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Research Article

EXPRESSION PATTERN OF CYCLIN D1 IN MAJOR TYPES OF BLADDER CANCER IN RELATION TO BILHARZIASIS

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ABSTRACT

Most investigators have accepted the association between schistosomiasis and bladder cancer since the work of Ferguson in 1911. Bladder cancer is the fourth most common cancer in men and the eighth most common in women, accounting for 8% of adult cancers. It is estimated that 54,300 new cases and 12,400 deaths were reported in the USA in 2001. Cell cycle proteins are important markers in predicting tumor behavior in urothelial carcinoma of the bladder. Over- expression of the D1 cyclins allows uncontrolled tumour cell proliferation. This study was designed to investigate the immunohistochemical expression of cyclin D1 in the two commonest types of bladder cancer in relation to tumor grade, to evaluate their role on the tumorgenesis and progression of bladder cancer (associated with bilharziasis or not associated). Our results showed it is generally over-expressed in urothelial carcinoma compared to non-malignant lesions and to squamous cell carcinoma. It's down regulation was associated with aggressive bladder cancers.

In conclusion, Cyclin D1 expression is not just a research tool. In bladder cancers, cyclin D1 expression may provide prognostic information important in the management of patients with these diseases. In the future, the cyclin D1 gene and/or protein may be a site for immunotherapy. Before such treatments are possible, the full significance of this molecule in different variants of bladder cancer needs to be examined.

INTRODUCTION

In the United States, bladder cancer is the fourth most common cancer [1], and it is estimated that bladder cancer will account for 72,570 new cases and 15,210 deaths in the US alone in 2013 [2]. Urothelial carcinoma of the bladder (UCB) is the most frequent histological subtype of bladder cancer and accounts for more than 90% of the cases in USA and Europe [3]. In Egypt, the high frequency of squamous cell carcinoma (SCC) is due to schistosomiasis-infested bladders that frequently show squamous metaplasia and dysplasia of the transitional epithelium [4,5].

UCB is classified into two major groups for treatment and prognostic purposes, namely: non-muscle invasive bladder cancer NMIBC (Ta/Tis/T1) and muscle invasive bladder cancer (MIBC) (T2-T4). A majority of patients with UCB present with NMIBC that although clinically heterogeneous is typically associated with a favorable prognosis and a relatively low risk for recurrence following cystectomy. However, NMIBC is associated with a high risk (60-75%) of recurrence in patients with intact bladders [6]. In contrast, MIBC accounts for only 25-30% of cases, yet the five-year survival rate, even following radical cystectomy, is greatly reduced as compared with NMIBC, particularly when patients have lymph node metastases [7, 8]. In both NMIBC and MIBC, available histopathological parameters, including tumor stage and grade do not always predict the course of disease in individual patients, including those who have an increased risk of post cystectomy recurrence. There is therefore an urgent need to identify novel biomarkers that indicate UCB cases with increased risk for recurrence and metastases. Currently, much interest centers on the molecular processes underlying the development and progression of transitional cell carcinoma of the bladder. It is already clear that molecular pathways involving the p53 tumors-suppressor gene are of great importance in bladder cancer, especially the more aggressive types of cancer at this site [9].

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Fundamental to the neoplastic process is deregulated cellular proliferation and this will almost certainly be a factor relevant to the variable biological behavior of bladder tumours. The cyclin family of proteins which includes the mitotic cyclins, A and B, and cyclins C, D and E (associated with G/S transition) have a central role in the control of the cell cycle [10]. Cyclin D1 contributes to regulate G1 progression by forming a complex with different cyclin dependent kinases. It has oncogenic properties and is frequently overexpressed in several human tumor types [11].

The retinoblastoma tumor suppressor gene (RB) encodes a nuclear phosphoprotein that plays a central role in regulating the cell cycle. RB regulates progression through the G1-to-S phase transition of the cell cycle [12]. Loss of RB is well documented in many human tumor types and it is probable that the p16-cyclin D1-CDK4/6RB pathway is disrupted in most human malignancies [13]. Signi cant proportion of bladder cancer cases showed that over-expression of the cyclin D1 gene and increased cyclin D1 expression were associated with poor prognosis and decreased postoperative patient survival [14, 15]. Aberrant cyclin D1 expression has been observed early in carcinogenesis as well [16]. Cyclin D1 is a key cell-cycle regulatory protein, playing a critical role in the G-to-S transition of the cell-cycle progression through binding to cyclin-dependent kinase 4 (CDK4) to phosphorylate [17] and inactivate the retinoblastoma protein (pRb; ref. 9), heterozygous deletion of which occurs in approximately 50% of human muscle-invasive bladder cancer. Thus, identifying a new anticancer drug targeting and down regulating cyclin D1 expression and function is one of the rst priorities in the eld of anticancer research.

Identifying a natural compound that speci cally inhibits bladder cancer invasion and metastasis is of tremendous importance for potentially reducing mortality as a result of this disease.

In this study we aimed to evaluate the expression of Cyclin D1 in different types and stages of bladder cancer and in relation to bilharzial infection in order to assess its value as a target for bladder cancer therapy.

MATERIAL AND METHODS

Patients

Our study included 62 cases (54 men and 8 women) between ages 55 and 72 years. The cases understudy consist of chronic cystitis (4cases), squamous cell carcinoma (12 cases) and urothelial carcinoma (46 cases). Patients were subjected to cystoscopic examination, and transurethral resection biopsies taken from the apparent lesions. Specimens were obtained from the urology department of Theodor Bilharz Research Institute (TBRI), Cairo, Egypt, and fixed in buffered formalin 10% and sent to the pathology department, TBRI. Serial sections were examined histopathologically diagnosed and assessed for tumor stage and grade. The 2002 tumor-node-metastasis (TNM) classification was used for pathological staging and the 1973 World Health Organization classification was used for pathological grading. Informed consents from all patients were taken.

Diagnosis of bilharzial infestation was based on detection of *Schistosoma* eggs in tissues and/or detection of circulating *Schistosoma* antibodies in sera of patients by enzyme-linked immunosorbent assay (ELISA). All specimens were processed into paraffin blocks; 5-micrometer thick sections were cut on positively charged slides for immunohistochemistry (IHC).

Immunohistochemical (IHC) Technique

A standard 3-layer protocol was used as follows:

The sections were deparaffinized with xylene and then dehydrated with 100%, 98%, and 70% ethanol. Endogenous peroxidase was blocked by immersing slides in methanol with 0.3% hydrogen peroxide for 30 minutes.

The antibody-binding epitope of the antigen was retrieved by microwave treatment for 30 minutes in boiling 10 mM citrate buffer (pH 6.0). The slides were allowed to cool for 20 minutes in the citrate buffer before further treatment. After a quick rinse in phosphate buffered saline, 2 sections were covered by cyclin D1 primary antibodies and were used at a dilution of 1:100, for 24 hours ina humid chamber. (purchased from Santa Cruz Biotechnology Company, California.) The sections were then incubated for 30 minutes with the secondary biotinylated antibody followed by avidin peroxidase complex for another 30 minutes according to the manufacturer's instructions (Universal Detection Kit, Dako, Denmark). A brown color was developed with diaminobenzidine for 2-4 minutes, washed in distilled water, and counterstained with Mayer's hematoxylin for 1 minute. The entire procedure was performed at room temperature. In addition, negative controls in which the primary antibody was omitted and replaced by phosphate buffered saline were also used. Positive controls sections consist of colonic mucosa known to express cyclin D1 were added to be processed with the bladder tissue sections in the same run for precision and standardization of the elaborated IHC result.

The expression of cyclin D1 was measured in 10 successive high-power fields (x400) according to Tut *et al*., 2001. Cyclin D1 expressed as nuclear and cytoplasmic brown color. Two pathologist analyzed the intensity, distribution, and pattern, and evaluated cyclin D1 immunoexpressions independently. The percentage of positively stained cells was determined semi quantitatively by assessing the whole tumor section. [18].

The scoring of all the tumor cores was performed and later reanalyzed. An intensity score was assigned and represented the average intensity of positively stained cells (0=no staining, 1=weak, 2=intermediate and 3=strong staining). The fraction of cells stained with antibodies to cyclin D1 were counted as a percentage and scored (0%=0, <15%=1, 15-50%=2, >50%=3) [19].

Statistical Analysis

The statistical analysis of the results was done with analysis of variance (ANOVA) to compare cyclin D1 scores in different groups. Results were given as mean ±SD. Distribution of negative and positive cases was studied with cross tables (Pearson's Chi square-test). To investigate a possible correlation of cyclin D1 scores with tumor grade and stage, the Spearman rank correlation coefficient was used (SPSS software program, version 20). In all tests, $P < .05$ was considered to indicate significant.

RESULTS

Our study consists of 62 urinary bladder biopsy specimens from patients suffering of urinary symptoms coming to hospital of Theodor Bilharz Research Institute (TBRI), Cairo, Egypt, seeking medical advice and were cystoscopically examined and biopsed for histopathological diagnosis. We got tissue sections from their archival material kept in the pathology department of TBRI.

The cases understudy consist of chronic cystitis (4cases), squamous cell carcinoma (SCC) (12 cases) and urothelial carcinoma (UC) (46 cases).

The mean age of different groups was studied statistically and proved by ANOVA test to be significant ($p < 0.01$). The vast majority of studied cases were males (54 cases) with female patients constitute only 12.9% of cases (8 patients). Age and sex distribution was listed in (Table 1).

Table 1 Sex and Age distribution of studied cases

			Sex		Total	Age (Mean ± S.D.)
			female	male		
diagnosis	cystitis	Count	0_a	4_a	4	63.5000 ± 1.73205
		%	0.0%	100.0%	100.0%	
	scc	Count	6_a	6_b	12	67.8333 ± 5.06024
		%	50.0%	50.0%	100.0%	
	uc	Count	2_a	44_b	46	61.0870 ± 6.40078
		%	4.3%	95.7%	100.0%	
Total		Count	8	54	62	62.5484 ± 6.49256
		%	12.9%	87.1%	100.0%	

Each subscript letter denotes a subset of sex categories whose column proportions do not differ significantly from each other at the .05 level. (Pearson Chi-Square)

SCC: Squamous cell carcinoma
UC: Urothelial carcinoma

All cases of squamous cell carcinoma showed high tumor grade and positive muscle invasion, while most cases of urothelial carcinoma were of low grade malignancy (65.2%) and showed negative muscle invasion (69,6%). The difference was statistically significant ($p<0.001$). (Table 2)

Table 2 Difference in tumor grade and muscle invasion between UC and SCC groups

			M. Invasion		Tumor Grade		Total
			Negative	Positive	Low	High	
Diagnosis	scc	Count	0_b	12_a	0_a	12_b	12
		%	0.0%	100.0%	0.0%	100.0%	100.0%
	uc	Count	32_b	14_a	30_b	16_a	46
		%	69.6%	30.4%	65.2%	34.8%	100.0%

Each subscript letter denotes a subset of M. invasion and tumor grade categories whose column proportions do not differ significantly from each other at the .05 level.

Pearson Chi-Square: $p < 0.001$

Half of the chronic cystitis cases and the squamous cell carcinoma cases were associated with bilharziasis, while only 4.3% of the urothelial carcinoma cases were positive for bilharziasis. The difference between groups was statistically significant ($p<0.001$). (Table 3).

Immunohistochemical study shows significant difference between groups considering the intensity and percentage of cellular expression of cyclin D1 ($p < 0.01$ and $p <0.001$ respectively) Urothelial carcinoma showed higher score of cyclin D1 expression compared to both the chronic cystitis and the squamous cell carcinoma groups, with statistically significant difference ($p<0.001$ by ANOVA test). (Table 4)

Table 3 Difference in bilharzial association in the studied groups

			Bilharziasis		Total
			Negative	Positive	
diagnosis	cystitis	Count	2_a	2_a	4
		% within diagnosis	50.0%	50.0%	100.0%
	scc	Count	6_a	6_b	12
		% within diagnosis	50.0%	50.0%	100.0%
	uc	Count	44_a	2_b	46
		% within diagnosis	95.7%	4.3%	100.0%
Total		Count	52	10	62
		% within diagnosis	83.9%	16.1%	100.0%

Each subscript letter denotes a subset of bilh categories whose column proportions do not differ significantly from each other at the .05 level.

Pearson Chi-Square: $p < 0.001$

Table 4 Difference in RAGE expression parameters between studied groups

Diagnosis		Cyclin D1 percent	Cyclin D1 intensity	Cyclin D1 score
cystitis	Mean	.5000	1.0000	.5000
	N	4	4	4
	Std. Deviation	.57735	1.15470	.57735
scc	Mean	8.0000	1.3333	.8333
	N	12	12	12
	Std. Deviation	10.87115	.98473	.71774
uc	Mean	46.7391	2.0000	2.2609
	N	46	46	46
	Std. Deviation	28.54270	.73030	.68101
Total	Mean	36.2581	1.8065	1.8710
	N	62	62	62
	Std. Deviation	30.75868	.86534	.94927
P value (ANOVA)		< 0.001	< 0.01	< 0.001

Cases of chronic cystitis and squamous cell carcinoma showed negative and lower scores of cyclin D1 expression compared to cases of urothelial carcinoma that mostly showed high scores of cyclin D1 expression. The difference between groups was statistically significant ($p<0.001$) (Table 5).

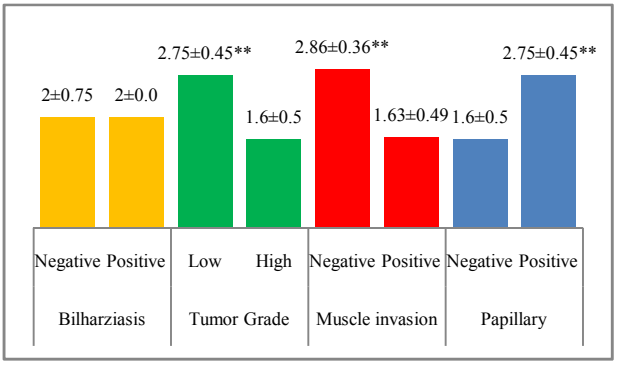
Table 5 Difference in the score of cyclin D1 expression between studied groups

			Cyclin D1 score				Total
			.00	1.00	2.00	3.00	
Diagnosis	cystitis	Count	2_a	$2_{a,b}$	0_b	$0_{a,b}$	4
		% within diagnosis	50.0%	50.0%	0.0%	0.0%	100.0%
	scc	Count	4_a	$6_{a,b}$	$2_{b,c}$	0_c	12
		% within diagnosis	33.3%	50.0%	16.7%	0.0%	100.0%
	uc	Count	0_a	6_a	22_b	18_b	46
		% within diagnosis	0.0%	13.0%	47.8%	39.1%	100.0%
Total		Count	6	14	24	18	62
		% within diagnosis	9.7%	22.6%	38.7%	29.0%	100.0%

Each subscript letter denotes a subset of cyclind1score categories whose column proportions do not differ significantly from each other at the .05 level.

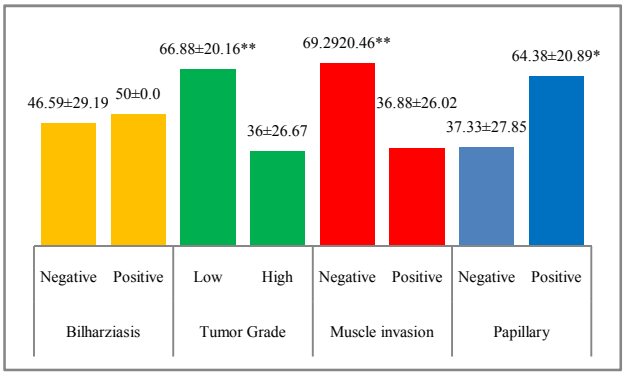
Pearson Chi-Square: $p < 0.001$

High grades of urothelial carcinoma and tumor associated with muscle invasion as well as non-papillary pattern showed significantly lower intensity and percentage of cyclin D1 expression compared to their counterparts. ($p<0.001$). However, no significant differences were achieved between bilharzial and non-bilharzial associated urothelial carcinoma ($p>0.1$) (Graphs 1 & 2).



Graph 1 Intensity of Cyclin D1 expression (Mean± S.D.) in urothelial carcinoma in relation to papillary pattern, muscle invasion, tumor grade and bilharzial association

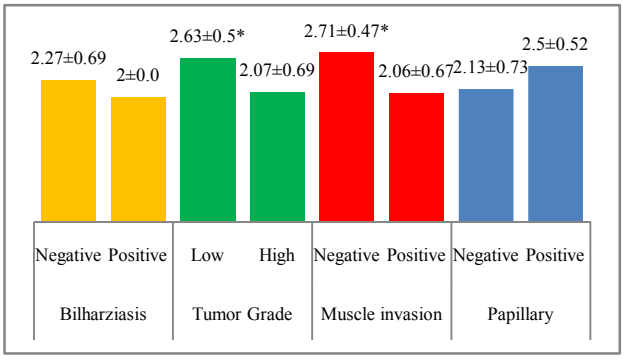
*: Significant difference with the corresponding group (p<0.01)
**: (High significant difference with the corresponding group (p<0.001)



Graph 2 Percentage of Cyclin D1 expression (Mean± S.D.) in urothelial carcinoma in relation to papillary pattern, muscle invasion, tumor grade and bilharzial association

*: Significant difference with the corresponding group (p<0.01)
**: (High significant difference with the corresponding group (p<0.001)

The mean score of cyclin D1 expression was significantly higher in low grade urothelial carcinoma compared to high grade one (p<0.01), and it was also significantly lower in invasive UC compared to non-invasive UC. On the other hand non-papillary UC and bilharzial associated UC showed lower score of cyclin D1 expression compared to papillary and bilharzial non-associated UC, however, the differences were statistically non-significant (p>0.05 and p>0.1 respectively). (Graph 3).



Graph 3 Difference in cyclin D1 expression score between groups of urothelial carcinoma (UC)

*: Significant difference with the corresponding group (p<0.01)

Spearman's rho test showed that all parameters of cyclin D1 expression were correlated inversely with high statistical significance to both the grade and stage of urothelial carcinoma (p<0.001).

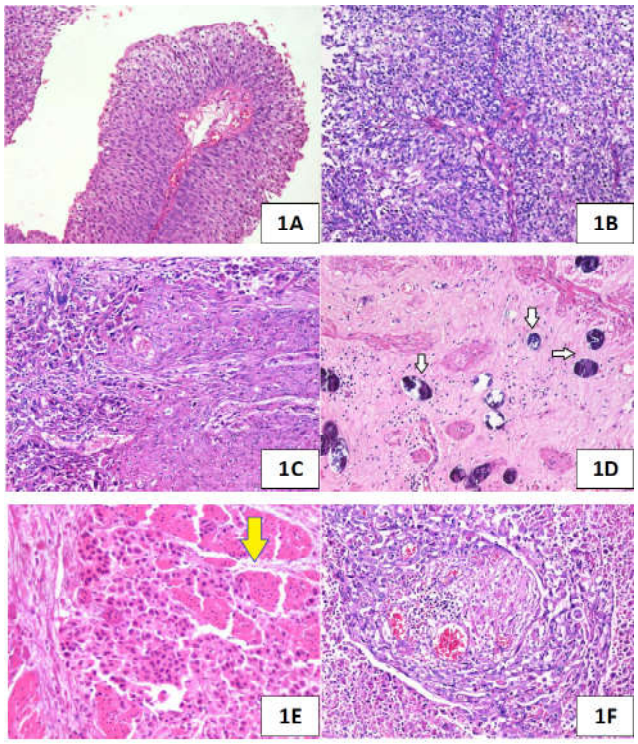


Fig (1A) Section in low grade papillary urothelial carcinoma (H&E stain, X200) **Fig (1B):** Section in high grade papillary urothelial carcinoma invading the lamina propria (T1) (H&E stain, X200). **Fig (1C):** Section in moderately differentiated invasive squamous cell carcinoma (H&E stain, X200). **Fig (1D):** Multiple calcified bilharzia ova (arrows), frequently associated with squamous cell carcinoma of the urinary bladder in Egyptian patients (H&E stain, X200). **Fig (1E):** Section in case of urothelial carcinoma invading the muscle propria (arrow) (T2) (H&E stain, X400). **Fig (1F):** Section in case of poorly differentiated urothelial carcinoma invading the muscle propria (arrow) (T2) (H&E stain, X400)

DISCUSSION

In the present study, we examined 62 urinary bladder biopsy specimens from Egyptian patients suffering of urinary symptoms and were cystoscopically examined and biopsed for histopathological diagnosis. The cases understudy consist of chronic cystitis (4cases), squamous cell carcinoma (12 cases) and urothelial carcinoma (46 cases). All cases of squamous cell carcinoma showed high tumor grade and positive muscle invasion, while most cases of urothelial carcinoma were of low grade malignancy (65.2%) and showed negative muscle invasion (69,6%). The difference was statistically significant (p<0.001). Half of the chronic cystitis cases and the squamous cell carcinoma cases were associated with bilharziasis, while only 4.3% of the urothelial carcinoma cases were positive for bilharziasis. The difference between groups was statistically significant (p<0.001).

In Egypt, bladder cancer accounts for about 30% of all cancers, where it is the most common malignancy in men and the second most common malignancy in women after breast cancer, and has been associated with many pathogenetic factors - most commonly bilharzial infestation, which is an endemic infection in the Nile River Valley [20, 21].

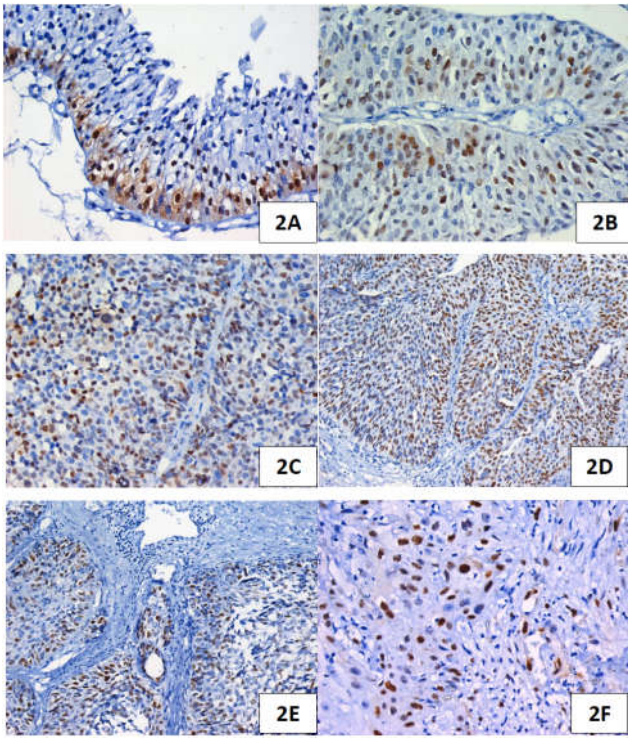


Fig (2A): Full-thickness urothelium in case of chronic cystitis showing positive nuclear expression of cyclin D1 in the basal layer. (IHC stain for cyclin D1, X400). **Fig (2B):** Low grade papillary urothelial carcinoma showing positive nuclear expression of cyclin D1 mostly in the basal layer. (IHC stain for cyclin D1, X400). **Fig (2C):** Moderately differentiated papillary urothelial carcinoma showing moderate positive nuclear expression of cyclin D1 dispersed within all layers. (IHC stain for cyclin D1, X400). **Fig (2D):** Low grade papillary urothelial carcinoma invading the lamina propria, showing high nuclear expression of cyclin D1. (IHC stain for cyclin D1, X200). **Fig (2E):** Non papillary urothelial carcinoma invading the lamina propria, showing moderate nuclear expression of cyclin D1. (IHC stain for cyclin D1, X200). **Fig (2F):** High grade non-papillary urothelial carcinoma invading the muscle propria, showing moderate nuclear expression of cyclin D1. (IHC stain for cyclin D1, 400)

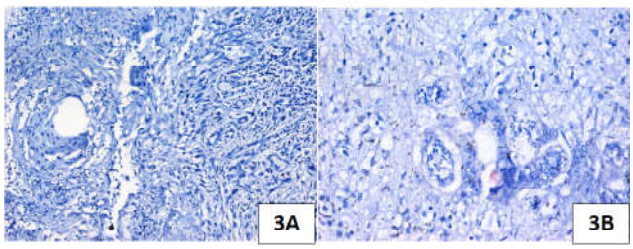


Fig (3A): Section in a case of squamous cell carcinoma invading the lamina propria, showing negative expression of cyclin D1. (IHC stain for cyclin D1, X200). **Fig (3B):** Many recently deposited bilharzia ova in a case of invasive squamous cell carcinoma showing negative expression of cyclin D1 within the surrounding tumor cells. (IHC stain for cyclin D1, X400)

Over expression of the cyclin D1gene has been reported in many human tumors and preneoplastic lesions including bladder transitional cell carcinoma (TCC) [22, 23]. In several animal model systems, deregulated expression of cyclinD1 has been shown to contribute to tumorigenesis [24, 25]. Ohtsuba *et al*, 1993 and Quelle *et al*, 1993[26, 27], found that alteration in cyclin D1 expression is an early event in bladder tumorigenesis. Our study shows significant difference between groups considering the intensity and percentage of cellular expression of cyclin D1. Urothelial carcinoma showed higher score of cyclin D1 expression compared to both the chronic cystitis and the squamous cell carcinoma groups, with statistically significant difference. Cases of chronic cystitis and squamous cell carcinoma showed negative and lower scores of cyclin D1 expression compared to cases of urothelial carcinoma that mostly showed high scores of cyclin D1 expression. The difference between groups was statistically significant. Previous results showed that the overall expression of cyclin-D in malignant cases (TCC & SCC) was 51.1% which was significantly higher than its expression in bilharzial cystitis (20%) and it was not expressed in control cases.

We found that urothelial carcinoma showed higher score of cyclin D1 expression compared to both the chronic cystitis and the squamous cell carcinoma groups, with statistically significant difference. Overexpression of cyclin D1 is a prognostic factor for good PFS in bladder cancer patients. The clinical implication of this finding is to help us to identify the subjects at high risk of progression after surgery. Patients with lower expression of cyclin D1 may be treated more carefully and followed closely [28].

Loss of cyclin D1 expression was associated with an increased probability of disease recurrence and bladder cancer-specific mortality in univariate analyses, however, this association was not significant when tested in a multivariate analysis that adjusted for the effects of standard pathologic features. These findings are consistent with previous reports showing that alteration in cyclin D1 is an early event in bladder tumorigenesis, but it does not add any prognostic significance [29].

On the other hand, Zhao *et al*.(2012)[30] reported that cyclin D1 expression level detected by IHC is associated with worst clinicopathological features and prognosis for esophageal squamous cell carcinoma. Rainsbury's study indicated that nuclear cyclin D1 may be a prognostic biomarker of survival in oropharyngeal squamous cell carcinoma. However, in the field of bladder cancer, we achieved different results; high grades of urothelial carcinoma and tumor associated with muscle invasion as well as non-papillary pattern showed significantly lower intensity and percentage of cyclin D1 expression compared to their counterparts. Also, no significant differences were achieved between bilharzial and non-bilharzial associated urothelial carcinoma. This was in agreement with others who found that Cyclin-D1 expression in low-grade and superficial tumors was higher than its expression in high-grade and invasive tumors, these results are in agreement with the results of [31], who found that over expression of cyclin D1 was associated with less aggressive disease and better survival in univariate analysis Cyclin-D and with the results of Hammam *et al*. (2009) who reported that cyclin D1 expression in non-bilharzial and bilharzial associated cancer was almost similar [32].

Our results showed also that all parameters of cyclin D1 expression were correlated inversely with high statistical significance to both the grade and stage of urothelial carcinoma.

In conclusion, Cyclin D1 expression is not just a research tool. In bladder cancers, cyclin D1 expression may provide prognostic information important in the management of patients with these diseases. Our results showed it is generally over-expressed in urothelial carcinoma compared to non-

malignant lesions and to squamous cell carcinoma. It's down regulation was associated with aggressive bladder cancers. In the future, the cyclin D1 gene and/or protein may be a site for immunotherapy. Before such treatments are possible, the full significance of this molecule in different variants of bladder cancer needs to be examined. In the words of Winston Churchill: "It is a mistake to look too far ahead. Only one link in the chain of destiny can be handled at a time."

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