

Original Article

G15 sensitizes epithelial breast cancer cells to doxorubicin by preventing epithelial-mesenchymal transition through inhibition of GPR30

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Abstract: Resistance to single or multiple chemotherapeutic drugs is a major obstacle in breast cancer therapy. Recent studies have suggested that GPR30 is implicated in mediating cancer cell proliferation. The aim of this study was to examine the anti-tumor effects of the GPR30 antagonist G15 in breast cancer. We found that low concentrations of G15 had little effect on breast cancer cell viability, but could enhance doxorubicin sensitivity in MDA-MB-231 and MCF-7 cells with epithelial phenotypes. In addition, G15 prevented epithelial breast cancer cells undergoing epithelial-mesenchymal transition (EMT) after doxorubicin induction. Moreover, downregulation of GPR30 suppressed the EMT in breast cancer cells. These results support that G15 enhanced doxorubicin sensitivity and prevented the EMT in epithelial breast cancer cells by inhibiting GPR30 expression.

Keywords: Breast cancer, GPR30, G15, epithelial-mesenchymal transition

Introduction

Breast cancer is currently one of the most common malignant tumors among women all over the world. It has become the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths [1, 2]. At present, combination chemotherapy serves as one of the important adjuvant therapies for breast cancer after surgery in the clinic [3]. Although chemotherapy improves survival rates in the adjuvant treatment, nearly 50% of treated patients will relapse due to the acquired resistance against the anticancer drugs [4, 5]. Therefore, improving the chemotherapy sensitivity is imperative for optimized treatment for breast cancer.

It has been acknowledged that the pathogenesis of breast cancer is a multistep process reg-

ulated by aberrantly protein expression and alterations of morphological and molecular features during malignant progression [6-8]. Epithelial-mesenchymal transition (EMT) is a complex, reversible process which induces epithelial cells to transform to mesenchymal phenotype [9, 10]. Although accumulating evidences suggest that EMT plays an important role in regulating the chemoresistance properties of breast cancer, but the molecular mechanism still remains elusive [11-13]. The G-protein coupled receptor 30 (GPR30), a seven-transmembrane domain protein, was recently identified as a novel estrogen receptor structurally distinguished from the classic ER α and ER β [14, 15]. In keratinocytes, GPR30 enhances cell proliferation by promoting cyclin D expression and inhibits oxidative stress-induced apoptosis by stimulating Bcl-2 expression [16, 17]. Furthermore, GPR30 has been shown to regulate the

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proliferative effects of tamoxifen and estrogen in endometrial cancer cells [18]. A recent study has demonstrated that GPR30 activates Mitogen-activated protein (MAP) kinases and contributes to tamoxifen resistance development in breast cancer [19]. G15 is a tetrahydro-3H-cyclopenta[c]quinoline analog that inhibits GPR30 with high affinity. It has been reported to be active against proliferation of uterine epithelial cells and induce apoptosis and autophagy in human oral squamous carcinoma cells [20, 21]. In the present study, we aimed to evaluate the effects of G15 on the chemoresistance of breast cancer cells to doxorubicin.

Materials and methods

Cell culture and reagents

Human breast cancer cell lines MCF-7, MDA-MB-231 and Bcap-37 were purchased from the ATCC (Manassas, VA, USA) and cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained at 37°C in 5% CO₂ incubator. Doxorubicin and G15 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The GPR30 siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

CCK-8 assay

Breast cancer cells were seeded onto 96-well plates at 3.0×10^3 cells/well. The medium was replaced with the corresponding serum-free medium for 24 h to synchronize the cell cycle, then serum-free medium was replaced with complete medium containing the drugs at the indicated concentrations for 48 h. Then 10 μ L/well CCK8 solution (Dojindo, Kumamoto, Japan) was added, the plates incubated for 3 h, and absorbance was measured at 450 nm using an MRX II microplate reader (Dynex, Chantilly, VA, USA).

EdU incorporation assay

Cell viability was calculated as a percentage of untreated control. Measurement of inhibitive rate of cell proliferation was carried out using a Click-iT EdU Imaging Kit (Invitrogen, Carlsbad, CA, USA) according to the procedure provided by the manufacture.

siRNA transfection

Breast cancer cells were transfected with GPR30 siRNA or negative control siRNA (Invitrogen) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instruction. The transfection medium (Opti-MEM; Gibco, USA) was replaced with complete medium 12 h after transfection, and the cells were incubated for the indicated times.

Immunofluorescence

Breast cancer cells were seeded into 48-well plates at 6.0×10^3 cells/well. Cells were fixed with 4% formaldehyde for 15 min, washed with PBS, treated with 5% BSA for 30 min at room temperature, and incubated with mouse anti-human vimentin or anti-human E-cadherin primary antibodies (Cell Signaling Technology, USA) at 4°C overnight. The cells were incubated with goat anti-mouse FITC-conjugated secondary antibody (Abcam) at 4°C for 2 h, incubated with DAPI (Sigma-Aldrich) for 2 min at room temperature, washed twice with PBS, and observed using an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Western blot analysis

Breast cancer cells were lysed in 50 μ L cell lysis buffer (Cell Signaling, Danvers, MA, USA) containing protease inhibitors (Sigma, USA). Whole cell lysates were prepared and fractionated were separated by 10% SDS-PAGE and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with anti-GPR30 (Abcam, Cambridge, USA), anti-E-cadherin, or anti-Vimentin (Cell Signaling Technology, USA) antibodies at 4°C overnight. The membranes were washed three times with TBST and then incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Protein expression was detected by chemiluminescence (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis

Each experiment was performed in triplicate, and repeated at least three times. All the data were presented as means \pm SD and treated for statistics analysis by SPSS program. Comparison between groups was made using

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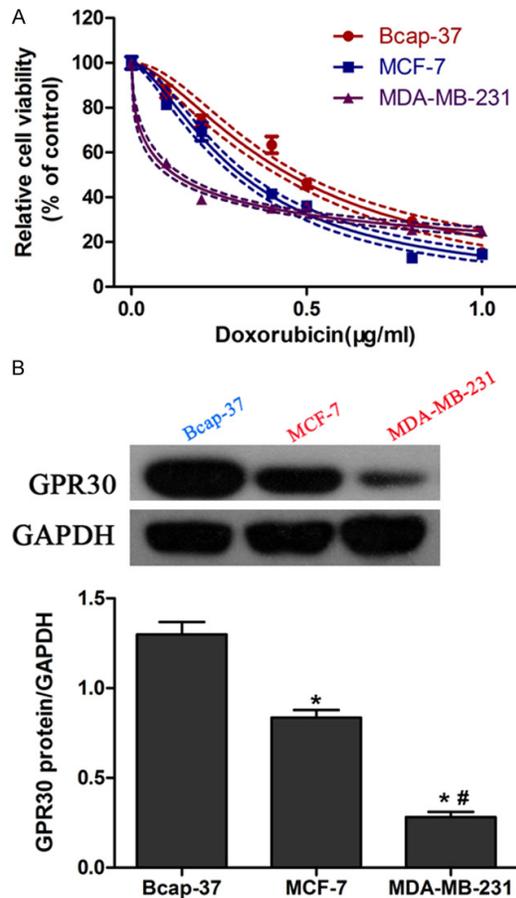


Figure 1. Different doxorubicin sensitivity in breast cancer cells. Three breast cancer cell lines including MCF-7, MDA-MB-231 and Bcap-37 were incubated with doxorubicin for 48 h. Cell viability was measured using CCK-8 method (A). Western blot was performed to determine GPR30 expression in MCF-7, MDA-MB-231 and Bcap-37 cells (B). * $P < 0.05$, compared with Bcap-37; # $P < 0.05$, compared with MCF-7.

ANOVA and statistically significant difference was defined as $P < 0.05$.

Results

Different doxorubicin sensitivity in breast cancer cells

Firstly, we performed CCK-8 assay to examine the sensitivity of different kinds of breast cancer cell types (MCF-7, MDA-MB-231 and Bcap-37) to doxorubicin. Compared with Bcap-37 cells, MCF-7 and MDA-MB-231 cells are more sensitive to doxorubicin (**Figure 1A**). Western blot was used to measure the expression of GPR30 in breast cancer cell lines. Interestingly, we observed the highest expression of GPR30 in Bcap-37 cells, and the lowest expression in

MDA-MB-231 cells (**Figure 1B**), suggesting that GPR30 may be involved in the chemoresistance to doxorubicin.

Low concentrations of G15 had little cytotoxicity on breast cancer cells

A series of G15 concentrations ranging from 0~5.0 (μM) were incubated with three breast cells lines (MCF-7, MDA-MB-231, Bcap-37) and data from CCK-8 assay showed that G15 exerted little cytotoxicity in cancer cells between 0 and 0.625 μM . However, higher concentrations of G15 (1.25, 2.5, 5.0 μM) significantly inhibited the viability of the three cell lines (**Figure 2A-C**). Therefore, 0.625 μM G15 was used for further coadministration with doxorubicin.

G15 enhanced doxorubicin sensitivity in epithelial breast cancer cells

To evaluate the synergistic cytotoxic effects of doxorubicin combined with G15, we used CCK-8 assay to measure cell viability treated with doxorubicin alone or doxorubicin plus G15 for 48 h. Surprisingly, the doxorubicin sensitivity was reduced in the mesenchymal Bcap-37 cells after co-administration with G15 (**Figure 3A**). By contrast, the breast cancer cells with epithelial features (MDA-MB-231 and MCF-7) showed a higher sensitivity to doxorubicin in combined treatment group (**Figure 3B and 3C**). In addition, EDU incorporation assay was used to measure cancer cell proliferation. As shown in **Figure 3D**, doxorubicin and G15 co-treatment resulted no significant differences in Bcap-37 cell growth. But the proliferation of MCF-7 (**Figure 3E**) and MDA-MB-231 (**Figure 3F**) cells was reduced after cotreatment with doxorubicin and G15. These results suggested that G15 could enhance the cytotoxicity of doxorubicin in epithelial breast cancer cells.

G15 regulated doxorubicin-induced EMT in epithelial breast cancer cells

In order to investigate whether doxorubicin induced EMT in breast cancer cells, the expression levels of epithelial/mesenchymal markers in breast cancer cells were examined. Western blot analysis showed that doxorubicin treatment led to significant down-regulation of E-cadherin and up-regulation of Vimentin in MCF-7, MDA-MB-231, Bcap-37 cells (**Figure 4A**). These results suggested that doxorubicin could induce EMT in breast cancer cells. In

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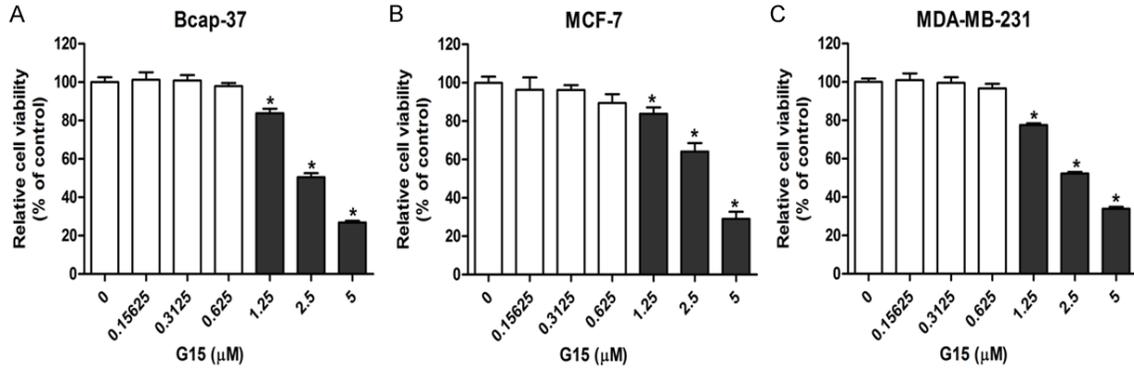


Figure 2. Low concentrations of G15 had little cytotoxicity on breast cancer cells. Three breast cancer cell lines including Bcap-37 (A), MCF-7 (B), and MDA-MB-231 (C) were incubated with different concentrations of G15 for 48 h. The CCK-8 values of the treated breast cancer cells were normalized to the control group with the absence of G15. *P < 0.05.

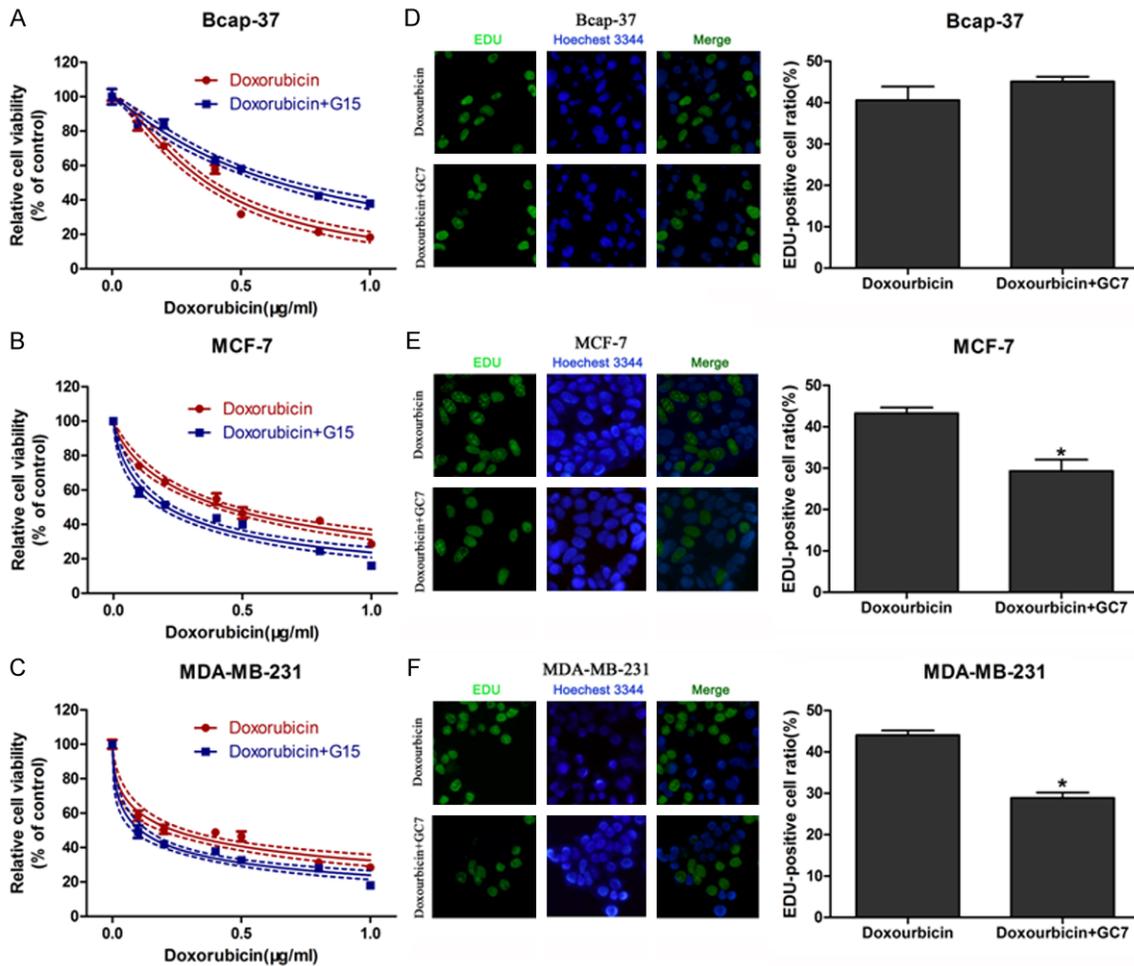


Figure 3. G15 enhanced doxorubicin sensitivity in epithelial breast cancer cells. G15 (0.625 μM) significantly reduced the cytotoxicity of doxorubicin in the mesenchymal Bcap-37 cells (A), but increased the doxorubicin sensitivity in epithelial MCF-7 (B) MDA-MB-231 (C) cells. Photomicrographs and bar charts depict the EdU staining and relative EdU-positive ratio, respectively, of Bcap-37 (D), MCF-7 (E) and MDA-MB-231 (F) cells after treatment with doxorubicin or doxorubicin plus G15 for 48 h. *P < 0.05.

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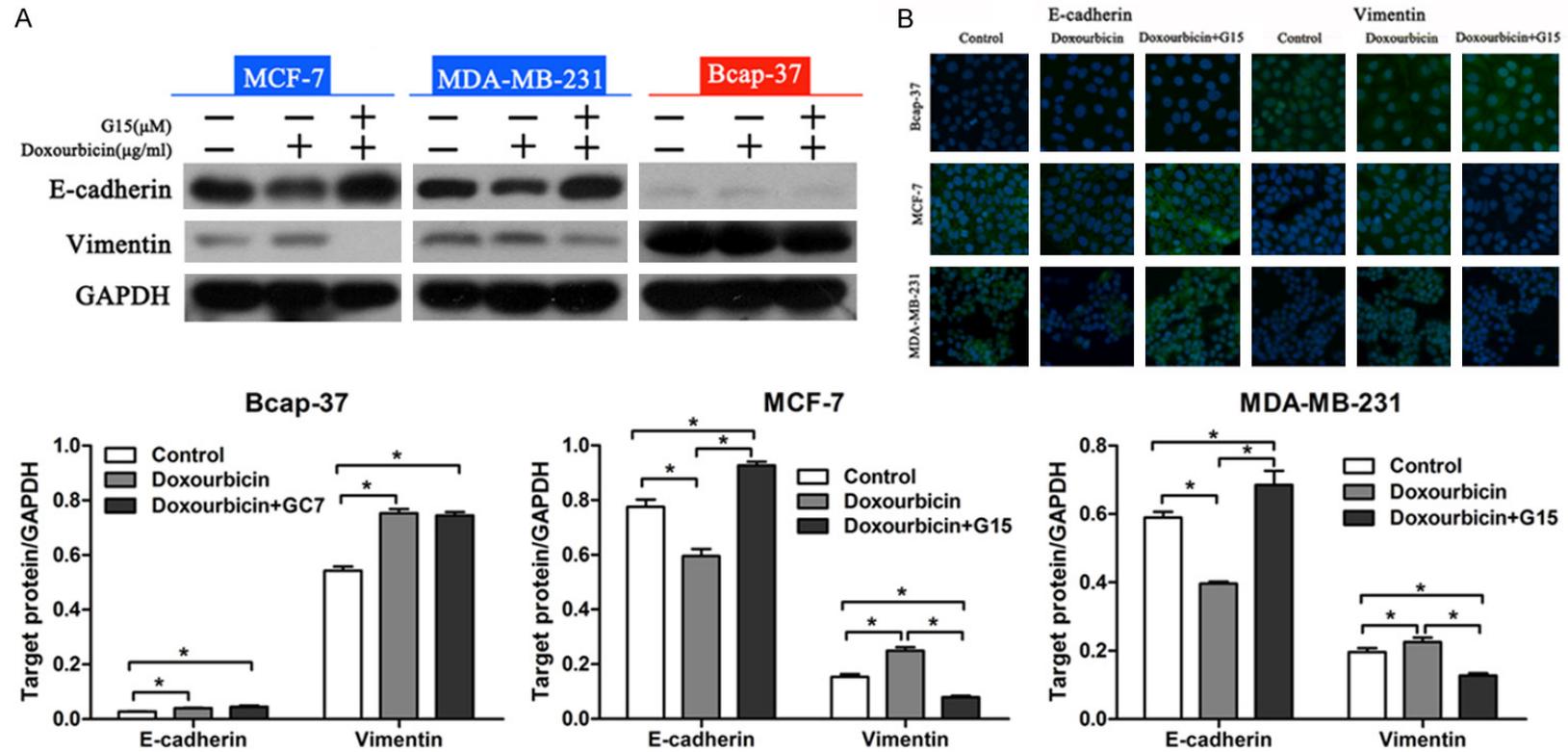


Figure 4. G15 prevented EMT in epithelial breast cancer cells. A. Western blot analysis of E-cadherin and Vimentin expression in breast cancer cells. Relative protein expression in Bcap-37, MCF-7, and MDA-MB-231 cells was quantified by band density with GAPDH served as control. *P < 0.05. B. EdU staining of Bcap-37, MCF-7, and MDA-MB-231 cells after treatment with doxorubicin or doxorubicin plus G15 for 48 h.

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addition, combined treatment with G15 reversed such changes in MDA-MB-231 and MCF-7 cells (epithelial phenotype), but not in Bcap-37 cells (mesenchymal phenotype). Immunofluorescent staining also showed similar results consistent with the western blotting analysis (**Figure 4B**). These data suggested that G15 could reverse the doxorubicin-induced EMT in breast cancer cells with epithelial features.

G15 enhanced doxorubicin sensitivity via inhibition of GPR30

In order to investigate the mechanism by which G15 enhanced doxorubicin sensitivity, RNAi was applied to knockdown the expression of GPR30 in breast cancer cells. We found that downregulation of GPR30 increased the sensitivity to doxorubicin in Bcap-37 (**Figure 5A**), MCF-7 (**Figure 5B**) and MDA-MB-231 (**Figure 5C**) cells. We then treated these transfected cells with doxorubicin alone, or doxorubicin combined with G15, and carried out CCK-8 cell viability assays. Results showed that there were little changes in the doxorubicin sensitivity between two groups (**Figure 5D-F**), suggesting that GPR30 was involved in the chemosensitivity. In order to verify whether GPR30 was a key factor in G15 regulation of EMT, we transfected GPR30 siRNA into breast cancer cells without G15 treatment. Western blot analysis indicated that GPR30 knockdown led to upregulation of E-cadherin and downregulation of vimentin in cancer cells (**Figure 5G**). Taken together, these data demonstrated that downregulation of GPR30 could suppress EMT in breast cancer cells.

Discussion

Accumulating evidences suggest that the acquired drug resistance to traditional chemotherapeutics has become a major obstacle to the triumph of chemotherapy [22, 23]. In this study, we examined whether G15, a GPR30 antagonist, could enhance the cytotoxicity of doxorubicin in human breast cancer cells. Interestingly, our data showed that G15 increased the doxorubicin sensitivity in epithelial breast cancer cells (MDA-MB-231 and MCF-7) but not in mesenchymal cells (Bcap-37).

GPR30, a novel estrogen receptor at the plasma membrane, has been proposed as a candi-

date for triggering a broad range biological activities, including mobilization of intracellular calcium stores, stimulation of adenylyl cyclase, and activation of mitogen-activated protein kinase and phosphoinositide 3-kinase signaling pathways [24, 25]. GPR30 is recognized as a promising target for cancer treatment because it is expressed in many types of cancer, such as breast, endometrial and ovarian cancer [26-28]. In our study, results showed that the doxorubicin sensitivity and GPR-30 expression varied in different cancer cells. Specifically, Bcap-37 cells with highest expression of GPR30 exhibited lowest sensitivity to doxorubicin, while MDA-MB-231 cells with lowest expression of GPR30 showed greatest sensitivity. These results implied that GPR30 may be involved in the drug resistance in breast cancer cells.

G15, a GPR30-specific antagonist, has been investigated in several cancers. Incubation with G15 promotes cell death, G2/M arrest as well as autophagy in human oral squamous carcinoma cells [21]. Combination treatment with the G15 and tamoxifen induces cytotoxic action *in vitro* and anti-tumor progression *in vivo*, suggesting that GPR30 might be a useful target in developing better treatments for breast cancer patients [19]. In the present research, we found that G15 co-treatment enhanced the cytotoxicity of doxorubicin in epithelial breast cancer cells (MDA-MB-231 and MCF-7) but not in mesenchymal cells (Bcap-37).

Many studies have shown that EMT plays a key role in carcinogenicity, metastasis, progression and acquired chemoresistance in many kinds of cancers, including breast cancer [29, 30]. During EMT, epithelial markers such as Ecadherin decrease, while mesenchymal markers such as vimentin increase [31]. In our study, we found that doxorubicin treatment induced EMT in breast cancer cells. However, combined administration with G15 reversed EMT in MDA-MB-231 and MCF-7 cells (epithelial phenotype), but not in Bcap-37 cells (mesenchymal phenotype). These results demonstrated that G15 regulated EMT in breast cancer cells with epithelial phenotype.

The etiology of breast cancer involves a complex interplay of various factors, of which the accumulation of oncogenes and loss of tumor repressors are crucial events in the initiation

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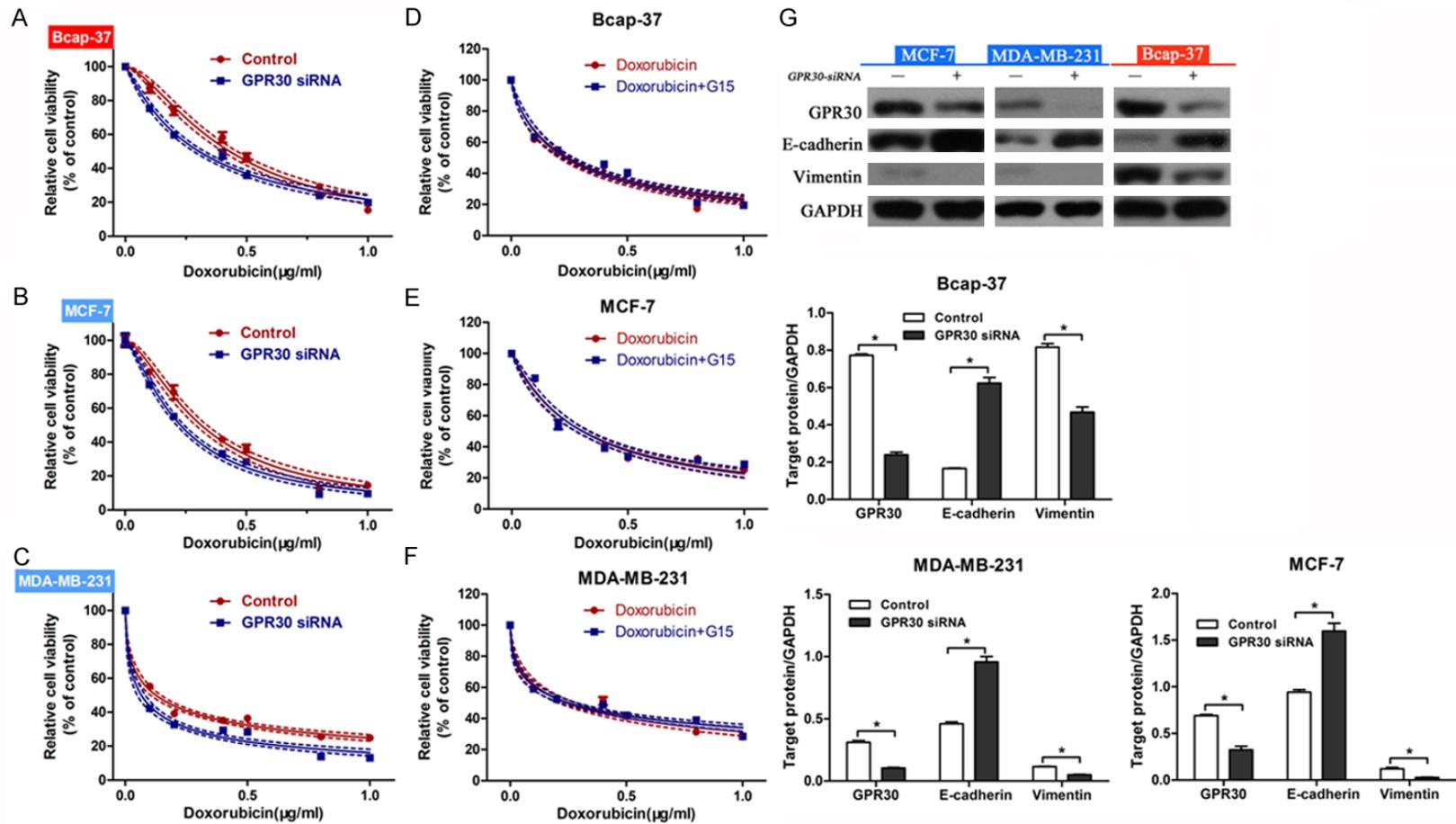


Figure 5. G15 enhanced doxorubicin sensitivity through inhibition of GPR30. Knockdown of GPR30 enhanced the doxorubicin sensitivity of Bcap-37 (A), MCF-7 (B), and MDA-MB-231 (C) cells. After transfection with GPR30 siRNA, CCK-8 cell viability assays were performed to determine the chemosensitivity of Bcap-37 (D), MCF-7 (E), and MDA-MB-231 (F) cells treated with doxorubicin alone or doxorubicin plus G15. (G) Western blot analysis of E-cadherin and Vimentin expression in GPR30 siRNA transfected cells. Relative protein expression in Bcap-37, MCF-7, and MDA-MB-231 cells was quantified by band density with GAPDH served as control. *P < 0.05.

and progression of breast cancer [32, 33]. EMT has been recognized as a critical procedure regulating the pathogenesis of breast cancer [34]. In order to explore the molecular mechanism by which G15 modulated EMT, GPR30 was downregulated in breast cancer cells. We found that depletion of GPR30 increased the doxorubicin sensitivity. However, there were no significant differences in the doxorubicin sensitivity with or without G15, demonstrating that G15 enhanced doxorubicin sensitivity via inhibition of GPR30. In addition, our results indicated that GPR30 knockdown suppressed EMT in tumor cells. These data suggested that GPR30 might be an upstream factor regulating EMT and thereby plays an important role in EMT phenotypic changes.

In conclusion, the present study demonstrated that G15 prevented epithelial breast cancer cells from undergoing EMT changes via inhibition of GPR30. Furthermore, these results suggested that GPR30 plays a key role in EMT and drug resistance in breast cancer. Therefore, inhibition of GPR30 could enhance the anti-tumor drugs sensitivity and prevent EMT of breast cancer cells. These findings may provide a novel therapeutic strategy for breast cancer therapy.

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Disclosure of conflict of interest

None.

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