Expression of p16, p15 and Cyclin D1 in bladder cancer and correlates with cancer progression and clinical out come

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Citation

Abstract

The biological behavior of urinary bladder neoplasms cannot be adequately predicted by histological criteria alone. Dysregulation of cell cycle control may lead to genomic instability, neoplastic transformation and tumor progression. The present study evaluated the prognostic significance of p16 and p15 gene deletion and cyclin D1 expression in bladder carcinoma among Egyptian patients, in relation to different clinicopathological features of the tumors and the present or absence of schistosomiasis. Tissue specimens from 86 patients with bladder carcinoma undergoing surgery were prospectively included and evaluated. Tumors samples were collected after surgery and stored at –800 C until assayed. The benign group consisted of 30 patients with benign schistosomiasis bladder tissues and the control samples group was collected from safety margin from the same cancer patients. P16 and p15 gene deletions were examined by polymerase chain reaction (PCR) and Cyclin D1 was detected by Westen blotting technique (WB). The data on primary tumor stage, age, grade and lymph node status were reviewed and recorded. Clinical follow-up information was available in only 56 patients. P16 was deleted in (55.8%) bladder patients, (16.7%) benign samples and (13.3%) control samples, while p15 was deleted in (50%) bladder patients, (16.7%) benign samples and (0.0%) control samples. The deletion of both genes was associated with superficial low grade and schistosomiasis. Cyclin D1 was expressed in (54%) of bladder tumor was significantly higher (p=0.001) than that in the control group (6.7%) and in the benign group (0.0%), where its expression was correlated to early stage, low grade, schistosomiasis and deletions of both p15 and p16 gene. Deletion of Cyclin D1 and p16 were linked to the risk of recurrence higher than high grade and stage. Cell cycle is dysregulated in bladder carcinoma and responsible for initiation and recurrence. This was evident from the increased expression of cyclin D1, the deletion of p15 and p16 genes. Moreover, p16 and p15 gene deletion and cyclin D1 expression appears to be an early event in bladder cancer and might explain bilharzial associated bladder carcinogenesis, and tumor without p16, p15 was recurrent more than the tumors with these genes.

ABBREVIATIONS

BC, bladder cancer; SCC, squamous cell carcinoma; TCC, transitional cell carcinoma; SABC, schistosomal associated bladder cancer; NSABC, non schistosomal associated bladder cancer.

INTRODUCTION

Urinary bladder cancer is a multifactorial and complex disease involving both environmental and genetic factors. Most bladder cancers are transitional cell carcinomas (TCC). The risk factors of bladder cancer include cigarette smoking, chemical exposure, and viral infection in addition to Schistosomiasis. Schistosomiasis (bilharziasis) is an infections parasitic disease, multiple factors are involved in the process of carcinogenesis in bilharzial bladder, including irritation of the urothelium by Schistosomal eggs, and a spontaneous chromosomal error in regenerative cells could then become initiating carcinogenic focus. Also, urine retention may result from incomplete bladder evacuation due to bladder neck abstraction caused by bilharzial cystitis (Aly and Khaled, 1999). Therefore, there is a need for additional objective information on the aggressiveness of these tumors. After cystectomy about 30% will recurrences. A better prediction of the individual prognosis at the time of the initial biopsy would greatly facilitate therapeutic decision-making in these patients. The study of the cell cycle regulation elements represents the most reliable and reproducible method that has proved validity in the assessment of the bladder tumors and the current concepts in the molecular biology of cancer emphasize a combination of genetic events, in particular, oncogene activation and tumor suppressor gene inactivation as a critical element in the

emergence of the neoplastic phenotype (Mhawech et al., 2004).

Cyclin D1 forms a holoenzyme with a cyclin-dependent kinase (CDK), either CDK4 or CDK6 that phosphorylates the retinoblastoma gene product pRb. The phosphorylation of pRb results in the release of E2F transcription factors, freeing them to stimulate transcription of growth-promoting target genes thus, the overexpression of cyclin D1 promotes progression through the G1 phase of the cell cycle in cells grown on substratum (Sherr and Roberts ,1999) . This overexpression is associated with a variety of neoplasms, including bladder cancer (Stacey, 2003). Inhibition of pRb phosphorylation, therefore, represents a potent form of growth inhibition. Such inhibition has been exemplified through the characterization of cyclin-dependent kinase inhibitor proteins. To dates, these proteins exist as two functionally and structurally distinct groups typified by p21 and its homologues p27 and p57, as well as p16 and p15 and their related homologues. As potential tumor suppressors, the cyclin-dependent kinase inhibitor genes have been studied extensively to evaluate the possible contribution of cyclin-dependent kinase inhibitor-specific genomic mutations to neoplastic transformation. In particular, the gene coding p16 and p15, on Chromosome 9p21 have been postulated to encode a tumor suppressor and demonstrated to deleted and methylated in a wide variety of tumors including bladder cancer (Chatterjee et al., 2004; Hopman et al., 2002). Therefore, unregulated phosphorylation of pRb by CDKs due to the lack of CDKIs inhibiting activity of proteins like p16 and p15 or in response to over expressed cyclin D1, could lead to loss growth control and might indeed be one of the earliest, clonally selected genetic changes during malignancies and recurrence (Peschos et al., 2004). The aim of the present work is to identify the alterations in the balance between p16/p15 genes and cyclin D1 protein in normal, benign, and bladder tumors tissues therefore to understand the impact of p16/p15 genes and cyclin D1 protein in tumor formation and recurrence, particularly in schistosomal bladder cancer being an endemic possible risky factor in Egypt.

PATIENTS, MATERIALS AND METHODS

PATIENTS

The tumor tissues were obtained from transurethral resections or radical cystectomy samples from 86 patients suffering from primary bladder cancer. Demographic data on this cohort may be summarized as follows: 72 patients were males and 14 were females, and the median patient age was 63 years. Tumor staging was classified according to TNM system (Doller and Gospodarowics, 1996). Tumor grading was classified according to World Health Organization System. Normal tissues were also obtained from 30 patients from a tumor free area of the same bladder specimen, and served as control. Thirty samples were obtained from patients with benign schistosomiasis lesions. All specimens were stored at –800 C. Representative hematoxylin-eosin stained section of each frozen block were examined microscopically to confirm the presence of tumor, and only lesions with more than 50% neoplastic cells were included in the study. Only 56 patients were followed up by cytoscopy every 3 months in the first year, every 4 months in the second and third year and annually thereafter if there was no recurrence. Recurrence was defined as the occurrence of a positive cytoscopy with a biopsy-proven lesion. There were 14 additional patients died of causes unrelated to bladder cancer post operatively or during the course of the study. Three patients died with or due to bladder cancer. Finally, 39 patients were alive and being followed at the time that the study concluded for 30 months, containing 26 patients with recurrence.

DETECTION OF P16 AND P15 GENE DELETION BY POLYMERASE CHAIN REACTION (PCR

Tissues of our study were digested with proteinase K. and high molecular weight genomic DNA was extracted by using DNA isolation Kit (provided by PURGene kit, Minneapolis-USA), and subjected to PCR using 50 ng each of genomic DNA extracted from the bladder tissue and from human placental DNA included as positive control. The primers listen in Table 1 were used to amplify a 430 bp product from exon 2 of p15 gene and 167 bp product from an intron-exon boundary of p16 gene. The cycling conditions for PCR amplification are also shown in Table 1

PCR amplification for p15 the reaction mix consisted of 67 mmol/l Tris pH 8.8, 16.6 mmol/l ammonium sulphate, 6 mmol/l magnesium chloride, 10 mmol/l B-mercaptoethanol, 6% (v/v) dimethylsuphoxide (DMSO), 1.25 mmol/l dNTPs, 1 μM of each oligonucleotide primers, 5 units of thermo stable Taq polymerase (Promega, USA).

PCR amplification for p16 the reaction mix consisted of 10 mM Tris pH 8.3, 50 mM potassium chloride, 0.2 mM dNTPs, 2 mM magnesium chloride, 0.15 μM of each oligonucleotide primers and 1 unit of thermo stable Taq polymerase (Promega, USA).

PCR products from individual experiments were run on 2% agarose gels and visualized with UV light after staining with ethidium bromide by using UV-Gel Documentation 2000 (BioRad Lab., Italy).

Figure 1

Table 1: Oligonucleotide sequences

Gene	Primer	Position	Sequence	Cycling conditions
P15	p15(2)F	Forward	5'-CCTTAAATGGCTCCACCTGC-3'	95° C for 2 min
	p15(2)R	Reverse	5'-CGTTGGCAGCCTTCATCG-3'	95° C, 30 s — 66° C, 1 min **30 cycles** — 70° C, 1 min — 70° C 5 min
P16	p16f	Forward	5'-GGAAATTGGAAACTGGAAGC-3'	95° C, 1 min — 62° C, 30 s **40 cycles** — 72° C, 30 s — 72° C, 10 min
	p16r	Reverse	5'-CTGCCCATCATCATGACCTG-3'	

DETECTION OF CYCLIN D1 PROTEIN BY WESTERN BLOTTING TECHNIQUE

Tissues of the studied groups were homogenized and lysed on ice in an extraction buffer: [HEPES 0.1 mol/L, Glycerol 10%, K2 EDTA 1 mol/L, Triton-X-100 10 ml/L, NaCl 0.5 mol/l] containing protease inhibitors, including (benzamidine 10 mmol/l, ?-mercaptoethanol 10 mmol/l, a protenin 5 mg/l, and PMSF 0.39 mmol/l) were freshly added to the lysing buffer before use (Eissa et al., 2000). Then the western blots were performed according to Sambrook et al. 1989 as follows. Lysates were spun down, and the supernatant fluid was quantified using Bradford method. Then, 40-60 μg of total protein per lane were ran on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for cyclin D1, followed by electrophoretic transfer to nitrocellulose membranes. Cyclin D1 was probed with anti-cyclin D1 monoclonal antibodies (Oncogene, Science, Boston, USA) at a dilution of 1:1000 at room temperature for 1 h, and horseradish peroxidase-conjugated antimous immunoglobulin G (IgG) antibody was used for visualization of positive bands at 35 kDs.

STATISTICAL ANALYSIS

Chi-square and fisher exact tests were used for comparison of different variables among various groups. Survival curves were calculated using the Kaplan-meier method, and the log-rank test was used for the analysis. Univariate and multivariate relative risks were calculated using Cox propartional hazards regression. All analysis was performed using the statistical package for the social sciences (SPSS, version 9).

RESULTS

The current study included 30 normal bladder tissue specimens, 30 benign bladder tissue specimens, and 86 bladder carcinoma tissue specimens. Forty-seven patients with bladder cancer were bilharziasis positive. Twenty five cancer patients had SCC while 61 had TCC. The clinicopathological features of the malignant group are shown in table 2. Bilharziasis was more associated (X2 = 9.1, p= 0.003) with SCC (20/25) than TCC (27/61).

Figure 2

Table 2: Clinicopathological data of bladder cancer group.

Parameter	Number	Percentage
Sex		
Males	72	83.7
Females	14	16.3
Stage		
Ta	10	11.6
T1	13	15.2
T2	15	17.4
T3(a-b)	48	55.8
Lymph node		
-ve	71	82.6
+ve	15	17.4
Grade		
1	7	8.1
2	51	59.3
3	28	32.6
Pathological type		
TCC	61	70.9
SCC	25	29.1
Schistosomiasis		
-ve	39	45.3
+ve	47	54.7

P16 AND P15 GENE DELETION

Using the specific P15 and P16 primers, the expected 430bp and 167bp sized single product were amplified by PCR from human placental DNA (included as positive control) and with DNA from 86 bladder carcinoma samples, 30 benign samples and 30 normal controls for both P15 and P16 genes as shown in figures (1,2). Deletion of p15 gene was not observed in normal control but observed in 16.7% of benign group and in 50% of malignant bladder group (p=0.001). On the other hand there was 55.8% of malignant cases with P16 gene deletion and this deletion was also observed at lower frequencies in both control and benign group (13.3% and 16.7%, respectively, (p=0.001) (Table 3).

A statistically significant (p=0.02) association was observed between p15 gene deletion and the tumor stage. While 78.3 %of superficial tumors showed p15 deletions, only 39.7% of invasive lesions were deleted, in addition, a highly significant (p=0.000) association was found between tumors grade and p15 deletions: 70.2% of low and intermediate

grade tumors had p15 deletions; such alterations were detected in only 11.5% of high grade lesions. Similarly, p16 deletions were statistically associated (p=0.011) with tumor stage, whereas 78.3% of superficial tumors showed these deletions. P16 deletions were slightly more common among low and intermediate grade tumors (61.4%) than high grade tumors (44.8%), but did not reach statistical significance (p=0.099). Thus, deletions of p15 and p16 genes were more frequent in lower stage and lower grade bladder lesions. A statistically significant (p=0.009) association was observed between p15 gene deletion and SCC type of bladder cancer, as 72% of SCC showed p15 gene deletion, but only 41% showed these deletion in TCC type. P16 deletion showed non-significant difference in frequency between both types as (60% in SCC and 54.1% in TCC) (p=0.62) as shown in Table 3.

P15 and p16gene deletions were significantly associated (p=0.001 and p=0.05 respectively) with schistosomiasis (SABC, p15: 76.7% and p16: 74.5%; NSABC, p15: 17.9 and p16: 33.3%), and were not significantly associated (p=0.127, 0.06) with smoking, Tables 3.

Figure 3

Table 3: Relationship between p15 and p16 gene deletion and clinicopathological features of bladder cancer.

Clinicopathological features	No of Samples P16+ve	P16-ve	No of Samples P15+ve	P15-ve
Malignant (86)	38	48	43	43
Benign (30)	25	5	25	5
Control (30)	26	4	30	0
	X^2= 24.8, p= 0.001**		X^2= 29.7, p= 0.001**	
SCC (25)	10	15	7	18
TCC (61)	28	33	36	25
	X^2 =0.25, p= 0.62		X^2=6.8, p= 0.009**	
Stage				
Super. (Ta-T1) (23)	5	18	5	18
Invasive(T2-T4) (63)	33	30	38	25
	X^2= 6.4 , p=0.011*		X^2 =10 , p=0.002**	
Lymph node				
+ve	12	3	13	2
-ve	26	45	30	41
	X^2= 9.4, p= 0.002**		X^2=9.7, p= 0.003**	
Grade				
Low (G1-G2)(57)	22	35	17	40
High (G3) (29)	16	13	26	3
	X^2= 4.6, p= 0.099		X^2= 27.3, p= 0.000**	
Schistosomiasis				
+ve (47)	12	35	11	36
-ve (39)	26	13	32	7
	X^2= 3.6, p= 0.05*		X^2= 13.3, p= 0.000**	

** highly significant difference between groups by chi-square test.
* significant difference between groups by chi-square test.

CYCLIN D1 PROTEIN EXPRESSION

Cyclin D1 protein was detected in bladder tumers by WB (fig 3). In bladder cancer speciments, 54.7% were cyclin D1 positive and 6.7% in the control group, while all the benign tissues were negative. Within the malignant group, cyclin D1 positivity was significantly associated with low stage and grade (Table 4). Cyclin D1positivity was seen in 95.7% of superficial bladder lesions and in 39.7% of invasive bladder lesions. In addition cyclin D1 positivity was shown in 66.7% of low grade and in 31%of high-grade tumors. All the 15 lymph node positive tumors did not show cyclin D1 (Table 4).

The frequency of cyclin D1 expression was higher in SABC (68.1%) than in NSABC (38.5%), but this association did not reach statistical significance (p=>0.05). In case of SCC the expression of this protein was shown in (60%) in NSABC while it was shown in (50%) in SABC but without statistical significance (p=>0.05). In TCC type of bladder lesions, this expression was significantly (p=0.000) associated with SABC (81.5% SABC vs. 35.3 % NSABC).

We examined 49 cases expressing cyclin D1 (including 47 malignant and 2 normal control) for P15/P16 gene deletions: 70.2% of the 47positive cyclin D1 malignant cases showed p15 deletion where {81.8% in low stage and 60% in high stage; 84.2% in low grade and11.1% in high grade tumors}. On the other hand, 36 of the same 47 positive cyclin D1 tumors showed p16 deletion where {77.2% in low stage and 72% in high stage; 76.3% in low grade and 66% in high grade while 50%in control group}. The deletion of both P15/P16 genes was observed in 25 of 47 positive cyclin D1 malignant tumors whereas 4 of 39 cyclin D1-negative tumors showed such P15/P16 deletions, p=0.001.

## PROGNOSTIC SIGNIFICANCE OF CYCLIN D1, P15 AND P16 STATUS IN BLADDER CANCER

Only 56 patients continued the follow up in the urology clinic.

Twenty-two of the 35 bladder malignant tumors (60%) with negative p16 gene and 9 of the 21 (43%) with positive p16 tumors recurred in our series of tumors during the period of follow-up. Thus, recurrence was more frequent in negative p16 tumors, and the Kaplan-Meier curves of disease-free survival showed a non significant separation (p=0.28 by log-rank test=1.2) (Figure 4A), on the other hand recurrence was observed in 15 out of 28 (53.5%) tumors with both positive and negative p15 gene and the Kaplan-Meier curves showed a non significant separation (p=0.78 by log rank test=0.08)(Figure 4B).

Twenty one of the 28 bladder malignant tumors (75%) with negative Cyclin D1 and 9 of the 28 of cyclinD1 positive samples (32.1%) recurred in our series during the period of follow up. Thus, recurrence was less frequent in positive-cyclin D1 tumors and the Kaplan-Meier curves of disease-free survival showed a highly significant separation (p=0.004) (Figure 4C).
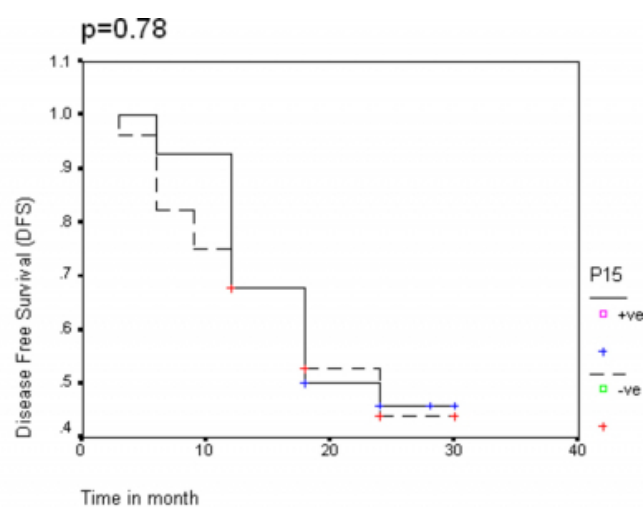
Figure 4

Figure 4: Kaplan-Meier curves for disease-free survival in 56 patients who continued the follow up in the urology clinic was stratified according to P16 (a) , P15 (b) and Cyclin D1 (c) status
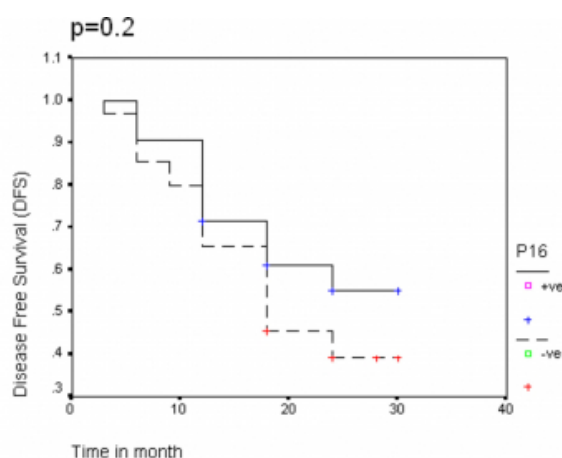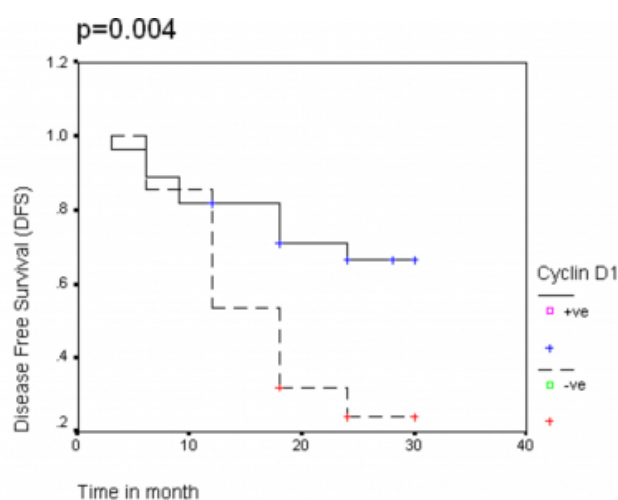

P 16.	Total	Patient with Recurrence		DFS period in months
		No	%	Mean (Range)
-ve	35	21	60	19.9 (16.8-23.0)
+ve	21	9	43	22.7 (18.8-26.0)
Total	56	30	53.5	

Figure 5

P15	Total	Patient with Recurrence		DFS period in months
		No	%	Mean (Range)
-ve	28	15	53.5	20.5 (16.8-24.2)
+ve	28	15	53.5	21.4 (18.2-24.8)
Total	56	30	53.5	

Figure 6

Cyclin D1	Total	Patient with Recurrence		DFS period in months
		No	%	Mean (Range)
-ve	28	21	75	17.7 (14.6-20.8)
+ve	28	9	32.1	24.2 (20.8-27.6)
Total	56	30	53.5	

Figure 7

Table 4 : Cyclin D1 expression in relation to clinicopathological features of bladder carcinoma and p15 and p16 gene deletion

Parameter (NO)	Cyclin D1		Statistics	
	Negative	Positive	X^2	p
Malignant	39	47	25.3	0.001**
Benign	30	0		
Control	28	2		
SCC	12	13	0.1	0.75
TCC	28	33		
Stage				
Super. (Ta-T1)	1	22	21.3	0.001**
Invasive (T2-T4)	33	30		
Lymph node				
+ve	15	0	21.9	0.001**
-ve	24	47		
Grade				
Low (G1-G2)	19	38	9.8	0.002**
High (G3)	20	9		
Schistosomiasis				
+ve	15	32	1.55	0.21
-ve	24	15		
P15-ve	10	33	16.9	0.001**
P15+ve	29	14		
P16-ve	13	35	14.6	0.001**
P16+ve	26	12		

** highly significant difference between groups by chi-square test.

When a Cox proportional hazards model was constructed that included age of patients at the diagnosis, tumor grade and stage, cyclin D1, P16 and P15 status, negative P16 gene (p=0.15; RR=2.8) and negative P15 gene (p=0.24; RR=1.5) confirmed to be independent indicators of disease-free survival second to negative cyclin D1 expression (p=0.000; RR=4.8),as shown in Table 5.
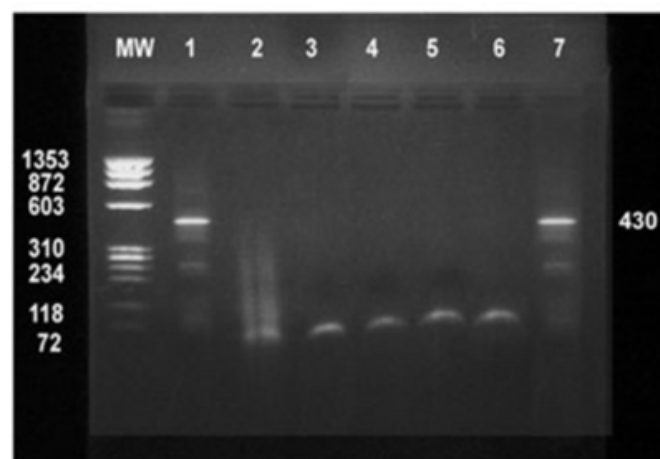
Figure 8

Table 5: Contribution of various potential prognostic factors to disease-free survival by Cox Regression analysis in 56 bladder cancers.

Variable	Risk Ratio	95% confidence interval	p value
Age[1]	1.3	0.6 -3.03	0.47
Grade[1]	0.68	0.23-2.00	0.45
Stage[1]	1.4	0.36-5.7	0.62
P16[2]	2.8	1.18-6.70	**0.015**
P15[2]	1.5	0.74-3.30	0.24
Cyclin D1[2]	4.8	2.00-11.8	**0.000**

[1] The risk ratio is given as high vs. low. [2] The risk ratio given as negative vs. positive expression.

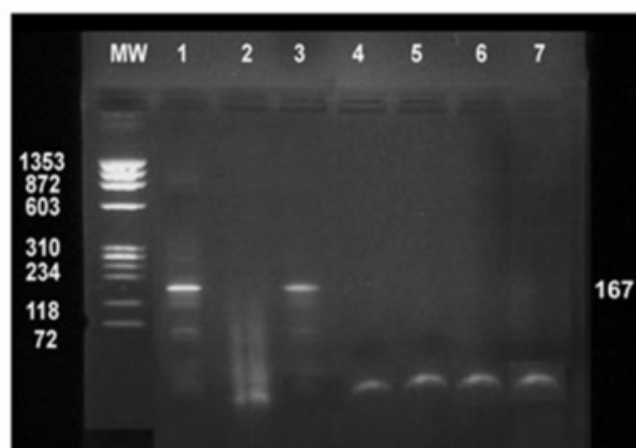## Figure 9

Figure 1: Agarose gel showing PCR product obtained with primers specific for p15. MW: # X 174 DNA/ Hae III markers; lane 1: Positive control; lane 2: Negative control. Lane 7 tissue from invasive high grade TCC bladder patient (+ve band for p15 DNA at 430 bp). Lanes 3,4,5,6 tissues from non-schistosomiasis invasive low grade SCC, scistosomiasis super. low grade SCC, schistosomiasis invasive low grade SCC, schistosomiasis superficial low grade TCC bladder patients (-ve bands for p15 DNA).


Figure 10

Figure 2: Agarose gel showing PCR product obtained with primers specific for p16. Lane MW: # X 174 DNA/ Hae III markers; lane 1: Positive control; lane 2 negative control. Lane 3 tissue from non-schistsomiasis invasive high grade TCC bladder patient (+ve band for p16 DNA at 167 bp). Lanes 4,5,6,7 tissues from non-schistosomiasis invasive low grade TCC, schistosomiasis superficial low grade TCC, non-schistosomiasis invasive low grade SCC, schistosomiasis superficial low grade SCC bladder patients (-ve bands for p16 DNA).

Figure 11

Figure 3: Western blotting analysis of Cyclin D1in malignant group. Expression of Cyclin D1 was seen in lanes 2(schistosomiasis invasive low grade SCC), 4(non-schistosomiasis invasive high grade SCC), 5,6 (schistosomiasis superficial low grade TCC), 7(non-schistosomiasis invasive low grade TCC),8(non-schistosomiasis invasive high grade TCC), while lanes 1,3,9(non-schistosomiasis invasive low grade SCC, schistosomiasis invasive high grade TCC tissue sample, control tissue sample respectively) were represented the Â–ve lanes for cyclin D1. The first lane corresponds to molecular weight standards.
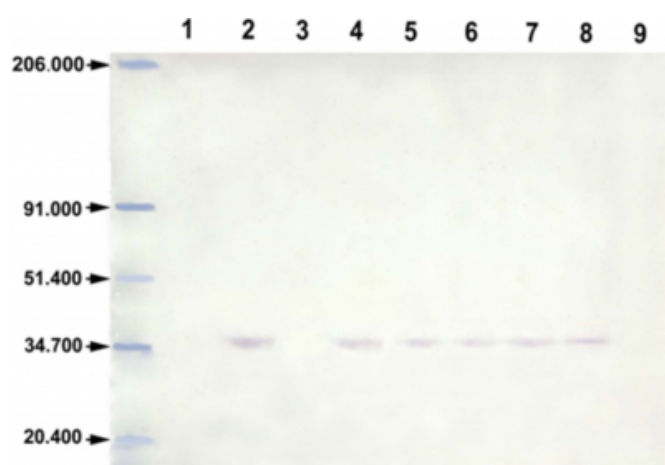
DISCUSSION

Bladder cancer is one of the most common malignancies in developed countries, ranking as the sixth most frequent neoplasm. It constitutes 30% of all cancers. The incidence is particularly high along the Nile river valley secondary to schistomiasis that is frequently associated with SCC (Khaled et al., 2005). The inactivation of both RB and p53 pathways has been shown to be required for the transformation and immortalization of uroepithelial cells, and their alterations are common and of predictive nature in clinical studies of bladder cancer (Brandau and Bohle, 2001).

Losses of tumor suppressor genes are involved in many types of cancer, including bladder cancer, although deletions involving chromosome 9 are frequently reported in TCC of the bladder (Simoneau et al., 1996), It is clear that p16 (CDKN2) and p15 (CDKN1) on 9p21, was altered and deleted in a variety of primary tumors, including bladder carcinomas (Van Oers et al, 2006).Our study reported an overall frequency of deletions for both p16 and p15 genes in bladder cancer in a nearly similar frequency of approximately 55.8 and 50%, respectively. These frequencies are higher than previously reported (Eissa et al.,

2000; Aveyard and Knowles, 2004). The higher frequency of p15, p16 deletion in the present work in comparison with previous work is due to inclusion of a larger number of tumors in low stage, low grade that are expected to show higher frequency of p15/p16 deletions.

The present study is carried out on a unique cohort of patients including SCC and SABC. The bladder tumors showed a higher frequency of p15 and p16 deletions in SCC than TCC (72% SCC vs. 41% TCC; 60% SCC vs. 54.1% TCC, respectively). These results are in agreement with other finding (Eissa et al., 2000; Tsutsumi et al., 1998) and suggesting that the SCC component is more genetically unstable than the TCC component and the genetic alteration of 9p21 containing p16/p15 may therefore have a positive role in the formation of SCC from TCC (Tsutsumi et al., 1998). The current study of schistosome-related tumors showed a higher frequency of p15 and p16 loss than non schistosomal-related tumors this may be explained by the chromosome instability mediated by either reactive oxygen species or urinary nitrosamines as a result of chronic inflammation and irritation in the urinary bladder by schistosomal infection (Rosin et al., 1994). On the other hand, the schistosome-related tumors showed a significant association and a higher frequency of p15 loss in SA-SCC tumor type than SA-TCC tumor type (80% p15 in SA-SCC versus 74% in SA-TCC; 40% p15 in NSA-SCC versus 14.7% in NSA-TCC), and a higher frequency of p16 loss in SA-TCC than SA-SCC (65% p16 in SA-SCC versus 81.5% in SA-TCC; 40% p16 in NSA-SCC versus 45.8% in NSA-TCC) these observation are in agreement with other findings (Muscheck et al., 2000; Gutierrez et al., 2004) and indicated that bladder carcinogenesis with this etiology follows a more narrow pathogenetic pathway (loss of 9p21) than is the case for BC in the industrialized world. The present analysis also reveals that deletion in the two genes are associated with low-stage, low-grade bladder cancer confirming previous finding (Eissa et al., 2000). Concerning the lymph node metastasis, our study showed a significant association between p16, p15 genes and lymph node metastasis (p=0.002, 0.003 respectively). These results are in harmony with previous report (Berggren et al., 2003). Thus we suggesting that the loss of p16 and p15 genes may be an early event in bladder carcinogenesis and not related to tumor invasion.

Chromosome 11q13 is the locus of cyclin D1 and EMS1 genes and has been reported as commonly amplified in

bladder carcinomas, the cyclin D1 protein plays an important role in the regulation of cell cycle transition in normal cells (Zaharieva et al., 2003).

In our series, 54.7% of BC were cyclin D1 positive. Similar results was obtained using immunohistochemical (Tut et al., (2001). In the current study, cyclin D1 we observed in low stage, low and intermediate grade bladder tumors. This finding is in agreement with previous studies reporting over expression of cyclin D1 in association with papillary, low-grade, non-invasive bladder tumors (Wang et al., 2002; Sgambato et al., 2002). The association between expression of cyclin D1 and the presence of bilharziasis suggests a possible role of this cell cycle stimulatory protein in schistosomiasis-induced carcinogenesis. Osman et al. 1997 reported similar findings using immunohistochemistry, with lower frequencies of cyclin D1 overexpression in schistosome-related SCC (22%) compared with schistosome-related TCC (38%), but their study reported a significant association between cyclin D1 overexpression and deep muscle invasion, although this reported association which was not observed in our study and others may be attributed to the inclusion of Adenocarcinoma tumors in their study and the use of immunohistochemical technique which is dependent on the type of fixative, monoclonal antibody and observer.

The relation between cyclin D1 as a positive cell cycle regulator and p15, p16 as negative cell cycle regulators have not been reported before and needs to be emphasized. In the current study, expression of cycline D1 was associated with p15 and p16 gene deletion which support the hypothesis that loss of p16 and p15 functions could be related to the overexpression of cyclin D1 found in bladder cancer whatever tumor stage or grade and this may extend to other tumor types as well (Yurakh et al., 2006).

Conversely, we often found cyclin D1 overexpression in p16 negative carcinomas, and vice-versa, which is in agreement with other studies reporting abnormalities of cyclin D1 and p16 the concomitant occurrence in human cancer cell lines, including bladder cancer (Niehans et al., 1999; Yang et al., 2002). Experimentally, they were able to demonstrate in human diploid fibroblast strains that microinjection of Glutathion S transferase-p16 protein and a neutralizing antibody to cyclin D1 synergistically enhanced G1 arrest, leading them to hypothesize that in Rb-competent cells it is the balance between functional cyclin D1 and p16 which determines whether or not cells will proceed through the G1

checkpoint (Li et al., 2005). Our results are consistent with their conclusion that while Rb defects eliminate the G1 checkpoint completely, aberrations of the upstream components such as p16 and cyclin D1 can cooperate in multistep tumorigenesis.

Evaluation of prognostic value of investigated parameters revealed that increased expression of cyclin D1 and retaining of p15 and p16 genes in tumor cells were associated with improved overall survival, while negative P16 gene (p=0.15) and negative P15 gene (p=0.24) confirmed to be independent indicators of disease-free survival second to negative cyclin D1 expression (p=0.000). Our results are in agreement with previous study that also found that overexpression of cyclin D1 is associated with less aggressive disease and better survival in bladder cancer patients (Sgambato et al., 2002). When p16 and cyclin D1 status were combined, patients with cyclin D1 negative and p16 gene negative have a significantly shorter disease-free interval. Conversely, in the subset of patients with cyclin D1 positive and p16 gene positive displayed a significantly longer disease-free survival. Thus the presence or absence of cyclin D1 and p16 in the tumor cells represents the most effected event in the recurrence in that tumor. P16 deletion occurs as an early event in tumor cells followed by increased cellular level of cyclin D1, which enhances other processes such as apoptosis, which limit tumor growth and prevent recurrence. In this context, it is interesting that head and neck carcinomas with the highest levels of cyclinD1 expression also display that highest percentage of apoptotic cells when subjected to in-situ end labeling (Kotelnikov et al., 1997). Conversely, bladder carcinomas with G1 checkpoint aberrations other than cyclinD1 over expression could have a higher frequency of mutations that promote tumor spread (e.g. a pro-metastatic phenotype).

Multivariate analysis showed that p16gene and Cyclin D1 protein were significant independent prognostic factors for disease free survival as previously demonstrated by Niehans et al. 1999 and Yang et al. 2002. Patient's with negative p16 gene were 2.8 times susceptible to recurrence than p16 +ve patient's and patient's with cyclin D1–ve tumors were 4.8 susceptible to recurrence than cyclin D1+ve tumors.

In Summary, our results reveal that expression of the p16 gene and p15 gene are inversely proportional to the expression of the cyclin D1protein and the down- regulation of p15 and p16 and cyclin D1 expression are a frequent event in early neoplastic transformation of the urothelium

which provide the first evidence for the possible involvement of these genes and protein in carcinomas, particularly those associated with schistosomiasis and could potentially be exploited in urinary tract cytology to increase the sensitivity for detection of low-grade and stage bladder tumors.On the other hand the higher frequency of recurrence associated with low expression of cyclin D1 and p16 deletion, which suggested that these cell cycle regulators gave prognostic information in addition to tumor stage and grade. Moreover, these findings underscore the importance of evaluating cell cycle regulators concurrently rather than independently and are of extreme interest considering the scarcity of useful prognostic factors able to accurately predict the clinical outcome of these patients. It is the hope that they will help to identify patients with bladder cancers who are at risk for recurrence and might benefit from a selective aggressive adjuvant treatment while sparing low-risk patients from unnecessary therapies.

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