate reader (ELx800; Bio-Tek China). The IC₅₀ of DOX and Liq was calculated using SPSS software (version 17.0; SPSS, Inc.). Assays were performed in triplicate.

Lentivirus production and cell infection. shRNA targeting ER β or negative control (NC) scramble sequence were sub-cloned into the GV112 vector (Shanghai GeneChem Co., Ltd.), respectively.

The shRNA sequences were designed by Shanghai GeneChem Co., Ltd. (shER β , 5'-GCTGAATGCCACGTGCTT-3'; shNC; 5'-TTCTCCGAACGTGTACGT-3'). For the production of lentivirus, the expression vectors (20 μ g) were co-transfected with packaging plasmid pHelper 1.0 vector (15 μ g) and envelope plasmid pHelper 2.0 vector (10 μ g; Shanghai Genechem Co., Ltd.) into 293T cells using TransIT[®]-LT1 (Mirus Bio, LLC). The supernatant was collected 72 h after transfection, concentrated by ultracentrifugation at 60,000 x g for 90 min at 4°C and resuspended with OptiMEM (Gibco; Thermo Fisher Scientific, Inc.). MDA-MB-231 cells (2×10^4 cells/well) were cultured in 12-well plates for 24 h at 37°C before transduction. The shER β or shNC lentivirus particles (multiplicity of infection, 10) were respectively added into the medium. After 24 h at 37°C, the culture medium was removed and replaced with complete medium (Gibco; Thermo Fisher Scientific, Inc.) containing puromycin. The cells were incubated for 7 days at 37°C to obtain stable ER β knockdown (ER β -KD)-MDA-MB-231 cells (an ER α -/ER β - cell model). Subsequently, the ER β -KD-MDA-MB-231 cells were divided into three groups for subsequent experiments. Western blot analysis was used to assess the efficiency of transduction.

Colony formation assay. Colony formation assays were performed to evaluate the effect of the different treatments on cell proliferation. MDA-MB-231 and ER β -KD-MDA-MB-231 cells were selected. Single cell suspensions were prepared using 0.25% trypsin at 37°C for 30 sec. Subsequently, cells at a density of 1×10^3 cells/ml were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 40 μ g/ml Liq, 1 ng/ml DOX or 1 ng/ml DOX and 40 μ g/ml Liq. Cells were incubated at 37°C with 5% CO₂ for 10-14 days until macroscopic clones appeared. The colonies were fixed with 2% paraformaldehyde at room temperature for 15 min, and stained with 0.1% Giemsa (AppliChem GmbH) at room temperature for 30 min. Colonies containing >50 cells were counted using an inverted light microscope (magnification, x100). The assay was performed in triplicate.

Western blotting. Western blotting was used to evaluate the protein expression levels of ER β , AKT and mTOR. MDA-MB-231 and ER β -KD-MDA-MB-231 cells were washed twice with PBS and lysed using a protein gel buffer (60 mM Tris-HCl, 10% SDS and 10% glycerol) supplemented with 1 mM phenylmethanesulfonyl fluoride for 20 min at 4°C. Cell lysates were centrifuged at 14,000 x g for 10 min at 4°C and the supernatants were collected. Protein concentration was quantified using a Nanodrop nd-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Protein (20 μ g) was resolved by 12% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature. Subsequently, the membranes were incubated for 24 h at 4°C with primary antibodies targeted against: Phosphorylated (p)-mTOR (cat. no. 2974; 1:1,000; Cell Signaling Technology, Inc.), mTOR (cat. no. 2983; 1:1,000; Cell Signaling Technology, Inc.), ER β (cat. no. sc-8974; 1:2,000; Santa Cruz Biotechnology, Inc.), β -actin (cat. no. 4970; 1:2,000; Cell Signaling Technology, Inc.), phosphorylated (p)-AKT (cat. no. 4060; 1:2,000; Cell Signaling Technology, Inc.) and total AKT (cat. no. 4691; 1:2,000; Cell Signaling Technology,

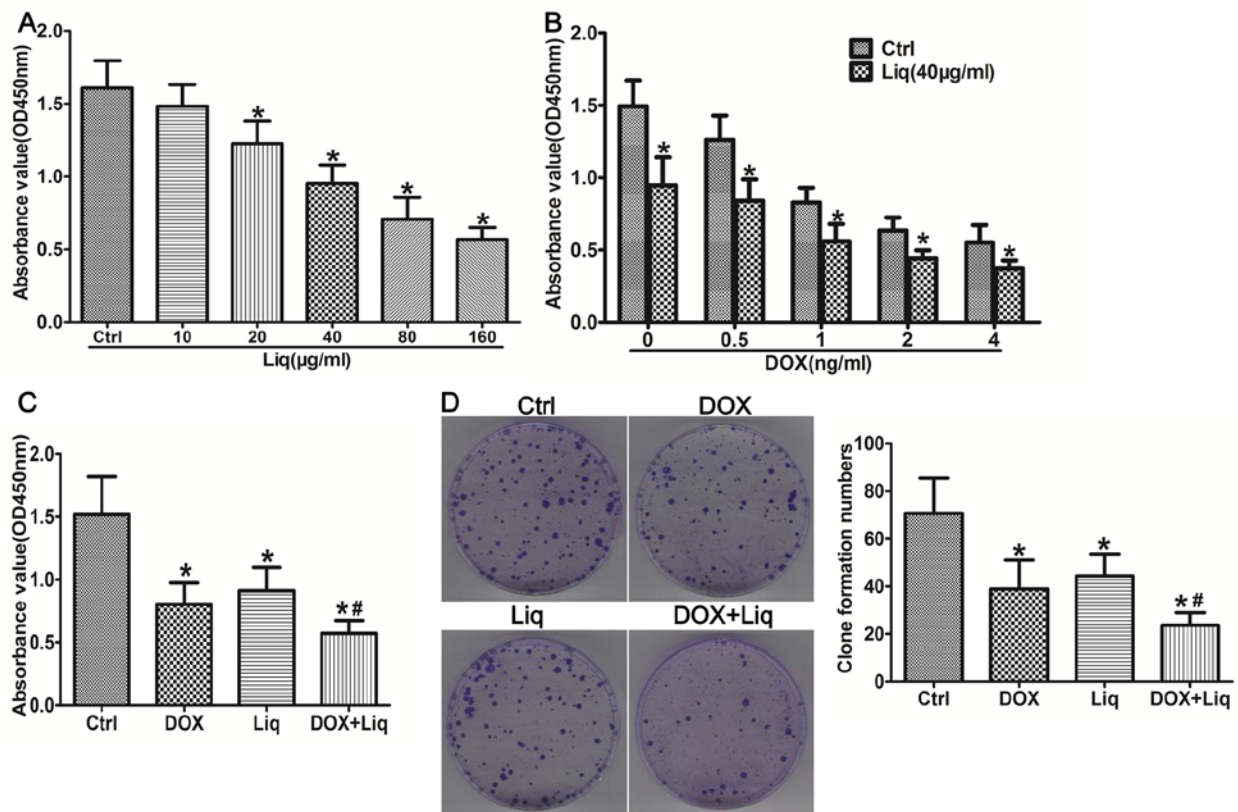


Figure 1. Liq treatment inhibits the viability and promotes the sensitization of MDA-MB-231 cells to DOX treatment. (A) MDA-MB-231 cells were treated with (A) Liq (0-160 $\mu\text{g/ml}$), (B) DOX (0-4 ng/ml) or a combination of DOX (0-4 ng/ml) and Liq (40 $\mu\text{g/ml}$), and (C) DOX (1 ng/ml), Liq (40 $\mu\text{g/ml}$) or a combination of DOX (1 ng/ml) and Liq (40 $\mu\text{g/ml}$) for 48 h. Subsequently, cell viability was determined using the Cell Counting Kit-8 assay in triplicate. (D) MDA-MB-231 cells were treated with DOX (1 ng/ml), Liq (40 $\mu\text{g/ml}$) or a combination of DOX (1 ng/ml) and Liq (40 $\mu\text{g/ml}$). Subsequently, cell proliferation was assessed using a clone formation assay. Magnification, $\times 100$. * $P < 0.05$ vs. the negative control group. # $P < 0.05$ vs. the DOX-treated group. Liq, liquiritigenin; DOX, doxorubicin; OD, optical density; Ctrl, control.

Inc.). Following primary incubation, the membranes were washed three times with TBST and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. AS014; 1:5,000; ABclonal Biotech Co., Ltd.) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence kit (EMD Millipore). Blots were performed in at least triplicate. The protein expression levels were quantitatively analyzed using the Image lab 6.0 software (Bio-Rad Laboratories, Inc.) and normalized against β -actin loading control.

Statistical analysis. Data are presented as the mean \pm SD. One-way ANOVA followed by Tukey's post-hoc test was used to analyze the data. Comparisons between multiple groups and across multiple factors were made using two-way ANOVA followed by Bonferroni's post-hoc test. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Liq inhibits the proliferation and promotes the sensitization of TNBC cells to DOX treatment. To investigate the role of Liq in regulating the therapeutic response to DOX treatment, the MDA-MB-231 and BT549 TNBC cell lines were used. The

CCK-8 assay suggested that the viability of MDA-MB-231 cells decreased in a dose-dependent manner following treatment with different concentrations of Liq for 48 h (Fig. 1A). The results also indicated that Liq concentrations $\geq 20 \mu\text{g/ml}$ significantly decreased the viability of MDA-MB-231 cells compared with the control group (Fig. 1A). Therefore, 40 $\mu\text{g/ml}$ Liq (Liq $\text{IC}_{50} = 69.28 \mu\text{g/ml}$) was used for subsequent experiments. Additionally, the effects of DOX and combination treatment (1 ng/ml DOX and 40 $\mu\text{g/ml}$ Liq) on the viability of MDA-MB-231 cells were assessed. DOX treatment alone did not significantly alter the viability of MDA-MB-231 cells compared with the negative control group. By contrast, the viability of MDA-MB-231 cells was significantly decreased by the combination treatment, even with low concentrations of DOX, compared with the negative control group (DOX IC_{50} combination treated group = 0.60 ng/ml vs. DOX IC_{50} DOX treated group = 1.72 ng/ml ; Fig. 1B). Compared with the control group, DOX (1 ng/ml), Liq (40 $\mu\text{g/ml}$) and combination treatment (1 ng/ml DOX and 40 $\mu\text{g/ml}$ Liq) significantly reduced the viability of MDA-MB-231 cells. Furthermore, combination treatment (1 ng/ml DOX and 40 $\mu\text{g/ml}$ Liq) significantly decreased the viability of MDA-MB-231 cells compared with DOX treatment alone (1 ng/ml) ($P < 0.05$; Fig. 1C). Compared with the control group, the number of cell colonies was found to be significantly reduced in both DOX-treated and Liq-treated groups, whilst the number of cell colonies was significantly

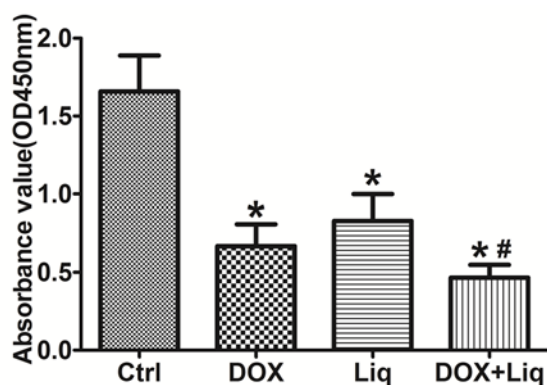


Figure 2. Liq treatment inhibits the viability and promotes the sensitization of BT549 cells to DOX treatment. BT549 cells were treated with DOX (1 ng/ml), Liq (40 μ g/ml) or a combination of DOX (1 ng/ml) and Liq (40 μ g/ml) for 48 h. Subsequently, cell viability was determined using the Cell Counting Kit-8 assay in triplicate. * $P < 0.05$ vs. the negative control group. # $P < 0.05$ vs. the DOX-treated group. Liq, liquiritigenin; DOX, doxorubicin; OD, optical density; Ctrl, control.

decreased in the combination treated group compared with that in the DOX-treated group ($P < 0.05$; Fig. 1D). Similar results were obtained for BT549 cells. DOX (1 ng/ml), Liq (40 μ g/ml) and combination treatment (1 ng/ml DOX and 40 μ g/ml Liq) significantly reduced the viability of BT549 cells, compared with that in the control group. The viability of BT549 cells was significantly decreased in the combination treatment group, compared with that in the DOX-treated group ($P < 0.05$; Fig. 2).

Liq enhances the therapeutic efficacy of DOX by inhibiting the PI3K/AKT/mTOR signaling pathway. To investigate whether Liq enhanced DOX sensitivity by modulating the PI3K/AKT/mTOR signaling pathway, the protein expression levels of ER β , p-AKT, AKT, p-mTOR and mTOR were assessed by western blotting in DOX-treated, Liq-treated and combination-treated MDA-MB-231 cells. MDA-MB-231 cells treated with Liq or the combination treatment displayed increased ER β expression levels, and decreased levels of AKT and mTOR phosphorylation, compared with the control group (Fig. 3A). Subsequently, the ratio of ER β / β -actin, p-AKT/AKT and p-mTOR/mTOR was calculated. MDA-MB-231 cells treated with Liq or the combination treatment displayed significantly increased expression levels of ER β , but a significantly decreased ratio of p-AKT/AKT and p-mTOR/mTOR, compared with the control group (Fig. 3B). ER β expression was significantly increased, and the ratio of p-AKT/AKT and p-mTOR/mTOR was significantly decreased in the combination treatment group compared with the DOX-treated group (Fig. 3B).

ER β knockdown inhibits the effects of Liq on proliferation and the therapeutic efficacy of DOX in TNBC cells. To identify the role of ER β in regulating DOX sensitivity in TNBC cells, ER β knockdown in MDA-MB-231 cells was performed using lentiviral particles. Subsequently, the viability and proliferation of MDA-MB-231 (ER α -/ER β +) and ER β -KD (ER α -/ER β -) cells were assessed. The protein expression levels of ER β were significantly decreased in the ER β -KD group compared with the NC-KD group (Fig. 4A). ER β -KD-MDA-MB-231 cells treated with Liq (40 μ g/ml) or the combination treatment

(1 ng/ml DOX and 40 μ g/ml Liq) displayed increased cell viability compared with the corresponding NC-KD group (Fig. 4B). The number of cell colonies was also significantly increased in the Liq-treated (40 μ g/ml) and combination-treated (1 ng/ml DOX and 40 μ g/ml Liq) ER β -KD groups compared with the corresponding NC-KD groups (Fig. 4C).

Liq-mediated effects on the PI3K/AKT/mTOR signaling pathway are ER β -dependent. Western blotting was used to further investigate the relationship between the expression of ER β and the regulation of the PI3K/AKT/mTOR signaling pathway. MDA-MB-231 cells treated with Liq (40 μ g/ml) or the combination treatment (1 ng/ml DOX and 40 μ g/ml Liq) displayed significantly decreased expression levels of p-AKT and p-mTOR compared with the control group (Fig. 5A). However, ER β -KD cells treated with Liq or the combination treatment displayed significantly increased levels of AKT and mTOR phosphorylation compared with the corresponding NC-KD groups ($P < 0.05$; Fig. 5B).

Discussion

DOX is one of the most active conventional chemotherapeutic drugs used for breast cancer in neoadjuvant, adjuvant and palliative settings (12,13). DOX, as a cytotoxic agent affiliated with anthracycline, can inhibit DNA and RNA synthesis, topoisomerase II enzymatic activity, and block DNA transcription and replication (14-16). Due to a lack of effective endocrine-based and HER2-targeted therapies, doxorubicin-containing chemotherapy plays an important role in patients with TNBC in an adjuvant setting (17). However, the clinical outcome of conventional chemotherapy is not satisfactory in patients with TNBC, as resistance to standard anthracycline- and taxane-based chemotherapy results in treatment failure in some cases (18). In the present study, the ER β specific agonist Liq decreased the proliferation of MDA-MB-231 cells and enhanced the cytotoxic chemotherapeutic effects of DOX. Liq is a natural compound isolated from the roots of *Glycyrrhizae uralensis* (19). Similar to other ER β specific agonists, including diarylpropionitrile and WAY200070, Liq upregulates ER β expression and displays inhibitory effects in TNBC cells (20). ER β -KD-MDA-MB-231 cells treated with the combination treatment did not display increased sensitivity to DOX compared with ER β -positive cells. The results suggested that the synergistic effect of DOX and Liq in TNBC was dependent on ER β . ER β activation caused by Liq does not induce cell apoptosis and proliferation of TNBC cells, but does contribute to cell cycle arrest (21). In 2017, Reese *et al* reported that the activation of ER β resulted in the decreased expression of a number of cell cycle-related genes, including cyclin B and cyclin-dependent kinase 1 (CDK1), both *in vitro* and *in vivo* (22). The inhibition of CDK1 induced G₂/M phase cell cycle arrest, which led to decreased proliferation of MDA-MB-231 cells (22). Collectively, the aforementioned studies suggest that doxorubicin and ER β agonists display synergistic antitumor activity in TNBC, which provides strong rationale for the combined use of ER β agonists and conventional chemotherapeutic agents for the treatment of TNBC.

A number of previous studies investigating TNBC have focused on the therapeutic value of ER β in endocrine therapy, or

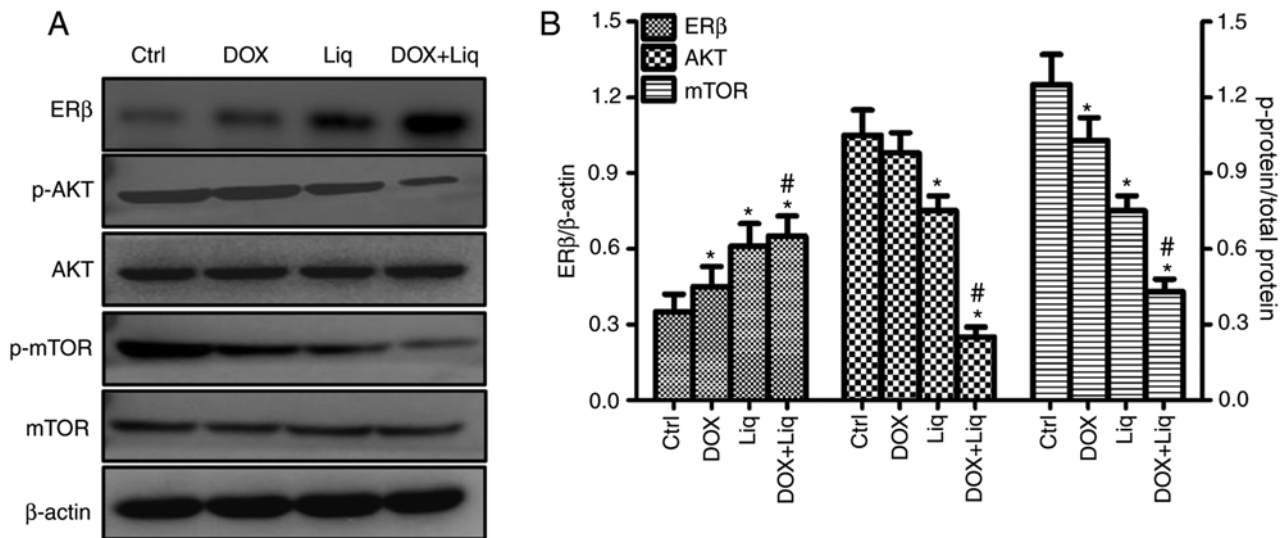


Figure 3. Liq treatment enhances the protein expression of ERβ and inhibits the activity of the PI3K/AKT/mTOR signaling pathway in TNBC cells. MDA-MB-231 cells were treated with DOX (1 ng/ml), Liq (40 μg/ml) or a combination of DOX (1 ng/ml) and Liq (40 μg/ml). Subsequently, the protein expression levels of ERβ, p-AKT, AKT, p-mTOR and mTOR were (A) determined by western blotting and (B) quantified. ERβ expression levels were increased in MDA-MB-231 cells treated with Liq (40 μg/ml) or the combined treatment, whereas the ratio of p-AKT/AKT and p-mTOR/mTOR was decreased, compared with the control group. *P<0.05 vs. the negative control group. #P<0.05 vs. the DOX-treated group. Liq, liquiritigenin; ERβ, estrogen receptor β; DOX, doxorubicin; p, phosphorylated; Ctrl, control.

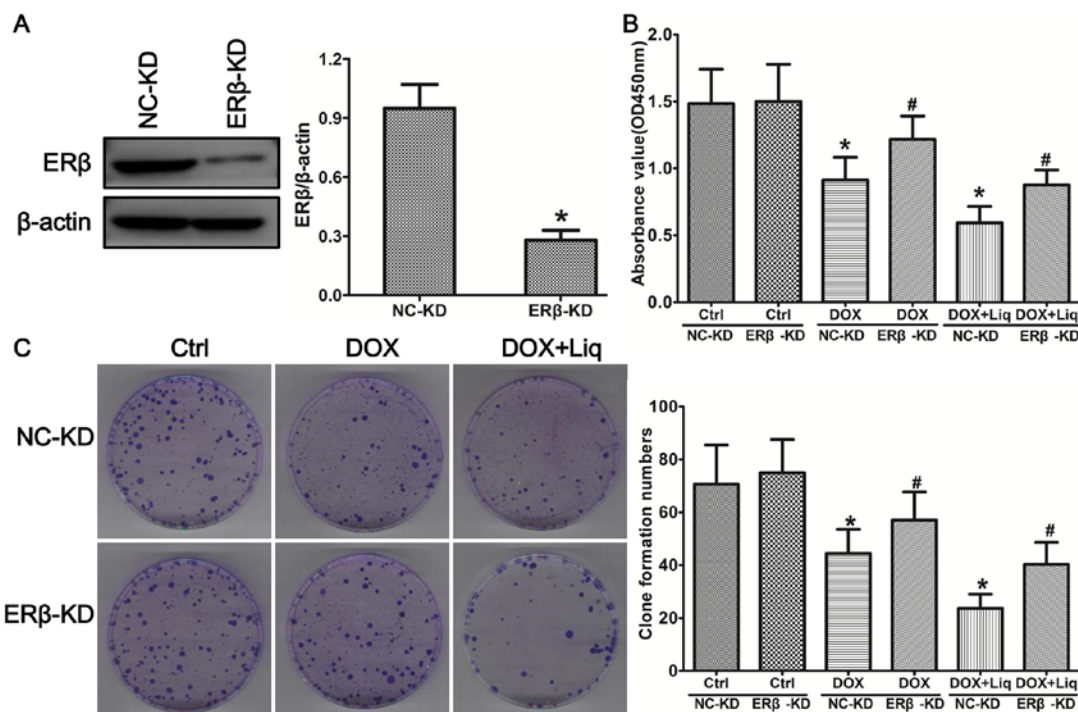


Figure 4. Liq function is ERβ-dependent. (A) The expression of ERβ in NC-KD and ERβ-KD MDA-MB-231 cells was assessed using western blotting. (B) NC-KD and ERβ-KD MDA-MB-231 cells were treated with Liq (40 μg/ml) or a combination of DOX (1 ng/ml) and Liq (40 μg/ml). Subsequently, cell viability was determined using the Cell Counting Kit-8 assay. (C) Proliferation of ERβ-KD and NC-KD cells was assessed using a colony formation assay. *P<0.05 vs. the negative control group. #P<0.05 vs. the NC-KD group. Liq, liquiritigenin; ERβ, estrogen receptor β; shRNA, short hairpin RNA; NC, negative control; KD, knockdown; DOX, doxorubicin; Ctrl, control; OD, optical density.

the role of ERβ in tumor invasion and metastasis. For example, Hinsche and Girgert (21) co-cultured MG63 osteoblast-like cells with the HCC1806 TNBC cell line (ERα-/ERβ+), and reported that the ERβ agonists Liq and ERB-041 increased the expression of ERβ, and inhibited bone-directed invasion. Thomas *et al* (23) reported that ERβ1 inhibits EMT

and invasion in TNBC cells *in vitro* and *in vivo*. The present study further suggested that the ERβ agonist Liq increased the sensitivity of TNBC cells to conventional chemotherapeutic agents. ERβ agonist-induced chemical sensitization has also been observed in various types of malignant tumors, including TNBC (24). Furthermore, Liu *et al* suggested that Liq treatment

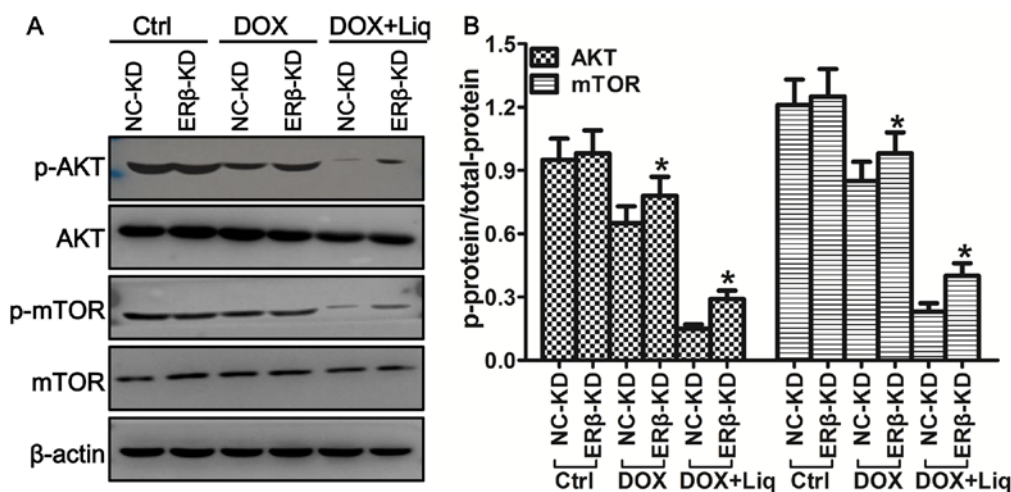


Figure 5. ER β KD increases the activity of the PI3K/AKT/mTOR signaling pathway in MDA-MB-231 cells. Protein expression levels of p-AKT, AKT, p-mTOR and mTOR in NC-KD and ER β -KD cells were (A) determined by western blotting and (B) the ratio of phosphorylated/total protein was quantified. * $P < 0.05$ vs. the NC-KD group. ER β , estrogen receptor β ; p, phosphorylated; NC, negative control; KD, knockdown; Ctrl, control; Liq, liquiritigenin; DOX, doxorubicin.

increased the susceptibility of glioma cells to temozolomide by inhibiting the mTOR signaling pathway (25).

The PI3K/AKT/mTOR signaling pathway plays a critical role in regulating cell metabolism, growth, survival, proliferation, migration and differentiation (26). The inappropriate activation or overactivation of the signaling pathway can result in the progression of tumors in several malignancies, including TNBC (27,28). AKT interacts with the DNA-protein kinase catalytic subunit and induces DNA double-strand break repair (29). In TNBC, the PI3K/AKT/mTOR signaling pathway serves as an oncogenic driver (30). PI3K mutations were reported in 73.9% cfDNA samples and 57.1% tumor samples obtained from patients with metastatic TNBC (31). In addition, overexpression of PI3K and overactivation of the PI3K/AKT/mTOR signaling pathway are associated with chemical drug resistance in breast cancer cells (32,33). Therefore, some have hypothesized that combined treatment, including standard chemotherapy and specifically target components of the PI3K/AKT/mTOR signaling pathway could be used to effectively treat TNBC (31). However, Park *et al.* (31) previously found that the addition of the mTOR inhibitor everolimus to the gemcitabine/cisplatin treatment strategy did not result in a synergistic effect in patients with metastatic TNBC. In addition, the toxicities of everolimus, including stomatitis and hematologic toxicities, should be considered (31,34). The identification of other inhibitors of the PI3K/AKT/mTOR signaling pathway, which display increased tolerance and decreased toxicity, is essential for the effective treatment of TNBC. The present study suggested that increased ER β expression levels decreased the level of AKT and mTOR phosphorylation in TNBC cells. The result was consistent with a previous study, which reported that ER β 1+/pAKT- status in TNBC tumor samples predicted the most favorable prognosis. The previous study also suggested that ER β activation was associated with inhibition of the PI3K/AKT/mTOR signaling pathway (11). An explanation for the association could be that increased ER β expression results in decreased cell proliferation, which is primarily controlled by the PI3K/AKT/mTOR signaling

pathway in TNBC cells (35). Furthermore, downregulation of the signaling pathway results in decreased cell proliferation (35). Alternatively, the ER β -mediated inhibition of the PI3K/AKT/mTOR signaling pathway may be associated with downstream actions that influence the secretion of amphiregulin and Wnt-10b, which may form part of a cascade that could potentially regulate the signaling pathway (36). However, the mechanism underlying how ER β activation modulates the activity of the PI3K/AKT/mTOR signaling pathway requires further investigation. Therefore, Liq, which can specifically target ER β -positive cells, displays characteristics of a therapeutic agent with improved tolerance and reduced toxicity. Furthermore, Liq may display increased specificity compared with general PI3K/AKT/mTOR signaling pathway inhibitors, which could result in improved patient outcomes when used in combination with chemotherapy. To conclude, the *in vitro* results of the present study suggested that Liq increased the sensitivity of TNBC cells to DOX, and indicated that ER β agonists in combination with chemotherapy may serve as a novel therapeutic strategy for TNBC. Additionally, Liq enhanced the sensitivity of TNBC cells to DOX by inhibiting the PI3K/AKT/mTOR signaling pathway, in an ER β -dependent manner.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Health and Family Planning Commission of Hunan Province (grant no. 20180726), and the Ren Shu Foundation from Hunan Provincial People's Hospital (grant no. RS201707).

Availability of data and materials

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

Authors' contributions

SL, PF and AW designed the study. SL, YJ and MF performed the experiments. MW, CZ, SH and ZH analyzed the data. SL and AW drafted the manuscript. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Foulkes WD, Smith IE and Reis-Filho JS: Triple-negative breast cancer. *N Engl J Med* 363: 1938-1948, 2010.
- Papa A, Caruso D, Tomao S, Rossi L, Zaccarelli E and Tomao F: Triple-negative breast cancer: Investigating potential molecular therapeutic target. *Expert Opin Ther Targets* 19: 55-75, 2015.
- Oakman C, Viale G and Di Leo A: Management of triple negative breast cancer. *Breast* 19: 312-321, 2010.
- Foulkes WD, Smith IE and Reis JS: Triple-negative breast cancer. *New Engl J Med* 363: 1938-1948, 2010.
- Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P and Narod SA: Triple-negative breast cancer: Clinical features and patterns of recurrence. *Clin Cancer Res* 13: 4429-4434, 2007.
- Imamov O, Lopatkin NA and Gustafsson JA: Estrogen receptor beta in prostate cancer. *N Engl J Med* 351: 2773-2774, 2004.
- Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson JA: Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 93: 5925-5930, 1996.
- Nilsson S and Gustafsson JA: Estrogen receptors: Therapies targeted to receptor subtypes. *Clin Pharmacol Ther* 89: 44-55, 2011.
- Chen JQ and Russo J: ER alpha-negative and triple negative breast cancer: Molecular features and potential therapeutic approaches. *Biochim Biophys Acta* 1796: 162-175, 2009.
- Sakamoto G and Honma N: Estrogen receptor beta status influences clinical outcome of triple negative breast cancer. *Breast Cancer* 16: 281-282, 2009.
- Wang J, Zhang C, Chen K, Tang H, Tang J, Song C and Xie X: ERβ1 inversely correlates with PTEN/PI3K/AKT pathway and predicts a favorable prognosis in triple-negative breast cancer. *Breast Cancer Res Treat* 152: 255-269, 2015.
- von Minckwitz G, Raab G, Caputo A, Schütte M, Hilfrich J, Blohmer JU, Gerber B, Costa SD, Merkle E, Eidtmann H, *et al*: Doxorubicin with cyclophosphamide followed by docetaxel every 21 days compared with doxorubicin and docetaxel every 14 days as preoperative treatment in operable breast cancer: The GEPAR DUO study of the German Breast Group. *J Clin Oncol* 23: 2676-2685, 2005.
- Gasparini G, Dal Fior S, Panizzoni GA, Favretto S and Pozza F: Weekly epirubicin versus doxorubicin as second line therapy in advanced breast cancer. A randomized clinical trial. *Am J Clin Oncol* 14: 38-44, 1991.
- Mompalmer RL, Karon M, Siegel SE and Avila F: Effect of adriamycin on DNA, RNA, and protein synthesis in cell-free systems and intact cells. *Cancer Res* 36: 2891-2895, 1976.
- Fornari FA, Randolph JK, Yalowich JC, Ritke MK and Gewirtz DA: Interference by doxorubicin with DNA unwinding in MCF-7 breast tumor cells. *Mol Pharmacol* 45: 649-656, 1994.
- Tewey KM, Rowe TC, Yang L, Halligan BD and Liu LF: Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* 226: 466-468, 1984.
- Mamounas EP, Bryant J, Lembersky B, Fehrenbacher L, Sedlacek SM, Fisher B, Wickerham DL, Yothers G, Soran A and Wolmark N: Paclitaxel after doxorubicin plus cyclophosphamide as adjuvant chemotherapy for node-positive breast cancer: Results from NSABP B-28. *J Clin Oncol* 23: 3686-3696, 2005.
- Fan Y, Li M, Ma K, Hu Y, Jing J, Shi Y, Li E and Dong D: Dual-target MDM2/MDMX inhibitor increases the sensitization of doxorubicin and inhibits migration and invasion abilities of triple-negative breast cancer cells through activation of TAB1/TAK1/p38 MAPK pathway. *Cancer Biol Ther* 5: 617-632, 2019.
- Shanle EK, Hawse JR and Xu W: Generation of stable reporter breast cancer cell lines for the identification of ER subtype selective ligands. *Biochem Pharmacol* 82: 1940-1949, 2011.
- Reese JM, Suman VJ, Subramaniam M, Wu X, Negron V, Gingery A, Pitel KS, Shah SS, Cunliffe HE, McCullough AE, *et al*: ERβ1: Characterization, prognosis, and evaluation of treatment strategies in ERα-positive and -negative breast cancer. *BMC Cancer* 14: 749, 2014.
- Hinsche O, Girgert R, Emons G and Gründker C: Estrogen receptor β selective agonists reduce invasiveness of triple-negative breast cancer cells. *Int J Oncol* 46: 878-884, 2015.
- Reese JM, Bruinsma ES, Monroe DG, Negron V, Suman VJ, Ingle JN, Goetz MP and Hawse JR: ERβ inhibits cyclin dependent kinases 1 and 7 in triple negative breast cancer. *Oncotarget* 8: 96506-96521, 2017.
- Thomas C, Rajapaksa G, Nikolos F, Hao R, Katchy A, McCollum CW, Bondesson M, Quinlan P, Thompson A, Krishnamurthy S, *et al*: ERbeta1 represses basal-like breast cancer epithelial to mesenchymal transition by destabilizing EGFR. *Breast Cancer Res* 14: R148, 2012.
- Shanle EK, Zhao Z, Hawse J, Wisinski K, Keles S, Yuan M and Xu W: Research resource: Global identification of estrogen receptor β target genes in triple negative breast cancer cells. *Mol Endocrinol* 27: 1762-1775, 2013.
- Liu X, Wang L, Chen J, Ling Q, Wang H, Li S, Li L, Yang S, Xia M and Jing L: Estrogen receptor β agonist enhances temozolomide sensitivity of glioma cells by inhibiting PI3K/AKT/mTOR pathway. *Mol Med Rep* 11: 1516-1522, 2015.
- Khan KH, Yap TA, Yan L and Cunningham D: Targeting the PI3K-AKT-mTOR signaling network in cancer. *Chin J Cancer* 32: 253-265, 2013.
- Bitting RL and Armstrong AJ: Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer. *Endocr Relat Cancer* 20: R83-R99, 2013.
- Martelli AM, Evangelisti C, Chiarini F and McCubrey JA: The phosphate-dylinositol 3-kinase/Akt/mTOR signaling network as a therapeutic target in acute myelogenous leukemia patients. *Oncotarget* 1: 89-103, 2010.
- Toulany M, Lee KJ, Fattah KR, Lin YF, Fehrenbacher B, Schaller M, Chen BP, Chen DJ and Rodemann HP: Akt promotes post-irradiation survival of human tumor cells through initiation, progression, and termination of DNA-PKcs-dependent DNA double-strand break repair. *Mol Cancer Res* 10: 945-957, 2012.
- Costa RLB, Han HS and Gradishar WJ: Targeting the PI3K/AKT/mTOR pathway in triple-negative breast cancer: A review. *Breast Cancer Res Treat* 169: 397-406, 2018.
- Park IH, Kong SY, Kwon Y, Kim MK, Sim SH, Joo J, and Lee KS: Phase I/II clinical trial of everolimus combined with gemcitabine/cisplatin for metastatic triple-negative breast cancer. *J Cancer* 9: 1145-1151, 2018.
- Chock KL, Allison JM, Shimizu Y and ElShamy WM: BRCA1-IRIS overexpression promotes cisplatin resistance in ovarian cancer cells. *Cancer Res* 70: 8782-8791, 2010.
- O'Brien NA, Browne BC, Chow L, Wang Y, Ginther C, Arboleda J, Duffy MJ, Crown J, O'Donovan N and Slamon DJ: Activated phosphoinositide 3-kinase/AKT signaling confers resistance to trastuzumab but not lapatinib. *Mol Cancer Ther* 9: 1489-1502, 2010.
- Xu J and Tian D: Hematologic toxicities associated with mTOR inhibitors temsirolimus and everolimus in cancer patients: A systematic review and meta-analysis. *Curr Med Res Opin* 30: 67-74, 2014.
- Vivanco I and Sawyers CL: The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2: 489-501, 2002.
- Hamilton N, Márquez-Garbán D, Mah V, Fernando G, Elshimali Y, Garbán H, Elshoff D, Vadgama J, Goodlick L and Pietras R: Biologic roles of estrogen receptor-β and insulin-like growth factor-2 in triple-negative breast cancer. *Biomed Res Int* 2015: 925703, 2015.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.