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Studying the Role of P-glycoprotein in Resistance to Tamoxifen in Human Breast Cancer T47D Cells by Immunocytochemistry

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Abstract: In the present study, resistant subline of human breast cancer T47D cells was isolated by stepwise exposure to increasing concentrations of TAM (10^{-8} - 10^{-6} M). The subline that showed resistance to 10^{-6} M of TAM, named T47D/TAMR-6, was compared to the parental cells with respect to the growth characteristics, cross-resistance to Adriamycin (ADR) and P-gp expression. A significant increase in doubling time of the T47D/TAMR-6 cells was observed when compared to the parental T47D cells. This indicates a relatively slow growth rate pattern of the resistant cells. The T47D/TAMR-6 cells also showed cross-resistance to 1×10^{-8} M concentration of ADR. Immunocytochemical analysis using monoclonal antibody (C494; Dako-Denmark) against human P-gp and LSAB2 detection kit (Dako-Denmark) revealed a significant increase in P-gp level in resistant cells when compared to parental cells. Therefore, present results indicate that P-gp can play an important role in development of resistance to TAM and cross-resistance to ADR in the human breast cancer T47D cells.

Key words: Breast cancer, T47D, tamoxifen, resistance, P-glycoprotein, adriamycin

INTRODUCTION

Breast cancer remains a leading cause of women morbidity and mortality worldwide^[1]. At present despite early diagnosis, a significant percentage of patients with breast cancer die from metastatic disease. Therefore, systemic treatment is required to prevent disease progression. Since, in most cases growth of breast cancer is due to hormones such as estrogens, hormone therapy plays a significant role in breast cancer management. Tamoxifen (TAM), a non-steroidal antiestrogen, is being used for prevention and treatment of all stages of breast cancer^[2-5]. Mechanisms of actions of TAM includes: competition with estrogens on binding to Estrogen Receptor (ER); inhibition of calmodulin; alteration in membrane fluidity; stimulation of expression of TGF- β ; induction of apoptosis and interaction with P-gp^[6]. Unfortunately, the majority of initially responsive tumors acquire resistance due to several reasons such as alterations in ER structure and function; alterations in post-receptor interactions; pharmacological alterations and P-gp over-expression^[7-12].

The development of Multi-drug Resistance phenotype (MDR) is one of the most important reasons for treatment failure in patients with leukemia, colon,

adrenal, ovary, lung and breast cancer^[13,14]. This phenotype shows a broad spectrum of resistance to structurally and functionally unrelated anticancer drugs *in vitro* and *in vivo*. MDR is frequently associated with the over-expression of a 170 kDa membrane protein, P-glycoprotein (P-gp), which is encoded by the MDR1 gene. This protein acts as an energy dependent drug-efflux pump that decreases cytotoxic drug accumulation in cancer cells. It has been demonstrated that occurrence of MDR phenotype severely limits the usefulness of chemotherapeutic drugs such as Adriamycin (ADR) in some patients^[14-19].

Several studies have indicated that TAM reverses MDR associated resistance and strongly acts as P-gp substrate^[14,20-22]. There are also other reports that indicate the alterations in the expression of P-gp in TAM resistant tumor samples^[9-11]. In addition, there are conflicting data on relationship between TAM resistance and changes in the levels of estrogen receptor^[8,23,24]. Therefore, these findings together possibly suggest an association between over-expression of P-gp and resistance to TAM.

In this study, we looked at the involvement of MDR phenomenon in resistance of T47D breast cancer cells to TAM by determining expression of P-gp in parent cells and its isolated subline that shows resistance to 1×10^{-6} M

of TAM (T47D/TAMR-6) using immunocytochemical techniques. We observed a significant over-expression of P-gp in the process of development of TAM resistance in T47D/TAMR-6 cells. These cells were significantly resistant to growth inhibitory effects of TAM and ADR alone or in combination as opposed to the parent T47D cells.

MATERIALS AND METHODS

Chemicals and reagents: Tamoxifen citrate was a gift of Iran- Hormone Pharmaceutical Co. (Tehran-Iran). Culture medium components including RPMI-1640, Fetal Bovine Serum (FBS) and Penicillin-streptomycin (Pen/strep) were purchased from Gibco (UK). Trypsin-EDTA was obtained from Boehringer (Germany) and monoclonal mouse IgG against P-gp (C494) and LSAB2 kit were purchased from Dako (Denmark). All other chemicals and reagents purchased from Sigma (UK).

Cell culture: The T47D human breast cancer cell line (ATCC HTB-133, USA) was obtained from Pasteur Institute cell-bank (Tehran-Iran). These cells were maintained in monolayer culture in RPMI-1640 with L-glutamine supplemented with 10% FBS, 100 units/mL penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin in a humidified incubator in 5% CO_2 at 37°C. Cells were routinely sub-cultured once a week using 0.05% Trypsin – 0.02% EDTA in $\text{Ca}^{+2}/\text{Mg}^{+2}$ - free phosphate buffer saline (1.5 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 , 2.7 mM KCl and 140 mM NaCl).

Establishment of TAM resistant subline of T47D cells: Resistance to TAM was established by exposing drug-sensitive T47D cells to increasing concentrations of TAM *in vitro*. TAM was dissolved in ethanol 96% and PBS (1:1 v/v) at 1×10^{-3} M concentration as stock solution, light protected, stored at 4°C and serially diluted in culture medium. The final ethanol concentration never exceeded 0.05% in either Blank (B) or treated samples. Resistance was initiated against a concentration of 1×10^{-8} M of TAM (TAM-8). Following three successive passages of cells at each concentration, viable cells were exposed to the next higher concentration of TAM. In this experiment, the highest concentration in which cells were still proliferative was determined to be 1×10^{-6} M of TAM (TAM-6). The cells were grown continuously in the medium containing TAM-6 for further 3 months to get more stable resistant cells that named T47D/TAMR-6 cells.

Resistance stability assay: The T47D/TAMR-6 cells were grown in the RPMI medium without TAM-6 for a period of 50 days (8 passages) and named T47D/8P. The T47D,

T47D/TAMR-6 and T47D/8P cells were seeded at 1×10^5 cells/well in the RPMI or medium containing TAM-6 for 7 days. Medium was changed every three days. The cell number of each well was determined using trypan blue dye exclusion method. Five wells were used for each cells (n=5).

Growth rate assay: Growth curves for T47D and T47D/TAMR-6 cells were obtained by seeding 4×10^4 viable cells into 24-well plates in the absence and presence of TAM-6. Cells were counted after 24 h of seeding (day 0) and at different intervals following drug exposure, using trypan blue dye exclusion and MTT methods. For each experiment, six wells were used at each time point (n=6). The doubling time for each cell population was determined from its growth curve.

Cross-resistance assay: The T47D and T47D/TAMR-6 cells (5×10^4 cells/well) were grown in the absence and presence of TAM-6 and 1×10^{-8} M ADR (ADR-8) alone or in combination. This experiment was repeated twice in triplicate culture wells. The medium was changed every three days with the corresponding assay medium and after 1 week of exposure, the cells in each well were harvested and counted using trypan blue dye exclusion method.

Immunocytochemical assay. The T47D and T47D/TAMR-6 cells were seeded (2×10^5 cells/well) in Lab-Tek chamber slides (4-wells glass slide, Nunc, USA). After 24 h, medium was removed and cells were rinsed with cold PBS. Cells were fixed in methanol: acetone (9:1) for 30 min at -20°C and then rinsed again with cold PBS at room temperature. For immunocytochemical assay, the cells were incubated with primary mouse monoclonal antibody [C494; 1:100 in 1% BSA in Tris-HCl buffer (TB)] for 18 h at 4°C. Cells were then washed in TB and incubated with biotinylated rabbit anti-mouse immunoglobulin secondary antibody for 30 min. Following incubation with secondary antibody, the results was visualized by adding streptavidine conjugated horseradish peroxidase (30 min), 3-amino-9-ethylcarbazole (AEC) as chromogen substrate (30 min) and finally Meyer's hematoxylin (3-4 min) for counter-staining according to the manufacturer's instructions (LSAB-2 kit, Dako-Denmark). The slides were then mounted using Faramount and P-gp immunostains as red-brown dots were examined by two observers under light microscope. In each series, a slide in which incubation with the primary antibody was omitted used as negative control.

Statistical analysis: Data for growth curves were expressed as mean±SD while other values were expressed as mean±SE. Data were analyzed by student's t-test and mean differences were considered to be statistically significant if $p < 0.05$.

RESULTS

Growth characteristics of T47D and T47D/TAMR-6 cells: To study the growth characteristics of T47D and T47D/TAMR-6 cells, their proliferation rates were determined in the absence and presence of TAM-6 at different time points (Fig. 1). T47D cells grown in drug-free culture medium had a doubling time of 50 h and T47D/TAMR-6 cells grown in TAM-6 were divided relatively slowly with a doubling time of 63 h. According to our results growth of T47D/TAMR-6 cells was significantly increased ($p < 0.0001$) in drug-free medium indicating of hormone responsiveness of cells. The stability of the resistant cells was confirmed upon removal of the TAM-6 from culture medium for 50 days and re-exposure to TAM-6 (Fig. 2). The results were the same in both MTT and trypan blue dye exclusion assays. The concentration of 0.05% of ethanol used as vehicle of TAM had no effect on cell growth in all experiments.

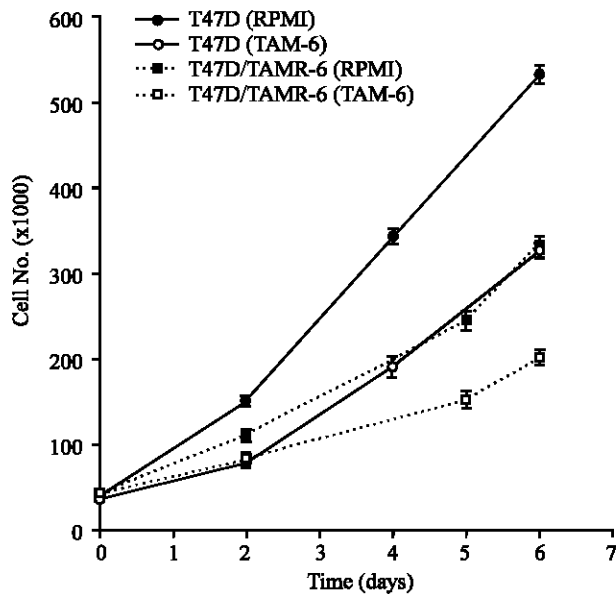


Fig. 1: Growth characteristics of T47D and T47D/TAMR-6 cells. T47D and T47D/TAMR-6 cells were seeded at 4×10^4 cells/well in the presence and absence of TAM-6. Medium was changed every three days. The cell number of each well was determined using trypan blue dye exclusion method at different intervals up to six days. Each point represents the mean±SD of six wells (n=6) *** indicates significant difference between T47D and T47D/TAMR-6 cells in corresponding medium ($p < 0.001$)

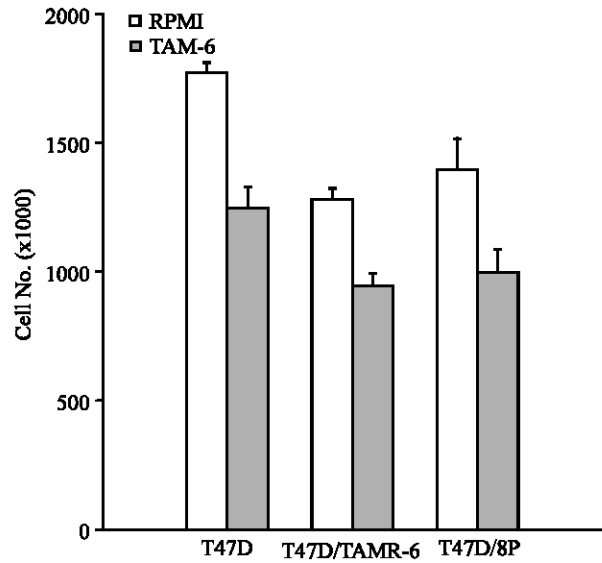


Fig. 2: Stability of resistant cells. The T47D, T47D/TAMR-6 and T47D/8P cells were seeded at 1×10^5 cells/well in the RPMI or medium containing TAM-6 for 7 days. Medium was changed every three days. The cell number of each well was determined using trypan blue dye exclusion method. Each point represents the mean±SD of six wells (n=6) * indicates significant difference from T47D cells in corresponding medium ($p < 0.05$)

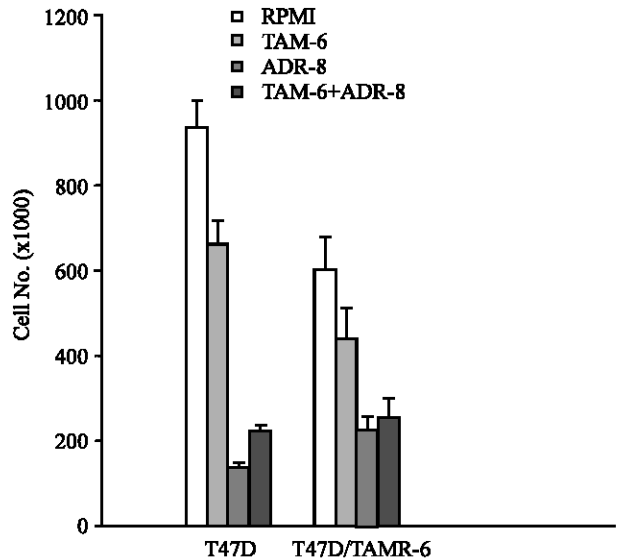


Fig. 3: Effect of TAM-6, ADR-8 on growth of T47D and T47D/TAMR-6 cells. T47D and T47D/TAMR-6 cells were seeded at 5×10^4 cells/well. After 24h, RPMI medium was changed with fresh medium or medium containing TAM-6, ADR-8 and TAM-6+ADR-8 in corresponding wells. The medium was changed accordingly every three days and cell viability was measured after 1 week using trypan blue dye exclusion method. Values are mean±SE of triplicate determination from two individual experiments (n=6) ** indicates significant difference between T47D and T47D/TAMR-6 cells in ADR-8 ($p < 0.01$) # indicates significant difference compare to TAM-6 +ADR-8 ($p < 0.001$)

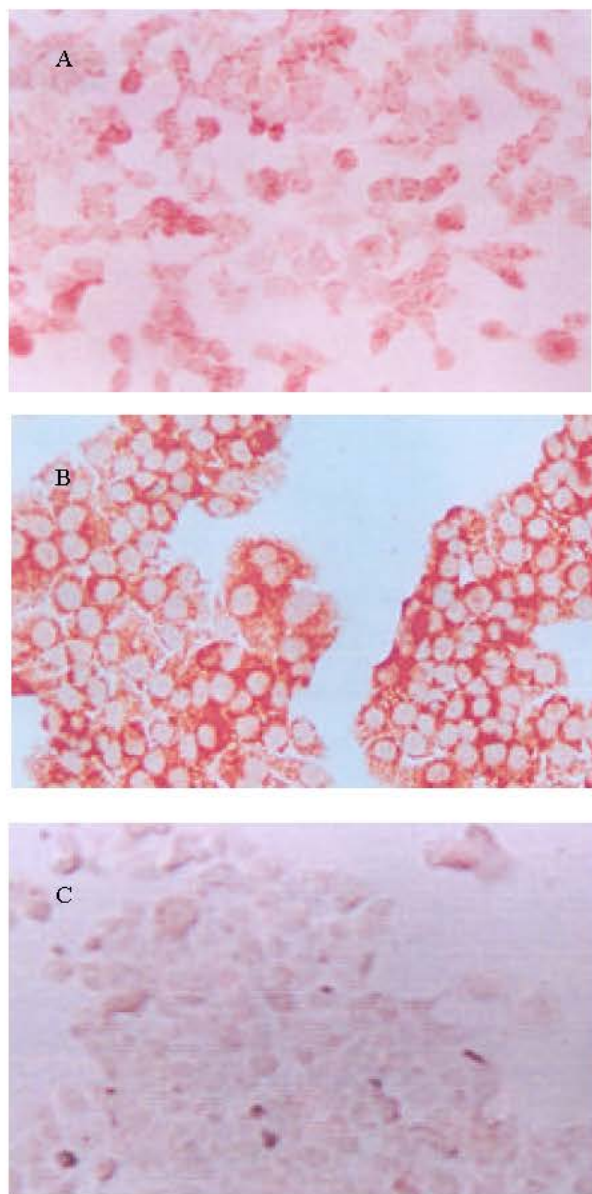


Fig 4: Immunocytochemical Analysis of P-gp. T47D (A) and T47D/TAMR-6 (B) cells were seeded (2×10^5 cells/well) in 4-wells Lab-Tek chamber slides. After 24 h, the medium was removed and cells were fixed in methanol/acetone (9:1) and incubated with primary antibody against human P-gp (C494). The results were visualized using LSAB2 kit according to the manufacturer's instructions. [Chromogen: AEC; magnification 200X (A, B) or 100X (negative control, C)]

Cross-resistance of T47D/TAMR-6 cells to ADR: Cells were grown in the presence and absence of ADR-8 and TAM-6 alone or in combination for 1 week. The growth of parent and resistant cells was significantly decreased in the presence of ADR-8 alone to 14% ($p < 0.00001$) and 38% ($p < 0.02$) of untreated controls, respectively (Fig. 3). Therefore, the T47D/TAMR-6 cells were resistant to

ADR-8 compared to parent cells, which possibly indicates a cross resistance between these drugs. A combination of TAM-6 with ADR-8 compounds was examined to determine whether this shows more inhibition than either compound alone. The results showed that in the presence of combination of TAM-6 and ADR-8, growth of cells was significantly increased ($p < 0.001$) in T47D cells when compared to ADR alone, whereas there was no significant difference in case of resistant subline.

Immunocytochemical analysis of P-gp: The P-gp expression was determined based on the percentage of positive cells and the intensity of immunostains. The results of immunocytochemical analysis (Fig. 4) revealed a significant increase in P-gp level in TAM resistant cells (B), when compared to parental T47D cells (A). No staining was observed in the negative control (C). The results of the same analysis on T47D cells that were resistant to TAM at concentrations of 1×10^{-8} (TAM-8) and 1×10^{-7} (TAM-7) showed gradual but not significant increase in P-gp expression compared to parent T47D cells (data not shown).

DISCUSSION

This study examined the involvement of P-gp in the process of development of TAM resistance in the human breast cancer T47D cells. We isolated a resistant subline of T47D cells to TAM, a non-estroidal antiestrogen drug. The process of selection of resistant subline from the parent cells was based on the previous experiment in establishing TAM-resistant ZR-75-1 cell line^[25] with some modifications. In agreement with previous studies^[25,26], in this study the development of resistance was associated with an increase in the population doubling time. Unlike other studies including the present study, Nawata *et al.*^[27] reported the isolation of a TAM resistant subline from MCF-7 cells that had the same growth rate as parent cells. The difference in the results of doubling time might be due to the differences in the process of selection of resistant cells between Nawata study and others.

Present data showed that the T47D/TAMR-6 cells remained stably resistant to TAM-6, even without being in exposure of TAM-6 for 8 passages (50 days). This is in contrast to the selected TAM-resistant variants R3 and R27 from MCF-7 cells, which change their resistant characteristics if not kept under selective pressure of TAM^[25]. On the other hand, Sipila *et al.*^[28] selected a TAM resistant cell clone that was stable both *in vitro* and *in vivo*. Also LY-2 cells selected for MCF-7 ability to grow in the presence of the potent antiestrogen LY117018 showed cross resistance to TAM that appeared to be

stable even in the absence of TAM exposure^[29]. Since in our study, isolated T47D/TAMR-6 cells retained its responsiveness to growth stimulatory effects of estrogen, it can be concluded that development of TAM resistance is not necessarily due to alterations in the gene expression, structure or function of estrogen receptor. Wosikowski *et al.*^[26] have also reported that only 6 of the 15 resistant sublines derived from parental MCF-7, ZR75B and T47D human breast cancer cell lines showed decrease or loss of expression of estrogen receptor.

Immunocytochemical analysis indicated that long-term TAM treatment induces an increase in specific immunostaining of P-gp in cell membrane of resistant subline. The level of P-gp expression was correlated with the degree of cellular resistance to TAM (data not shown). It seems that TAM might interfere with normal function of P-gp pump and therefore, cells increase the content of P-gp protein in response to new conditions as an adaptation mechanism. However, our findings have not determined if overexpression of P-gp is cause or a consequence of TAM resistance. There are conflicting data regarding the alterations in *mdr1* gene expression during TAM therapy^[30-34]. Kellen *et al.*^[35,36] reported that administration of TAM significantly lowers the P-gp content of the tumor sections. The variation in the level of P-gp expression among these studies may reflect the different methodologies used and/or the generally low levels of P-gp expression found in different cell types.

Most previous researchers have used TAM as a reversal agent that prevents P-gp function and therefore leads to the accumulation of drugs within resistant cells. In such cases the activity of Mitoxantrone, vinblastin (VBL) and ADR in drug-resistant subline (MCF-7/ADR) reported to be synergistically potentiated by TAM^[37,38]. Leonessa *et al.*^[37] demonstrated that different concentrations of TAM ($1-5 \times 10^{-6}$ M) show antagonistic or additive interactions with ADR and VBL in MCF-7 compared to MCF-7/ADR cells. Similar to Leonessa *et al.*^[37] report, in this experiment concomitant exposure of parent T47D cells to TAM and ADR showed an antagonistic effect between these drugs. In the present study, TAM resistant cells were also more resistant to ADR compared to the parental T47D cells. This phenomenon of cross resistance to ADR might be due to the P-gp overexpression in cell membrane as a result of TAM resistance that reduces intracellular ADR accumulation.

In conclusion, during this study we have isolated and characterized a TAM resistant subline derived from T47D human breast cancer cells that is different from parental cells with respect to the characteristics such as decrease in growth rate, over expression of P-gp and hormone responsiveness along with stable TAM

resistance. Furthermore, we have observed that these cells are cross resistant to ADR. Therefore, the established T47D/TAMR-6 cells may be a useful model to further study different aspects of the role of P-gp in resistance to TAM and other cytotoxic drugs in treatment of the human breast cancer.

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