International Journal of Cancer Research 1 (1-2): 16-20, 2005
ISSN 1811-9727

Expression of Cyclin D1 in Tamoxifen Resistant Subline of Human Breast Cancer T47D Cells

[1]Shamileh Fouladdel, [2]Zahra Motahari and [1,2]Ebrahim Azizi
[1]Molecular Research Laboratory, Department of Pharmacology and Toxicology
[2]National Center of Excellence of Toxicology, Faculty of Pharmacy,
Tehran University of Medical Sciences, Tehran, Iran

Abstract: In the present study, the protein expression of cyclin D1 was determined in human breast cancer T47D cells and its 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 cyclin D1 in both parent and resistant cells. The slow growing T47D/TAMR-6 cells showed lower level of expression of cyclin D1 protein compare to parent T47D cells. Expression of cyclin D1 protein was significantly decreased in both cell types following exposure to TAM (1 μM). Therefore, it can be suggested that TAM can regulate the expression of cyclin D1 protein that affects the cell proliferation. In conclusion, determination of protein expression of cyclin D1 in tumor samples of patients would be helpful in tumor prognosis and prediction of outcome of chemo-hormonal therapy of breast cancer.

Key words: Cyclin D1, Tamoxifen resistance, breast cancer, immunocytochemistry

INTRODUCTION

The cyclin-dependent kinases are a family of serine/threonine kinases that play an important role in controlling progression through the cell cycle[1,2]. Dysregulation of the cell cycle control system is an almost uniform aberration in tumorigenesis[3]. The cyclins encode regulatory subunits of the kinases which phosphorylate specific proteins, including the retinoblastoma (pRB) protein, to promote transition through specific cell cycle checkpoints[2,4]. Cyclin D1 plays a crucial role in G1/S phase of cell cycle progression in fibroblasts and mammary epithelial cell proliferation[5,6]. Overexpression of cyclin D1 is found in 1/3 of human breast cancers that correlates with poor prognosis[7]. Several different oncogenic signals including mutations of the Ras and α-catenin pathway induce cyclin D1 expression[2,8]. Mammary-targeted expression of cyclin D1 is sufficient for the induction of mammary adenocarcinoma and cyclin D1-/- mice are resistant to ErbB2-induced tumorigenesis[9,10]. In addition to binding cyclin-dependent kinases 4 and 6 (cdk4 and cdk6) and pRB, cyclin D1 forms physical associations with P/CAF (p300/CBP-associated factor), Myb, MyoD and the cyclin D1 myb-like binding protein (DMP1)[11,14]. Cyclin D1 binds to the estrogen receptor alpha (ERα) and enhances reporter gene activity but inhibits androgen receptor reporter gene activity[14-16]. The *in vivo* or genetic evidence indicating a requirement for cyclin D1 in nuclear receptor function needs to be more elucidated.

Anti-estrogens, especially tamoxifen, are the drugs of choice for the treatment of all stages of breast cancer[17-20]. Tamoxifen as a classical anti-estrogen and its new congeners as well as compounds known as Specific Estrogen Receptor Modulators (SERMs) are regarded as competitive inhibitors of estrogen receptors[17,21,22]. As results of hormonal therapy, gene transcription, DNA synthesis and cellular proliferation are lowered or prevented[23,24]. Unfortunately, in the systemic treatment of patients with advanced breast cancer, the occurrence of drug resistance in patients is a major problem. Therefore, despite the tremendous therapeutic and commercial success of tamoxifen since its introduction, intrinsic or acquired resistance in almost all patients during prolonged treatment needs to think of the important question that is which therapeutic regimen could be most effective after the occurrence of tamoxifen resistance[25, 26]. Answer to this important question requires

Corresponding Author: Ebrahim Azizi, Ph.D., Director of Molecular Research Laboratory, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
Tel/Fax: +9821 6959100 E-mail: aziziebr@sina.tums.ac.ir

comprehensive knowledge about gene and protein expression of key elements of cell growth and proliferation such as cyclin D1.

The aim of the present study was to investigate the effect of TAM resistance on expression of cyclin D1 and thus the breast tumor cell proliferation that would be helpful in disease prognosis and also prediction of the efficacy of chemo-hormonal therapy in breast cancer.

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 100 ng mL^{-1} of streptomycin (Sigma, UK) at 37°C in 5% CO_2 incubator.

Cytotoxicity assay: The T47D cells were seeded in two 24-well plates ($4x10^5$ 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.

Establishment of a tamoxifen-resistant subline: Based on the results of the cytotoxicity assay, the Tamoxifen-resistant subline was isolated by continuous exposure of T47D cells to TAM at concentrations starting from $1x10^{-8}$ M and increasing in a stepwise manner to $1x\ 10^{-6}$ M within 6 months. Cells that were capable of sustained growth in medium containing $1x10^{-6}$ M of TAM were considered to be resistant to Tamoxifen and are referred to T47D/TAMR-6 cells here after. Cell viability was determined after each step using trypan blue dye exclusion method.

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.

Immunocytochemical analysis of Cyclin D1 protein expression: 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 cyclin D1 rabbit monoclonal antibody SP4. Lab. Vision Corporation that recognizes nuclear expression of the human cyclin D1 protein. The primary antibody was used at dilution of 1:50. The results were visualized using the streptavidine-biotin immunoperoxidase detection kit (LSAB2; Dakocytomation-Denmark) and AEC 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 expression of cyclin D1. Cells were scored as 4+ if they had strong nuclear staining in more than 50% of cells, 3+ if they had moderate nuclear staining in 25-50% of cells, 2+ if they had mild nuclear staining in 5-25% of cells, 1+ if they had 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 cyclin D1 antibody: The parent and TAM resistant T47D cells were immunostained with primary antibody for cyclin D1 as described in the methods. In the absence of TAM, the nuclear staining of

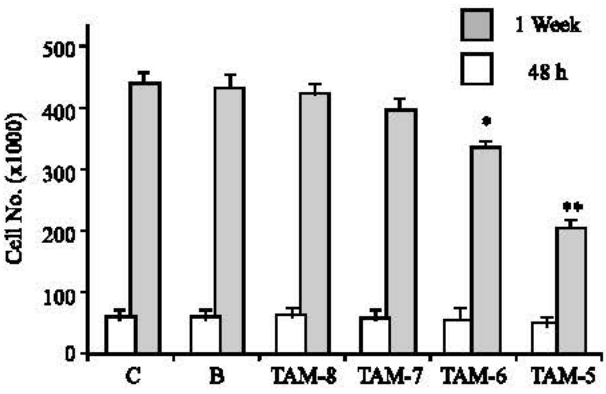

Fig. 1: Cytotoxicity of tamoxifen concentrations on T47D cells. The T47D cells were seeded in two 24-well plates ($4x10^5$ cells/well) in the absence (Blank) and presence of TAM ($1x10^{-8}$ - $1x10^{-5}$ M) for 48 h and 1 week. The cell number in each group was then determined using trypan blue dye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

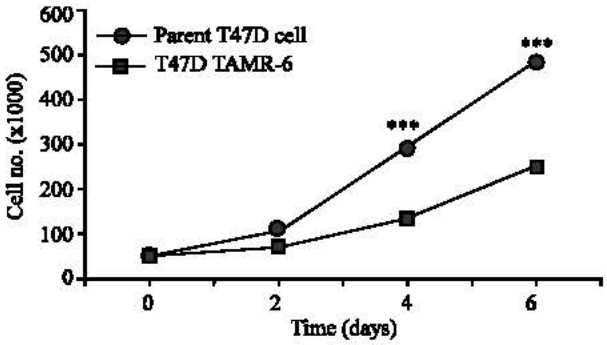

Fig. 2: Growth rate characteristic of T47D and T47D/TAMR-6 cells. The T47D and T47D/TAMR-6 cells were seeded in 24-well plates at $5x10^4$cells/well in RPMI 1640 culture medium. Cells were then counted using trypan blue dye exclusion method every 48 h for 6 days. Data are mean±SE of the determination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

cyclin D1 was more in parent T47D cells compare to T47D/TAMR-6 cells [Fig. 3A (3+) vs B (2+)]. Following exposure to TAM, the nuclear immunostaining of cyclin

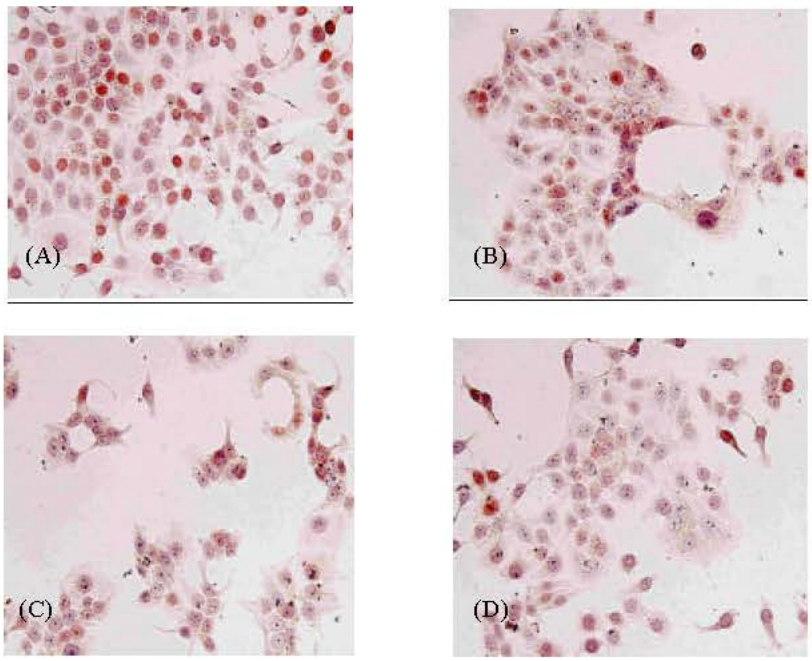

Fig. 3: Immunostaining of cyclin D1 in T47D and T47D/TAMR-6 cells. The parent T47D (Panel A and C) and TAM-resistant T47D/TAMR-6 cells (Panel B and D) were immunostained with primary antibody for cyclin D1 in the absence (Panel A and B) and presence of TAM (Panel C and D) as described in the methods (Chromogen AEC, magnification 400X)

D1 was significantly less in both cell types and in particular in parent cells [Fig. 3C (1+) and D (1+)]. In all conditions a heterogeneous pattern of immunostaining was seen in both cell types.

DISCUSSION

It is known that cyclins and CDKs play important roles in cell proliferation which can affect patients' tumors prognosis[3-7]. On the other hand, 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[17-20]. In the systemic treatment of patients with advanced breast cancer, the occurrence of drug resistance in some patients is a major problem. The reasons for tamoxifen resistance have not yet been fully elucidated, but several possibilities have been suggested. These include metabolic changes during transport to and into the tumor cells, disturbance of binding to the estrogen receptor or during receptor dimerization, modifications in the binding process of the homodimer/estrogen complex to DNA and/or modulation in the activation of the transcription cascade[27,28]. Possibilities to overcome (acquired) tamoxifen resistance arose from the use of newly developed anti-estrogens[29], progestins[29] or aromatase inhibitors[30]. However, none of these agents could completely replace tamoxifen so far from first-line clinical use[28,31].

In addition to necessity to further elucidate the molecular mechanisms underlying tamoxifen resistance, it is quite important to address which therapeutic regimen could be most effective after the occurrence of tamoxifen resistance[25,26]. Therefore, in the present study we studied the expression of cyclin D1 as a proliferative marker in sensitive and resistant human breast cancer T47D cells. It is well known that specific chemotherapeutic drugs interfere with cellular pathways in one way or another. In the breast cancer cells used in our study, cyclin D1 protein expression level was different among parent and TAM-resistant cells. In addition, the growth rate of parent cells was seen to be faster than TAM-resistant cells. Therefore, observed decrease in cyclin D1 protein expression in slow growing TAM-resistant cells compared to parent cells further indicates the role of cyclins in tumor cell proliferation. It has been reported that endocrine treatment is more effective in slowly proliferating breast cancers[25]. Therefore, the anti-proliferative effect of tamoxifenon tumor growth via ER blockade may likely be due to regulation of expression of some cyclins and in particular the cyclin D1 protein.

Our results also suggest that treating breast cancer patients should be individually-based considering the tumor levels of markers such as cyclin D1. In addition, for hormone-resistant tumors, chemotherapeutic regimen should be selected based on the level of expression of tumor markers including cyclin D1 to increase the efficacy of such chemotherapy.

ACKNOWLEDGMENTS

The authors would like to thank the financial support of National Center of Excellence of Toxicology (NCET) and also kind assistance of office of vice-chancellor for research of TUMS.

REFERENCES

1. Pestell, R.G., C. Albanese, A.T. Reutens, J.E. Segall, R.J. Lee and A. Arnold, 1999. The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. Endocrine Rev., 20: 501-534.
2. Sherr, C.J. , 1996. Cancer cell cycles. Science, 274: 1672-1677.
3. Sherr, C.J. and J.M. Roberts, 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev., 13:1501-1512.
4. Weinberg, R.A., 1995. The retinoblastoma protein and cell cycle control. Cell, 81: 323-330.
5. Lukas, J., J. Bartkova and J. Bartek, 1996. Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D-cyclin dependent kinase-pRb-controlled G1 checkpoint. Mol. Cell. Biol., 16: 6917-6925.
6. Zwijsen, R.M.L., R. Klompmaker, E.B.H.G.M. Wientjens, P.M.P. Kristel, B. van der Burg and R.J.A.M. Michalides, 1996. Cyclin D1 triggers autonomous growth of breast cancer cells by governing cell cycle exit. Mol. Cell. Biol., 16: 2554-2560.
7. Kenny, F.S., R. Hui, E.A. Musgrove, J.M. Gee, R.W. Blamey, R.I. Nicholson, R.L. Sutherland and J.F. Robertson, 1999. Overexpression of cyclin D1 messenger RNA predicts for poor prognosis in estrogen receptor positive breast cancer. Clin. Cancer Res. 5: 2069-2076; Mol. Cell, 3: 799-804.
8. Shtutman, M., J. Zhurinsky, I. Simcha, C. Albanese, M. D'Amico, R. Pestell and A. Ben-Ze'ev, 1999. The cyclin D1 gene is a target of the-catenin/LEF-1 pathway. Proc. Natl. Acad. Sci. USA, 96: 5522-5527.
9. Wang, T.C., R.D. Cardiff, L. Zukerberg, E. Lees, A. Arnold and E.V. Schmidt, 1994. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature, 369: 669-671.

10. Yu, Q., Y. Geng and P. Sicinski, 2001. Specific protection against breast cancers by cyclin D1 ablation. Nature, 411:1017-1021.
11. Ganter, B., S.L. Fu and J.S. Lipsick, 1998. D-type cyclins repress transcriptional activation by the v-Myb but not the c-Myb DNA-binding domain EMBO J., 17: 255-268.
12. Inoue, K. and C.J. Sherr, 1998. Gene expression and cell cycle arrest mediated by transcription factor DMP1 is antagonized by D-type cyclins through a cyclin-dependent-kinase-independent mechanism. Mol. Cell. Biol., 18: 1590-1600.
13. McMahon, C., T. Suthiphongchai, J. DiRenzo and M.E. Ewen, 1999. P/CAF associates with cyclin D1 and potentiates its activation of the estrogen receptor. Proc. Natl. Acad. Sci. USA., 96: 5382-5387.
14. Reutens, A.T., M. Fu, G. Watanabe, C. Albanese, M.J. McPhaul, S.P. Balk, O.A. Janne, J.J. Palvimo and R.G. Pestell, 2001. Cyclin D1 binds the androgen receptor and regulates hormone-dependent signaling in a p300/CBP-associated factor (P/CAF)-dependent manner. Mol. Endocrinol., 15: 797-811.
15. Neuman, E., M.H. Ladha, N. Lin, T.M. Upton, S.J. Miller, J. DiRenzo, R.G. Pestell, P.W. Hinds, S.F. Dowdy, M. Brown and M.E. Ewen, 1997. Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. Mol. Cell. Biol., 17: 5338-5347.
16. Zwijsen, R.M.L., E. Wientjens, R. Klompmaker, J. van der Sman, R. Bernards and R.J.A.M. Michalides, 1997. CDK-independent activation of estrogen receptor by cyclin D1. Cell, 88: 405-415.
17. Gajdos, C.J., 2002. VC. Selective estrogen receptor modulators as a new therapeutic drug group: Concept to reality in a decade. Clin. Breast Cancer, 2: 272-281.
18. Chan, S., 2002. A review of selective estrogen receptor modulators in the treatment of breast and endometrial cancer. Semin Oncol., 29: 129-133.
19. Chew, H.K., 2002. Medical management of breast cancer: today and tomorrow. Cancer Biother Radiopharm, 17: 137-149.
20. Clemons, M., S. Danson and A. Howell, 2002. Tamoxifen, ('Nolvadex'): A review. Cancer Treat Rev., 28: 165-180.
21. Jordan, V.C., 1998. Antiestrogenic action of raloxifene and tamoxifen: today and tomorrow. J. Natl. Cancer Inst., 90: 967-971.
22. Favoni, R.E. and A. de Cupis, 1998. Steroidal and nonsteroidal oestrogen antagonists in breast cancer: basic and clinical appraisal. Trends Pharmacol. Sci., 19: 406-415.
23. Jordan, C., 2002. Historical perspective on hormonal therapy of advanced breast cancer. Clin Ther., 24 : A3-A16.
24. Katzenellenbogen, B.S., 1996. Estrogen receptors: bioactivities and interactions with cell signaling pathways. Biol. Reprod., 54: 287-293.
25. Spyratos, F., P.M. Martin, K. Hacène, S. Romain, C. Rieu, M. Ferrero-Poüs, S. Deytieux, V. Le Doussal, M. Tubiana-Hulin and M. Brunet, 1992. Multiparametric prognostic evaluation of biological factors in primary breast cancer. J. Natl. Cancer Inst. (Bethesda), 84: 1266-1272.
26. Swain, S.M., M. Lippman, E.F. Egan, J.C. Drake, S.M. Steinberg and C.J. Allegra, 1989. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. J. Clin. Oncol., 7: 890-899.
27. Johnston, S.R., 1997. Acquired tamoxifen resistance in human breast cancer: Potential mechanisms and clinical implications. Anticancer Drugs, 8: 911-930.
28. Katzenellenbogen, B.S., M.M. Montano, K. Ekena M.E. Herman, E.M. McInerney, L. William, 1997. McGuire Memorial Lecture. Antiestrogens: Mechanisms of action and resistance in breast cancer. Breast Cancer Res. Treat, 44: 23-38.
29. Levenson, A.S. and V.C. Jordan, 1999. Selective oestrogen receptor modulation: Molecular pharmacology for the millennium. Eur. J. Cancer, 35: 1628-1639.
30. Hamilton, A. and M. Piccart, 1999. The third-generation non-steroidal aromatase inhibitors: A review of their clinical benefits in the second-line hormonal treatment of advanced breast cancer. Ann Oncol., 10: 377-384.
31. Braun, M., 2002. Has tamoxifen had its time? Breast Cancer Res., 4: 213-217.