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Full length article

Estrogen receptor positive breast tumors resist chemotherapy by the overexpression of P53 in Cancer Stem Cells

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ABSTRACT

Background and Objectives: Breast cancer (BC) is classified according to estrogen receptor (ER) status into ER^+ and ER^- tumors. ER^+ tumors have a worse response to chemotherapy compared to ER^- tumors. *BCL-2, TP53, BAX* and *NF-κB* are involved in drug resistance in the ER^+ tumors. Recently it was shown that Cancer Stem Cells (CSCs) play an important role in drug resistance. In this study we tested the hypothesis that CSCs of the ER^+ tumors resist drug through the overexpression of *BCL-2, TP53, BAX* and *NF-κB*.
Methods: CSCs were isolated by anoikis resistance assay from MCF7 (ER^+) and MDA-MB-231 (ER^-) cell lines. Isolated CSCs were treated with doxorubicin (DOX) and the mRNA expression levels of *BCL-2*, *TP53*, *BAX* and *NFKB* were investigated by quantitative real time PCR (qPCR) with and without treatment.
Results: BCL-2, BAX and *NF-κB* showed decreased expression in MCF7 bulk cancer cells after DOX treatment whereas only *BCL-2* and *BAX* showed decreased expression in MDA-MB-231 bulk cancer cells. Interestingly *TP53* was the only gene showed a considerable increase in its expression in CSCs of the ER^+ MCF7 cell line compared to bulk cancer cells. Moreover, *TP53* was the only gene showing exceptionally higher level of expression in MCF7-CSCs compared to MDA-MB-231-CSCs.
Conclusion: Our results suggest that CSCs in the ER^+ cells escape the effect of DOX treatment by the elevation of p53 expression.
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Introduction

BC is one of the most common causes of cancer death in women worldwide. Although BC is one of the most susceptible tumors to chemotherapy, some patients relapse and develop resistance to a wide range of drugs [1]. Drug resistance is a multifactorial process and considered the major barrier against successful cancer chemotherapy [2].

BC patients are classified according to the status of their estrogen receptors (ER) into ER positive (ER^+) and ER negative (ER^-). Around two-thirds of breast cancer patients are ER^+ and their tumors depend on estrogen for growth and development [3]. It was reported that ER^+ tumors have worse responses to chemotherapy compared to ER^- tumors [4,5]. This may be due to the presence of estrogen which may reverse the effects of chemotherapy [6]. It was found that when MCF-7 and ZR-75-1, both are ER^+ breast cancer cell lines, were exposed to paclitaxel, there was an increase in cell apoptosis and this response was inverted in the presence of estradiol [7]. Differential gene expression between ER^+ and ER^- tumors before and after drug treatment shows that BCL-2, P53, BAX and NF-κB play a role in drug resistance [6,8–11].

Cancer Stem Cells (CSCs) are tumor initiating cells with high ability to self-renew [12]. Alhaj et al. (2003) was the first to show that breast cancer originates from CSCs [13]. Several studies showed that CSCs play a role in drug resistance. Antiproliferative chemotherapeutic agents were shown to be less effective on CSCs, as they are slow-dividing compared to bulk cancer cells [14,15]. Therefore chemotherapy targets tumor cells, but not CSCs, which eventually will give rise to tumor relapse. Chen et al. (2013) have reviewed and summarized different approaches that have been developed for the elimination of CSCs and subsequently minimizing tumor recurrence [16].

In this study, we hypothesized that ER^+ tumor cells acquire resistance to therapy through activation of *BCL-2*, *TP53*, *BAX* and *NF-κB* genes in CSCs. If this is proven true, it means that targeting CSCs, by any of the means which have been already reported, in ER^+ tumors is enough to make these tumors sensitive to chemotherapy and no need to specifically target these genes' products.

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Thus we investigated the effect of doxorubicin (DOX) treatment on the mRNA expression levels of the aforementioned genes in bulk tumor cells and CSCs in MCF7 and MDA-MB-231 cell lines. We chose DOX due to its ability to induce cell death in both dividing and non-dividing cells [17]. We detected the change in gene expression after DOX treatment in both cell lines. Moreover, we have shown that overexpression of *TP53* in CSCs may be the main reason behind the poor response of ER$^+$ tumors to therapy.

Materials and methods

Cell lines

MCF7 and MDA-MB-231 cell lines were obtained from VACSERA (Cairo, Egypt) and maintained in RPMI and DMEM high glucose media (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Seralab, West Sussex, United Kingdom), 25 μM HEPES (Lonza, Walkersville, MD, USA) and 1% (v/v) penicillin/streptomycin (Lonza, Walkersville, MD, USA). Cells were cultured in a humid cell culture incubator at 37 °C and 5% CO_2.

Cancer Stem Cells enrichment: Anoikis-resistance assay

CSCs were isolated based on their ability to survive anoikis [18]. Briefly, cells were seeded at a density of 2000 cells/cm^2 in agarose-coated tissue culture flasks [19] in mammosphere media (RPMI without phenol red and L-Glutamine, complemented with B27 supplement without vitamin B (1x final concentration) (Gibco life technologies Carlsbad, CA, USA), 1% penicillin/streptomycin and 10 ng/ml epidermal growth factor (Sigma Aldrich, St. Louis, MO, USA). After 12 h, anoikis-resistant cells (representing CSCs and early progenitors) were collected, centrifuged, washed by PBS and RNA was then extracted.

Drug treatment

Both bulk tumor cells and anoikis resistant cells were divided into two groups; Control (cells cultured in fresh media without any treatments) and DOX-treated (cells cultured in media containing 1 μM doxorubicin) (EIMC united pharmaceuticals, Cairo, Egypt). The groups of MCF7 cells were also treated with 17-β estradiol (Sigma Aldrich, St. Louis, MO, USA) re-suspended in ethanol at a final concentration of 10 nM to activate ER. All cell groups were incubated for 12 h at 37 °C and 5% CO_2.

RNA extraction and cDNA synthesis

Total RNA from each group was extracted using iTRAZOL reagent (ITSI Biosciences, Johnstown, PA, USA) following manufacturer instructions. RNA was reverse transcribed using Revert aid first strand cDNA kit (Thermo Fisher scientific, Waltham, MA, USA) following manufacturer instructions.

Real time PCR (qPCR)

The mRNA expression levels of *BCL-2*, *TP53*, *BAX* and *NF-KB* were assessed in all groups by qPCR using the Quantitect SYBR green PCR kit (QIAGEN, Hilden, Germany). Reactions were prepared at a total volume of 25 μl and run on the qPCR machine (MX3005P Stratagene, San Diego, CA, USA). Reactions were run for 40 cycles of 94 °C for 15 sec, 52 °C, 62 °C, 56 °C, or 59 °C (depending on gene under study) for 30 sec, and 72 °C for 30 sec and then a final extension step of 72 °C for 10 min. Primers sequences are listed in Table 1. GAPDH was used as a housekeeping gene. The relative quantification method ($\Delta\Delta$Ct) was used to identify changes in gene expression among groups.

Results

Expression levels of BCL-2, BAX and NF-KB decrease in the ER$^+$ MCF7 cells after DOX treatment

We investigated the effect of DOX treatment on the expression levels of *BCL-2*, *TP53*, *BAX* and *NF-KB* in bulk cancer cells from both ER$^+$ and ER$^-$ cell lines. We found that DOX treatment decreased the expression levels of *BCL-2*, *BAX* and *NF-KB* and slightly increased p53 levels in MCF7 bulk cells after 12 h. In contrast, in the MDA-MB-231 cells, DOX treatment caused subtle changes in the gene expression of genes under study compared to that in MCF7 cell line. Whereas it decreased the expression levels of *BCL-2* (0.81 fold) and *BAX* (0.91 fold) and increased the expression levels of *TP53* (1.14 fold) and *NF-KB* (1.86 fold) in MDA-MB-231 cells (Fig. 1).

TP53 is overexpressed in CSCs of MCF7 after DOX treatment

Changes in the expression levels of all genes under study were detected in DOX-treated bulk and CSCs and compared to their cor-

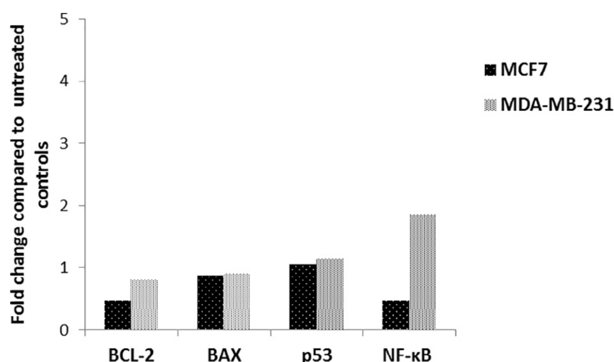

Fig. 1. Gene expression of *BCL-2*, *BAX*, *TP53* and *NF-KB* in MCF7 and MDA-MB-231 bulk cells after 12 h DOX treatment. Cells were incubated with 1 μM DOX for 12 h. Changes in gene expression of *BCL-2*, *BAX*, *TP53* and *NF-KB* after treatment were detected in both cell lines. All genes showed decrease in MCF7 after both 12 h of treatment except *TP53* which showed slight increase. In MDA-MB-231 cell line, only *TP53* and *NF-KB* after 12 h showed considerable increase. The rest of the genes showed subtle change.

Table 1
Primers information.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′to 3′)	Melting temperature
GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT	52 °C
NF-κB	ATGGACAAGACAGCAGGCTC	CTCCACAGCTTCTCTTACCTTT	59 °C
BAX	GCCCTTTTGCTTCAGGGTTTC	CTGATCAGTTCCGGCACCTT	62 °C
P53	ACCTATGGAAACTACTTCCTGAAA	ACCATCGCTATCTGAGCAGC	62 °C
BCL-2	GAACTGGGGGAGGATTGTGG	CATCCCAGCCTCCGTTATCC	56 °C

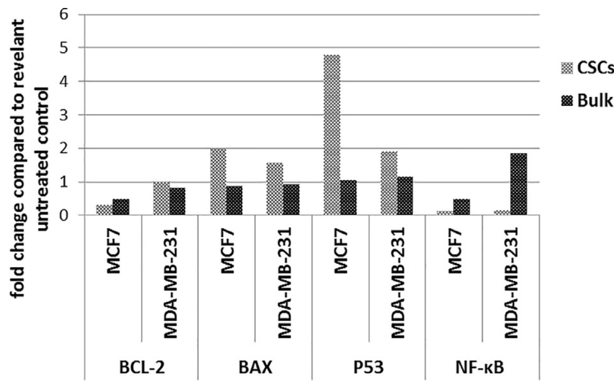

Fig. 2. Gene expression of *BCL-2*, *BAX*, *TP53* and *NF-KB* in MCF7 and MDA-MB-231 bulk and CSCs after 12 h of DOX treatment. CSCs were isolated and incubated with 1 μM DOX for 12 h. Gene expression of *BCL-2*, *BAX*, *TP53* and *NF-KB* were detected in both treated CSCs and bulk tumor cells and compared to their corresponding untreated controls in both cell lines. Interestingly, *TP53* showed the most striking increase in its expression in the treated MCF7-CSCs compared to both treated MCF7 bulk tumor cells and treated MDA-MB-231-CSCs and bulk tumor cells.

Table 2
Fold change between CSCs and bulk cells after 12 h of DOX treatment in MCF7 and MDA-MB-231.

Gene	Fold change in CSCs compared to bulk tumor cells		Fold change in MCF7-CSCs compared to MDA-MB-231-CSCs
	MCF7	MDA-MB-231	
BCL-2	0.66	1.25	0.3
BAX	2.24	1.7	1.25
P53	4.53	1.67	2.5
NF-κB	0.27	0.07	0.93

responding untreated control groups in both MCF7 and MDA-MB-231 cell lines. Interestingly, in MCF7 cell line (ER^+), *TP53* expression showed a striking increase (4.8 fold) in DOX-treated compared to untreated CSCs whereas, it showed only 1.06 fold increase in bulk cancer cells after DOX treatment. None of the other genes showed a striking change in its expression in CSCs compared to bulk cancer cells. In the ER^- MDA-MB-231 cell line, none of the genes showed considerable differences in CSCs compared to bulk tumor cells after DOX treatment (Fig. 2, Table 2).

We then calculated the difference in the expression of all genes between CSCs of MCF7 and those from the MDA-MB-231. Interestingly, *TP53* was shown to show the highest increase in MCF7 CSCs (2.5 fold increase) compared to those from MDA-MB-231 cells (Table 2).

Discussion

In this study we tested the hypothesis that CSCs, and not bulk cancer cells, in the ER^+ tumors may manipulate the expression of *BCL-2*, *TP53*, *BAX* and *NF-KB* to resist therapy. So we investigated the effect of DOX treatment on the mRNA expression of *BCL-2*, *TP53*, *BAX* and NF-KB in bulk cancer cells and CSCs isolated from both the ER^+ MCF7 and the ER^- MDA-MB-231 cell lines. When the two cell lines were treated with DOX for 12 h, the expression levels of BCL-2, BAX and NF-KB were decreased while p53 mRNA expression level was increased in MCF7 bulk cells. Our data agree with other studies revealed that DOX treatment down-regulates BCL-2 mRNA expression levels [20,21]. The decrease in BAX expression is in contrast to other studies showed that BAX is over-expressed in MCF7 cells after treatment with DOX [8,20]. The discrepancy between our data and others could be because of the short period of treatment with DOX in this study.

We showed an increase in NF-KB expression after 12 h of DOX treatment in MDA-MB-231 bulk cells. This agrees with other studies showed that anthracycline drugs cause activation of the anti-apoptotic actions of NF-KB [22]. Moreover, another study showed that treatment of MDA-MB-231 with DOX leads to activation of NF-KB [23]. Although we showed that the mRNA expression levels of BCL-2, BAX, TP53 and NF-KB changed in MCF7 bulk cancer cells, it is not clear whether this change may lead to apoptosis or not.

Taking into consideration the key role of CSCs in drug resistance, we then asked the question whether the detected changes in the expression of these genes occur mainly in CSCs as part of their resistance mechanisms. We isolated CSCs using anoikis resistance assay, measured the mRNA expression of these genes and compared them with those of corresponding controls of bulk cancer cells. Interestingly we found that only TP53 showed a striking increase in its expression in CSCs of the ER^+ MCF7 cell line compared to bulk cancer cells (4.8 fold) suggesting a role for the CSCs in drug resistance through TP53. Moreover, TP53 was the only gene showing exceptionally higher level of expression in MCF7-CSCs compared to MDA-MB-231-CSCs (2.5 fold) suggesting that the proposed TP53 mediated role of CSCs in drug resistant is a characteristic of ER^+ tumors. P53 inhibits tumorigenesis by arresting cell cycle to allow DNA repair [24]. After drug treatment, if the level of DNA damage is beyond the capacity of the DNA repair machinery, P53 induces apoptosis through the activation of pro-apoptotic proteins including BAX [25]. Since CSCs are slowly dividing, it is expected that drug mediated DNA damage is not overwhelming in CSCs. So drug treatment induces the expression of TP53 in CSCs to a level enhances their DNA repair machinery but not to induce apoptosis. This highlights the important role of P53 in CSCs response to drugs in ER^+ tumors. It suggests that when ER^+ tumors are treated, their CSCs tremendously elevate TP53 expression which subsequently activates their DNA repair machinery to resist drug effect.

In conclusion, our data have shown that TP53 is overexpressed in CSCs of the ER^+ MCF7 cells after DOX treatment. Moreover, TP53 level of expression after DOX treatment is tremendously higher in MCF7-CSCs compared to the ER^- MDA-MB-231-CSCs. This suggests that CSCs in ER^+ tumors escape the effect of DOX treatment by stimulating their DNA repair machinery through the elevation of TP53 expression. More studies investigating the gene expression profiles of CSCs isolated from patients with ER^+ tumors after therapy is highly recommended to better understand how CSCs resist therapy in this group of patients. Identification of these genes may facilitate targeting CSCs which eventually reduce the risk of tumor recurrence.

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Potential conflict of interest

The authors have no conflict of interest.

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