

Immunohistochemical Study of Caspase 3 and Cyclin D1 In Bilharzial Bladder Cancer and Their Significance

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Abstract: Tumor growth is the result of cell excessive proliferation and lack of apoptosis. Apoptosis has been considered to be closely associated with Caspase-3. Cell cycle proteins are important markers in predicting tumor behavior in urothelial carcinoma of the bladder over expression of the D1 cyclins allow uncontrolled tumour cell proliferation. This study was designed to investigate the expression of Caspase-3 and cyclin D1 to evaluate their role on the tumorigenesis and progression in bladder cancer (associated with bilharziasis or not associated).The immunohistochemistry (SP method) was used to determine the expression of caspase-3 and cyclin D1 in 45 cases of Bladder cancer (30 TCC and 15 SCC), 10 control and 10 bilharzial cystitis. Caspase 3 was present as faint intracytoplasmic brownish staining of the urothelial cells in 90% of control cases, 80% in benign conditions and 48% of bladder cancer cases. The protein expression level of Caspase-3 was 48% of Bladder cancer cases (24/45) which was significantly lower than that in normal bladder samples ($P < 0.001$). Caspase-3 expression level was decreases in SCC compared to TCC cases $P < 0.05$, also caspase 3 showed decrease expression in bilharzial compared to non bilharzial cancer cases $P < 0.01$. Caspase-3 expression level was correlated with the grades of TCC ($P < 0.01$), and with clinical stages ($P < 0.05$). The overall expression of cyclin-D in malignant conditions was 50.6%. Cyclin-D was not expressed in control cases and was expressed in only 10% in bilharzial cystitis. Expression of cyclin-D was more significant in low-grade and superficial tumors than in high-grade and invasive tumors. In conclusion, Caspase 3 and cyclin-D expression in bladder tissue may be of help in detection of bladder cancer and can be used more effectively in combination with other tumor markers.

Key words:

INTRODUCTION

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 (El-Mawla *et al.*, 2001 & El-Sebaie *et al.*, 2005). Most investigators have accepted the association between schistosomiasis and bladder cancer since the work of Ferguson in 1911 (Ferguson, 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 (Greenlee *et al.*, 2001).

The development of bladder cancer in a younger age group affects males more, is usually associated with schistosomal infection, and shows a high mortality rate and clinicopathologic features of schistosomal-associated bladder cancer (SABC) (El-Bolkainy *et al.*, 1981, Mostafa *et al.*, 1999, & Zimmermann *et al.*, 1999). 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 (El-Bolkainy *et al.*, 1981 & Zimmermann *et al.*, 1999).

Apoptosis, which is the most common form of cell death, is gene directed, coordinated self-destruction under the influence of a number of well characterized genes and proteins. Failures in normal apoptosis pathways contribute to carcinogenesis by creating a permissive environment for genetic instability and accumulation of gene mutations, promoting resistance to immune-based destruction, facilitating growth factor/hormone-independent cell survival, supporting anchorage-independent survival during metastasis, reducing dependence on oxygen and nutrients, and conferring resistance to cytotoxic anticancer drugs and radiation. (American Society of Clinical Oncology,1999).

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Caspase activation has been documented in several types of tumor cells when they have been successfully induced to undergo apoptosis by various chemotherapeutic drugs (Zapata *et al*, 1998 & Yamamoto *et al*, 1998). Caspases are a group of proteolytic effector molecules that induce morphological changes seen in apoptosis. More than a dozen caspases, of which most are homologous in structure, have been characterized in humans (Hengartner, 1998). The group consists of initiator upstream caspases, e.g. caspase-8 and caspase-9, which may be activated by internal stress signals involving cytochrome c or from interaction with cell surface death receptors via their large pro-domains. These pro-domains contain 2 distinct interaction domains. Procaspase-8 contains DEDs that interact with adaptor proteins such as FADD. In contrast, procaspase-9 contains caspase recruitment domains, which interact with other Caspases and adaptor proteins such as APAF-1 to amplify the signal and interact with effector Caspases. DED and caspase recruitment domain structures allow the exposure of large protein interaction domains for binding to the other proteins involved. The recruitment of adapter proteins and downstream Caspases result in close proximity autocatalyzation of the downstream signal.

Downstream or effectors caspases, eg caspase-3 and caspase-7, which lack the protein interaction motifs of upstream Caspases, are responsible for the specific proteolytic activity that results in cell disassembly (Nicholson, 1999). In addition to their direct proteolytic activity, effector Caspases can also cleave and activate initiator Caspases, causing amplification of the original signal (Slee *et al*, 1999).

Cellular proliferation was involved in all carcinogenesis processes including initiation, progression, and metastasis (Brandau & Bohle, 2001). Cellular proliferation is strictly regulated by complexes of proteins formed by a cyclin and a cyclin dependent kinase (cdk), that induce the cell to move from one phase to the next one; in particular, the G1/S phase transition is activated upon the binding of the cyclin D1 to its corresponding kinase (cdk4) (Harrington *et al*. 1998; Donnellan and Chetty 1998, Sherr and Roberts 1999). It has been suggested that modifications in the expression of cell cycle regulatory proteins are among the most promising markers in determining the outcome of cancer disease (Galmozzi *et al*, 2006).

Cyclins D and E are responsible for the initial and terminal phases of G1 respectively (Ohtsubo & Roberts, 1993, Donnellan & Chetty, 1999). Normally, when quiescent cells enter the cell cycle, the cyclin D/Cdk4/6 complex initiates phosphorylation of pRB, which is then further phosphorylated by cyclin E/Cdk2. Phosphorylation of pRB initiates the steps required for the cell to enter S phase. A future function of the cyclin D/Cdk4 complex is the sequestration of the Clip/Kip inhibitors, p27Kip1 and p21Cip1, which normally inhibit the activity of cyclin E/Cdk2. cyclin D1 and E1 expression is altered in various cancers, suggesting that their deregulation contributes to tumorigenesis (Spruk *et al*, 1999 & Schraml *et al*, 2003).

Our aim, was to study the expression of both caspase 3 and cyclin D1 in bladder cancer associated with bilharziasis or not associated, and to find their role in tumorogenesis and correlate them with the clinico-pathological parameters (grade and stage).

MATERIAL AND METHODS

Patients:

Demographic data on this study is summarized as follows: 65 cases (51 men and 14 women) between ages 25 and 75 years. Normal tissues were also obtained from 10 cancer free patients and served as control. Samples were obtained from 10 patients with benign bilharzial lesions (cystitis) and 45 bladder cancer cases (30 TCC and 15 SCC).

Patients were subjected to detailed history taking, full and complete clinical examination, urine analysis and routine laboratory investigations, urine cytology abdominal-pelvic ultrasonography, excretory urography, 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 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. Tumor specimens were taken by cystoscopy (Transurethral resection biopsies, TUR) and from cystectomy specimens. Informed consent from all patients and control were taken. The study protocol was approved by the Ethics Committee of TBRI according to the Institutional Committee for the Protection of Human Subjects and adopted by the 18th World Medical Assembly, Helsinki, Finland. Diagnosis of schistosomal 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 slides, which were treated with TESPA (3-aminopropyl-triethoxysilane, Sigma) for immunohistochemistry (IHC).

IHC Procedures:

For IHC, a standard 3-layer protocol was used, as previously described. Unstained sections were processed for immunostaining with caspase 3 and cyclin D1 monoclonal antibodies as follows: Positive control sections were added to be processed with the bladder tissue sections in the same run for precision and standardization of the elaborated IHC result. 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 sections were incubated in 5% skim milk for 30 minutes at room temperature. 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 with Caspase 3 and cyclin D1 primary antibodies and were used at a dilution of 1:100, 1:50 and incubated for 24 hours in a 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. Colonic mucosa known to express Caspase 3 and cyclin D1 and was used as a positive control.

The expression of caspase 3 and cyclin D1 was measured in 10 successive high-power fields (x400) according to Shen *et al.*, 2004 and Tut *et al.*, 2001 respectively. Caspase 3 showed mostly cytoplasmic expression, while cyclin D1 expressed as nuclear and cytoplasmic brown color. Pathologist analyzed the intensity, distribution, and pattern, and evaluated caspase 3 and cyclin D1 immunoeexpressions independently. The percentage of positively stained cells was determined semiquantitatively by assessing the whole tumor section. (Vallmanya Llena *et al.*, 2006).

Statistical Analysis:

The statistical analysis of the results was done with analysis of variance (ANOVA) to compare caspase 3 and cyclin D1 scores. Results were given as mean \pm SD. Distribution of negative and positive cases was studied with cross tables (Z-test). To investigate a possible correlation of caspase 3 and cyclin D1 scores with tumor grade and stage, the Spearman rank correlation coefficient was used (SPSS software program, version 9). In all tests, $P < .05$ was considered to indicate significant.

RESULTS AND DISCUSSION

Results:

This study included 55 patients and 10 control cases. 10 chronic cystitis patients, 30 TCC patients (12 with bilharziasis and 18 not associated with bilharziasis), 15 SCC patients all associated with bilharziasis. The 30 cases of TCC were stratified according to histopathological grades into low-grade (n=12), high-grade (n=18) and stratified according to histopathological stage into superficial tumors (n=10) and invasive tumors (n=20). The overall expression of caspase 3 in malignant cases (TCC & SCC) was 48% which is much less than its expression in control cases (90%) and benign cases (cystitis) 80%. Caspase 3 expression was higher in TCC cases 60% than in SCC cases 40% (Table 1). Caspase 3 expression in bilharzial-associated cancer was 50% in 12 TCC cases (6/12) and 40% in all SCC cases (6/15), which was less than caspase 3 expression in non-bilharzial-associated cancer which was 66.6% (18 TCC cases, 12/18) (Table 1). Caspase 3 expression in low-grade TCC cases was 75% (9/12) and in superficial TCC cases was 80% (8/10) which was higher than its expression in high-grade and invasive TCC which was 50% (9/18 and 10/20 respectively) (Table 2, Figures 1A-D).

The overall expression of cyclin-D in malignant cases (30 TCC cases and 15 SCC) was 51.1% (23/45) while it was 20% in 10 bilharzial cystitis (2/10) and was not expressed in the control cases (Table 3). Cyclin-D expression in bilharzial-associated and non-bilharzial-associated cancer was almost similar; where its expression in bilharzial-associated cancer was 50% in 12 TCC cases (6/12), 53.3% in 15 SCC cases (8/15) and was 50% in 18 cases of non-bilharzial-associated TCC (9/18) (Table 3). Cyclin-D expression in 12 low-grade TCC cases was 66.6% (8/12) and in 10 superficial TCC cases was 60% (6/10), which was higher than its expression in 18 high-grade TCC which was 38.8% (7/18) and it was 45% in 20 invasive TCC (9/20) (Table 4, Figures 2A-D).

Table 1: Expression of caspase 3 in the studied cases.

Groups	Number of positive cases		Caspase 3		
	No	%	+	++	% of positive cells
Normal control (n=10).	9	90	5	4	90
Cystitis (n=10).	8	80	4	4	80
TCC (n=30).	18	60	10	8	51.5±10.2 ^{‡,§}
Bilharzial associated TCC (n=12).	6	50	3	3	33.3±5.3 ^{‡,§§,##}
Non Bilharzial associated TCC (n=18)	12	66.6	7	5	66.6±12.4 ^{*,§}
SCC (n=15).	6	40	1	5	46.6±6.4 ^{‡,§,##}

*Significant difference in comparison to control at p<0.05.

‡Highly significant difference in comparison to control at p<0.001.

§ Significant difference in comparison to cystitis at p<0.05.

§§ Highly significant difference in comparison to cystitis at p<0.01.

Significant difference in comparison to Non bilharzial TCC at p<0.05.

Highly significant difference in comparison to Non bilharzial TCC at p<0.01.

Table 2: Caspase 3 expression in tissue and distribution of positive cases in different histopathological grades and stages of urothelial carcinoma cases.

Parameters	Number of cases		Caspase 3		
	No	%	+	++	% of positive cells
Histopathological Grade (n=30)					
Low grade urothelial carcinoma (n=12)	9	75	4	5	83.3±16.3*
High Grade urothelial carcinoma (n=18)	9	50	6	3	55.5± 4.5
Histopathological Stage					
Superficial tumor (n=10)	8	80	4	4	70.3±11.5 [‡]
Invasive tumor (n=20)	10	50	6	4	40.2±1.8

*Significant difference in comparison to high-grade tumor at p<0.05.

‡Highly significant difference in comparison to invasive tumor at p<0.001

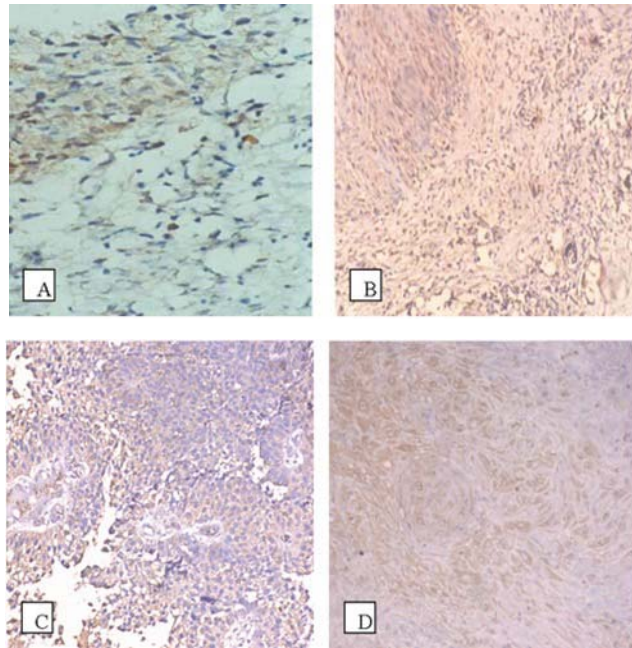


Fig. 1: A) Control case, showing faint expression of caspase 3 monoclonal antibody (IHC, DAB, x100). B) Mild cytoplasmic expression of caspase 3 in a case of chronic cystitis (IHC, DAB, x100).C) Strong cytoplasmic expression of caspase 3 in a grade 1, superficial tumor (IHC, DAB, x200). D) Moderate cytoplasmic expression of caspase 3 in SCC (IHC, DAB, x400).

Table 3: Expression of Cyclin D1 in the studied cases

Groups	Number of positive cases		Cyclin-D1		
	No	%	+	++	% of positive cells
Normal control (n=10).	0	0	0	0	0
Cystitis (n=10).	2	20	0	0	15.6±2.2
TCC (n=30).	15	50	5	10	60.5±8.2 ^{‡, \$\$}
Bilharzial associated TCC (n=12).	6	50	0	6	80.6±10.5 ^{‡, \$\$}
Non Bilharzial associated TCC (n=18)	9	50	5	4	50±7.3 ^{‡, \$, ##}
SCC (n=15).	8	53.3	3	5	66.6±9.4 ^{‡, \$\$, #}

‡Highly significant difference in comparison to control at p<0.001.

\$ Significant difference in comparison to cystitis at p<0.05.

\$\$ Highly significant difference in comparison to cystitis at p<0.01.

Significant difference in comparison to Non bilharzial TCC at p<0.05.

Highly significant difference in comparison to Non bilharzial TCC at p<0.01

Table 4: Cyclin D1 expression in tissue and distribution of positive cases in different histopathological grades and stages of urothelial carcinoma cases.

Parameters	Number of cases		Cyclin-D		
	No	%	+	++	% of positive cells
Histopathological Grade (n=30)					
Low grade urothelial carcinoma (n=12)	8	66.6	2	6	55.3± 2.1*
High Grade urothelial carcinoma (n=18)	7	38.8	3	4	30.3±7.3
Histopathological Stage					
Superficial tumor (n=10)	6	60	1	5	76.9±4.8 [‡]
Invasive tumor (n=20)	9	45	4	5	40.7±3.6

*Significant difference in comparison to high-grade tumor at p<0.05.

‡Highly significant difference in comparison to invasive tumor at p<0.001

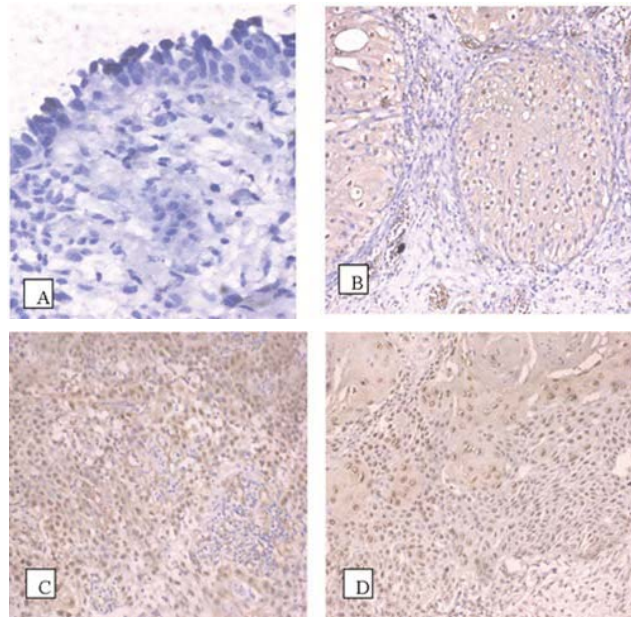


Fig. 2: A) Control case, negative for cyclin D1 monoclonal antibody (IHC, DAB, x400). B) Mild nuclear and cytoplasmic expression of cyclin D1 in burn's nest from a case of chronic cystitis(IHC, DAB, x200).C) Strong nuclear and cytoplasmic expression of cyclin D1 in a grade 2, superficial tumor (IHC, DAB,x200). D) Moderate nuclear expression and cytoplasmic of cyclin D1 in SCC(IHC, DAB, x200).

Discussion:

Dysregulation of cell cycle control may lead to genomic instability, neoplastic transformation and tumor progression (Eissa *et al.*, 2004). Caspase-3 is a downstream apoptosis effector molecule that causes cellular disassembly (Arai *et al.*, 2005). Kaufmann, 2000 and Varghese, 2003 found in their studies that loss of caspase 3 expression was associated with a high pathological grade, advanced pathological stage and lymph node

metastasis. Furthermore, they found that this marker was an independent predictor of survival after radical cystectomy. Activated caspase 3 constitutes an important downstream step in both intrinsic and extrinsic apoptotic pathways (Kaufmann & Earnshaw, 2000 and Varghese *et al*, 2003).

In the current study, we found that caspase 3 expressions in malignant cases (TCC and SCC) was 48% which was significantly less than its expression in control cases (90%) and benign cases (cystitis) (80%). The bilharzial associated cancer cases, 12 TCC cases and all SCC cases showed a 50% and 40% caspase expression respectively which was less than its expression in non-bilharzial-associated cancer (18 TCC cases) which was 66.6%. The bilharzia associated TCC and SCC showed more decrease in the expression of caspase 3 compared to non-bilharzial ($P < 0.01$). Also caspase 3 expression is significantly higher in low-grade and superficial tumors compared to high-grade and invasive tumors, our findings are in agreement with the results of Karam *et al*, 2007, Verghese 2003 and Kaufmann, 2000 and do not correlate with Burton *et al*, 2000.

Burton *et al*, 2000, evaluated caspase-3 expression in 34 patients with carcinoma in situ, of which 41% developed invasive bladder cancer. They reported that activated caspase-3 overexpression was associated with higher rates of disease invasiveness. Conversely, a more recent study by Karam *et al*, 2007 involving 226 consecutive patients treated with radical cystectomy specimens reported that 49% of the patients had loss of caspase-3 expression, which was associated with higher pathologic grade and stage, and presence of lymph node metastasis. Moreover, loss of caspase-3 was an independent predictor of bladder cancer-specific survival after radical cystectomy (Karam *et al.*, 2007). Giannopoulou *et al.*, 2002 studied caspase-3 expression in 53 patients with bladder cancer and did not find a correlation between caspase-3 expression and tumor grade or stage.

The deregulation of G1/S transition with the disappearance of restriction point is the hallmark of cancer, leading to continuous, uninhibited cell proliferation. Cyclins D and E are responsible for the initial and terminal phases of G1, respectively. In agreement with previous studies (Del Pizzo *et al.*, 1999, Richter *et al.*, 2000). Overexpression of cyclin D1 may be the result of one or more of four following reasons: chromosomal rearrangement, chromosomal translocation, activation by retroviral insertion, and DNA amplification. The direct relation between cyclinD1 overexpression and chromosome 11q13 amplification in human bladder tumor has been reported (Bringuier *et al*, 1996). Overexpression of the cyclin D1 gene has been reported in many human tumors and preneoplastic lesions including bladder TCC (Arber *et al*, 1996 & Oya *et al*, 1998). In several animal model systems, deregulated expression of cyclinD1 has been shown to contribute to tumorigenesis (Bodrug *et al*, 1994 & Lovec *et al*, 1994). Ohtsuba *et al*, 1993 and Quelle *et al*, 1993, found that alteration in cyclin D1 expression is an early event in bladder tumorigenesis. Tut *et al*, 2001 reported that this protein was independent prognostic factor for survival in patients with MITCC of the bladder and found that a cyclin D1 index $< 8\%$ was associated with significantly better survival (Tut *et al*, 2001).

Lumbiao *et al*, 2006 showed that the levels of cyclin D1 expression in both nodular hyperplasia (PN) hyperplasia and neoplasms were significantly higher than that of control epithelia. Moreover, the degree of overexpression of cyclin D1 was higher in the older PN hyperplasia, papilloma, and carcinoma (Lumbiao *et al*, 2006).

In our study, 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. Cyclin-D expression in non-bilharzial and bilharzial associated cancer was almost similar which around 50% was. Cyclin-D 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 Niehans *et al.*, 1999, who found that over expression of cyclin D1 was associated with less aggressive disease and better survival in univariate analysis. While Shariat *et al.*, 2009, reported that cyclin D1 immunoreactivity was elevated in bladder cancer patients compared to controls but within bladder cancer patients it was not associated with clinical or pathologic characteristics. While loss of cyclin D1 expression was associated with an increased probability of disease recurrence and bladder cancer-specific mortality in univariate analyses, 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 (Shariat *et al.*, 2009).

Lopez-Beltran *et al*. 2006, reported that cyclin D3 overexpression was associated with larger tumor size, tumor grade, and increased risk of disease progression (together with cyclin D1) in patients with Ta/T1 bladder cancer (Lopez-Beltran *et al*. 2006).

Conclusion:

Caspase 3 and cyclin-D expression in bladder tissue may be of help in detection of bladder cancer and can be used more effectively in combination with other tumor markers.

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