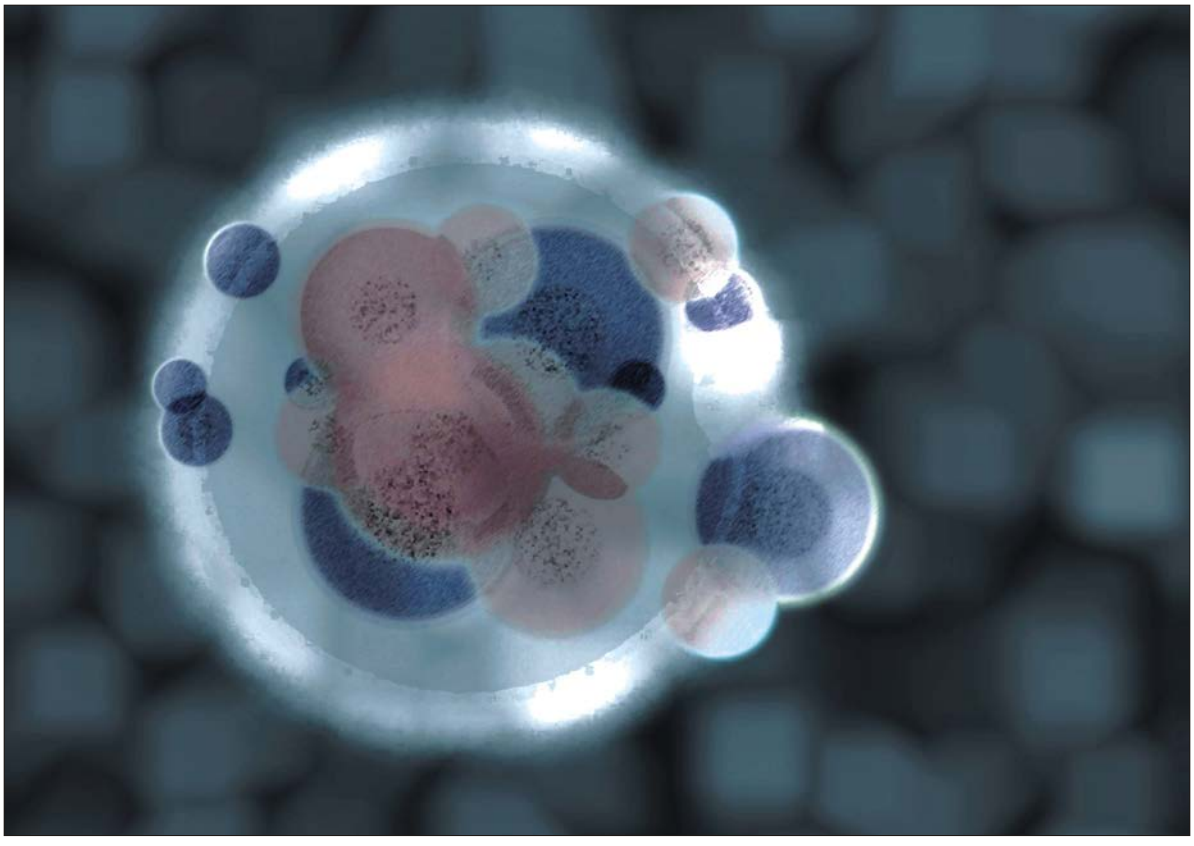

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p53 Expression in MCF7, T47D and MDA-MB 468 Breast Cancer Cell Lines Treated with Adriamycin Using RT-PCR and Immunocytochemistry

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Abstract: Numerous mutated genes have been shown to be involved in breast cancer. The p53, a well-known tumor suppressor gene, is the most commonly mutated gene that plays an important role in directing cells with DNA damage into apoptosis. On the other hand, Estrogen Receptor (ER), an important prognostic factor is differentially expressed in breast cancer cells. Therefore, we decided to study the p53 gene and its protein in MCF7, T47D and MDA-MB-468 breast cancer cell lines with different ER status following exposure to Adriamycin (ADR). Cytotoxicity of ADR on these cell lines was determined using MTT assay. The mRNA and protein levels were also analyzed in cell lines using RT-PCR and immunocytochemistry (ICC) assays, respectively. ADR cytotoxicity was highest on ER negative MDA-MB-468 cells and lowest on ER positive MCF7 cells. The p53 mRNA level was highest before or after treatment with ADR in MDA-MB-468 and lowest in T47D cells. It is noteworthy to mention that the p53 mRNA level slightly increased in T47D cells but decreased in other two cell lines after ADR treatment. Interestingly, higher level of p53 protein expression was detected after ADR exposure in all three cell lines. In conclusion, these three cell lines with different ER status showed differential molecular responses to Adriamycin that is important in tumor-targeted cancer therapy.

Key words: Breast cancer, p53, adriamycin, RT-PCR, immunocytochemistry

INTRODUCTION

Breast cancer, one of the most common malignancies affecting women today (Jemal *et al*., 2003), is associated with different types of genetic alterations such as mutations in oncogenes and tumor suppressor genes. The p53, a putative tumor suppressor gene, is expressed in a variety of malignancies including breast cancer (Borresen-Dale, 2003), often showing missense point mutations and less frequently deletions or loss of heterozygosity (Olivier *et al*., 2002). Most p53 alterations found in breast carcinomas lead to the synthesis of a stable and non-degradable protein that accumulates in tumor cells and thus can be detected by Immunohistochemistry (IHC) (Borresen-Dale, 2003). p53 gene encodes a 53 kDa nuclear phosphoprotein and transcription factor with multiple functions including regulation of the cell cycle, inhibition of angiogenesis, DNA repair and apoptosis (Lacroix *et al*., 2006). p53 plays an important role in cellular response to a variety of environmental and intracellular stresses including DNA damage, UV radiation, hypoxia and hyper-proliferation (Vousden and Lu, 2002; Meek, 2004). The p53 signaling pathway is in standby mode under normal cellular conditions. Activation occurs in response to cellular stresses and leads to an increase in the level of p53 protein due to reduced MDM2-dependent proteolytic degradation and increased affinity of p53 for DNA. As a result, the wild-type protein acquires sequence-specific DNA binding activity to a large number of genes that are known as transcriptional targets of wild-type p53 (Shu *et al*., 2007). Since p53 exerts its anti-proliferative action by inducing cell cycle arrest or apoptosis, loss of p53 activity, which often occurs in cancer, disrupts apoptosis and accelerates the appearance of tumors. Breast tumors expressing a high level of p53 (as measured by IHC) are more frequently ER-negative and associated with a higher proliferation rate and poorer survival (Feki and Irminger-Finger, 2004; Putti *et al*., 2005).

Estrogen and Estrogen Receptor (ER) play important roles in the genesis and malignant progression of breast cancer. ERα regulates the transcription of various genes

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as a transcription factor. It binds to Estrogen Response Elements (ERE) upstream of the target genes (Hayashi *et al*., 2003). On the other hand, there are numerous factors that affect ERα expression including effectors of chromatin structure, hormones and other relevant agents. p53 has the ability to regulate ERα expression (Angeloni *et al*., 2004). Increasing evidence indicates that p53 dysfunction is an important event in breast cancer (Soussi and Beroud, 2001; Miller *et al*., 2005). It is possible that interaction between p53 and ER resulting in their reciprocal regulation, plays an important role in regulating normal breast epithelial cell proliferation and aberration of this control may lead to breast cancer onset and progression (Liu *et al*., 2006).

The aim of the present study is therefore, to investigate the expression of p53 tumor suppressor gene and its protein following exposure to Adriamycin, an important antitumor drug, in MDA-MB-468, T47D and MCF7 breast cancer cell lines with different ER status.

MATERIALS AND METHODS

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 (Gibco, UK) supplemented with 10% fetal bovine serum (Gibco, UK) and 100 U mL^{-1} of penicillin and 100 ng mL^{-1} of streptomycin at 37°C in 5% humidified CO_2 incubator. This study was conducted in 2006.

Cytotoxic effect of different concentrations of adriamycin on MCF7, T47D and MDA-MB-468 breast cancer cell lines: The cells were seeded in 96-well plates at 1×10^4 cells/well in RPMI 1640 culture medium and incubated at 37°C in 5% CO_2 incubator for 48 hours. The cells were then exposed to Adriamycin (EBEWE Pharma, Austria) at different concentrations (100, 250, 500 and 1000 nM) for 48 and 72 h. The medium was changed every 48 h with corresponding assay medium and the anti-proliferative effect of Adriamycin was evaluated using colorimetric MTT assay (Carmichael *et al*., 1987).

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_2 incubator (5% CO_2, 37°C). After 48 h culture medium was changed and Adriamycin [MCF7 (500 nM), T47D and MDA-MB 468 (250 nM)] was added to the corresponding flasks. The medium was changed every 48 h with corresponding assay medium. After 72 h total RNA was isolated using TriPure isolation reagent (Roche, Germany) according to the method, previously described by Chomczynski (Chomczynski and Sacchi, 1987).

RT-PCR: cDNA was synthesized from 1.5 μg total RNA by incubation for 1 h at 42°C with M-MLV reverse transcriptase (Fermentas, Lithuania) and oligo$(dt)_{18}$ primer according to the manufacturer's instruction. Then 2.5 μL of the reaction mixture was subjected to Polymerase Chain Reaction (PCR) to amplify sequence of p53 using specific primers (sense, 5′-CTG AGG TTG GCT CTG ACT GTA CCA CCA TCC-3′; antisense, 5′-CTC ATT CAG CTC TCG GAA CAT CTC GAA GCG-3′) for p53. As an internal control, the house keeping gene β-actin (sense, 5′-TGA CGG GGT CAC CCA CAC TGT-3′; antisense, 5′-CTA GAA GCA TTT GCG GTG GAC-3′) was co-amplified in each reaction. The PCR reactions were carried out in final volumes of 50 μL containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM of each oligonucleotide primer and 2.5 U of Taq DNA polymerase. PCR conditions for p53 and β-actin amplification were: 35 cycles of 95°C for 1 min, 69°C (p53) and 57°C (β-actin) annealing for 1 min and 72°C extension for 1 min and 20 sec. The PCR products were visualized using 1.2% agarose gel electrophoresis with ethidium bromide staining. In negative control, template cDNA was replaced by DEPC water.

Immunocytochemical analysis of p53 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_2 incubator (5% CO_2, 37°C). After 48 h culture medium was changed and Adriamycin [MCF7 (500 nM), T47D and MDA-MB-468 (250 nM)] was added to wells of each chamber slide while the medium of the control wells remained RPMI 1640. The medium was changed every 48 h with corresponding assay medium. The cells were then washed with PBS and fixed in methanol: acetone (9:1) at -20°C. Endogenous peroxidase activity and non-specific binding sites were blocked by incubating fixed cells in 3% H_2O_2 in methanol for 30 min followed by UltraV block (Labvision, USA) for 10 min. Cells were then incubated overnight at 4°C with p53 antibody. The primary antibody for p53 (Clone: DO-7, Dako, Denmark) was used at dilution of 1:50. The results were visualized using the streptavidine-biotin immunoperoxidase detection kit and AEC chromogen (Labvision, USA) based on the manufacturer's instruction with necessary modifications. Finally, cells were counterstained with Meyer's hematoxyline (DakoCytomation, Denmark),

mounted and studied under light microscope. A section in which incubation with the primary antibody was omitted used as negative control.

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 different concentrations of Adriamycin on MCF7, T47D and MDA-MB-468 breast cancer cell lines: The cytotoxic effects of different concentrations of Adriamycin on each cell line were evaluated using MTT assay at 48 and 72 h after drug exposure. ADR showed a significant dose-dependent antiproliferative effect on MCF7, T47D and MDA-MB-468 cells (Fig. 1 A and B).

Effect of adriamycin on the mRNA level of p53: After treatment of cells with ADR, total RNA was isolated from treated and untreated samples and effect of Adriamycin on the expression level of p53 mRNA was analyzed using RT-PCR. The expression of p53 mRNA was highest in MDA-MB-468 and lowest in T47D cell line. The p53 mRNA expression increased slightly in T47D cells but decreased in two other tested cell lines after ADR treatment (Fig. 2, Table 1).

Immunostaining of cell lines with p53 antibody: The cell lines were immunostained with primary antibody for p53 in the presence and absence of ADR as described in the methods. Immunocytochemical analysis showed an increase in p53 protein expression in all cell lines following exposure to ADR. Significant p53 protein accumulation was found in ADR-treated MDA-MB-468 and T47D cells. In contrast, p53 expression increased weakly in treated MCF7 cell line (Fig. 3).

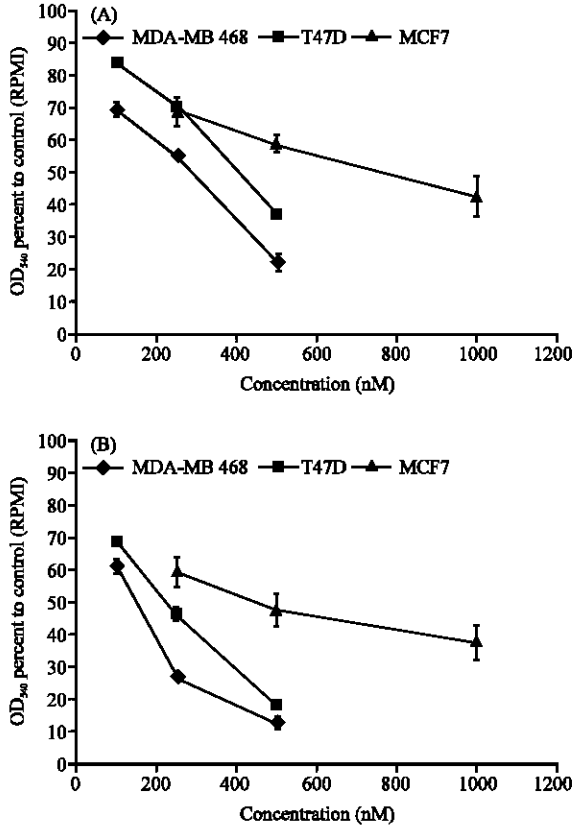

Fig. 1: Cytotoxic effects of different concentrations of Adriamycin on breast cancer cell lines. Cells were seeded in 96-well plates at 1×10^4 cells/well in RPMI 1640 culture medium and incubated at 37°C in 5% CO_2 incubator. After 48 h culture medium was changed and different concentrations of Adriamycin were added to each well. Cytotoxicity was then measured after 48 h (A) and 72 h (B) following drug exposure using MTT method. Data are presented as mean±SE of the average of 4 wells in three independent experiments

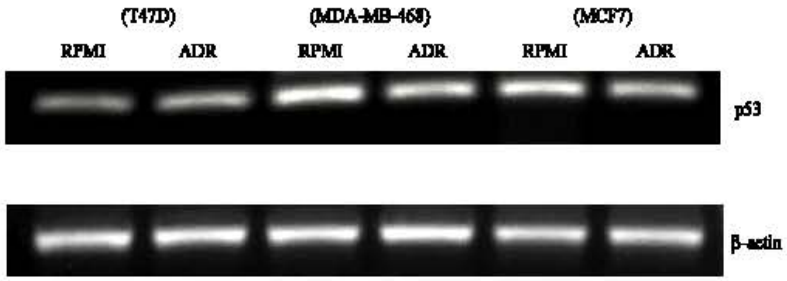

Fig. 2: Effect of Adriamycin on the mRNA levels of p53 in T47D, MDA- MB-468 and MCF7. After incubation with ADR, total RNA was isolated from treated and untreated samples and effect of Adriamycin on the expression levels of p53 mRNA was analyzed using RT-PCR followed by agarose gel electrophoresis and EtBr staining

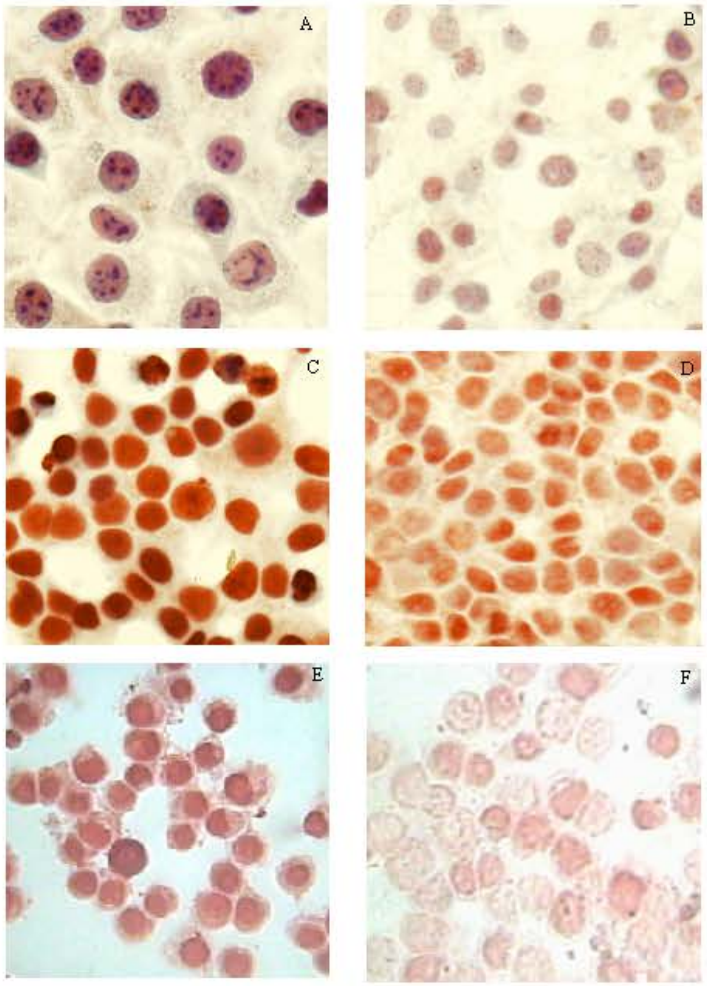

Fig. 3: Immunostaining of MCF7, T47D and MDA-MB-468 cells with p53 antibody. ADR treated MCF7 (A), T47D (C) and MDA-MB-468 (E) and untreated MCF7 (B), T47D (D) and MDA-MB-468 (F) cells were immunostained with primary antibody for p53, visualized by labvision detection system using AEC chromogen, mounted and studied under light microscope (Magnification 400X)

Table 1: Comparison of the mRNA expression levels of p53 in ADR treated and untreated T47D, MDA-MB-468 and MCF7 breast cancer cell lines

	Cell lines and treatments					
	T47D		MDA-MB-468		MCF7	
Gene	RPMI	ADR	RPMI	ADR	RPMI	ADR
p53	0.89±0.026	0.99±0.027	1.32±0.002	1.20±0.005	1.29±0.01	1.16±0.013

mRNA expression was analyzed by RT-PCR and relative intensity of each band was measured and normalized with β-actin

DISCUSSION

The p53 is mutated in the majority of human cancers including breast cancer in which mutations in the p53 gene is the most frequently observed single gene alteration (Cetin-Atalay and Ozturk, 2000; Michalak *et al*., 2005). The p53 is involved in the cellular response to DNA damage and has been implicated as an important determinant of tumor cell sensitivity to cytotoxic agents. Tumors that lack p53 function often respond poorly to radiation and chemotherapy (Michalak *et al*., 2005). Although more recent evidence of p53 induced chemoresistance has also been described (Anelli *et al*., 2003). The wild type p53 protein has a very short half life and does not usually accumulate in normal cells so it is undetectable. In contrast, mutation in p53 gene results in conformationally altered and nonfunctional protein with a prolonged half life. Therefore, it usually leads to accumulation of mutant p53 in tumor cells which can be detected by immunochemical techniques (Montero *et al*., 2001). However, all mutations do not yield a stable protein and some mutations lead to a truncated protein not detected by IHC. On the other hand, wild type p53 may accumulate in some tumors as a result of a response to DNA damage or binding to other cellular proteins leading to a positive IHC result (Lacroix *et al*., 2006).

In the present study, p53 mRNA and protein expressions were measured in ADR treated and untreated breast cancer cell lines using RT-PCR and immunocytochemistry, respectively. RT-PCR analysis showed that the p53 mRNA level was highest before or after treatment with ADR in MDA-MB-468 and lowest in T47D cells. It is noteworthy to mention that the p53 mRNA level slightly increased in T47D cells but decreased in other two cell lines after ADR treatment. Interestingly, higher level of p53 protein expression was detected after ADR exposure in all three cell lines.

ADR is a topoisomerase IIα poison. Topoisomerase IIα is a nuclear enzyme that transiently breaks and rejoins the phosphodiester backbone of both strands of the double helix. Therefore, it is essential for DNA replication, chromosome segregation and maintenance of chromosome structure. ADR forms a stable complex with DNA and topoisomerase IIα resulting in inhibition of the normal function of the enzyme. The complexed enzyme is unable to relegate DNA, so DNA strand breaks and DNA damage would be increased (Gewirtz, 1999).

Therefore, increase in p53 protein expression level in MCF7, T47D and MDA-MB-468 cells after ADR exposure is a response to ADR induced DNA damage in these cell lines and mild increase of p53 protein expression in ADR treated MCF7 in contrast to significant p53 protein accumulation detected in ADR treated MDA-MB-468 and T47D cells could be explained by the expression of wild type p53 in MCF7 and mutant p53 in MDA-MB-468 and T47D cells.

Unexpectedly, the p53 mRNA levels were relatively equal in untreated ER^- MDA-MB-468 and ER^+ MCF7 cell lines and higher than that of T47D cell line. ERα is a ligand inducible transcription factor that activates the transcription of genes that contain an estrogen response element in their promoter region. Physical interaction between wild type p53 and ERα (Liu *et al*., 2006) suggests a function for ERα-p53 complex in cellular biology. It has been shown that p53 increase the expression of human double minute-2 (hdm-2) that controls the level of p53 in cells via the ubiquitin proteasome pathway. It is also shown that the presence of ERα in cells protects p53 from hdm-2 targeted degradation and leads to an increase in the level of p53. As an evidence, transfection of ERα into the MCF-10A human breast epithelial cell line which normally does not contain ERα created an ER^+ stable cell line, 139B6. A western blot of the proteins in transfected cells demonstrated that the presence of ERα is accompanied by increasing levels of both p53 and hdm2 proteins above those detected in the parental ER^- cell line. Therefore, although the expression level of p53 is usually higher in ER^- cell lines compared to ER^+ ones (Putti *et al*., 2005), the relatively equal levels of p53 mRNA expression in MDA-MB-468 and MCF7 cells could probably be due to protective role of ERα on p53 in MCF7 cell line. On the other hand the lower level of p53 expression in T47D cell line might be due to p53 mutation which can disrupt physical interaction between p53 and ERα and thus the protective role of ERα.

In the present study, we studied the expression of p53 a putative tumor suppressor gene, at both mRNA and protein levels following exposure to a DNA damaging agent (Adriamycin) in breast cancer cells. In conclusion, these three cell lines with different ER status showed differential molecular responses to Adriamycin that is important in tumor-targeted cancer therapy.

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