

## Molecular analysis of Bcl-2 and cyclin D1 expression in differentially expressing estrogen receptor breast cancer MCF7, T47D and MDA-MB-468 cell lines treated with adriamycin

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### ABSTRACT

**Background and purpose of the study:** Bcl-2 and Cyclin D1 (CCND1) are key elements in cancer development and progression. Bcl-2 acts as a cell death suppressor and is involved in apoptosis regulation. Cyclin D1 is an important regulator of G1/S phase of the cell cycle progression. In addition, estrogen receptor (ER) is an important prognostic factor in breast cancer cells. Therefore it is important to determine the Bcl-2 and CCND1 expression in MCF7, T47D and MDA-MB-468 breast cancer cell lines with different ER status following Adriamycin (ADR) treatment.

**Methods:** Cytotoxicity of ADR (250 and 500nM) after 1-5 days exposure of the cell lines was evaluated by MTT assay. The mRNA and protein levels of Bcl-2 and cyclin D1 in tested cell lines were also analyzed by RT-PCR and immunocytochemistry (ICC) methods.

**Results:** ADR cytotoxicity was highest in MDA-MB-468 and lowest in MCF7 cells in a time-dependent manner. Bcl-2 mRNA increased in MCF7 and decreased in MDA-MB-468 after exposure to ADR but it was less detectable in T47D cells. The expression of CCND1 in MCF7 with high level of ER expression was higher than the other two cell lines in untreated conditions. However, CCND1 mRNA did not show significant changes after ADR treatment. Immunocytochemical analysis did not show significant differences between Bcl-2 protein expression in the presence or absence of ADR in MDA-MB-468 cell line while in T47D and MCF7 cells its expression decreased after exposure to ADR. In addition to nuclear expression of cyclin D1 in all cell lines, strong cytoplasmic expression of cyclin D1 protein was observed only in MCF7 and T47D cells.

**Conclusion:** The tested cell lines with different levels of ER expression showed differential molecular responses to ADR that is important in tumor-targeted cancer therapy.

**Keywords:** Breast Cancer, Bcl-2, CCND1, Adriamycin, RT-PCR, Immunocyto-chemistry

### INTRODUCTION

Breast cancer is one of the leading causes of mortality among women worldwide. A large number of mutated genes play important roles in the pathogenesis as well as breast cancer response to chemotherapy. The Bcl-2 oncoprotein functions to suppress or delay the induction of apoptosis in a number of systems, including mammary glands (1). Bcl-2 belongs to a family of proteins that includes both pro apoptotic and anti apoptotic members that are expressed differentially among different cell types and are believed to regulate apoptosis. Bcl-2 has been investigated in various cancers and is associated with poor prognosis. However, in breast cancer Bcl-2 expression has been reported to be associated with better

outcomes in patients who treated either with hormone- or chemotherapy (2). The expression of Bcl-2 in ER positive breast tumors is higher than in ER negative ones (3-6) which seems to be in contradiction with separate clinical observations that ER negative breast cancers, which express bcl-2 at low levels, show more aggressive phenotypes and greater resistance to endocrine and chemotherapy (4) suggesting a complex interaction between Bcl-2 expression and clinical outcome.

Cyclin D1 is a key cell cycle regulatory protein which governs cell cycle progression from G1 to S phase (7). The cyclin D1 gene is one of the most frequently amplified genes observed in human tumors and plays a pivotal role in the

development of a subset of human cancers including breast cancer (8). Overexpression of cyclin D1 is correlated with the early onset of cancer and risk of tumor progression and metastasis (9). A strong positive correlation between cyclin D1 and ER expression has also been demonstrated in several studies (10-13).

Taking into consideration the important roles of Bcl-2 and cyclin D1 in cancer development and progression, it was decided to analyze the expression of Bcl-2 and CCND1 genes and proteins following exposure to Adriamycin, a putative antitumor drug, in MCF7 cells that highly express estrogen receptor (ER), in T47D cells that moderately express estrogen receptor and finally MDA-MB-468 cells that do not express estrogen receptor as examples of breast cancer cell lines with different ER status.

## MATERIALS AND METHODS

### Materials

RPMI 1640 culture medium and Fetal Bovine Serum (FBS) were purchased from Gibco, UK. MTT powder was obtained from Sigma, UK. Primary antibodies for Bcl-2 and cyclin D1 were purchased from Dako, Denmark and LAB VISION, USA, respectively. Doxorubicin was purchased from EBEWE Pharma, Austria.

### Cell lines and culture conditions

The human breast cancer cell lines (MCF7, T47D and MDA-MB-468) were obtained from National Cell Bank of IRAN (Pasteur Institute). Cells were maintained in RPMI 1640 culture medium supplemented with 10% FBS and 100 U ml<sup>-1</sup> of penicillin and 100 ng ml<sup>-1</sup> of streptomycin at 37°C in 5% humidified CO<sub>2</sub> incubator.

### Cytotoxic Effect of Adriamycin on MCF7, T47D and MDA-MB-468 Breast Cancer Cell Lines:

The cells were seeded in 96-well plates at 1×10<sup>4</sup> cells/well in RPMI 1640 culture medium and incubated at 37°C in 5% CO<sub>2</sub> incubator for 48 hours. After 48 hours culture medium was changed to RPMI with and without Adriamycin. Concentration of Adriamycin was obtained from previous report (14) as 250nM for T47D and MDA-MB-468 cells and 500nM for MCF7 cells. Cytotoxicity was then measured during 1-5 days of drug exposure by MTT method (15).

### RNA isolation

The cells (MCF7, T47D and MDA-MB-468) were seeded in T-25 flasks in RPMI 1640 culture medium and incubated in a humidified CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37°C). After 48 hours culture medium was changed and Adriamycin [MCF7

(500nM), T47D & MDA-MB-468 (250nM)] were added to the corresponding flasks. After 72 hours total RNA was isolated by TriPure isolation reagent (Roche, Germany) according to the previously reported method (16).

### RT-PCR

cDNAs were synthesized by M-MLV reverse transcriptase (Fermentas, Lithuania) and subjected to polymerase chain reaction (PCR) by specific primers (sense, 5'- CTG CTC GTG AAG AAG CTC -3'; antisense, 5'-CTC TGG AGA GGA AGC GTG TG- 3') for CCND1 and (sense, 5'- GTT CGG TGG GGT CAT GTG TGT GGA GA -3'; antisense, 5'- GCT GAT TCG ACG TTT TGC CTG AAG AC -3') for Bcl-2. The β-actin was used as house keeping gene (sense, 5'-TGA CGG GGT CAC CCA CAC TGT-3'; antisense, 5'-CTA GAA GCA TTT GCG GTG GAC-3'). PCR conditions for Bcl-2 amplification were: 35 cycles of 95°C for 1 min, 64°C annealing for 1 min and 72°C extension for 1 min and 20 s and for CCND1 and β-actin: 30 cycles of 95°C for 30 s, 57°C annealing for 30 s and 72°C extension for 30 s. The PCR products were visualized using 1.2% agarose gel electrophoresis and staining with ethidium bromide.

### Immunocytochemical analysis of cyclin D1 and Bcl-2 expression

The cells (MCF7, T47D and MDA-MB-468) were seeded in 8-well chamber slides (Lab Teck, USA) in RPMI 1640 culture medium and incubated in a humidified CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37°C). After 48 hours culture medium was changed and Adriamycin [MCF7 (500nM), T47D & MDA-MB-468 (250nM)] was added to corresponding wells for 72 hours. The cells were then fixed with methanol: acetone (9:1) and blocked for endogenous peroxidase activity and non-specific binding sites by 3% H<sub>2</sub>O<sub>2</sub> in methanol followed by UltraV block (Labvision, USA). Cells were then incubated overnight at 4°C with Cyclin D1 (Clone: SP4, Labvision, USA) and Bcl-2 (Clone: 124, Dako, Denmark) antibodies. The results were visualized by the streptavidine-biotin immunoperoxidase detection kit and AEC chromogen (Labvision, USA) according to the manufacturer's instruction with necessary modifications. Finally, cells were counterstained with Meyer's hematoxyline (Dako, Denmark), mounted and studied under light microscope.

### Statistical analysis

Results of the cell growth assay were presented as mean ± SE in three independent experiments and were analyzed using one way ANOVA followed by dunnett post test. Mean differences with P<0.05 were considered statistically significant.

## RESULTS

### Cytotoxic effects of Adriamycin on breast cancer cell lines

The cytotoxic effects of Adriamycin [MCF7 (500nM), T47D & MDA-MB-468 (250nM)] were evaluated by MTT assay during 1-5 days of drug exposure. ADR showed a significant time-dependent anti-proliferative effect on MCF7, T47D and MDA-MB-468 cells (Fig. 1).

### Effect of Adriamycin on the mRNA levels of Bcl-2 and CCND1

Following treatment of cells with ADR, total RNA was isolated from treated and untreated samples and effects of Adriamycin on the level of expression Bcl-2 and CCND1 mRNA was analyzed using RT-PCR. While Bcl-2 mRNA level of expression level increased in MCF7 and decreased in MDA-MB-468 after ADR exposure, it was not detectable in T47D cells, which means that the mRNA expression level of Bcl-2 in T47D is less than the other two cell lines (Fig. 2A-B and Table 1). The CCND1 mRNA level did not show significant changes after ADR treatment and relatively equal levels of CCND1 mRNA were detected in all cell lines in comparison to control. However, its expression in MCF7 cells was higher than the other two cell lines (Fig. 2A and Table 1).

### Immunostaining of cell lines with Bcl-2 and cyclin D1 antibodies

The cell lines were immunostained with primary antibodies for Bcl-2 and cyclin D1 in the presence and absence of ADR as described in the section of methods. Cytoplasmic expression of Bcl-2 was detected in the absence of ADR in all cell lines. After ADR exposure, Bcl-2 protein expression did not change significantly in MDA-MB-468 cells while it decreased in T47D and MCF7 cell lines (Fig. 3). Nuclear expression of cyclin D1 was detected in all control samples which was highest in MCF7 and lowest in MDA-MB-468. Following ADR treatment, in addition to nuclear staining, strong cytoplasmic expression of cyclin D1 protein was observed in MCF7 and to some extent in T47D cells. The MDA-MB-468 cells showed only a mild increase in cyclin D1 nuclear expression (Fig. 4).

## DISCUSSION

An important finding of the present study is the lower toxicity of Adriamycin, a well known anticancer drug, in highly expressing estrogen receptor breast cancer cell line (MCF7) in comparison to other two cell lines which have less (T47D) or no estrogen receptor (MDA-MB-468).

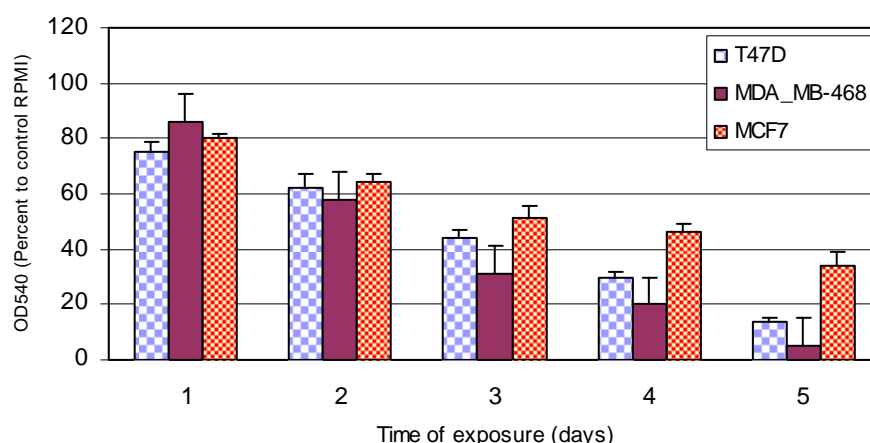
This is most probably due to higher levels of expression of Bcl-2 and cyclin D1 proteins in MCF7 cells than the other two tested cell lines. These findings indicate the importance of predictive values of certain biomarkers such as Bcl-2 and cyclinD1 in selection of chemotherapy regimen.

Although the Bcl-2 gene is overexpressed in many tumors, its precise role in tumor development is not well understood (1, 17-18). Different mechanisms might be involved in the regulation of Bcl-2 expression. For example, negative regulation of Bcl-2 transcription by the p53 tumor suppressor gene has been reported (19-21). Several studies suggest that resistance of breast cancer cells to chemotherapy is linked to Bcl-2 expression in these cells (22-24). In addition, it has been shown that the mean expression of Bcl-2 in ER positive breast cancers is higher than in ER negative cells (3-6). In T47D cells, a decrease in Bcl-2 mRNA level was observed after ADR exposure, confirming the results obtained by the immunocytochemistry study. In MDA-MB-468 cells, although Bcl-2 protein expression was low and did not show a significant change, at mRNA level there was a decrease after ADR treatment. When comparing MCF7 cells with high estrogen receptor and T47D cells with moderate estrogen receptor, it could be concluded that the lower Bcl-2 expression observed in this study in T47D cells might be due to lower expression of estrogen receptor in these cells.

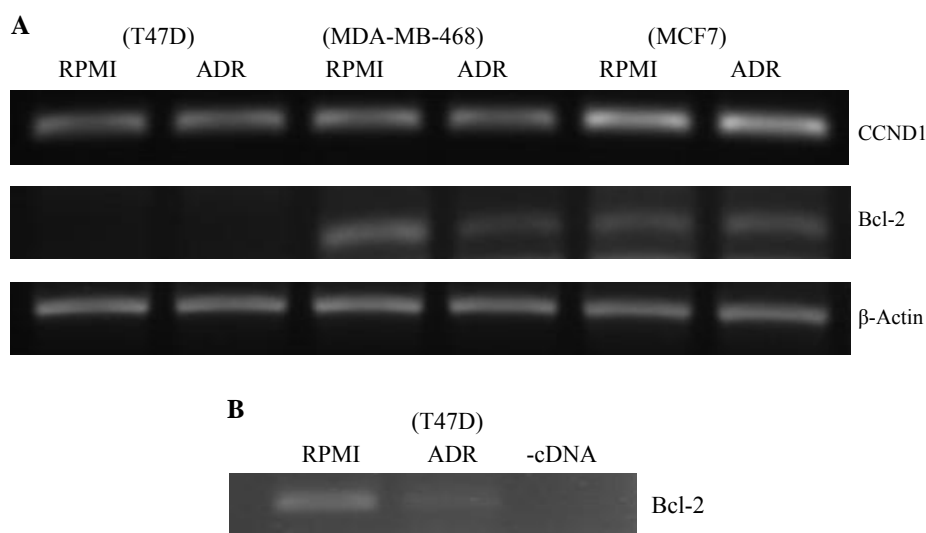
In MDA-MB-468 cells, a low level of Bcl-2 protein expression was observed even before ADR treatment that could be explained by estrogen receptor negativity of this cell line. Interestingly the Bcl-2 mRNA expression was not affected by ER status and was even higher than the Bcl-2 mRNA level in MCF7 which could be due to p53 mutation in this cell line. Decrease in the Bcl-2 mRNA expression in T47D and MDA-MB-468 cells after ADR treatment is consistent with the occurrence of drug induced apoptosis in these cell lines. Therefore, the role of Bcl-2 in breast cancer is complex and depends on tumor stage, chemotherapeutic regimens and co-expression of other factors such as ER and p53 that are critical for cancer cell growth, differentiation and apoptosis.

Deregulation of genes involved in the control of the cell cycle, such as cyclin D1, is also one of the most frequent alterations in tumor growth. Cyclin D1 overexpression has been observed in many human malignancies including breast carcinoma in which both nuclear and cytoplasmic cyclin D1 staining are well known events (8, 25).

It has been shown that cytoplasmic staining for cyclin D1 occurs during the G1/S transitional



**Figure 1. Cytotoxic effects of Adriamycin on breast cancer cell lines.** Cells were seeded in 96-well plates at  $1 \times 10^4$  cells/well in RPMI 1640 culture medium and incubated at 37°C in 5% CO<sub>2</sub> incubator. After 48 hours culture medium was changed to RPMI with and without Adriamycin (250nM for T47D and MDA-MB-468 cells and 500nM for MCF7 cells). Cytotoxicity was then measured during 1-5 days of drug exposure by MTT method. Data are presented as mean  $\pm$  SE of the average of 4 wells in three independent experiments.

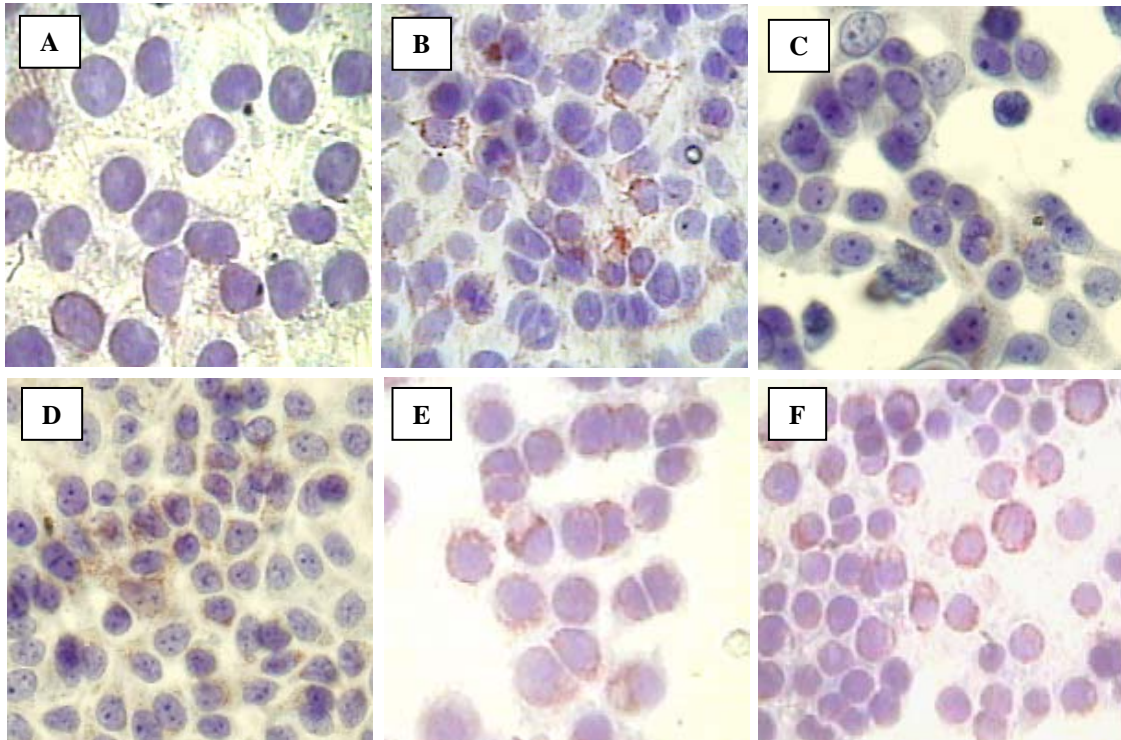


**Figure 2. Effects of Adriamycin on the mRNA expression of CCND1 and Bcl-2 in MCF7, T47D and MDA-MB-468 cells.** After incubation with ADR, total RNA was isolated from treated and untreated samples and effect of Adriamycin on the expression levels of CCND1 and Bcl-2 mRNA was analyzed by RT-PCR (A). The mRNA expression of Bcl-2 in ADR treated and untreated T47D cells using 5µl of cDNA (B).

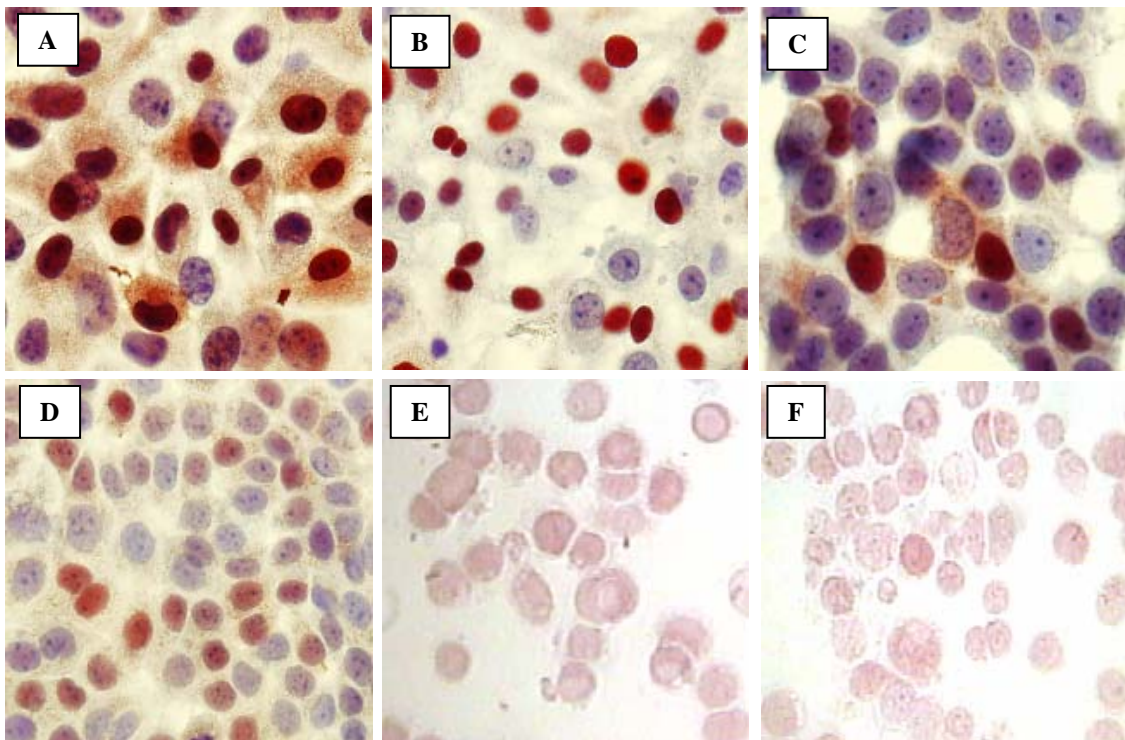
**Table 1.** Comparison of the mRNA expression levels of CCND1 and Bcl-2 in ADR treated and untreated T47D, MDA-MB-468 and MCF7 breast cancer cell lines. The mRNA expression was analyzed by RT-PCR and relative intensity of each band was measured and normalized with  $\beta$ -Actin using LabWork software for densitometric analysis of amplified cDNA bands resolved on agarose gel and stained with EtBr.

	T47D		MDA-MB-468		MCF7	
	RPMI	ADR	RPMI	ADR	RPMI	ADR
CCND1	1.457 $\pm$ 0.005	1.602 $\pm$ 0.002	1.566 $\pm$ 0.025	1.706 $\pm$ 0.083	2.859 $\pm$ 0.013	2.756 $\pm$ 0.01
Bcl-2	*0.173 $\pm$ 0.02(5µl)	*0.028 $\pm$ 0.004 (5µl)	0.397 $\pm$ 0.038	0.176 $\pm$ 0.007	0.179 $\pm$ 0.006	0.276 $\pm$ 0.006

\*: According to the data from figure 2-B



**Figure 3. Immunostaining of MCF7, T47D and MDA-MB-468 cells using mouse monoclonal antibody against Bcl-2.** ADR treated MCF7 (A), T47D (C) and MDA-MB-468 (E) and untreated MCF7 (B), T47D (D) and MDA-MB-468 (F) cells were immunostained with primary antibody for Bcl-2, visualized by labvision detection system using AEC chromogen, mounted and studied under light microscope. (Magnification 400X)



**Figure 4. Immunostaining of MCF7, T47D and MDA-MB-468 cells using rabbit monoclonal antibody against cyclin D1.** ADR treated MCF7 (A), T47D (C) and MDA-MB-468 (E) and untreated MCF7 (B), T47D (D) and MDA-MB-468 (F) cells were immunostained with primary antibody for cyclin D1, visualized by labvision detection system using AEC chromogen, mounted and studied under light microscope. (Magnification 400X)

phase of the cell cycle, in which nuclear staining is visualized only in G1. Cyclin D1 accumulation is required for progression through the G1 phase of the cell cycle and its degradation at the end of G1 phase is also necessary for progression into S phase. Elevated levels of cyclin D1 are required for continuation of the cell cycling. However, cells rapidly adjust the level of cyclin D1 protein by regulation of the rate of ubiquitin dependent degradation (26). Cyclin D1 is localized in the nucleus. At the end of the G1 phase, GSK3 $\beta$  migrates into the nucleus and phosphorylates cyclin D1, resulting in ubiquitination, nuclear export and degradation of the cyclin in the cytoplasm (27).

In the present study, Immunocytochemical analysis showed a strong cytoplasmic expression of cyclin D1 in addition to nuclear staining following ADR exposure in ER positive MCF7 cells and to some extent in T47D cell line. ER negative MDA-MB-468 cell line showed only a mild increase in cyclin D1 nuclear expression. MCF7 cells showed higher cyclin D1 protein expression compared to other two cell lines.

RT-PCR analysis revealed that ADR did not change CCND1 expression and relatively equal levels of CCND1 mRNA were detected in all cell lines after ADR treatment compared to untreated samples. However, it should be mentioned that the mRNA expression level of CCND1 in MCF7 cells was higher than the other two cell lines. The higher expression of cyclin D1 in MCF7 cells, both at protein and mRNA levels, confirms the results of the previous studies indicating the positive correlation between cyclin D1 expression and estrogen receptor status. On the other hand the cytoplasmic expression of the cyclin D1

protein in MCF7 and T47D cells after ADR treatment could be due to ubiquitin dependent degradation of the protein with the mechanism which was mentioned.

In normal cells there is a regulatory pathway between p53 and cyclin D1. The p53 induces cyclin D1 overexpression through induction of p21. It has been shown that some p53 mutations abolish this inductive effect of p53 on p21 resulting in loss of the effects of p53 (28) on the cell cycle.

Therefore, overexpression of cyclin D1 in MCF7 cells can also be explained by the presence of wild type p53 in this cell line compared to mutant p53 in T47D and MDA-MB-468 cells and is consistent with the results of a report that showed more frequent Cyclin D1 protein overexpression in tumors with wild type p53 and ER positive status (29).

Considering our findings in these cell lines with different estrogen receptor status which showed differential molecular and cellular responses to ADR treatment, it is essential to do similar studies in different available breast cancer cell lines for prediction of clinical effectiveness of ADR therapy. Taken together, these data on gene expression patterns as predictive biomarkers of sensitivity to chemotherapy could expand the horizons in treatment of breast cancer and allow choosing the most suitable, tumor-targeted regimen for each individual patient.

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