Prognostic significance of Cyclin D1 in urothelial carcinoma; correlation with p53 and clinicopathological parameters

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Abstract: Introduction: Dysregulation of the cell cycle is a key mechanism for the occurrence of various types of tumors including urinary bladder carcinoma [UBC]. Cyclin D1 critically targets the proliferative signals and has been implicated in progression of UBC. **Objectives:** to determine the expression of cell cycle protein [cyclin D1] in urothelial carcinoma; and to correlate the findings with p53 expression as well as various clinicopathological characteristics. **Material and Methods:** bladder carcinoma specimens (n=90) were immunostained using antibodies against cyclin D1 and p53. The association between cyclin D1 and clinicopathological parameters [including grade, stage, muscle invasion and lymph node metastasis] as well as p53 expression were evaluated. **Results:** Cyclin D1 expression showed a highly significant inverse association with MIBC and high grade urothelial tumors ($p<0.001$). UC with nodal metastasis showed significantly lower cyclin D1 expression compared to cases without nodal metastasis ($p=0.038$). No significant association between Cyclin D1 expression and age ($p=0.745$), gender ($p=0.777$), lympho-vascular invasion ($p=0.697$), or distant metastasis ($p=0.151$). Cyclin D1 expression was inversely proportional to P53 expression. **Conclusions:** Cyclin D1 expression may have a prognostic significance in UBC; as low expression of Cyclin D1 is associated with poorly differentiated, advanced stage and lymph node metastasizing tumors as well as high p53 expression.

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1. Introduction:

Bladder carcinoma is the most common urinary tract malignancy; it ranks the sixth most common malignancy among male gender and considereda leading cause of cancer-associated morbidity and mortality worldwide **(Siegel et al, 2012).** Urothelial carcinoma of the urinary bladder (UCB) is the most predominant histological pattern of bladder carcinoma; as it accounts for more than 90% of the diagnosed cases. Traditionally, UCB is classified into two major typesbased primarily on treatment planes and prognostic parameters as follows: *non-muscle invasive bladder cancer (NMIBC)* (corresponding to stages Ta, T1) and *muscle-invasive bladder cancer* (MIBC) (corresponding to stages T2-T4) **(Nargund etal, 2012).**

Urinary bladder carcinomaoccasionally arises through a complex, multistep processes that involve progressive disturbance of the normal mechanisms which control epithelial proliferation, inflammation and differentiation **(Koyuncuer, 2013).** Dysregulation of the cell cycle is a key mechanism for the occurrence of tumors. Cyclins are cell-cycle proteins that control normal cell-cycle progression; and hence affect epithelial proliferation pathways. Cyclin D1 has a key role in the regulation of cell cycle and critically targets the proliferative signals in G1-S phase progression; through forming a complex with different cyclin dependent kinases **(Shan and Tang, 2015).** Cyclin D1 is an oncogenic genethat contributes to cell-cycle dysfunction, *and is frequently over-expressed in numerous human malignancies* **(Kim and Diehl, 2009).** Many studies proved that cyclin D1 gene is usually over-expressed in human bladder carcinoma. However, its association with pathological parameter is conflicting **(Yuan et al, 2010).**

The p53 tumor suppressor gene is the most frequently mutated gene in all human cancers and has been largely described as the universal sensor of genotoxic stress **(Pflaum et al., 2014).** P53 belongs to unique protein family which includes three important members: *p53*, p63 and p73. As a tumor suppressor, p53 is essential for preventing inappropriate cellular proliferation and maintaining genome integrity following genotoxic stress. P53 activation involves an increase in overall p53 protein level as well as qualitative changes in the protein through marked posttranslational modification, leading to activation of p53-targeted genes **(Lane and Levine, 2010).** The p53 downstream targets are distinctively activated depending on the cell type, extent of the damage that has influenced p53 activation and other unidentified parameters. Alterations in the p53 pathway lead to urinary bladder tumor progression and are likely to provide relevant prognostic factor in the management of bladder cancer patients **(Goldar et al., 2015).**

Typing, grading and staging of bladder cancer are important for proper assessment and management of the UBC patients. The current study aims to determinethe expression of cell cycle protein [cyclin D1] in urothelialcarcinoma; and to correlate the findings with p53 expression as well as various sclinicopathological characteristics [including patient age, patient gender, tumor type, tumor grade, lympho-vascular invasion and nodal as well as distant metastasis].

2. Material and methods:

This study was performed during the period from December 2016 to December 2018. All procedures performed in the current study [specifically involving human participants] were accepted from the Local Research Ethics Committee of Faculty of Medicine, Tanta University.

Patient characteristics:

The present study was carried out on 90 cases primarily diagnosed as urothelialcarcinoma. The patients included were mainly of male gender (representing 57.78%) and 38of female gender (42.22%), aged from 45 to 72 years. Paraffin blocks of the patients were retrieved from the archives of pathology department, Faculty of Medicine, Tanta University.

Patient classification:

H & E stained sections were prepared for histopathological review and confirmation of the histopathological features. The histological types **(Eble et al., 2004)** and the pathological grading **(Seitz et al., 2004)** were classified according to the 2004 World Health Organization classification [WHO] of urothelial tumor.

Primary monoclonal antibodies:

- Anti-Cyclin D1 antibody: a rabbit monoclonal antibody with molecular weight of 36 kDa. (Kit no. **#2978**.). Isotype: IgG. (1:50 dilution, Clone EP12; Daco, USA).
- Anti-p53 antibody: a mouse monoclonal antibody with molecular weight of 53 kDa. (Kit no. M7001.). Isotype: IgG2b, kappa. (1:50 dilution; Clone DO-7; Daco, USA).

Methodology of immunohistochemical staining:

Paraffin-embedded sections were immunostained for anti-Cyclin D1 and anti-p53 antibodies. After dewaxing, inactivating endogenous peroxidase activity and blocking cross-reactivity with normal serum. An overnight incubation was done in a humidity chamber with Cyclin D1 and p53 antibodies followed by washing in PBS. Sections were then covered with 2-3 drops of secondary biotinilated antibody, incubated at room temperature for 10 min, then washed in PBS. Finally, sections were counter-stained with hematoxylin. As positive controls, sections from human tonsillar tissue (for Cyclin D1) and sections from colonic carcinoma (for p53) were used. Negative controls were prepared by primary antibody with PBS and normal mouse serum.

Interpretation of immunohistochemical results:

For Cyclin D1 nuclear and cytoplasmicbrownish staining was considered positive. The immunostaining intensity was scored as negative = 0, weak = 1, moderate = 2, or strong = 3. Staining intensity scores graded as low level immunoreactivity (score 0 and 1) and high level (score 2 and 3); according to **Kopparapu et al., 2013.** Regarding p53, tumors with nuclear immunoreactivity of more than 10% were considered positive according to **Compérat et al., 2006.**

Statistical analysis

Statistical presentation and analysis of the present study were conducted, using the mean and chi-square test, person correlation by SPSS (version 15.0; SPSS Inc., Chicago, Illinois, USA) software. Significant results were considered at p value < 0.05, highly significant results were considered at p value < 0.01.

3. Results:

Ninety patients [90] diagnosed as urothelial carcinoma were included in the current work, at the time of presentation, the patients' ages ranged from 45 to 72 years (mean 58.5 years). Fifty two (52) patients were of male gender (57.78%) and the remaining 38were female (42.22%). Cases were classified according to muscle invasion into 49 NMIBC (54.44%); and 41 MIBC (45.56%). Forty (40) cases showed high grade features [high grade urothelialcarcinoma] (44.44%),43cases were presented with nodal metastasis (47.78%) and only 35 cases showed evident distant metastasis (38.89%).

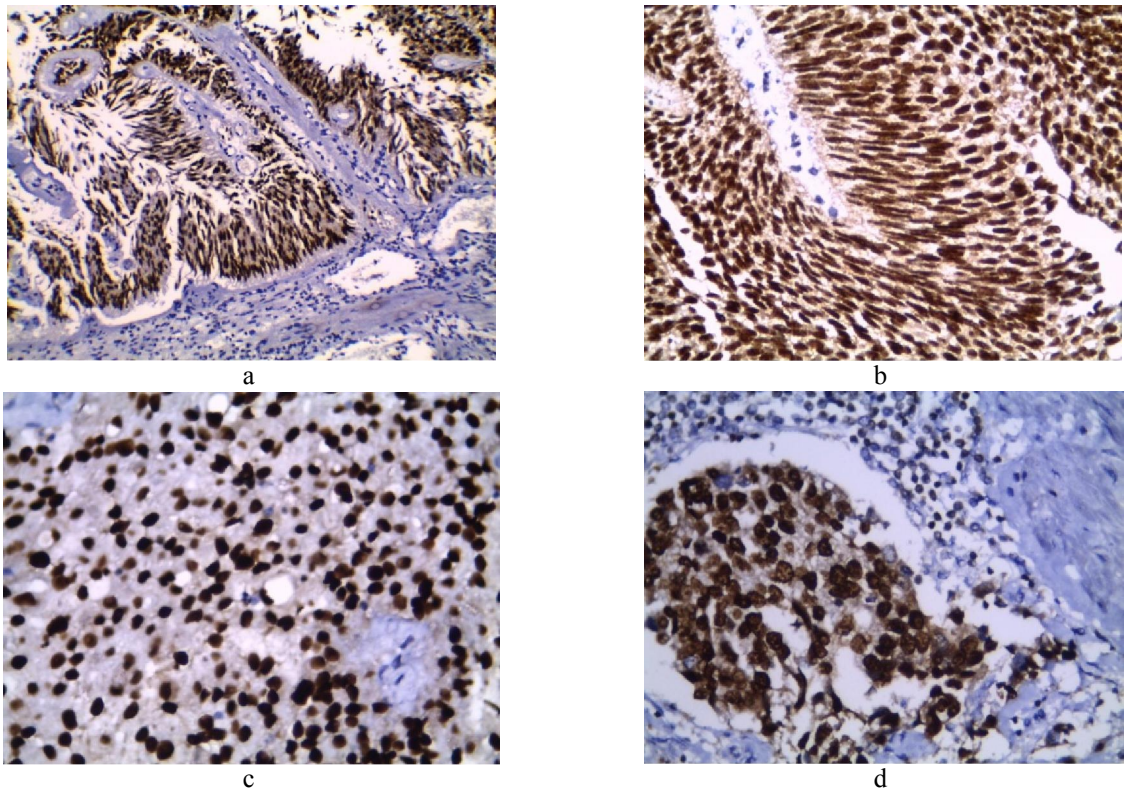
Immunohistochemistry results:

Table (1) summarizes the relation of Cyclin D1 expression to clinicopathological parameters and P53 immunohistochemical expression. Cyclin D1 showed high expression (+2 and +3) in 56 cases (62.22%) (Fig. 1 a, b, c and d). Cyclin D1was inversely correlated with tumor type, tumor grade and nodal status showing asignificant correlation with each, as low Cyclin D1 expression showed a highly significant association with MIBC and high gradeurothelial tumors ($p<0.001$). UC with nodal metastasis showed significantly lower cyclin D1 expression compared to cases without nodal metastasis ($p=0.038$). No significant association between Cyclin D1expression and age ($p=0.745$), gender ($p=0.777$), lympho-vascular invasion ($p=0.697$), ordistant metastasis ($p=0.151$). Cyclin D1expression was inversely proportional to P53 expression; as P53 positivity was observed in high

grades as well as deeply infiltrating tumors (p<0.001) (Fig. 2 a and b).

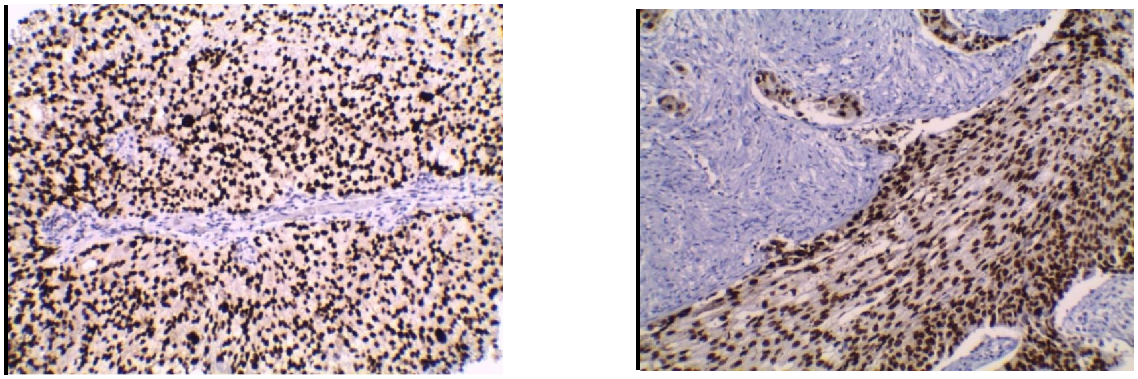
Table (1): Cyclin D1 expression in relation to patient and tumor characteristics as well as to p53 immunohistochemical expression

		Cyclin D1						Chi-square	
		Low expression		High expression		Total			
		N=34	%	N=56	%	N=90	%	^{2}	p-value
Age	<60	11	32.4	20	35.7	31	34.4	0.106	0.745
	>60	23	67.6	36	64.3	59	65.6		
Gender	Male	19	55.9	33	58.9	52	57.8	0.080	0.777
	Female	15	44.1	23	41.1	38	42.2		
Tumor type	NMIBC	5	14.7	44	78.6	49	54.4	34.791*	<0.001*
	MIBC	29	85.3	12	21.4	41	45.6		
Tumor grade	Low grade	9	26.5	41	73.2	50	55.6	18.721*	<0.001*
	High grade	25	73.5	15	26.8	40	44.4		
Lymphovascular invasion	Negative	18	52.9	32	57.1	50	55.6	0.151	0.697
	Positive	16	47.1	24	42.9	40	42.9		
Nodal status	Negative	13	38.2	34	60.7	47	52.2	4.284*	0.038*
	Positive	21	61.8	22	39.3	43	47.8		
Distant metastasis	Negative	24	70.6	31	55.4	55	61.1	2.065	0.151
	Positive	10	29.4	25	44.6	35	38.9		
P53 expression	Negative	7	20.6	47	83.9	54	60.0	35.365*	<0.001*
	Positive	27	79.4	9	16.1	36	40.0		



a b c d

Fig 1: Cyclin D1 expression in UBC; high expression in low grade papillary transitional cell carcinoma, (a [x200], b [x400]), high expression in NMIBC, (c [x400]), high expression in UBC with vascular emboli (d [x400])



a b

Fig 2: p53 expression in UBC; high expression in high grade papillary transitional cell carcinoma, (a [x200]), high expression in MIBC, (b [x200]).

4. Discussion:

Recently, molecular biomarkers have been examined for their prognostic role. Results remain inconclusive and controversial, and up till now, no molecular markers may be used in routine assessment of bladder cancer. There is a need to have immunohistochemical markers for prediction of the prognosis and selection of appropriate therapy with prediction of the response to this therapy for proper management of patients.

Cyclin D1 regulates the G0-G1 phase and promotes cell growth and proliferation. It is considered a proto-oncogene located on chromosome 11q13 that modulates a critical step in cell cycle control progression. Conflicting data on the significance of Cyclin D1 has been reported in urinary bladder tumors. Thus, the prognostic role of this protein remains controversial.

In the current study, majority of the included cases showed high immunohistochemical expression for Cyclin D1 (score +2 and +3) representing 62.22%, while only 37.78 % of cases with urothelial carcinoma showed low Cyclin D1 expression (score 0 and +1). Lower levels (51.6%, 56.5%) were observed by **Khabaz et al., 2016, Xu et al., 2015, respectively. Higher figures (68.6%) were noted by Shan and Tang, 2015.** These differences may be attributed to variations in the size of samples, difference in evaluation techniques, patient's circumstances, and included tumor variables.

Our results showed that Cyclin D1 low expression had associated with adverse clinicopathological parameters in patients with urothelial carcinoma, as low Cyclin D1 expression was significantly associated with invasive tumors ($p=<0.001^{*}$), high grades tumors ($p=<0.001^{*}$) as well as nodal metastasizing tumors ($p=0.038^{*}$). These findings were in agreement with **Kopparapu et al., 2013**; who reported that Cyclin D1 expression was significantly higher in non-invasive tumors than in muscle-invasive UCB. They claimed that nuclear cyclin D1 expression was absent from the majority of invasive tumors and from lymph node metastasis within MIBC group ($p<0.001$). **Khabaz et al., 2016** also reported a significant correlation between Cyclin D1 and various parameters [namely histological type of bladder tumor, grade, stage, muscularispropria invasion, vascular invasion and lymph node invasion].

In contrast to the current results; **Xu et al., 2015 noted that** Cyclin D1 expression was much higher in invasive carcinoma than superficially located tumors; the higher pathologic stage, the higher the positive rate; hence, they suggested that higher expression of Cyclin D1 is closely related to the progression of bladder carcinoma.

Cyclin D1 high expression in bladder malignancy has reported in low grade bladder cancer with no muscularispropria invasion (NMIBC). These results are consistent with other studies, which stated that high scores of cyclin D1 immunore activity is associated with NMIBC, while low level of Cyclin D1 observed in advanced stage, poorly differentiated tumors, MIBC tumors and tumors with vascular invasion, as well as lymph node involvement **(Lenz et al., 2012 and Galmozz et al.,2006).**

Controversy, other studies on Cyclin D1 immunohistochemical expression and its relation to clinicopathological parameter proved contradictory findings. They found that cyclin D1 was directly proportion with high grades and stage **(Lee et al.,2010).** Others reported that no significant correlation of Cyclin D1 with grade, stage and invasion **(Ioachim et al.,2004, Shariat et al., 2007, Seiler et al., 2014 and Shan and Tang, 2015).**

The association between low Cyclin D1 expression and poor prognostic parameters as linked in various reports**.** Not only detected in urothelial carcinoma; but among other tumors, as well **(Feakins et al., 2003 and Jovanovic et al., 2014).**

The p53 is the most frequently mutated gene in all human cancers and has been largely described as the universal sensor of genotoxic stress. In the current work, p53 expression was found to be inversely associated with Cyclin D1 expression. These findings were similar to the previous report of **Lee et al., 2000,** and in contrast to **Tut et al., 2001.** The latter argued that p53 often induces Cyclin D1 in UBC and may be suggested that p53 is involved in cell differentiation dedifferentiation pathways in UBC with different molecular pathways acting in UBC.

It has been accepted that high grade neoplasms of bladder have higher progression and invasiveness rate more than low-grade tumors **(Raman et al., 2005).** Hence, the phenotype of cyclin D1was correlated with the degree of cancer progression and invasiveness. Altered expression of cyclin D1 may lead to changes in the biological behavior of transformed cells, for instance growth, proliferation, invasion and metastasis.

Conclusion: Cyclin D1 expression may have a prognostic significance in UBC; as low expression of Cyclin D1 is associated with poorly differentiated, advanced stage and lymph node metastasizing tumors as well as high p53 expression. However, further studies are recommended to confirm its prognostic significance and to determine its purposed therapeutic implications.

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