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Gene expression alterations in doxorubicin resistant MCF7 breast cancer cell line

Shatha AbuHammad, Malek Zihlif *

Department of Pharmacology, Faculty of Medicine, University of Jordan, Amman 11942, Jordan

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ABSTRACT

Many molecular mechanisms contribute to the development of doxorubicin resistance and different cancers can express wide and diverse arrays of drug-resistance genes. The aim of this study was to identify the changes in gene expression associated with the development of doxorubicin resistance in MCF7 breast cancer cell line. The doxorubicin resistant MCF7 cell line was developed by stepwise selection of MCF7 cells and was tested using the MTT assay. The alterations in gene expression were examined using the real-time based PCR array. The findings showed an up-regulation of many phase I/II metabolizing genes, specifically, the CYP1A1 and the CYP1A2 that were up-regulated by 206- and 96-fold respectively. Drug efflux pump genes were also up-regulated profoundly. TOP2A was strongly down-regulated by 202-fold. Many other changes were observed in genes crucial for cell cycle, apoptosis and DNA repair. The findings of this project imply that the development of doxorubicin resistance is a multi-factorial process.

1. Introduction

Breast cancer is a serious global health problem being the second most common of all cancers and by far the most frequent cancer amongst women [1]. Doxorubicin is a naturally occurring anthracycline antibiotic [2] that is an essential component of many treatment regimens for solid and blood tumors [3], and it is broadly considered the most active single agent available for the treatment of breast cancer [4]. Doxorubicin induces cancer cell death by many mechanisms, most importantly is topoisomerase II-α (TOP2A) poisoning [5].

Although breast cancer is considered one of the most chemosensitive solid tumors, most initially responsive tumors relapse and develop resistance to a broad spectrum of drugs. Consequently, breast cancer becomes refractory to cytotoxic drugs and is typically incurable [6]. Response rates to single doxorubicin treatment range from 43% in previously untreated patients to 28% in patients previously exposed to the drug [7], which indicate that growing resistance to doxorubicin can lead to an unsuccessful outcome in nearly 50% of treated patients, making resistance a major cause of treatment failure.

Two forms of resistance to chemotherapy have been described: intrinsic and acquired resistance. Intrinsic resistance is a pre-existing resistance that present prior to the exposure to a given drug. In contrast, acquired resistance develops in tumors that were initially sensitive to the drug, after the exposure to this drug [8]. Drug resistance is a manifestation of cancer. The somatic mutations and genomic plasticity associated with cancer are the foundation of drug resistance [9]. Therefore, cancer heterogeneity explains the fact that every cancer expresses a different array of drug-resistance genes, and even cells within the same cancer, can exhibit enormous amount of heterogeneity with respect to drug resistance [10]. Many mechanisms could work simultaneously to protect cancer cells from chemotherapeutic agents such as doxorubicin [11]. The most frequently identified molecular changes in doxorubicin resistance are decreased intracellular concentration of the drug due to the expression of many membrane efflux pumps [12], increased drug metabolism enzymes such as glutathione S-transferase GST [13], reduced concentration and activity of TOP2A [14], and failure of the cellular apoptotic pathways [15].

Since multidrug resistance is a multi-factorial phenomenon, a large-scale expression analysis of drug resistant cells may provide information about new candidate genes that could contribute to the development of resistance [16]. In this study we identified the changes in gene expression associated with the development of doxorubicin-resistant phenotype in breast cancer cell line MCF7, employing the polymerase chain reaction (PCR) array technology (RT^2 Profiler™ PCR Array). The PCR array is a 96-well plate containing primers for a set of 84 genes involved in different cellular processes; to the best of our knowledge this is the first study that used the real time PCR array in studying doxorubicin resistance in breast cancer cell lines.

2. Results

2.1. Establishment and characterization of the MCF7/D_{320} cells

The doxorubicin resistant MCF7/D_{320} cell line was established after six sequential treatments with a 320 nM final concentration of doxorubicin.

* Corresponding author. Fax: +962 6 5356746.
E-mail address: M.zihlif@ju.edu.jo (M. Zihlif).

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2.2. Sensitivity to doxorubicin

In order to identify changes in gene expression in breast cancer cells that correlate with the establishment of resistance to doxorubicin, the sensitivity of the MCF7/D_{320} and the MCF7/WT cells to doxorubicin was compared.

According to the MTT assay results, the MCF7/D_{320} cells exhibited considerable resistance to the corresponding drug. The relative resistance of MCF7/D_{320} cells, as compared to the MCF7/WT, is demonstrated in Fig. 1. The IC_{50} values for MCF7/D_{320} and MCF7/WT were 4134.5 ± 9.2 nM (mean ± STD) and 286.2 ± 7.2 nM (mean ± STD), respectively. The degree of resistance is evaluated in terms of resistance index (R) which is calculated according to the relation: $R = IC_{50}$ resistant cells/IC_{50} sensitive cells. Therefore, the MCF7/D_{320} cells were approximately 14-fold more resistant to doxorubicin than the original cell line.

2.3. Morphological features

The development of the drug resistance phenotype is accompanied by changes in different biological features of the malignant cells, including morphological ones.

When compared to MCF7/WT, the MCF7/D_{320} cells were larger with less defined irregular, rounded shape and contained multiple nuclei in the cytoplasm. Multiple, relatively large vesicles were also observed in the cytoplasm. The shape of MCF7/WT and MCF7/D_{320} cells is shown in Fig. 2.

2.4. Intracellular doxorubicin analysis

Fluorescent cell imaging was used to determine intracellular doxorubicin localization and accumulation in MCF7/WT and MCF7/D_{320} cells. In the MCF7/WT cells doxorubicin was specifically concentrated in the nuclei. Conversely, MCF7/D_{320} cells showed no obvious fluorescence in their nuclei. Instead, multiple fluorescent vesicles were observed in the cytoplasm of the resistant cells; these vesicles were located preferentially around the nuclei; the high fluorescence indicates that these vesicles were filled with high concentration of doxorubicin (Fig. 3).

2.5. Gene expression changes upon selection for doxorubicin resistance

In order to identify genes and pathways important in the development of drug resistance, a gene expression profiling analysis was performed using the PCR array, as described in Materials and methods. To filter out unreliable data and identify genes with significantly different expression, a standard 3-fold change in expression was used as an arbitrary cut-off. Expression analysis revealed significant changes of 41 genes (49%), out of the 84 genes examined, in the MCF7/D_{320} cells compared to the MCF7/WT cells, Fig. 4. Twenty-seven of these genes (66%) were up-regulated (Table 1.A), and 14 genes (34%) were down-regulate (Table 1.B).

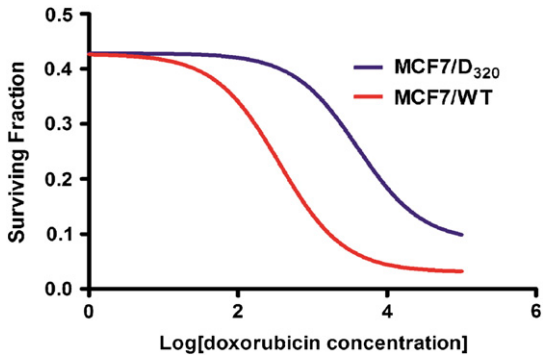

Fig. 1. The logarithmic curves for the response of MCF7/D_{320} cells to doxorubicin as compared to response of MCF7/WT cells.

2.6. Gene ontology and pathways analysis

Gene ontology categories for both up-regulated and down-regulated genes are shown in Table 2. The data are demonstrating that the selection for doxorubicin resistance led to overall changes in the patterns of gene expression, including mainly metabolism, transport, cell cycle, apoptosis and DNA repair. The most important pathways in which the up-regulated and the down-regulated genes participate in are presented in Table 3.

3. Discussion

The development of resistance towards doxorubicin in the MCF7 cells was accompanied by unique morphological changes. The resistant cells exhibited the presence of multiple nuclei in the cytoplasm, according to Yang et al. [19]; this observation was attributed, mostly, to increased cellular fusion between the cells under continuous doxorubicin stress. Additionally, many large vesicles were observed in the cytoplasm of resistant cells. These vesicles, when observed under the fluorescent microscopy, were found to be filled with doxorubicin and were clustered preferentially around the nuclei (Fig. 3). Chen et al. [20] suggested that the cells sequester the drug into these vesicles to avoid doxorubicin poisoning effect on the DNA, and to help in the extracellular clearance of the drug outside the cell. Moreover, Rajagopal and Simon [21] have suggested that these vesicles may play a role in the metabolic inactivation of doxorubicin, because of the high concentration of MRP transporters that was observed in the membranes of these vesicles. The MRP transporters are known to transport the inactivated glutathione-conjugated doxorubicin molecules.

One of the most common changes in doxorubicin resistance is the up-regulation of many transporters genes [12]. This up-regulation explains the reduced intracellular doxorubicin concentration inside the resistant cells that was observed in the intracellular drug analysis (Fig. 3).

The *ABCB1* gene, which encodes the P-gp, was the most up-regulated transporter gene by 24-fold. The P-gp is broad-spectrum multidrug efflux pump that is considered to be a major contributor to the development of multidrug resistance [12,22]. Moreover, two genes of the *ABCC* family, which encodes the MRP transporters, were also profoundly changed, namely *ABCC2* (MRP2) by 16.4-fold and *ABCC6* (MRP6) by 6.2-fold. Both of these transporters have also been detected in doxorubicin resistant cell lines [23,24].

Another finding on the transporters gene expression level was the up-regulation of the *ABCG2* gene, which encodes the BCRP, by 15.9-fold in the resistant cells. Several studies have shown that this transporter was over-expressed in doxorubicin resistant cells [25].

Finally, the *MVP* gene that encodes the LRP was up-regulated by 13.9-folds. The LRP is a non-ATP-dependent intra-cellular transporter, which is responsible for the drug efflux from the nucleus [26]. This up-regulation may be linked to the reduced accumulation of doxorubicin in the nuclei of the MCF7/D_{320} cells that was observed in the intracellular doxorubicin analysis (Fig. 3).

The TOP2A is the primary target for doxorubicin mechanism of action [5]. Thus, resistance to doxorubicin has been correlated reduction in quantity and quality in TOP2A activity [27]. Therefore, the massive reduction in the expression levels of *TOP2A* gene in the PCR array profiling data by 202.6-fold in MCF7/D_{320} cells was an expected finding. Interestingly, no considerable changes were observed in the expression levels of the other topoisomerase isomers, TOP1 and TOP2B. This selective down-regulation of the expression of TOP2A suggests a preferential activity of doxorubicin on the alpha isomer.

Many cytochrome P450 enzymes including CYP1A1, CYP1A2, CYP2C19, CYP2B6, CYP2C9, CYP3A4 and CYP3A5 were up-regulated

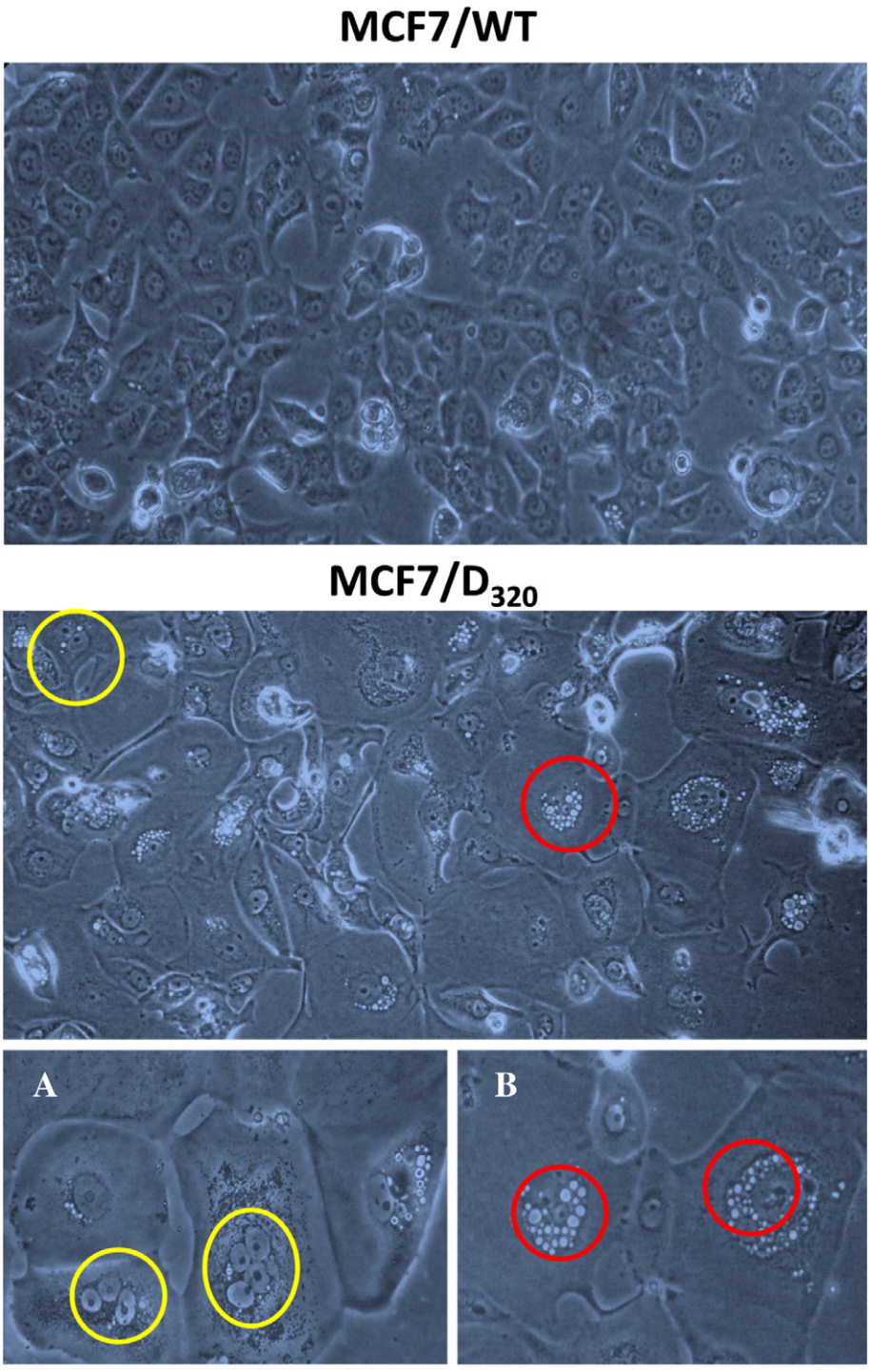

Fig. 2. Comparison of the morphology of MCF7/WT and MCF7/D_{320}. MCF7/WT cells appear small, spindle-shaped cells with single nucleus. MCF7/D_{320} cells appear large, irregular, rounded cells with multiple nuclei (highlighted by the yellow circles) and multiple vesicles (highlighted by the red circles). A and B, higher magnification.

in doxorubicin resistant cells. Remarkably, the *CYP1A1* and the *CYP1A2* genes were 206.1-fold and 96.2-foldup-regulated, respectively. Most of the studies concerning the relationship between doxorubicin and the CYP450 enzymes were conducted mainly on cardiac cell lines; therefore limited data are available concerning the effect of doxorubicin on the expression of these enzymes in cancer cells. Volkova et al. [28] concluded that the exposure of cardiac cells to doxorubicin increased the expression of many metabolizing enzymes including CYP1A1 and CYP1A2. This up-regulation was considered a protective response against doxorubicin cardiac toxicity. Likewise, the reported high up-regulation of these enzymes in the results of the current study could be regarded as an important resistant mechanism, which has been induced to deactivate doxorubicin and thereby increase the cell survival chances.

Additionally, the *CYP3A4* (10.5-fold) and *CYP3A5* (6.7-fold) gene expression levels were found to be up-regulated in the resistant cells. Rodriguez-Antona and Ingelman-Sundberg [29] suggested that tumor cells expressing high CYP3A enzymes showed low response to doxorubicin. This finding was related to the CYP3A oxidation role in the doxorubicin metabolic process.

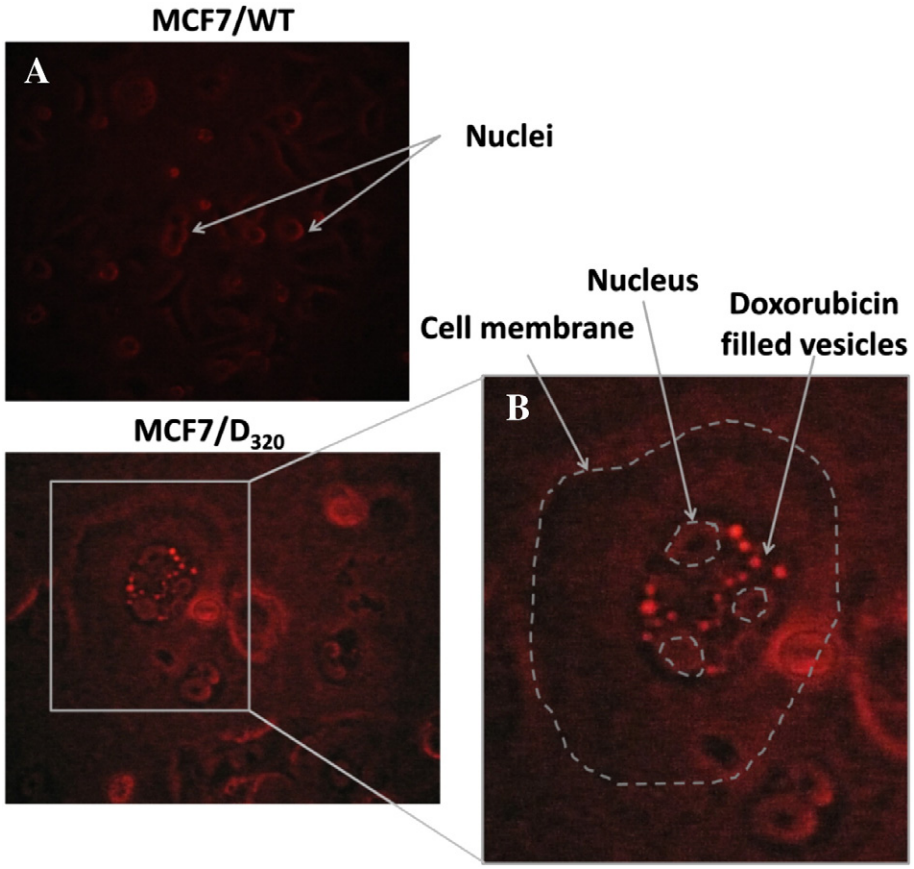

Fig. 3. Fluorescent microscopy images of MCF7/WT and MCF7/D_{320}. MCF7/WT cells: High fluorescence is seen inside the nuclei indicating high concentration of doxorubicin. MCF7/D_{320} cell: (A) Very low fluorescent both in the cytoplasm and in the nuclei of the cells. Fluorescent vesicles are seen indicating that these vesicles are doxorubicin-filled. (B) A closer view demonstrates the doxorubicin-filled vesicles.

Other metabolizing enzymes including CYP2C19 (34-fold), CYP2B6 (22.4-fold), and CYP2C9 (7.4-fold) were also up-regulated in MCF7/D_{320}. It is worth noting that an up-regulation of many *CYP450* genes was also observed clinically in two studies that addressed the resistance towards cyclophosphamide and doxorubicin combination regimen. However, the authors assumed that the

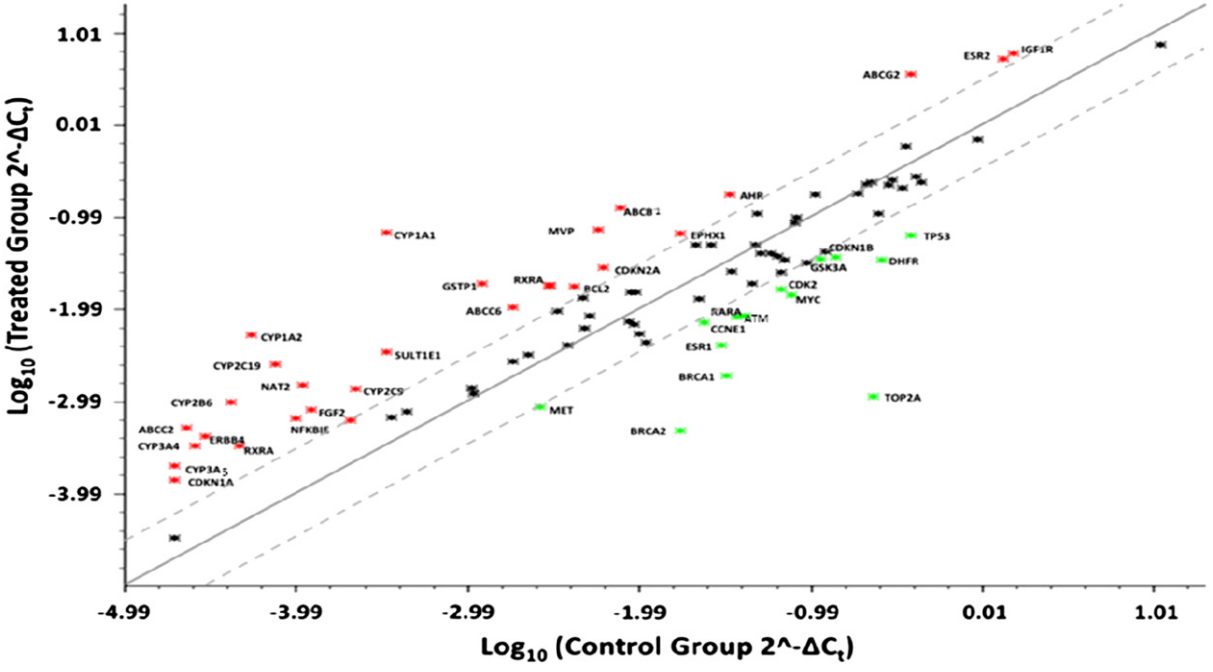

Fig. 4. Scatter plots of genes with altered expression. Up-regulated genes are in red and down-regulated genes are in green.

Table 1
Up-regulated (A) and down-regulated (B) genes in MCF7/D_{320}.

A		B	
Gene symbol	Fold regulation	Gene symbol	Fold regulation
CYP1A1	206.1	TOP2A	−202.6
CYP1A2	96.1	BRCA2	−35.8
CYP2C19	34.0	BRCA1	−16.7
ABCB1 (P-gp)	24.0	ESR1	−7.5
CYP2B6	22.4	DHFR	−7.3
ABCC2 (MRP2)	16.4	TP53	−5.9
ABCG2 (BCRP)	15.8	MYC	−5.3
GSTP1	14.2	CCNE1	−4.8
ERBB4	13.8	RARA	−4.5
MVP (LRP)	12.8	CDK2	−4.0
NAT2	12.8	CDKN1B (p27)	−3.8
CYP3A4	10.4	MET	−3.2
SULT1E1	10.4	GSK3A	−3.2
CDKN1A (p21)	9.4	ATM	−3.1
CYP2C9	7.4		
CDKN2A (p14)	7.1		
CYP3A5	6.7		
FGF2	6.7		
ABCC6 (MRP6)	6.2		
NFKBIE	6.2		
AHR	6.0		
EPHX1	5.8		
RXRA	5.2		
RXRB	4.7		
BCL2	4.3		
IGF1R	3.8		
ESR2	3.1		

up-regulation in the *CYP450* expression was recognized as a mechanism of resistance against cyclophosphamide rather than doxorubicin [30]. This conclusion was built on the documented fact that cyclophosphamide is inactivated by these enzymes, whilst limited data regarding role in doxorubicin deactivation are available.

A profound up-regulation of the expression of the *GSTP1* gene (14.3-fold) was also observed in resistant cells. The GSTP1, a phase II metabolizing enzyme, catalyses the conjugation of glutathione to a wide variety of drugs, rendering them more water-soluble, thereby facilitating their elimination from the cell [31]. Expression of *GSTP1* has been correlated with acquired resistance to doxorubicin [13]. Importantly, the up-regulation of the *GSTP1*gene expression is commonly accompanied by an up-regulation of the expression of the MRP (*ABCC*) transporters that expel the resultant doxorubicin-glutathione conjugates [32].

Additionally, the results showed that the *EPHX1* gene was up-regulated in MCF7/D_{320} cells by about 5.8-fold. The EPHX1enzyme inactivates many reactive species [33]. Hence, the up-regulation of this enzyme could enhance the resistance by inactivating the doxorubicin induced reactive species and reducing free radical formation. A similar finding was reported by Kudoh et al. [34], who observed an increase in the expression of this enzyme in doxorubicin resistant cells.

There was also a 10.5-fold increase in sulfotransferase (*SULT1E1*) gene expression level in MCF7/D_{320} cells. Cowan et al. [35] found an increase in the enzymatic activity of this enzyme in MCF7 cells resistant to doxorubicin. Finally, the expression level of another metabolizing enzyme, the N-acetyltrasferase 2 (NAT2), was elevated by 12.9-fold. This enzyme catalyzes the acetylation reaction of several aryl-amine and hydroxyl-amine xenobiotics [36].

The mRNA levels of many apoptotic pathway genes showed profound alterations. The *TP53* gene was down-regulated by 5.9-fold, while the *CDKN1A* (p21) gene, a major inhibitor of p53-dependent apoptosis [37], was up-regulated by 9.4-fold. Bunz et al. [38] found that expression of p21 protects cells from doxorubicin-induced apoptosis. Another upstream protein that regulates the p53 is the ataxia telangiectasia mutated (*ATM*) gene that was down-regulated by 3-fold. The ATM selectively activate p53, providing a mechanism for controlling cell cycle and apoptotic responses [39], so the up-regulation of the p21 gene and the down-regulation of the *ATM* gene will further suppress the p53 pathway.

Another important alteration in the apoptotic pathway was the up-regulation of the *BCL2* gene by 4.3-fold. The BCL2 is a potent anti-apoptotic protein [40]. The up-regulation of the expression of this gene is known to promote cell survival and has been correlated with the development of doxorubicin resistance [15]. Moreover, the BCL2/BAX (BCL2-associated X protein) ratio was high in the MCF7/D_{320} cells, as the BCL2 was up-regulated while the BAX level was almost unchanged. This ratio is a major indicator of the cell apoptotic fate [41].

Down-regulation of the expression of the glycogen synthase kinase-3 (*GSK3*) gene in MCF7/D_{320} cells by 3.1-fold was also observed. The product of this gene is a multi-tasking serine/threonine kinase known to regulate critical cellular functions such as gene expression, mobility, and apoptosis [42]. Studies have shown that the inhibition of GSK3 delays the apoptotic signaling pathway and thereby induces resistance to chemotherapeutic agents, such as camptothecin, etoposide, and doxorubicin [42]. Therefore, the observed *GSK3* gene down-regulation can be considered as an additional effort exhibited by the cells to escape doxorubicin-induced apoptosis.

Table 2
Gene ontology groups for up/down-regulated genes (≥3-fold) in MCF7/D_{320}.

Category	Count	Genes
Gene ontology groups for up -regulated genes (≥3-fold) in MCF7/D_{320}		
Metabolic process	19	EPHX1, NAT2, GSTP1, ABCC6, CDKN2A, RXRA, CYP1A1, CYP1A2, AHR, ERBB4, IGF1R, CYP3A5, CYP2B6, SULT1E1, FGF2, RXRB, CYP2C19, BCL2, CYP2C9
Regulation of cell proliferation	7	CDKN1A, IGF1R, ERBB4, FGF2, BCL2, CDKN2A, RXRA
Regulation of programmed cell death	7	CDKN1A, IGF1R, FGF2, BCL2, GSTP1, CDKN2A, RXRA
Transmembrane transport	5	ABCB1, ABCC2, ABCC6, ABCG2, MVP
Response to cyclic organic substances	5	CDKN1A, EPHX1, BCL2, RXRA, CYP1A1
Positive regulation of gene expression	4	AHR, FGF2, RXRB, RXRA
Gene ontology groups for down -regulated genes (≥3-fold) in MCF7/D_{320}		
Regulation of transcription, DNA-dependent	9	BRCA1, MYC, RARA, ESR1, CDKN1B, CCNE1, BRCA2, TP53, ATM
Regulation of cell cycle	7	BRCA1, MYC, CDK2, CDKN1B, BRCA2, TP53, ATM
Regulation of apoptosis	7	BRCA1, MYC, TOP2A, CDKN1B, BRCA2, TP53, ATM
Regulation of cell proliferation	6	BRCA1, MYC, CDK2, CDKN1B, BRCA2, TP53
Regulation of cellular metabolic process	5	BRCA1, GSK3A, CDKN1B, BRCA2, TP53
DNA repair	5	BRCA1, TOP2A, BRCA2, TP53, ATM

Table 3
Pathway analysis for up-regulated genes in MCF7/D_{320}.

Pathway	Count	Genes
Pathway analysis for up-regulated genes in MCF7/D_{320}		
Drug metabolism	10	CYP2B6, CYP3A5, CYP2C19, GSTP1, CYP2C9, CYP1A2, NAT2, CYP3A4, EPHX1, SULF1E1
Pathways in cancer	8	CDKN1A, IGF1R, FGF2, RXRB, BCL2, GSTP1, CDKN2A, RXRA
Transporters	5	ABCB1, ABCG2, ABCC2, ABCC6, MVP
Cell cycle: g1/s check point	2	CDKN1A, CDKN2A
Pathway analysis for up-regulated genes in MCF7/D_{320}		
Pathways in cancer	8	MYC, MET, RARA, CDK2, CDKN1B, CCNE1, BRCA2, TP53
Cell cycle	6	MYC, CDK2, CDKN1B, CCNE1, TP53, ATM
p53 signaling pathway	4	CDK2, CCNE1, TP53, ATM
DNA repair	3	ATM, BRCA1, BRCA2

Finally, the nuclear factor-kappa-B inhibitor (*NFKBIE*) gene was up-regulated by 6.2-fold. The nuclear factor-kappa-B (NFκB) plays an important role in regulating the expression of anti-apoptotic proteins [43]. Paradoxically, increasing *NFKBIE* expression might be expected to deactivate NFκB, thereby reducing expression of anti-apoptotic proteins and rendering cells more sensitive to doxorubicin. Whether the increased expression of *NFKBIE* observed in MCF7/D_{320} cells is important for doxorubicin resistance requires further study.

The changes in the gene expression profiles of the doxorubicin resistant cells involved many cell cycle controlling genes. Both *CDK2* and its target cyclin E (*CCNE1*) genes have been down-regulated in MCF7/D_{320} cells by 4.7- and 4-fold, respectively. The cyclin E/CDK2 complex is a crucial regulator of the G1/S transition [44]. Furthermore, the results showed alterations in the expression levels of *CDKN1A* (p21) and *CDKN1B* (p27) genes, which are inhibitors of the cyclin E/CDK2 complex [45]. *CDKN1A* was up-regulated by 9.4-fold, while *CDKN1B* was down-regulated by 3.7-fold; therefore it is clear that the cell is going more towards inhibiting the cyclin E/CDK2 complex. Another finding was the up-regulation of the expression level of *CDKN2A* (p16) by 7.1-fold. The p16 regulates the G1/S cell cycle transition, and has the capacity to arrest cells in the G1-phase [46]. Taken together, these changes in the cell cycle genes indicate that the resistant cells are trying to postpone the entrance into the S-phase in an attempt to prohibit doxorubicin from exerting its toxic effect.

Finally, the myelocytomatosis (*MYC*) transcription factor gene expression level was also decreased by 5.3-fold. The c-Myc has the ability to promote cell proliferation [47]. A study by Grassilli et al. [48] found that the absence of Myc impairs the apoptotic response to doxorubicin, thus conferring resistance to this drug.

The DNA repair genes, *BRCA1* and *BRCA2*, are well-established breast cancer susceptibility markers. These genes can serve as predictors of the response to chemotherapies that induce DNA damage [18]. *BRCA1* and *BRCA2* expression levels have been down-regulated in MCF7/D_{320} cells by 16.7- and 35.8-fold, respectively. There are great discrepancies in the studies that addressed the role of BRCA1 and BRCA2 proteins in doxorubicin resistance. Some studies found that absence of BRCA1 was accompanied by increased resistance to doxorubicin [4,18], whilst others found that tumor cells with low BRCA1 were highly sensitive to doxorubicin [49]. De Luca et al. [50] suggested that BRCA1 may have a role as a co-regulator in the transcriptional level, and found that BRCA1 mediates apoptosis and decreases viability in response to doxorubicin treatment in prostate cancer, it had also been concluded that loss of BRCA1 resulted in increased resistance towards doxorubicin. Consequently, the profound down-regulation of *BRCA1* and *BRCA2* gene expression level seen in MCF7/D_{320} cells may be considered as one of the main changes towards gaining the resistant phenotype.

4. Conclusions

In conclusion, changes in gene expression levels that accompany the establishment of doxorubicin resistance, in MCF7 breast cancer cells, were identified in a collective view. The findings of this project provide important insights into the possible biochemical pathways that enable breast cancer cells to acquire resistance to doxorubicin, such as the metabolic pathways, the drug efflux pathways, the apoptotic pathways, the cell cycle pathways, and the DNA repair pathways. In addition to alteration in TOP2A, that is the main target for doxorubicin.

5. Materials and methods

5.1. Cell culture growth conditions

The MCF7 cell line, originally obtained from the American Type Culture Collection (ATCC; USA), was kindly provided by the Molecular Biology Laboratory, University of Jordan. The MCF7 cells were maintained as an attached monolayer culture in the commercially defined RPMI 1640 medium (HyClone, USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (HyClone, USA), 2 mM L-glutamine, 100 U/mL and 100 μg/mL penicillin–streptomycin (HyClone, USA), and 25 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Lonza, USA).

The cells were grown on either 25 or 75 cm^2 attached types, filter-cap culture flasks (NunClon, Denmark). The cells were then incubated at 37 °C in a 90% humidified atmosphere of 5% CO_2.

5.2. Establishment of doxorubicin resistant sub-line

Doxorubicin 2 mg/mL (Ebewe, Austria) was used to induce resistance in the MCF7 cells. To resistant cells were selected by stepwise selection method [17]; initially the cells were grown in a cell culture media containing a doxorubicin concentration of 10 nM. When the cells were capable of growing and reaching appropriate confluency at a certain concentration, the cells were passaged and double the previous doxorubicin concentration was used for stepwise selection of resistant cells; a final concentration of 320 nM doxorubicin was applied.

The developed sub-line is regarded as MCF7/D_{320}, and the wild type (untreated control) MCF7 as MCF7/WT through this paper. To exclude the effects associated with long-term culture of MCF7 cells, the MCF7/WT cells were cultured under identical conditions and maintained in culture for the same period of time as the MCF7/D_{320} cells but in the absence of doxorubicin.

Before any further experiment, the MCF7/D_{320} cells were maintained in doxorubicin free medium for at least 2 days.

5.3. Cell proliferation assay

The antiproliferative effects of doxorubicin on MCF7/WT and MCF7/D_{320} cells were evaluated using the CellTiter Non-Radioactive Cell Proliferation Assay Kit® (Promega, USA), according to manufacturer's instructions. This assay is a colorimetric test based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a yellow tetrazole, to a purple formazan, a process that occurs in the mitochondria of viable cells.

The cells were seeded onto 96-well plates (Greiner, Germany) at a concentration of 10×10^3 cells/well and incubated for at least 18 h. After which, horizontal dilutions of the drug ranging from 40 to 1000×10^3 nM were added (100 μL/well). Each concentration was added in duplicates, and every plate contained a control of cells in plain medium. The cells were then incubated at 37 °C for 72 h.

After incubation, the media were aspirated from the wells and replaced by fresh media (100 μL/well); 15 μL of the MTT dye solution was added to each well. The plates were incubated at 37 °C for 4 h, and then 100 μL of solubilisation/stop solution was added to each well. Optical density (OD) at 570 nm wavelength was recorded 1 h later using a 96-well plate reader (Sunrise basic sciences, Austria). Measurements were performed in duplicates.

5.4. Data analysis

Results of the MTT cell proliferation assay were analyzed using the GraphPad PRISM®5.0 software (GraphPad Software, Inc.). The inhibitory concentration (IC_{50}) values, which are the drug concentration at which 50% of cells are viable, were calculated from the logarithmic trend line of the cytotoxicity graphs.

5.5. Analysis of intracellular doxorubicin

The distribution of doxorubicin associated with both the MCF7/WT and the MCF7/D_{320} cells was assessed based on the inherent fluorescence of doxorubicin [18].

Cells were incubated in 5 mM doxorubicin for 2 h at 37 °C. After incubation, cells were washed with cold PBS buffer and immediately run on a fluorescent microscope (Nikon, USA). Doxorubicin molecules associated with the cells were excited with an argon laser beam at 488 nm, and the emitted fluorescence was detected through 575 nm band pass filter.

5.6. RNA isolation

The RNA was isolated using an RNeasy® Mini kit (Qiagen, Germany) following the manufacturer's instructions. An RNase-free DNase set (Qiagen, Germany) was used according to the manufacturer's instructions to ensure complete genomic DNA elimination. Purity of isolated RNA was determined by measuring ratio of the optical density of the samples at 260 and 280 nm. The OD_{260}/OD_{280} ratio was ranging from 1.9 to 2.2 for all samples. Denaturing agarose gel electrophoresis was used for the visualization of the intactness of the isolated RNA.

5.7. cDNA synthesis

Complementary DNA strands were synthesized using RT^2 First Strand kit (Qiagen, Germany), according to the manufacturer's instructions; aliquots containing 1 μg of total RNA were used from each sample. The OD_{260}/OD_{280} ratio was calculated for purity and it was within 1.8–2.0.

5.8. Gene expression profiling

Pathway-focused gene expression profiling was done using a 96-well human cancer drug resistance and metabolism PCR array, RT^2 Profiler PCR array (PAHS-004A, Human Cancer Drug Resistance & Metabolism PCR Array, Qiagen, USA). In this array, 84 wells contained all the components required for the PCR reaction in addition to a primer for a single gene in each well. These genes are involved in the body response to chemotherapy, and encode important enzymes that contribute in drug resistance, metabolism, DNA repair, cell cycle, growth factors, hormone receptors, and transcription factors.

Before performing the procedures all the surfaces, plastic- and glassware were wiped down with RNaseZap® in order to eliminating RNase contamination.

A diluted cDNA aliquot, equivalent to 1 μg total RNA for each plate, was mixed with the RT^2 SYBR® green master mix (Qiagen, USA) according to the manufactures instructions, and loaded onto the 96-well array. Real-time PCR was performed using the iCycler (Bio-Rad, USA) thermo-cycler, by heating the plate to 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

5.9. Data analysis

The cycle threshold (C_t) values for each sample were given automatically by the iCycler according to the amplification curves. The baseline and threshold values were manually set as recommended by the PCR array user manual. The selected threshold was 20.0 and the baseline cycles are 2–10.

The PCR array data analysis was performed using the ΔΔCt method (delta delta cycle threshold); the analysis was performed automatically according to the SABiosciences company (Qiagen, USA) web portal (www.SABiosciences.com/pcrarraydataanalysis.php), and further re-calculated manually. Changes in gene expression were illustrated as a fold increase or decrease. The data were normalized, across all plates, to the following housekeeping genes: hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), ribosomal protein L13a (*RPL13A*), glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) and, actin beta *ACTB*. The threshold cycle values of the control wells were all within the ranges recommended by the PCR array user manual.

5.10. Gene functional annotation

The genes whose expression levels were altered by ≥3-fold were classified according to the web-based bioinformatics software, the Database for Annotation, Visualization and Integrated Discovery (DAVID) gene annotation website, http://david.abcc.ncifcrf.gov/, which provides biological classification of the genes according to their cellular function and the pathways in which they participate. Results are displayed in comparative tables that describe the most affected biological function by genes changes. By this means, it was possible to identify the biological pathways that were most disturbed by doxorubicin resistance, which could provide clues to the mechanisms by which the cells can develop resistance against doxorubicin.

For pathway analysis, two different databases were employed using DAVID, the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Biocarta databases.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2012.11.009.

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