Scholars Journal of Applied Medical Sciences (SJAMS) **ISSN 2320-6691 (Online)**
Sch. J. App. Med. Sci., 2017; 5(8D):3268-3273 **ISSN 2347-954X (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com DOI: 10.36347/sjams.2017.v05i08.055

Original Research Article

Semi-Quantitation of Urokinase Plasminogen Activator System (uPAS), And Bcl3 in Breast Tumors

Reyadh Salim Mohammed[1], Faeza Aftan Zghair[2]
[1]College of Dentistry, Ibn Sina University of Medical and Pharmaceutical Sciences, Baghdad, Iraq
[2]Department of pathology, College of Medicine, Aliraqia University, Baghdad, Iraq

***Corresponding author**
Reyadh Salim Mohammed
Email: reyadhsalim81@gmail.com

Abstract: Urokinase plasminogen activator (uPA) is a serine protease involved in cancer invasion and metastasis. uPA acts in vivo by binding to a membrane receptor known as uPAR. This study was aim to investigate the evaluation of uPA,uPAR, PAI-1 and BCL3 in Iraqi breast tumors and assessment the association of their expression with clinicopatholgical features. A total 54 or retrospective breast tumor were studied, 47 out of 45 were primary breast carcinoma, the rest 7 cases were benign. The malignant cases were graded according to the modified Bloom and Richardson criteria into three histological grades. uPA, uPAR, & PAI-1 levels were semiquantitated in these cases using immunohistochemistry and tissue microarray technigues. Association between clinicopathological variables and expression of biomarkers was evaluated by using the Fisher's exact test and Chi-square test as appropriate and a 2-tailed *p*-value <0.05 was considered statistically. From 47 infiltrative ductal carcinoma 76.6%, 78.7%, 89.3% and 67.6% were strongly positive for uPA, uPAR,PAI-1and Bcl3 respectively. In contrast, uPA was only weakly detected in benign tumors and was not detected in normal epithelia surrounding tumor or in areas of adenosis. uPA levels in both benign and malignant tumors were significantly correlated with those for uPAR, PAI-, and Bcl3. That both uPA and its receptor are mostly present in invasive breast carcinomas.
Keywords: urokinase plasminogen activator; urokinase plasminogen activator receptor; urokinase plasminogen inhibitor, BCl3, immunohistochemistry; breast carcinoma, Tissue microarray

INTRODUCTION

Breast cancer is the most common cancer in women. In the worldwide, more than one million women are suffered from this disease [1].

Metastasis is a multi-steps process that involves spreading of cancer cells from the primary to the secondary site. During this process, cancer cells must invade the surrounding tissue, penetrate the blood or lymphatic vessels, and form a new tumor mass at distant sites. For spreading, the cancer cells degraded the extracellular matrix (ECM) and basement membrane to produce a route for the cells to move out of the original site. This process is achieved by secretion of a variety of matrix-degrading enzymes including matrix metalloproteinases (MMPs) and serine proteases, such as plasminogen activator. Urokinase plasminogen activator (uPA) is one of the serine protease enzymes that involved in ECM breakdown, cancer invasion and metastasis by regulating the plasminogen/plasmin system. uPA is synthesized as a single-chain proenzyme which is activated by proteolytic cleavage to form the high-molecular-weight two-chain active uPA or the low-molecular-weight uPA through the action of plasmin, kallikrein, or cathepsin B [2]. Active uPA cleaves inactive plasminogen to produce active plasmin, which can dissolve a variety of ECM proteins. Besides, plasmin and uPA can also activate many types of matrix metalloproteinases (MMPs) which, in turn, breakdown ECM. Therefore, uPA amplifies proteolytic cascades in ECM degradation which is important step for cancer spreading. uPA do this effect by binding to its receptor (uPAR) on the cell membrane, which localizes uPA on the cell membrane and then finally promote its plasminogen activation capability[3]. Plasminogen activator inhibitor-1 (PAI-1), also member of the serine

protease inhibitor superfamily, it's doing a key role in regulation of extracellular matrix homeostasis, protecting the ECM from over degradation [4]. PAI-1 also interacts with the extracellular matrix component vitronectin and thus is believed to be a molecular switch that governs cell adhesion and migration [5]. From these biological properties, it can be concluded that PAI-1 may play an important role in cancer invasion and metastasis [4].

Bcl-3 is an established oncogene in hematologic malignancies, such as B-cell chronic lymphocytic leukemias. Nevertheless, recent research reported that it also participates in tumor progression [6]. The present study aim to assess Bcl3 and it is considered the first study that assesses immune expression of this marker on breast cancer.

PATIENTS AND METHODS

Fifty four paraffin blocks of breast tumors were randomly selected from archive files of Histopathology and Cytology Dept. in Tikreet Teaching Hospital-Salah Addin-Iraq and Private lab, Baghdad-Iraq. Forty seven were invasive ductal carcinoma, Not Otherwise Specified (NOS) type and seven were benign. For each case, an initial H&E-stained control section was reviewed to confirm an adequate tissue in donor block for transfer to the tissue microarray block and to select and mark the location points for cores to be taken. A semiautomatic tissue arrayer (Beecher TMA instrument-Beecher Instrument, Sun Prairie, WI 53590) was used to remove 2 cores of 0.6 mm from each donor block and transferred them to a recipient block, this step was achieved in Histopathology lab. Heath Hospital, UK. Cores were arranged in sectors, each containing 12 rows with 12 cores per row, the distance between each two cores 1mm and each two rows 1mm. TMA block was cut at a thickness of 5µm on a microtome cutter (Leica RM2135). Sections were placed on poly-L-lysine (PLL) coated slides (polysine, Thermo Fisher) and heated at 58°C for 24 hours after that the melting paraffin wax was added on the top of TMA section to prevent loss of cores. Slides were deparaffinized and rehydrated in graded alcohols, heat-induced epitope retrieval were done by immersing them in a 0.01-mol/L concentration of citrate buffer (pH 6.0) preheated to more than 90°C and left for 20 minutes, followed by 20minutes cool down period at 25-28 °C. Then slides were incubated with uPA, uPAR, PAI-1 and Bcl3 antibodies markers.

Scoring system of IHC

uPA, uPAR and PAI-1 scoring was employed according to Minisini*et al*. [7] and Dublin *et al*. [8]. It is semi-quantitive system and two parameters evaluated; (percentage of tumor cell stained and the intensity of stain), the following formula;

$$\Sigma\ (\%\text{Positive Cells}) \times (\text{Staining Score}) \times 100$$

The stain intensity negative =0; weak =+1 and strong= +2, cut off value 0%, from 0-10% was weak positive and more than 10% was strong positive.

Bcl3 was scored according to (Cardiff School of Bioscience Laboratory, Cardiff University, UK), two parameters evaluated in this score; proportion of stained tumor cells and intensity of stain, cut off value is 30%. Chi square, Fisher's Exact test, and ANOVA were used. $P < 0.05$ was considered as significant.

RESULTS

Patient's age ranged from 29-85 years with a mean of 50.7±11.8 years. The peak age frequency was in the age category 40-49 years. Of the tissue specimens, 6.4% were less than 2 cm in largest diameter and 93.6% were more than 2cm, positive lymph node metastasis were in 68.1%. Grade I infiltrative ductal carcinoma formed (12.8%) grade II (68.1%) and grade III (19.1%). 6.4% were stage I, 42.5% were stage II and 51.1% were stage III.

From 47 infiltrative ductal carcinoma 76.6%, 78.7%, 89.3% and 67.6% were strongly positive for uPA, uPAR,PAI-1and Bcl3 respectively (Table 1).

Positive immunostaining for uPA, uPAR, PAI-1and Bcl3 was observed in the cytoplasm of tumor cells as brown diffuse pigmentation (Figure-1).

Association of uPA with pathological staging was significant(Table -1).Significant association of uPA positive expression with (uPAR and PAI-1), as well as significant relation of Bcl3 with (uPA, uPAR and PAI-1) (Table 2).

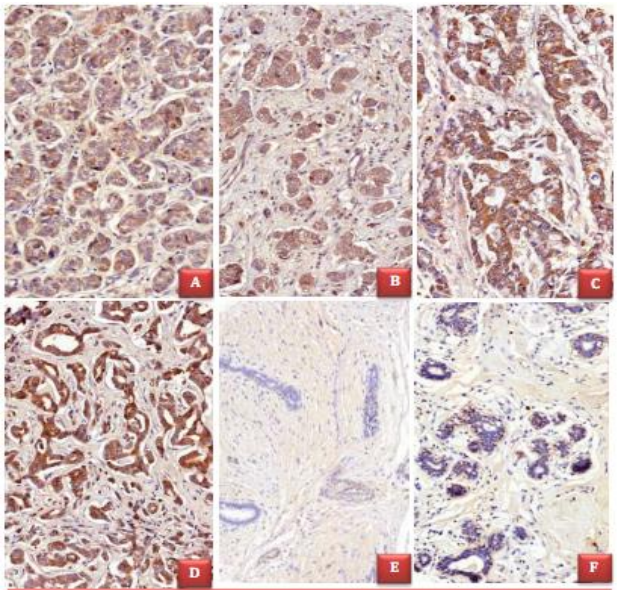

Fig-1: Immunohistochemical staining of uPA, uPAR and PAI-1 & Bcl3 in breast tumor. (A) Strong uPA expression in a grade III invasive ductal carcinoma; (B) strong uPAR expression in a grade III invasive ductal carcinoma; (C) strong PAI-1 expression in a grade II invasive ductal carcinoma; (D) Strong Bcl3 expression in a grade III invasive ductal carcinoma. (E) Negative Bcl3 expression in the epithelial cells of a fibroadenoma. (F) and weak uPA expression in the epithelial cells of a fibroadenoma. Original magnification, X10

Table 1: Association of uPA, uPAR, PAI-1& Bcl3 Expression with Clinicopathological Features

	uPA $^{+}$	uPA^{-}	uPAR^{+}	uPAR^{-}	PAI-1^{+}	PAI-1^{-}	Bcl3^{+}	Bcl3^{-}
Tumor largest diameter								
≤ 2cm	3(**100**)	0(**0**)	1(**33**)	2(**67**)	3(**100**)	0(**0**)	2(**67**)	1(**23**)
> 2cm	33(**75**)	11(**25**)	36(**82**)	8(**18**)	39(**89**)	5(**11**)	34(**77**)	10(**23**)
P value	**0.4**		**0.2**		**0.2**		**1.0**	
Nodal Status								
Negative	11(**73**)	4(**27**)	11(**73**)	4(**27**)	14(**93**)	1(**7**)	11(**73**)	4(**27**)
Positive	25(**78**)	7(**22**)	26(**18**)	6(**19**)	28(**88**)	4(**12**)	25(**78**)	7(**22**)
P value	**0.4**		**0.6**		**1.0**		**1.0**	
Histological grade								
I	3(**50**)	3(**50**)	5(**83**)	1(**17**)	6(**100**)	0(**0**)	5(**83**)	1(**17**)
II	27(**84**)	5(**16**)	25(**78**)	7(**22**)	27 (**84**)	5(**16**)	24(**75**)	8(**25**)
III	6(**67**)	3(**33**)	7(**78**)	2(**22**)	9(**100**)	0(**0**)	7(**78**)	2(**22**)
P value	**0.3**		**0.6**		**0.4**		**0.5**	
Pathological stage								
I	3(**100**)	0(**0**)	1(**33**)	2(**67**)	3(**100**)	0(**0**)	2(**67**)	1(**33**)
IIA	5(**56**)	4(**44**)	7(**78**)	2(**22**)	8(**89**)	1(**11**)	6(**67**)	3(**33**)
IIB	11(**100**)	0(**0**)	9(**82**)	2(**18**)	10(**91**)	1(**9**)	10(**91**)	1(**9**)
IIIA	11(**65**)	6(**35**)	13(**76**)	4(**24**)	14(**82**)	3(**18**)	12(**71**)	5(**29**)
IIIB	6(**86**)	1(**14**)	7(**100**)	0(**0**)	7(**100**)	0(**0**)	6(**86**)	1(**14**)
P Value	**0.024****		**0.2**		**0.4**		**0.8**	

** Significant association

Table 2: Association of immunohistochemical expression for four tumor markers.

Association	*P* value
uPA and uPAR	**0.003****
uPA and PAI-1	**0.005****
uPAR and PAI-1	**0.05 n.s**
Bcl3 and uPA	**0.001****
Bcl3 and uPAR	**0.04****
Bcl3 and PAI-1	**0.003****

** Significant association, n.s-no significant association

DISCUSSION

Extracellular proteolysis is an important step to tumor cell invasion and metastasis, not only because of its ability to break down the extracellular matrix, but also because of its effect on cell division, adhesion, migration, and angiogenesis [9]. uPA, uPAR and PAI-1 are important in extracellular proteolysis systems that lead to spread of tumor cells. High expression of PAI-1 and⁄or uPA protein in tumor tissues are correlated with aggressiveness of this tumor, also these two biomarkers considered as a strong predictors of poor prognosis of patients have different types of solid malignant tumors, including breast cancer [10]. As well uPAR expressed in breast cancer and also associated with adverse outcome [11]. A study from Germany on breast cancer type (invasive ductal carcinoma) reported that the positive expression of uPA, uPAR and PAI-1 in 96.6%, 93.3% and 93.3% respectively [12]. Study from UK showed the positive expression of uPA, uPAR and PAI-1 as a brown stain in the cytoplasmic of tumor cells with a positive rate of 90%, 97% and 93% respectively. Expression of all these three factors in the normal and benign breast lesions was similar. In the normal tissues the expression of these markers were either negative or may be weakly positive, while weak expression was common in the fibroadenomas and papillomas, with small, focally stronger areas in sclerosing adenomas [8]. The current study showed the lower expression of uPA, uPAR and PAI-1 than Germany and UK studies, this may be due to the difference in ethnic, genetic and environmental factors that may be affected on the expression of uPA, uPAR and PAI-1. The present study also showed no significant association between (uPA, uPAR and PAI-1) with patient's age, tumor largest diameter, lymph nodes status and histological grade. These results agreed with [13], who reported no significant association of expression between uPA, uPAR and PAI-1 with age, nodal status, histological grade. Other study found no significant correlation between uPA and uPAR levels with tumor size and nodal status [14]. Han *et al*. [15] revealed no significant correlation between uPA with tumor size and age, while documented a significant relationship with nodal status. An Italian study, found positive expression of uPA and PAI-1 in 92% and 91% respectively, also this study reported that the expression of PAI-1 was not associated with other classical predictive and prognostic factors in breast cancer [7]. Jahkola *et al*. [16] observed no association between uPA, PAI-1 with age, tumor size and histological grade. Other study found significant correlation between uPA/PAI-1 with histological grade while not significant with uPAR. Also other study found no significant correlation between uPA, uPAR, PAI-1 with tumor size and nodal status [8]. Furthermore, this study revealed no significant association between expression of Bcl3 and clinicopathological parameters such as (patien's age, tumor largest diameter, nodal status, histological grade and pathological stage). No previous study was assessed Bcl3 in breast tumors by immunohistochemistry technique, but by RT-PCR technique. The role of Bcl3 in breast cancer development has not been extensively investigated. There are, however, a small number of studies describing Bcl3 expression and function in human breast tumor, normal mammary epithelial cells and breast cancer cell lines. Cogswell *et al* [17] was considered the first to analyze Bcl3 expression in primary human breast tumors and human breast cancer cell lines. Overexpression of Bcl3 is characteristic of a growing cancer [17]. Rocha *et al*. [18] demonstrated that Bcl3 can activate in transcription of cyclin D, which in turn play a key role in driving cell cycle progression. However, assessment study of four breast cancer cell lines revealed that the levels of Bcl3, along with p50, p52 and c-Rel, were increased in all tumor samples in comparison with normal adjacent tissue. This was found to be regulated at the RNA level in two further samples, which also showed increases in *cyclin D1* expression. In support of these data, a more recent report found that 9 out of 12 human breast cancer tumors had higher Bcl3 protein levels than their corresponding adjacent tissue [19]. Another study revealed that the deletion of the Bcl3 gene in ErbB2 positive mice resulted in a 75% decreasing in metastatic

tumor burden in the lungs with a 3.6-fold decrease in cell turnover index in these secondary lesions with no significant effect on primary mammary tumor growth, cyclinD1 levels or caspase 3 activity. Direct inhibition of Bcl3 by siRNA in a transplantation model of an ErbB2-positive mammary tumor cell line confirmed the role of Bcl3 in malignancy suggesting that the Bcl3 was effect on the tumor cells. Bcl3 knockdown resulted in a 61% decrease in tumor cell motility and a concomitant increase in the cell migration inhibitors Nme1, Nme2, Nme3 and the metalloproteinase inhibitors Timp1 and Timp2. These results indicate for the first time the role of Bcl3 *in vivo*, affecting on metastatic progression rather than tumor growth [20]. The current results reported that increased expression of Bcl3, uPA, uPAR and PAI-1 in invasive ductal carcinoma, this proved the role of these markers in motility of cancer cells. Also this study showed a significant association between uPA and PAI-1, uPA and uPAR these results consisted with previous studies [7, 8]. Additionally, this study showed a significant association between Bcl3 expression with (uPA, uPAR and PAI-1), no previous study assessed this correlation, the direct correlation between Bcl3 and uPA system explain the role of these factors in invasion and motility of tumor cells and play an important role in invasion and metastasis. From this significant correlation between Bcl3 with uPA,uPAR and PAI-1 can be concluded that the expression of Bcl3 gene may be regulated the expression of uPA, uPAR and PAI-1 genes. We recommended studying the expression of these genes to validate our results.

ACKNOWLEDGMENTS

The authors are grateful to Iraqi ministry of higher education and scientific research for financial support. Also we would like to express our gratitude to Richard Clarkson, Cardiff university school of biosciences-Cardiff University-Wales-UK for hosting (Reyadh Salim) to finish this project, and many thanks to staff member of Clarkson's lab. for their technical assistance. Special thanks for consultant pathologist Bharat Jasani, Cardiff university school of medicine for his advices**.**

REFERENCES

1. Gluz O, Liedtke C, Gottschalk N. Triple-negative breast cancer-current status and future directions. AnnOncol. 2009;20:1913-27.
2. Stepanova VV, Tkachuk VA. Urokinase as a multidomain protein and polyfunctional cell regulator. Biochemistry (Mosc). 2002; 67: 109-118
3. Danø K, Behrendt N, Høyer-Hansen G, Johnsen M, Lund LR, Ploug M, Rømer J. Plasminogen activation and cancer. ThrombHaemost. 2005;93: 676-681
4. Durand MK, Bodker JS, Christensen A. Plasminogen activator inhibitor-I and tumor growth, invasion, and metastasis. ThrombHaemost. 2004; 91:438-49.
5. Deng G, Curriden SA, Hu G, Czekay RP, Loskutoff DJ. Plasminogen activator inhibitor-1regulates cell adhesion by binding to the somatomedin B domain of vitronectin. J Cell Physiol. 2001; 189:23-33.
6. Maldonado V, Melendez-Zajgla J. Role of Bcl3 in soild tumors. Mol Cancer. 2011; 10:152.
7. Minisini AM, Fabbro D, Di Loreto C, Pestrin M, Russo S, Cardellino GG, Andreetta C, Damante G, Puglisi F. Markers of the uPA system and common prognostic factors in breast cancer. Am J ClinPathol. 2007; 128:112-117.
8. Dublin E, Hanby A, Patel NK, Liebman R, Barnes D. Immunohistochemical expression of uPA, uPAR, and PAI-1 in breast carcinoma; fibroblastic expression has strong associations with tumor pathology. Am J Pathol. 2000; 157:1219-1227.
9. Noel A, Maillard C, Rocks N. Membrane associated proteases and their inhibitors in tumour angiogenesis. J ClinPathol. 2004; 57; 577-584.
10. Duffy M J. The Urokinase Plasminogen Activator System: Role in Malignancy. Curr Pharm Des. 2004; 10:39-49.
11. de Bock CE, Wang Y. Clinical significance of urokinase-type plasminogen activator receptor (uPAR) expression in cancer. Med Res Rev. 2004; 24:13-19.
12. Hildenbrand R, Schaaf A. The urokinase-system in tumor tissue stroma of the breast and breast cancer cell invasion.Int J Oncol. 2009; 34: 15-23.
13. Andres SA, Edwards AB, Wittliff JL. Expression of urokinase-type plasminogen activator (uPA), its receptor (uPAR), and inhibitor (PAI-1) in human breast carcinomas and their clinical Relevance. J Clin Lab Anal. 2012; 26: 93-103.
14. Kennedy S, Duffy MJ, Duggan C, Barnes C, Rafferty R, Kramer MD. Semi-quantitation of urokinase plasminogen activator and its receptor in breast carcinomas by immunohistochemistry. Br J Breast. 1998; 77(10): 1638-1641.
15. Han Y, Liu L, Yan D, Wang G. Correlation between Expression of P38 MAPK-Signaling and uPA in Breast Cancer. ClinOncol Cancer Res. 2008; 5: 161-164.
16. Jahkola T, Toivonen T, von Smitten K, Virtanen I, Wasenius VM, Blomqvist C. Cathepsin-D, urokinase plasminogen activator and type-1

plasminogen activator inhibitor in early breast cancer: an immunohistochemical study of prognostic value and relations to tenascin-C and other factors. Br J Cancer. 1999; 80(1/2): 167–174.
17. Cogswell PC, Guttridge DC. Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. Oncogene. 2000; 19(9): 1123-1131.
18. Rocha S, Martin AM, Meek DW, Perkins ND. p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-kappaB subunit with histone deacetylase 1. Mol Cell Biol. 2003; 23: 4713-27.
19. Choi HJ, Lee MJ. Bcl3-dependent stabilization of CtBP1 is crucial for the inhibition of apoptosis and tumor progression in breast cancer. Biochem Biophys Res Commun. 2010; 400(3): 396-402.
20. Wakefield AM, Soukupova J, Montagne A, Ranger JJ, French R, William M, Clarkson RWE. Bcl3 selectively promotes metastasis of ERBB2-driven mammary tumors. Cancer Res. 2013;73(2):745-755.