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Differential Expression of TS and TP in Tamoxifen Resistant Subline of Human Breast Cancer T47D Cells

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Abstract: In the present study, the protein expression levels of Thymidylate Synthase (TS) and Thymidine Phosphorylase (TP) were determined in human breast cancer T47D cells and its derived TAM resistant subline (T47D/TAMR-6 cells). The resistant subline was established in vitro following stepwise increase in TAM concentration in the culture medium of parent cells. Immunocytochemical method was used to determine the expression of TS and TP enzymes in both parent and resistant cells. The slow growing T47D/TAMR-6 cells showed lower level of expression of both proteins compare to parent T47D cells. Expression of TS was also observed to be more than TP in both studied cell types. In conclusion, in patients with tumor immunophenotype similar to these two cell lines, the efficacy of 5-FU chemotherapy and its congeners will be expected to be different. This in turn would affect the disease prognosis and therapeutic predictions. Therefore, the outcome for 5-FU-containing chemotherapy regimens when applied after tumor progression on hormonal therapy is better to be weighed against the status of TS and TP protein levels.

Key words: Thymidylate Synthase, Thymidine Phosphorylase, tamoxifen resistance, breast cancer, immunocytochemistry

INTRODUCTION

Thymidylate Synthase (TS) catalyzes the conversion of deoxyuridine monophosphate and 5, 10-methylenetetrahydrofolate to deoxythymidine monophosphate and dihydrofolate. This reaction is an essential step in DNA biosynthesis, as it provides the only *de novo* pyrimidine source of deoxythymidine monophosphate[1]. Because of its critical role in DNA synthesis, it has been an important target of cancer chemotherapy. In addition to biosynthetic role, data suggest that TS may be involved in other important regulatory pathways within the cell[2,3]. Because TS is a critical target enzyme of fluoropyrimidine cytotoxic drugs, the effect of expression of TS on the clinical response and prognosis has been investigated in patients with different cancers treated with 5-FU based chemotherapy[4-8]. Some published data could indicate that TS gene expression had a significant association with chemotherapeutic response and survival[5,6]. In node-positive breast cancer patients receiving adjuvant chemotherapy that includes 5-FU, TS expression was suggested to be related to a favorable response[7,8]. The efficacy of hormonal therapy might also be related to the tumor levels of DNA synthesis enzymes. In nude mouse MCF-7 breast cancer xenografts, tumor TS levels were reduced by treatment with a pure antiestrogen[9] and in breast cancer cells *in vitro* several genes have been shown to be under transcriptional control of estrogens and antiestrogens[10]. It seems that hormonal therapy targets the cell cycle with no preference for the *de novo* and salvage pyrimidine pathways of DNA synthesis[11].

Thymidine phosphorylase (TP) is the first enzyme in the pyrimidine salvage pathway[12,13] and its degradation products have angiogenic and anti-apoptotic effects *in vitro* and *in vivo*[14-18]. TP gene expression has been shown to be correlated with clinical stage, histologic grade and survival[19]. The relationship between TS and TP expression with survival has not adequately been examined. In one study on patients with colorectal cancer treated with 5-FU and leucovorin, only patients with tumors with low TS and TP gene expression profile had a significantly better clinical response and survival than the other patients[20]. However, these types of combined analyses with other chemotherapeutic regimens have not been extensively published.

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The aim of the present study was to investigate the relation of tumor TS and TP expression to predict the efficacy of polychemotherapy including 5-FU in breast cancer after the occurrence of tamoxifen resistance.

MATERIALS AND METHODS

Cell line and culture conditions: The human breast cancer T47D cell line (ATCC HTB-133, USA) was obtained from Pasteur Institute Cell Bank of IRAN (Tehran, IRAN). Cells were maintained in RPMI-1640 (Gibco, USA) culture medium supplemented with 10% Fetal Bovine Serum (Gibco, USA) and 100 U mL^{-1} of penicillin and 100ng mL^{-1} of streptomycin (Sigma, UK) at 37°C in 5% CO_2 incubator. TAM resistant cells were established and maintained in our lab as described previously[21].

Cytotoxicity assay: The T47D cells were seeded in two 24-well plates ($4x10^4$ cells/well) in the absence (Blank) and presence of TAM ($1x10^{-8}-1x10^{-5}$ M) for 48 h and 1 week. The cell number in each group was then determined using trypan blue dye exclusion method. Data are presented as mean±SE of the average determination of 4 wells in three independent experiments.

Determination of growth characteristics of T47D and T47D/TAMR-6 Cells: The T47D and T47D/TAMR-6 cells were seeded in 24-well plates at $5x10^4$cells/well in RPMI 1640 culture medium and incubated at 37°C in 5% CO_2 incubator. After washing with PBS, the cells were trypsinized and then counted using trypan blue dye exclusion method every 48 h for 6 days. The doubling time for each cell population was then determined from its growth curve, in which each point is the average determination of 4 wells in three independent experiments.

TS and TP immunocytochemical analyses: The parent T47D and TAM-resistant cells were seeded in 4-well chamber slides (LabTeck, USA) in RPMI 1640 for 48 h. Then cells were fixed in methanol:acetone (9:1) at -20°C. Endogenous peroxidase activity and nonspecific binding sites were blocked by incubating fixed cells in 3% hydrogen peroxide in methanol for 30 min and 5% BSA for 60 min, respectively. Cells were then incubated overnight at 4°C with TS mouse monoclonal antibody (TS 106, Lab Vision Corporation) that recognizes nuclear and cytoplasmic expression of the human TS protein. In case of TP we used the mouse monoclonal antibody (P-GF.44C, LabVision Corporation) that reacts with wild type TP protein and recognizes nuclear and cytoplasmic expression of the human TP protein. The primary antibodies were used at dilution of 1:40 for TS and 1:100 for TP. The results were visualized using the streptavidine-biotin immunoperoxidase detection kit (LSAB2; Dakocytomation -Denmark) and DAB chromogen (Dakocytomation- Denmark) based on the manufacturer's instruction with necessary modifications. Finally, cells were counterstained with Meyer's hematoxyline, mounted and studied under light microscope. A section in which incubation with the primary antibody was omitted used as negative control. Stained cells were then classified into 5 categories based on the nuclear and cytoplasmic expression of TS and TP. Cells were scored as 4+ if they had strong nuclear/cytoplasmic staining in more than 50% of cells, 3+ if they had cytoplasmic staining in 25-50% of cells, 2+ if they had cytoplasmic staining in 5-25% of cells, 1+ if they had cytoplasmic staining in less than 5% of cells and 0 in case of no cell staining.

Statistical analysis: SIGMASTAT™ (Jandel Software, San Raphael, CA) was used to perform statistical analysis of data. The students t-test was used to examine the differences among treatments. Mean differences with p-values less than 0.05 were considered to be significant.

RESULTS

Cytotoxicity of different TAM concentrations on T47D cells: The anti-proliferative effects of TAM on T47D cells was determined using trypan blue dye exclusion method as described in the methods. After 1 week, cells exposed to $1x10^{-6}$ and $1x10^{-5}$ M of TAM showed significant decrease in number compare to control RPMI (Fig. 1). There was no difference between control RPMI and Blank (%1 EtOH, used as co-solvent of TAM) in cell proliferation.

Growth characteristics of T47D and T47D/TAMR-6 Cells: The growth rate of T47D/TAMR-6 cells decreased significantly as compared with that of the parental T47D cells. This indicates the slow proliferation pattern of TAM-resistant cells (Fig. 2). The viability assay showed more than 95% viable cells in all steps of experiments.

Immunostaining of cells with TS and TP antibodies: The parent and TAM resistant T47D cells were immunostained with primary antibodies for TS and TP as described in the methods. Strong nuclear and cytoplasmic staining of TS was seen in parent T47D cells compare to T47D/TAMR-6 cells [Fig. 3A (3+) vs C (1+)]. Despite less staining for TP than TS in T47D cells [Fig. 3B (2+) vs A (4+)], the TP immunostaining was also more in the parent than resistant cells [Fig. 3B (2+) vs D (1+)].

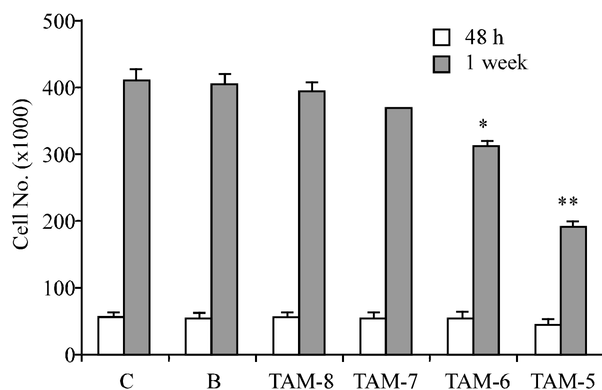

Fig. 1: Cytotoxicity of tamoxifen concentrations on T47D cells. The T47D cells were seeded in two 24-well plates ($4x10^4$cells/well) in the absence (Blank) and presence of TAM ($1x10^{-8}$ – $1x10^{-5}$ M) for 48 h and 1 week. The cell number in each group was then determined using trypan blue dye exclusion method. Data are presented as mean±SE of the average determination of 4 wells in three independent experiments.
*indicates significant difference compared to C ($p<0.01$)
**indicates significant difference compared to C ($p<0.001$)

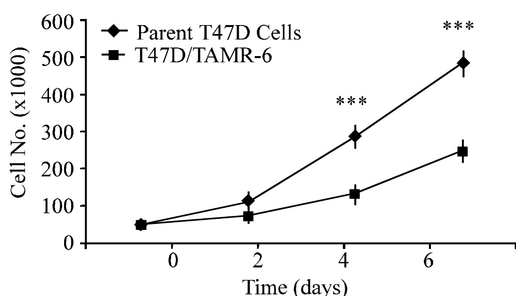

Fig. 2: Growth rate characteristic of T47D and T47D/TAMR-6 cells. The T47D and T47D/TAMR-6 cells were seeded in 24-well plates at $5x10^4$cells/well in RPMI 1640 culture medium. Cells were then counted using trypan blue dye exclusion method every 48 h for 6 days. Data are mean±SE of the determination of 4 wells in 3 independent experiments.
*** indicates significant difference compared to C ($p<0.0001$)

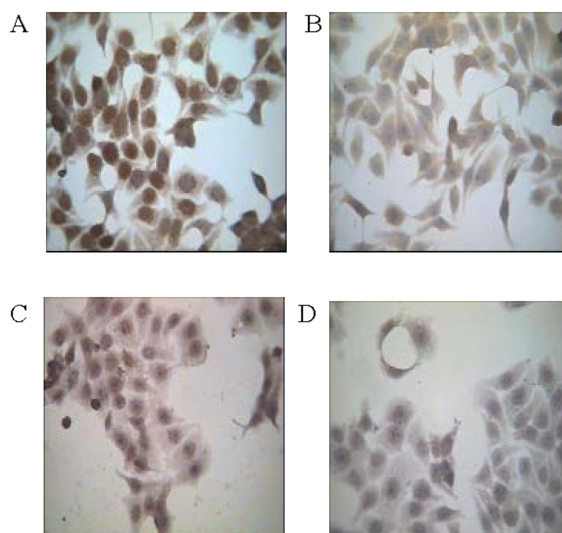

Fig. 3: Immunostaining of TS and TP in T47D and T47D/TAMR-6 cells. The parent T47D (Panel A and B) and resistant T47D/TAMR-6 (Panel C and D) cells were immunostained with primary antibodies for TS (Panel A and C) and TP (Panel B and D) as described in the methods (Chromogen DAB, magnification x400)

DISCUSSION

It is known that TS and TP play important roles in several cellular metabolism pathways which can affect patients' prognosis. In vitro studies demonstrated that cellular TS mRNA levels maximally occurred as a cell passes from G1 to S phase of the cycle and was greater in rapidly proliferating cells than in resting cells[22]. These studies suggested that TS expression is associated with increased cell proliferation. The availability of TS and TP antibodies made a great improvement in detection of expression level of these enzymes in tumor samples[23,24]. The TS immunostaining using TS 106 antibody has been shown to correlate with TS mRNA expression assessed by the reverse transcriptase-polymerase chain reaction (RT-PCR) technique in a series of gastrointestinal tumor samples[23]. In the present study we analyzed parent and TAM-resistant T47D human breast cancer cells by immunocytochemical (ICC) method using TS 106 antibody. Although there is no report about the TS expression in these cells using TS106 antibody, but reports on the expression of TS in patients with all stages of different cancers such as gastric cancer have shown that prognoses were poorer for groups with higher TS expression[20]. Most studies of advanced cancers have also reported worse prognoses and decreased responses to chemotherapy with higher levels of TS expression[7,20]. Overexpression of TS appears to be a major method of resistance to 5-FU and data from colorectal and breast cancer patients suggest an association with TS and resistance to 5-FU[4-8]. The benefits of hormonal therapy of

breast cancer have been widely accepted. Nevertheless, almost half of the tumors are less likely to respond effectively to anti-hormonal treatment. Similarly, some of the patients do not respond to chemotherapy. In the systemic treatment of patients with advanced breast cancer, the occurrence of drug resistance in some patients is a major problem. Most patients will initially be treated with hormonal therapy, mainly tamoxifen and the important question to be answered is which therapeutic regimen could be most effective after the occurrence of tamoxifen resistance[25,26]. In this report, we studied the enzymes TS and TP, which play key roles in the *de novo* pyrimidine synthesis and the salvage DNA synthesis pathways, respectively. It is well known that specific chemotherapeutic drugs interfere with these pathways in one way or another. In the breast cancer cells used in our study, TS and TP protein expression levels were different among parent and TAM-resistant cells. In addition, the growth rate of parent cells was faster than resistant cells. Therefore, observed decrease in TS and TP protein expression in slow growing TAM-resistant cells compared to parent cells further indicates the role of these enzymes in tumor cell proliferation. It has been reported that endocrine treatment is more effective in slowly proliferating breast cancers[25]. Therefore, the antiproliferative effect of tamoxifen on tumor growth via ER blockade may likely been abrogated in the presence of higher pyrimidine biosynthesis through salvage pathway. In hormone-refractory patients, subsequent 5-FU-based chemotherapy failed in the small group of patients with high tumor TS activity levels[26]. Combined analysis of TS and TP gene expression demonstrated that the relative risk of death for tumors with high TS and TP expression was greater than that for either only high TS or high TP gene expression.

Present results also suggest that treating patients individually should be based on the tumor levels of TS and TP. In addition, for hormone-resistant tumors, administration of 5-FU or its congeners, the level of expression of TS and TP should be considered as predictive markers of efficacy of such chemotherapy.

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