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Curcumin Suppresses Doxorubicin-Induced Epithelial–Mesenchymal Transition via the Inhibition of TGF-β and PI3K/AKT Signaling Pathways in Triple-Negative Breast Cancer Cells

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ABSTRACT: Triple-negative breast cancer (TNBC) is defined by a lack of expression of the estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (HER 2). Therefore, targeted therapy agents may not be used, and therapy is largely limited to chemotherapy. Doxorubicin treatment consequently acquires undesired malignance characteristics [i.e., epithelial–mesenchymal transition (EMT) and multi-drug resistance]. Our results illustrated that doxorubicin triggered EMT and resulted in the acquisition of a mesenchymal phenotype in TNBC cells. Moreover, we found that transforming growth factor-β (TGF-β) and PI3K/AKT signaling pathways were acquired for doxorubicin-induced EMT. Interestingly, we found that curcumin suppressed doxorubicin-induced EMT. Curcumin reversed doxorubicin-induced morphological changes, inhibited doxorubicin-induced downregulation of E-cadherin expressions, and inhibited doxorubicin-induced upregulation of vimentin expression. We also found that curcumin inhibited doxorubicin-induced EMT by inhibiting the TGF-β and PI3K/AKT signaling pathways. Moreover, curcumin enhanced the antiproliferative effects of doxorubicin in TNBC cells. In summary, our results suggest that doxorubicin in combination with curcumin may be a potential therapy for TNBC.

KEYWORDS: *Triple-negative breast cancer, doxorubicin, epithelial and mesenchymal transition, curcumin*

INTRODUCTION

Triple-negative breast cancer (TNBC) is an immunohistochemically defined subtype without the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). TNBC is noted by high risk of distant recurrence, death, visceral, and central nervous system (CNS) metastases.[1] There are no targeted agents developed specifically for TNBC at current therapy. The effective treatment choices are limited to chemotherapy, and the data about TNBC therapy are insufficient. Hence, it is necessary to establish a standard treatment regimen for TNBC.[2]

Doxorubicin is widely used in the treatment of patients with metastatic breast cancer.[3−5] However, doxorubicin is limited in clinical utility because of the number of troublesome side effects, including drug resistance and invasive potential in breast cancer cells.[6−9]

Transforming growth factor-β (TGF-β) regulates various cell behaviors, including cell proliferation, differentiation, migration, and apoptosis. TGF-β plays a dual role in cancer progression. During early stages of carcinogenesis, TGF-β acts as a tumor suppressor by inhibiting cell growth and promoting apoptosis; however, in the advanced stages of carcinogenesis, it acts as a tumor promoter by enriching the motility and invasiveness to promote epithelial–mesenchymal transition (EMT) and metastasis.[10,11] Previous studies implied that Twist, Snail, and Slug were transcriptionally induced by TGF-β during EMT in various cancer cells.[7,12,13]

β-Catenin is one of the adherent junction components anchored with E-cadherin to regulate cell–cell adhesion and cell migration. Phosphorylation of GSK3β by activation of AKT phosphorylation accumulated intracellular β-catenin. The stabilization and nuclear accumulation of β-catenin led to stimulation of EMT, stem cell maintenance, and self renewal.[14−16]

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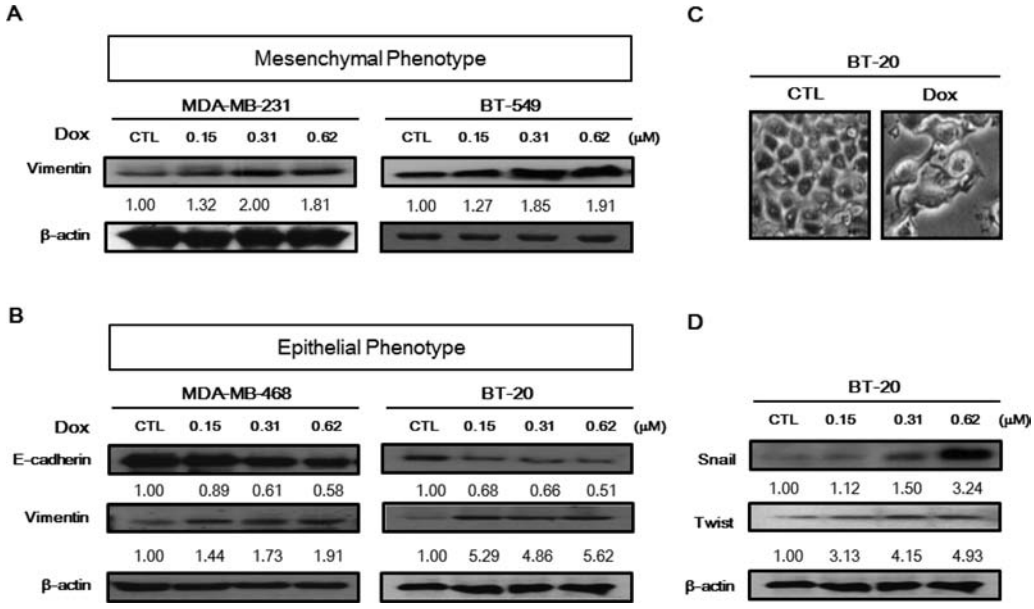

Figure 1. Stimulation of EMT by doxorubicin treatment in TNBC cells. (A) MDA-MB-231 and BT-549 cells were treated with DMSO (control) or increasing doxorubicin concentrations (0.15, 0.31, and 0.62 μM) for 48 h. The cells were then harvested and lysed for the detection of vimentin and β-actin. (B) MDA-MB-468 and BT-20 cells were treated with DMSO (control) or increasing doxorubicin concentrations (0.15, 0.31, and 0.62 μM) for 48 h. The cells were then harvested and lysed for the detection of E-cadherin, vimentin, and β-actin. (C) Phase-contrast images of BT-20 cells. The sub-confluent cultures were shown the morphological differences. BT-20 cells were treated with DMSO (control) or 0.15 μM doxorubicin for 48 h. (D) BT-20 cells were treated with DMSO (control) or increasing doxorubicin concentrations (0.15, 0.31, and 0.62 μM) for 48 h. The cells were then harvested and lysed for the detection of Snail, Twist, and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments. The lower panel presents the average of three independent experiments. The value of the control cells was set to 1.

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a major component of turmeric (*Curcuma longa* L.). Curcumin has several biological and pharmacological activities, such as anti-inflammatory, antioxidant, and chemotherapeutic properties. Curcumin exhibits non-toxicity even at high doses in laboratory animals.[17,18] Several studies indicated that curcumin decreases the ability of cancer cell motility, metastasis, and stem-like characteristics.[19−22]

Besides its therapeutic effects, doxorubicin also enhances the malignancy of treated cancers in clinical situations. Recently, EMT has attracted attention in studies of TNBC tumor progression. We aimed to test whether transient doxorubicin treatment induced EMT in TNBC cells and elucidated the role of TGF-β and PI3K/AKT signaling pathways in this process. In this study, we showed that doxorubicin exposure induced activation of p-Smad2 and β-catenin, which led to nuclear accumulation and consequence EMT. In addition, we found that curcumin reversed doxorubicin-induced EMT via inhibiting both TGF-β and PI3K/AKT signaling pathways. Curcumin also enhanced the chemosensitivity of TNBC cells to doxorubicin.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. MDA-MB-468, MDA-MB-231, BT-549, and BT-20 cells were purchased from the American Type Culture Collection (Manassas, VA). BT-549 and BT-20 cells were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) (Invitrogen Corporation, Carlsbad, CA). MDA-MB-231 and MDA-MB-468 were grown in Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12) (Invitrogen Corporation, Carlsbad, CA). Medium was supplemented with 2 mM L-glutamine, 100 μg of streptomycin, and 100 units of penicillin 10% fetal bovine serum (FBS) (Invitrogen Corporation, Carlsbad, CA). All cell lines were grown in a humidified incubator at 37 °C under 5% CO_2 in air.

Reagents and Antibodies. Doxorubicin, SB431542, and LY294002 were purchased from Sigma Chemical Co. (St. Louis, MO). Curcumin was purchased from Merck Co. (Darmstadt, Germany). Primary antibodies E-cadherin, Snail, Twist, phospho-AKT (S473), phospho-GSK3β (Ser9), β-catenin, and β-actin were purchased from Cell Signaling Technology (Beverly, MA). Primary vimentin was purchased from Abcam, Inc. (Cambridge, MA). Secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) and goat anti-rabbit IgG, were obtained from Millipore (Billerica, MA).

Western Blotting. Cells on 100 mm culture dishes (5×10^5/dish) were treated with various concentrations of doxorubicin, curcumin, or in combination and then incubated for 48 h. After treatment, the total proteins were extracted by adding 50 μL of gold lysis buffer [50 mM Tris−HCl at pH 7.4, 1 mM phenylmethylsulfonyl floride, 1 mM NaF, 1% NP-40, 150 mM NaCl, 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 10 mg/mL leupeptin] to the cell pellets. Lysate protein was measured by the Lowry protein assay (Bio-Rad Laboratories, Berkeley, CA). Membranes were blocked with 5% bovine serum albumin (BSA) (Sigma, St. Louis, MO) for 1 h at room temperature and probed with primary antibody for 1.5 h at room temperature or overnight at 4 °C followed by HRP-conjugated appropriated secondary antibodies.

Morphology Observation. BT-20 cells (1×10^4) were seeded in each well of a 24-well plate and incubated in a 37 °C incubator with 5% CO_2 overnight. BT-20 cells were treated with indicated concentrations of doxorubicin with or without curcumin or curcumin only, and then cells were incubated at 37 °C for 48 h. Representative photographs were taken at 200× magnification using a Nikon TE2000-U inverted microscope.

Cellular Fractionation Analysis. Cells were harvested with trypsinization and washed twice with ice phosphate-buffered saline (PBS). Cells were rapidly washed once with hypotonic buffer and allowed to swell on ice for 10 min. After centrifugation at 4 °C with 720*g* (3000 rpm) for 15 min, the supernatant was saved for the cytoplasmic fraction. The nuclear pellet was added to the same buffer. After sonication, the suspension was spun at 10000*g* (8000 rpm) for

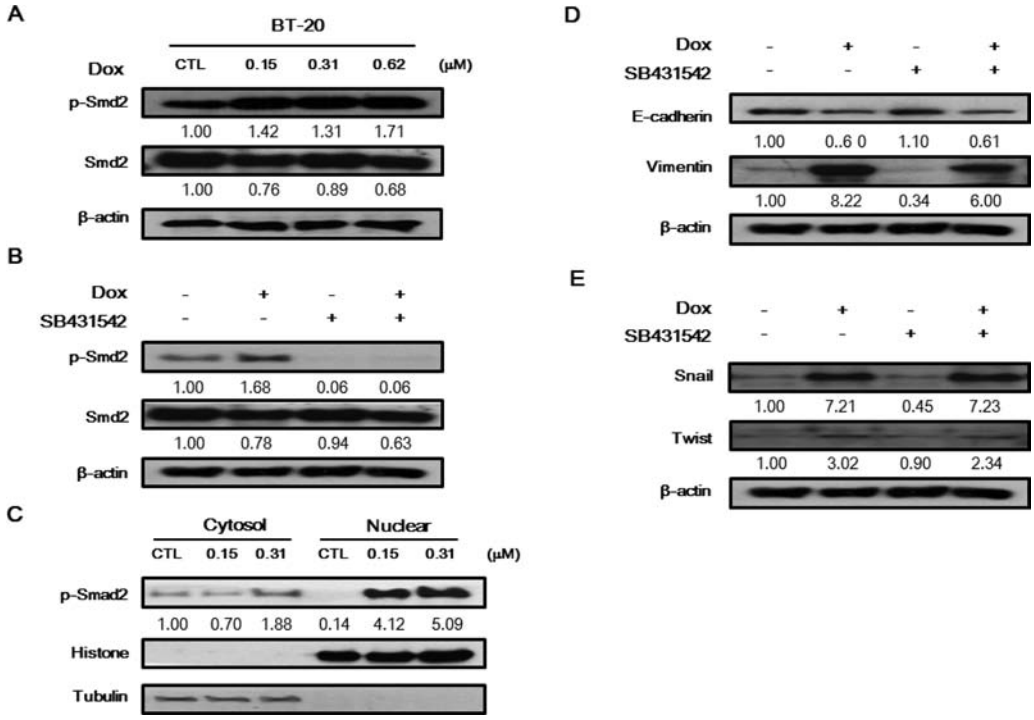

Figure 2. Doxorubicin increased p-Smad2 expression and nucleus accumulation in BT-20 cells. (A) BT-20 cells were treated with DMSO (control) or increasing doxorubicin concentrations (0.15, 0.31, and 0.62 μM) for 48 h. The cells were then harvested and lysed for the detection of Smad2, p-Smad2, and β-actin. (B) BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 10 μM SB431542 alone or in combination for 48 h. The cells were then harvested and lysed for the detection of Smad2, p-Smad2, and β-actin. (C) BT-20 cells were treated with DMSO (control) or doxorubicin (0.15 or 0.31 μM) for 36 h. The cells were then harvested and lysed for the detection of p-Smad2, Histone 3 (H3), and tubulin. H3 and tubulin served as loading controls. (D) BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 10 μM SB431542 alone or in combination for 48 h. The cells were then harvested and lysed for the detection of E-cadherin, vimentin, and β-actin. (E) BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 10 μM SB431542 alone or in combination for 48 h. The cells were then harvested and lysed for the detection of Snail, Twist, and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments. The lower panel presents the average of three independent experiments. The value of the control cells was set to 1.

20 min, and the supernatant was saved as the nuclear fraction. Equal proteins from cytoplasmic and nuclear fractions were used for western blot analysis.

Growth Inhibition Assay. Cells were seeded in a 24 well plate (1×10^4 cells/well) overnight and then treated with different concentrations of doxorubicin with or without curcumin or curcumin only for 48 h. Cell growth inhibition was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 80 μL of MTT solution (2 mg/mL, Sigma Chemical Co., St. Louis, MO) was added to each well and incubated for 1.5 h at 37 °C. The supernatant was removed and added to 500 μL of dimethyl sulfoxide (DMSO) to dissolve the MTT−formazan crystals, which formed by metabolically viable cells. Finally, the absorbance at an optical density (OD) of 570 nm was detected by an enzyme-linked immunosorbent assay (ELISA) reader.

Statistical Analysis. One-way analysis of variance (ANOVA) was used for the comparison of more than two mean values. Results represent at least two to three independent experiments and are shown as averages ± standard error of the mean (SEM). Results with a p value less than 0.05 were considered statistically significant (*, $p < 0.05$).

RESULTS

Doxorubicin Treatment Induces EMT in TNBC Cells. Recently, doxorubicin exposure not only causes apoptosis but also induces multi-drug resistance in breast cancer cells.[7] TNBC is an aggressive form of breast cancer and resistant to available treatments. Therefore, we first aimed to examine whether doxorubicin induced EMT in TNBC cells. Following treatment with various concentrations of doxorubicin (0.15, 0.31, and 0.62 μM, respectively) for 48 h, vimentin (mesenchymal marker) was upregulated in mesenchymal phenotype (MDA-MB-231 and BT-549) and epithelial phenotype (BT-20 and MDA-MB-468) TNBC cells. However, E-cadherin (epithelial marker) was downregulated in BT-20 and MDA-MB-468 cells (panels A and B of Figure 1). MDA-MB-231 and BT-549 did not show E-cadherin expression (data not shown). BT-20 was chosen for further experiments. The morphological change is another critical characteristic for EMT. In comparison to untreated cells, doxorubicin exposure induced a loose cell contact and acquired a fibroblast-like appearance in epithelial phenotype BT-20 cells (Figure 1C). We further examined whether EMT-inducing regulators, Snail and Twist, were involved in doxorubicin-induced EMT. Western blot analysis showed that doxorubicin increased Snail and Twist expressions (Figure 1D). Our results exhibited that doxorubicin contributed TNBC to acquire EMT characteristics.

Effect of the TGF-β Pathway on Doxorubicin-Induced EMT. TGF-β has been shown to induce EMT and to attribute metastatic progression in various cancer cells.[10] We questioned whether doxorubicin-induced EMT involved the TGF-β pathway. Because TGF-β induces Smad2 Ser465/467-phosphorylation, we examined the effect of doxorubicin exposure on Ser465/467-phosphorylation of Smad2 expression. Doxorubicin exposure significantly increased the Ser465/467-phosphorylation of Smad2 in BT-20 cells (Figure 2A). We used a specific inhibitor of TGF-β receptor kinase, SB431542, to confirm that doxorubicin activated the TGF-β pathway in BT-20 cells. BT-

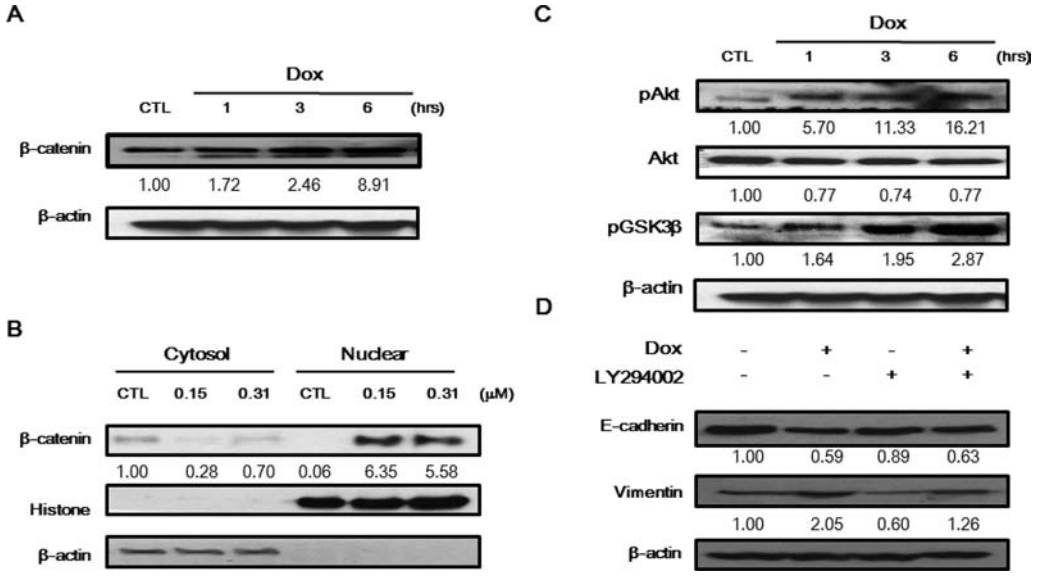

Figure 3. Doxorubicin-induced EMT via the activation of β-catenin in BT-20 cells. (A) BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin for various times (1, 3, and 6 h). The cells were then harvested and lysed for the detection of β-catenin and β-actin. (B) BT-20 cells were treated with DMSO (control) or doxorubicin (0.15 or 0.31 μM) for 36 h. The cells were then harvested and lysed for the detection of β-catenin, H3, and tubulin. H3 and tubulin served as loading controls. (C) BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin for various times (1, 3, and 6 h). The cells were then harvested and lysed for the detection of p-GSK3β, p-AKT, AKT, and β-actin. (D) BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 10 μM LY294002 alone or in combination for 48 h. The cells were then harvested and lysed for the detection of E-cadherin, vimentin, and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments. The lower panel presents the average of three independent experiments. The value of the control cells was set to 1.

20 cells were treated with doxorubicin and SB431542 alone or in combination for 48 h. We found that SB431542 abrogated doxorubicin-increased Ser465/467-phosphorylation of Smad2 (Figure 2B). Phosphorylation of Smad2 translocates to the nucleus and participates in transcriptional activation of responsive genes for EMT. Thus, we also confirmed the subcellular location of p-Smad2 in BT-20 cells under the doxorubicin treatment. In comparison to untreated cells, treatment with doxorubicin elevated the expression of p-Smad2 in the nuclear fraction (Figure 2C). Next, we confirmed whether doxorubicin-induced EMT via activating TGF-β signaling. BT-20 cells were treated with doxorubicin and SB431542 alone or in combination for 48 h. SB431542 did abrogate doxorubicin-induced vimentin upregulation but did not abrogate doxorubicin-induced E-cadherin downregulation (Figure 2D). We then questioned whether SB431542 affected doxorubicin-induced EMT-inducing factors, Snail and Twist. Our data showed that SB431542 only abrogated the doxorubicin-increased Twist protein level but did not inhibit the doxorubicin-increased Snail protein level (Figure 2E). Taken together, doxorubicin increased p-Smad2 expression and nuclear accumulation, which led it to take part in EMT induction.

Doxorubicin Induces Activation of β-Catenin in TNBC Cells. Aberrant activation of β-catenin in breast cancer was related with poor prognosis and is another important regulator for possessed EMT.[14−16] We questioned whether doxorubicin-induced EMT via the activation of β-catenin. Doxorubicin exposure increased the expression of β-catenin in a temporal response observed at 1−6 h post-doxorubicin treatment interval (Figure 3A). Nuclear accumulation of β-catenin participates in transcriptional activation of responsive genes critical for maintenance EMT. We next questioned whether doxorubicin increased β-catenin nuclear accumulation and western blot analysis of cytoplasmic and nuclear fractions from doxorubicin (0.15 and 0.31 μM) treated in BT-20 cells. β-Catenin elevated accumulation in the nuclear fraction of doxorubicin-treated cells compared to untreated cells (Figure 3B). In our results, doxorubicin modulated β-catenin expression and activity. GSK3β is a downstream target of phosphatidylinositol 3 kinase (PI3K)/AKT, and its activity is inhibited by phosphorylation, leading to modulation of downstream targets. GSK3β-mediated phosphorylation of β-catenin causes its degradation in the ubiquitin-dependent proteasome pathway. We further investigated whether GSK3β inactivation is involved in mediating the ability of doxorubicin to stabilize β-catenin. Doxorubicin exposure increased significantly the phosphorylation of GSK3β in a temporal manner. We also examined whether doxorubicin-mediated GSK3β inactivation and involvement of EMT properties included AKT. Following exposure of doxorubicin for 1, 3, and 6 h, the phosphorylation of AKT was increased at 1−6 h (Figure 3C). We also use LY294002, a highly selective inhibitor of PI3K, to determine if inhibition of AKT activation repressed the doxorubicin-induced EMT. Treatment of LY294002 reversed E-cadherin expression and reduced vimentin expression in the presence of doxorubicin treatment (Figure 3D). Taken together, β-catenin activation participated in doxorubicin-induced EMT related with the PI3K/AKT pathway.

Curcumin Inhibits Doxorubicin-Induced EMT Properties. Apart from inhibiting cell proliferation in various cancer cells, curcumin has the potential to inhibit the invasion and metastasis of cancer cells.[23] We examined whether curcumin abrogated doxorubicin-induced EMT properties. MDA-MB-231, BT-549, BT-20, and MDA-MB-468 cells were treated with doxorubicin and curcumin alone or in combination for 48 h. Our results revealed that doxorubicin-induced upregulation of vimentin expression was inhibited by treatment with curcumin

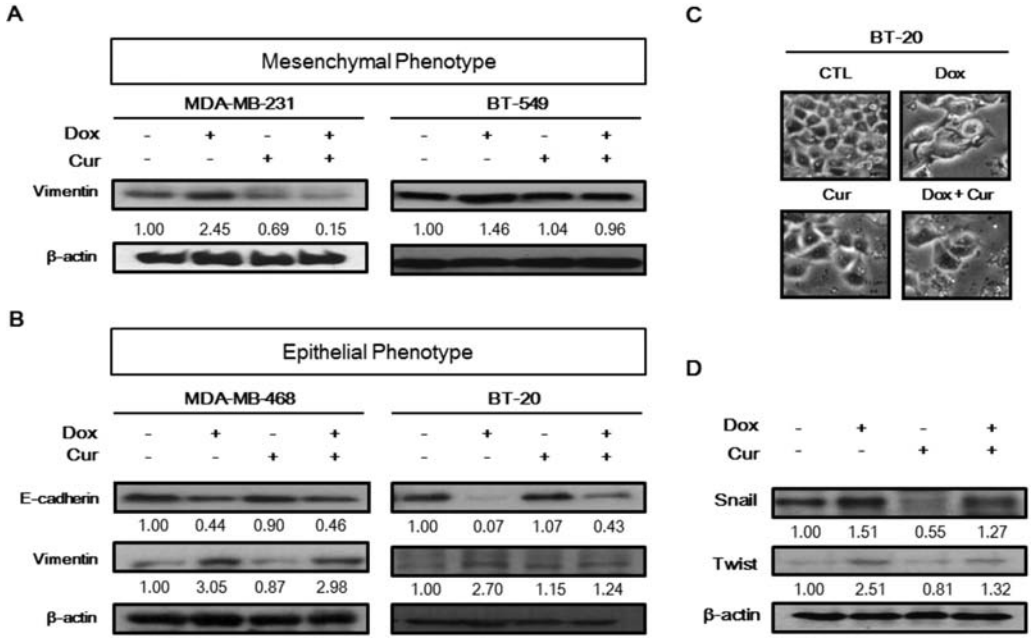

Figure 4. Curcumin inhibited doxorubicin-induced EMT characteristics. (A) MDA-MB-231 and BT-549 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 20 μM curcumin alone or in combination for 48 h. The cells were then harvested and lysed for the detection of vimentin and β-actin. (B) MDA-MB-468 and BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 20 μM curcumin alone or in combination for 48 h. The cells were then harvested and lysed for the detection of E-cadherin, vimentin, and β-actin. (C) Phase-contrast images of BT-20 cells. The sub-confluent cultures were shown the morphological differences. BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 20 μM curcumin alone or in combination for 48 h. (D) BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 20 μM curcumin alone or in combination for 48 h. The cells were then harvested and lysed for the detection of Snail, Twist, and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments. The lower panel presents the average of three independent experiments. The value of the control cells was set to 1.

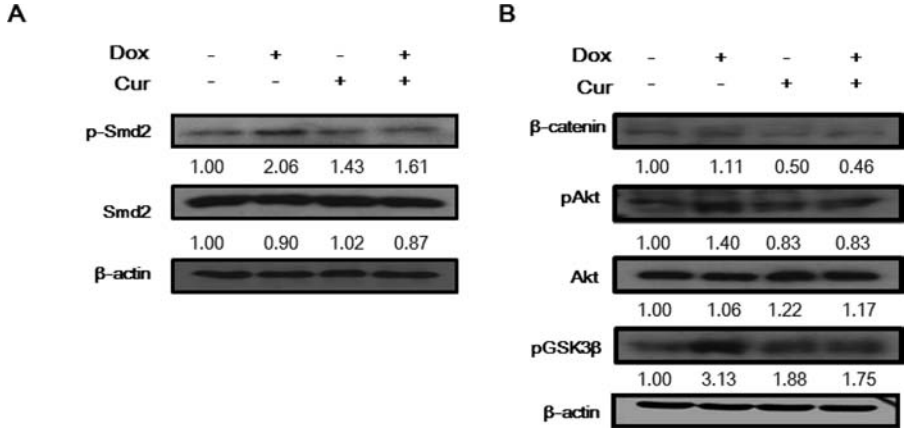

Figure 5. Curcumin blocked doxorubicin-induced EMT via inhibiting TGF-β and PI3K/AKT signaling pathways. (A) BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 20 μM curcumin alone or in combination for 48 h. The cells were then harvested and lysed for the detection of p-Smad2, Smad2, and β-actin. (B) BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 20 μM curcumin alone or in combination for 1 h. The cells were then harvested and lysed for the detection of β-catenin, p-AKT, AKT, p-GSK3β, and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments. The lower panel presents the average of three independent experiments. The value of the control cells was set to 1.

in MDA-MB-231, BT-549, MDA-MB-468, and BT-20 cells (panels A and B of Figure 4). Treatment with curcumin inhibited doxorubicin-induced downregulation of E-cadherin expressions in MDA-MB-468 and BT-20 cells (Figure 4B). We observed that curcumin maintained cell contact and epithelial phenotype even exposure in doxorubicin (Figure 4C). Moreover, curcumin suppressed doxorubicin-induced expressions of Snail and Twist (Figure 4D) in BT-20 cells. Taken together, our results shown that curcumin prevented the EMT characteristic induction by doxorubicin.

Curcumin Inhibits Doxorubicin-Induced EMT via Modulation of p-Smad2 and β-Catenin Expressions. Our result showed that doxorubicin increased p-Smad2 expression. We next identified whether curcumin inhibited doxorubicin-induced EMT via the inhibition of p-Smad2 activation. Our data showed that curcumin surpressed the doxorubicin-increased p-Smad2 protein level (Figure 5A). We

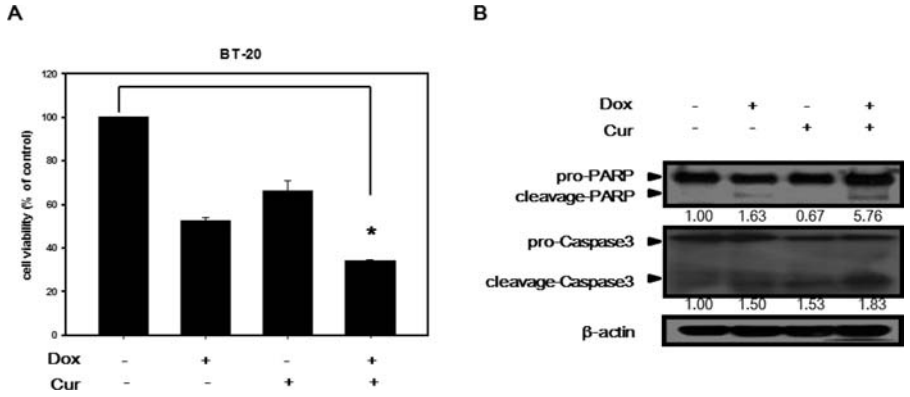

Figure 6. Curcumin sensitized the antiproliferative effect of doxorubicin in BT-20 cells. (A) BT-20 cells were treated with doxorubicin (0.31 μM) and curcumin (20 μM) alone or in combination treatment for 48 h. Growth inhibition was determined by the MTT assay. The percentage of cell growth inhibition was calculated by the absorption of control cells as 100%. Experiments were performed in triplicate. (*) $p < 0.05$. (B) BT-20 cells were treated with DMSO (control) or 0.31 μM doxorubicin and 20 μM curcumin alone or in combination for 48 h. The cells were then harvested and lysed for the detection of PARP, caspase 3, and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments. The lower panel presents the average of three independent experiments. The value of the control cells was set to 1.

next questioned whether curcumin inhibited doxorubicin-induced β-catenin, p-AKT, and p-GSK3β protein levels. Our data showed that curcumin diminished doxorubicin-increased β-catenin, p-AKT, and p-GSK3β protein levels (Figure 5B). Our results showed that curcumin inhibited doxorubicin-induced p-Smad2, β-catenin, p-AKT, and p-GSK3β upregulation. Taken together, the effect of curcumin on doxorubicin-induced EMT mediated TGF-β and PI3K/AKT signaling pathways.

Curcumin Enhanced the Antiproliferative Effect of Doxorubicin in BT-20 Cells. To test whether curcumin could enhance the antiproliferative effect of doxorubicin, the antitumor effects of doxorubicin and curcumin were assessed in BT-20 cells by the MTT assay at 48 h. Treatment with doxorubicin and curcumin alone reduced cell viability, and in combination treatment with 20 μM curcumin enhanced the antiproliferative effect of doxorubicin in BT-20 (Figure 6A). Subsequently, we also measured the cleaved forms of PARP and caspase 3 (both apoptosis markers). We observed that curcumin enhanced the doxorubicin-induced cleaved forms of PARP and caspase 3 (Figure 6B). Taken together, these results suggested that the ability of curcumin was to sensitize BT-20 cells to doxorubicin, and it is a potential approach.

■ DISCUSSION

Chemotherapy not only erases cancer cells but also faces undesired effects, including enhancing the aggressive ability of the treated cancer cells, resulting in chemotherapy failure. To find out a promising approach to eliminate cancer without opposite effects is essential. It is widely believed that a combination treatment may have a potential therapeutic benefit for improving cancer therapy. TNBC present with higher rates of visceral metastases has a relatively shorter medial survival and has limited duration of response to successive lines of chemotherapy. In this study, we found out a potential effective approach for TNBC treatment using a combination of curcumin and doxorubicin.

EMT, a physiological process of switching from epithelial phenotype into mesenchymal phenotype, has been indicated to increase the aggressive ability of the tumors (i.e., tumor cell migration, invasion, and dissemination).[12,23] Our data had shown that both epithelial and mesenchymal phenotypes of TNBC gained EMT characteristics after treatment with doxorubicin (Figure 1). It was consistent with previous publications that chemotherapy agents, such as doxorubicin and paclitaxel, have the undesired effects to induce drug resistance and EMT of the treated cancers.[7−9,24,25]

Accumulated evidence suggested that TGF-β plays a regulator of the EMT process and accelerates the tumor-promoting activity in various cancer progressions involving the progression of metastatic breast cancer. Administration of doxorubicin in a MMTV/PyVmT transgenic animal model had shown to elevate TGF-β circulating levels, which were a prometastatic signal in tumor cells.[27] Furthermore, doxorubicin treatment enhanced the properties of migration and invasion in murine 4T1, human MCF-7, and MDA-MB-231 breast cancer cells.[7,8,30] Hence, we hypothesized that doxorubicin exposure stimulated TGF-β signaling and caused EMT in BT-20 cells. Our data had shown that doxorubicin treatment did accumulate p-Smad2 in the nucleus and blocked TGF-β receptor kinase inhibitor, SB431542. We next questioned whether doxorubicin-induced EMT was Smad-dependent signaling. Snail and Twist are two of the concomitant TGF-β signaling-induced EMT. Our data showed that SB431542 did inhibit doxorubicin-induced upregulation of vimentin and Twist, but it is unexpected that SB431542 could not inhibit doxorubicin-induced E-cadherin and Snail alternation (Figure 2). Our results showed that Smad-dependent signaling may play a minor role in doxorubicin-induced EMT. Except EMT, TGF-β regulates cell proliferation, cell cycle arrest, extracellular matrix production, and other tumorigenicities.[26] The effect of Smad-dependent signaling induced by doxorubicin in BT-20 cells needs to be further investigated.

TGF-β-induced EMT not only goes through Smad-dependent mechanisms but also goes through Smad-independent mechanisms.[27,28] Activation of AKT phosphorylated Twist1 on S42 caused enhancement of TGF-β signaling, which kept PI3K/AKT hyperactivated, and then cancer cells acquired more aggressiveness.[29] Our results had shown that doxorubicin increased Snail and Twist expressions (Figure 1D). We questioned whether PI3K/AKT participated in doxorubicin-induced EMT. Our data had shown that doxorubicin treatment induced EMT through phosphorylated AKT and GSK3β and improved β-catenin accumulation in the nucleus and inhibited

by PI3K inhibitor, LY294002 (Figure 3). We considered that doxorubicin-induced EMT might be through triggering the PI3K/AKT pathway.

Doxorubicin is widely used in chemotherapy involving metastatic breast cancer. However, previous studies and our data indicated that doxorubicin treatment caused tumors more malignancy. Therefore, to search for a chemopreventive or chemotherapeutic agent to diminish the chemoresistance to doxorubicin via inhibition EMT will help to improve cancer therapy. Increasing evidence suggested that curcumin reversed chemoresistance,[17] invasion,[19] and metastasis.[20] In this study, we chose curcumin, an agent known to lessen tumor motility, invasion, and metastasis, to investigate whether curcumin could prevent doxorubicin-induced EMT. Our results showed that curcumin inhibited doxorubicin-acquired EMT properties involving downregulation of vimentin, upregulation of E-cadherin, and holding on the cell−cell contact in TNBCs. Although the effect of curcumin in E-cadherin and vimentin protein level expressions was not so effective in MDA-MB-468, curcumin may regulate other EMT-related proteins, such as Zeb1, Twist1, Snail, Slug, and N-cadherin in MDA-MB-468. We also found that curcumin inhibited doxorubicin-increased expressions of Snail and Twist in BT-20 (Figure 4). To address further mechanisms by curcumin-inhibited doxorubicin-induced EMT, we question whether curcumin has the ability to block doxorubicin-increased p-Smad2 and β-catenin expressions. Our results showed that curcumin inhibited doxorubicin-induced p-Smad2, β-catenin, p-AKT, and pGSK3β expressions (Figure 5). TGFβ signaling plays a mediator to regulate EMT through MEK/Erk, JNK/p38 MAP kinases, Rho GTPase, and PI3K/AKT.[31] Inhibition of the signaling of PI3K by LY294002 and MEK1/2 by UO126 inhibited TGF-β-1-induced EMT, supporting the fact that PI3K/AKT and MAPK/Erk1/2 may play a regulator role in TGF-β-1-induced EMT in A549 human lung cancer cells.[32]

During the past few decades, the chemopreventive efficacy of curcumin has been extensively studied; several molecular targets by curcumin have been found.[18] For instance, curcumin prevented paclitaxel-induced EMT through inhibiting NF-kB signaling.[20] Our results showed that curcumin could repress both TGF-β and PI3K/AKT signaling pathways. Our data also showed that curcumin enhanced doxorubicin-triggered apoptosis (Figure 6).

In summary, our data showed that doxorubicin-triggered apoptosis came with EMT via TGF-β and PI3K/AKT signaling pathways in TNBC. We found a chemopreventive agent, curcumin, which suppressed doxorubicin-induced EMT and enhanced doxorubicin-triggered apoptosis. On the other hand, curcumin could reduce the dosage of doxorubicin and abolish the undesired effects during clinical treatment of TNBC. Doxorubicin in combination with curcumin may be a potential therapy for TNBC.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

TNBC, triple-negative breast cancer; EMT, epithelial−mesenchymal transition; TGF-β, transforming growth factor-β; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ER, estrogen receptor; HER2, epidermal growth factor receptor-2; PR, progesterone receptor; DMSO, dimethyl sulfoxide; DMEM/F12, Dulbecco's modified Eagle's medium/nutrient mixture F12; RPMI 1640, Roswell Park Memorial Institute 1640

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