

RESEARCH ARTICLE

Editorial Process: Submission:10/03/2017 Acceptance:01/15/2018

Impact of Cyclin D1 and Heterogeneous Nuclear Ribonucleoprotein-K (HnRNP-K) on Urinary Bladder Carcinogenesis

Tarek Aboushousha^{1*}, Olfat Hammam¹, Noha Helal¹, Samir El Dahshan²

Abstract

Objective: This study aimed to investigate the expression of cyclin D1 and hnRNP-K in relation to the pathological findings in bladder cancer including the type, grade, muscle invasion and bilharzial association. **Methods:** We studied the immunoexpression; as regard the percentage, intensity and score of both cyclin D1 and hnRNP-K in different bladder lesions including 10 cases of cystitis; 10 cases of carcinoma insitu (CIS), 20 cases of Squamous cell carcinoma (SCC) and 66 cases of urothelial carcinoma (UC). **Results:** High expression of cyclin D1 was found in UC compared to other groups ($p < 0.001$) and in UC with low grade, non-muscle invasive and papillary tumors compared to their counterparts ($p < 0.05$, < 0.01 and < 0.05 respectively), however, bilharzial association does not affect cyclin D1 expression. Higher hnRNP-K expression was found in SCC compared to other groups ($p < 0.001$) and in UC with high grade, muscle invasive and non-papillary tumors compared to their counterparts ($p < 0.001$ each). Bilharzial-associated UC showed higher expression of hnRNP-K percent ($p < 0.05$) compared to non-bilharzial cases. **Conclusion:** This study elucidated a possible contribution of cyclin D1 and hnRNP-K expression in the initiation and progression of urinary bladder carcinoma, so, both of them can be used in predicting progression of urinary bladder carcinoma and to differentiate between UC and SCC in high grade tumors. The possible role of both markers in immunotherapy deserves supplementary studies.

Keywords: Bladder cancer- cystitis- bilharziasis- cyclin D1- hnRNP-K- immunohistochemistry

Asian Pac J Cancer Prev, **19** (2), 513-519

Introduction

Bladder cancer is one of the major foundations of morbidity and mortality worldwide, accounting for nearly 429,800 new incidence cases and 165,100 deaths per year (Torre et al., 2015). In Egypt, bladder malignancies are the commonest among urinary system malignant tumors (90.71%) and the third among all malignancies (Harb et al., 2017).

Urothelial carcinoma (UC) is the most frequent histological subtype and accounts for more than 90% of the cases in USA and Europe (Moreira et al., 2010). UC 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 UC patients present were NMIBC (Isharwal and Konety, 2015), which although clinically heterogeneous, is typically associated with a favorable prognosis and a relatively low risk for recurrence following cystectomy (Nargund et al., 2012). In contrast, MIBC accounts for only 25-30% of cases (Veeratterapillay et al., 2016), yet the five-year survival rate, even following radical cystectomy, is greatly reduced

as compared with NMIBC (Proctor et al., 2010; Meeks et al., 2012). 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. So there is an urgent need to identify novel biomarkers that indicate UC cases with increased risk for recurrence and metastases. Currently, much interest centers on the molecular processes underlying the development and progression of UC of the bladder (Waddell and Keegan, 1999).

Squamous cell carcinoma (SCC) is the most common type in Egypt due to endemicity of bilharziasis that exposes transitional epithelial lining of bladder to squamous metaplasia and dysplasia (Reuter, 2010). SCC is categorized into two subtypes; SCC associated with bilharziasis (B-SCC) and SCC not associated with bilharziasis (NB-SCC). Both differ in their epidemiology, natural history and clinic-pathological features (Martin et al., 2016).

The cyclin family of proteins, which includes the mitotic cyclins (A and B) and cyclins C, D and E (associated with G/S transition), has a central role in

¹Pathology Departments, ²Urology Departments, Theodor Bilharz Research Institute, Cairo, Egypt. *For Correspondence: t.aboushousha@tbri.gov.eg

the control of the cell cycle (Sherr, 1996). Cyclin D1 contributes to regulate G1-S phase progression by forming a complex with different cyclin dependent kinases. It has oncogenic properties and is frequently overexpressed in several human tumor types (Kim and Diehl, 2009). A significant proportion of bladder cancer cases showed that increased cyclin D1 expression and overexpression of its gene were associated with poor prognosis and decreased postoperative patient survival (Sherr and Roberts, 1995; Sgambato et al., 1998). Heterozygous deletion of cyclin D1 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 first priorities in the field of anticancer research (Fang et al., 2013).

Heterogeneous nuclear ribonucleoprotein K (hnRNP-K), a member of the hnRNP family, is an essential RNA- and DNA-binding protein (Chaudhury et al., 2010). HnRNP-K is multifunctional, involved in the development of heterogeneous nuclear RNA into mature RNA (Dinh et al., 2013). It interacts with diverse molecules involved in gene expression and signal transduction, including chromosome remodelling, DNA transcription, RNA processing, RNA splicing, and RNA stability and translation (Wu et al., 2014). HnRNP-K protein is aberrantly overexpressed in various human cancers including colorectal, pancreatic, hepatic, prostate and renal cancer (Lu and Gao, 2016). It plays a vital role in cancer prognosis and its aberrant expression correlates with unfortunate clinical outcome (Yang et al., 2016).

Early detection and proper typing, grading and staging of bladder cancer are critically important to alleviate morbidity and mortality rate, associated with the disease, and to meliorate the chances of a prosperous outcome. So this study aims to evaluate expression of cyclin D1 and hnRNP-K in bladder lesions and analyze their correlation with the pathological characteristics of bladder cancer including tumor type, grade, stage, as well as bilharzial association and to characterize their potential role as diagnostic markers and/or target therapy for bladder cancer.

Materials and Methods

Tissue samples

106 archival urinary bladder paraffin blocks from Pathology Department of Theodor Bilharz Research Institute were included in this study; including transurethral resections or radical cystectomy specimens. The study protocol was approved by the Ethics committee of Theodor Bilharz Research Institute, for the protection of human subject and adopted by the 18th world medical assembly, Helsinki, Finland.

Paraffin blocks consisted of 10 cases of chronic cystitis, 10 cases of carcinoma in situ (CIS), 20 cases of squamous cell carcinoma (SCC) and 66 case of urothelial carcinoma (UC).

They belong to 77 males and 29 females (mean age 62.55 ± 6.49 years, range 29–72 years) (Table 1).

Histological Study

Specimens

Tissue sections were stained by Hematoxylin-eosin and histopathologically diagnosed by expert pathologists, with special reference to type of malignancy, tumor grade and stage as well as positive or negative association with bilharziasis.

Histological grading

Urinary bladder tumors were histologically divided into 3 grades (I–III). Tumors of grade I are considered of low grade, while those in grades II and III are of high grade (Moch et al., 2016).

Pathological staging

Staging of bladder tumors followed WHO classification (Moch et al., 2016). Tumors of pathological stage T1 are considered superficial and that > T1 are muscle invasive.

Diagnosis of bilharziasis

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).

Immunohistochemical (IHC) technique

Immunohistochemistry for Cyclin D1 and hnRNP-K was performed on tissue sections cut from the paraffin blocks at 4µm onto positively charged slides (Superfrost plus, Menzel-Glaser, Germany) and stained on an automated platform the (Dako Autostainer Link 48) using anti-human hnRNPK and Cyclin D1 monoclonal primary antibodies (Santa Cruz Biotechnology, CA, USA) at 1:100 dilution. Heat induced antigen retrieval was used for 30 min at 97°C in the high-PH EnVision™ FLEX Target Retrieval Solution.

The antigen was localized by the addition of 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate chromogen solution (Universal Detection Kit, Dako, Denmark). Finally, slides were counterstained with hematoxylin, dehydrated in alcohol and mounted.

For each setting, positive and negative control slides were included. As a negative control, bladder tissue was processed in the above mentioned sequences but the primary antibodies were not added and instead add non-immune immunoglobulin G (IgG; DAKO, Glostrup, Copenhagen, Denmark). Colonic mucosa known to express cyclin D1 used as positive control for cyclin D1. Human prostate cancer tissues were used as positive control for hnRNP-K antibody.

Interpretation of immunostaining and scoring analysis

Immunohistochemical analysis of bladder tissue sections was blind-quantified by two pathologists. The sections were examined by using light microscope [Scope A1, Axio, Zeiss, Germany]. Photomicrographs were taken using a microscope-camera [AxioCam, MRc5, Zeiss, Germany].

For Cyclin D1: The expression of cyclin D1 was measured in 10 successive high-power fields (x400). Cyclin D1 expressed as nuclear and cytoplasmic brown

color. The immunostaining intensity was graded as negative = 0, weak = 1, moderate = 2, or strong = 3. The fraction of cells stained with cyclin D1 were counted as a percentage and scored (1 <15%, 2=15-50%, 3>50%) (Kopparapu et al., 2013).

For hnRNP-K: The nuclear immunostaining intensity was graded as negative = 0, weak = 1, moderate = 2, or strong = 3. The proportion of positively staining cells was assessed as a percentage. The score was then calculated as the intensity score multiplied by the percentage of cells stained (score = intensity X % of positive cells). The samples were classed as low (score <140) or high (score ≥140) hnRNP-K expression (Chen et al., 2017).

Statistical analyses

The immunohistochemical results were analyzed using SPSS version 20 (IBM Corporation, Armonk, New York, USA). Data are presented as the mean ± S.D. Two-tailed Student's t-tests and one-way ANOVA were used to evaluate the data. Comparison of difference in percentage between groups was evaluated using two tailed Fischer's exact test. Differences were considered statistically significant at $P < 0.05$.

Results

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 (77 cases) with female patients constitute only 27.36% of cases (29 patients). Age and sex distribution was listed in (Table 1).

All cases of SCC showed high tumor grade and positive muscle invasion, while most cases of UC were of low grade (65.2%) and showed negative muscle invasion (69.6%). The difference was statistically significant ($p < 0.001$). Eighty percent of SCC cases were associated

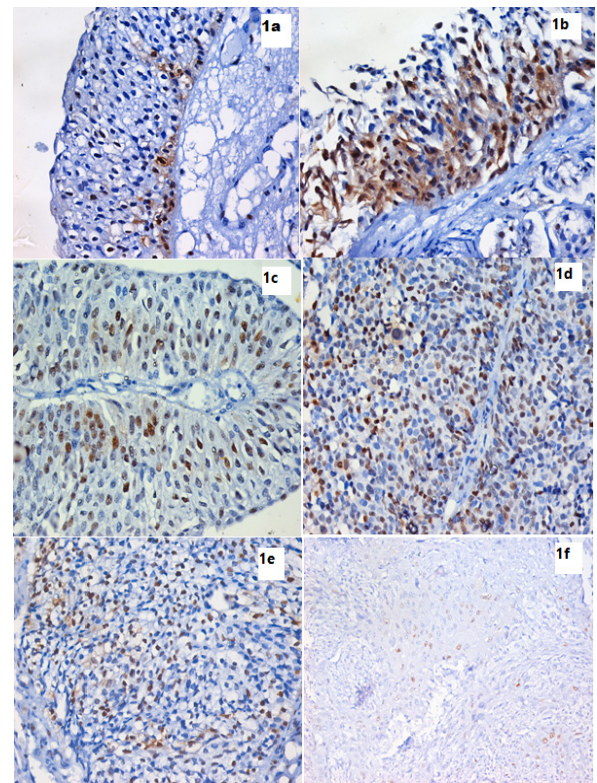


Figure 1. IHC Using Anti-Cyclin D1 Monoclonal Antibody and DAB in Bladder Tissue Sections Expressed as Brown Mostly Nuclear Staining. 1a. Full-thickness urothelium in case of chronic cystitis showing positive nuclear and cytoplasmic expression of cyclin D1 in the basal layer. (IHC stain for cyclin D1, X400). 1b. Full-thickness urothelium in case of CIS showing positive nuclear and cytoplasmic expression of cyclin D1 in all layers. (IHC stain for cyclin D1, X400). 1c. Low grade superficial papillary UC showing moderate positive nuclear expression of cyclin D1. (IHC stain for cyclin D1, X200). 1d. Higher grade of non muscle invasive papillary UC showing increased positive nuclear expression of cyclin D1 compared to previous photo. (IHC stain for cyclin D1, X400). 1e. Non papillary muscle invasive UC showing lower positive nuclear expression of cyclin D1 compared to non invasive UC. (IHC stain for cyclin D1, X400). 1f. Section in a case of invasive SCC showing weak positive nuclear expression of cyclin D1. (IHC stain for cyclin D1, X400).

Table 1. Sex and Age Distribution of Studied Cases

Lesion (n)	Female n (%)	Male n (%)	Age (Mean±S.D.)
Cystitis (10)	5 _a (50.0%)	5 _a (50.0%)	63.50 ± 1.73
CIS (10)	2 _a (20.0%)	8 _b (80.0%)	64.19 ± 3.34
SCC (20)	10 _a (50.0%)	10 _b (50.0%)	67.83 ± 5.06
UC (66)	12 _a (18.18%)	54 _b (81.82%)	61.08 ± 6.40
Total (106)	29 (27.36%)	77 (72.64%)	62.54 ± 6.49

Each subscript letter denotes a subset of sex categories whose column proportions do not differ significantly from each other at the .05 level. (Chi-Square); CIS, Carcinoma In Situ; SCC, Squamous cell carcinoma; UC, Urothelial carcinoma; n, number of cases

with bilharziasis, while only 4.3% of UC cases were positive for bilharziasis. The difference between groups was statistically significant ($p < 0.001$) (Table 2).

Cyclin D1 Immunoexpression

UC showed higher parameters of cyclin D1 expression (percentage, intensity and score) compared to chronic

Table 2. Difference in Tumor Grade, Muscle Invasion and Bilharzial Association between UC and SCC groups

	Tumor Grade		Muscle Invasion		Bilharziasis	
	Low n (%)	High n (%)	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)
SCC (20)	0 (0.0%)	20 (100.0%)	0 (0.0%)	20 (100.0%)	4 (20.0%)	16 (80.0%)
UC (66)	40 (60.61%)	26 (39.39%)	42 (63.64%)	24 (36.36%)	54 (81.82%)	12 (18.18%)
Fisher's exact test	$p < 0.0001$		$p < 0.0001$		$p < 0.0001$	

SCC, Squamous cell carcinoma; UC, Urothelial carcinoma; n, number of cases

Table 3. Difference in Expression Parameters of HnRNP-K and Cyclin D1 in Studied Bladder Lesions

Diagnosis (n)		Cyclin D1			HnRNP-K		
		percent	intensity	score	percent	intensity	score
Cystitis (10)	Mean	0.5	1	0.5	29	1.8	53.5
	S.D.	0.52	1.05	0.52	5.67	0.42	17.64
CIS (10)	Mean	25.64	1.62	1.48	90	3	270
	S.D.	17.36	0.42	0.46	5.27	0	15.81
SCC (20)	Mean	7.85	1.4	0.85	78	2.2	171
	S.D.	10.2	0.94	0.67	5.23	0.41	29.45
UC (66)	Mean	47.12	1.98	2.27	66.66	2.28	163.18
	S.D.	28.56	0.71	0.66	27.1	0.67	78.59
p value (ANOVA)		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

CIS, carcinoma insitu; SCC, squamous cell carcinoma; UC, urothelial carcinoma; S.D, standard deviation; n, number of cases.

cystitis, CIS and SCC groups, with statistically significant difference ($p < 0.001$) (Table 3).

UC of high grade, muscle invasion and non-papillary pattern showed significantly lower parameters of cyclin D1 expression compared to their counterparts. UC with bilharzial association show higher cyclin D1 percentage and intensity, while exhibit lower expression score compared to non-bilharzial cancers, however, no significant differences in cyclin D1 expression were achieved between bilharzial and non-bilharzial associated

bladder cancer (Table 4).

HnRNP-K Immunohistochemistry

All cases of cystitis showed lower parameters of hnRNP-K expression compared to cases of CIS, UC, SCC with significant difference ($p < 0.001$). On the contrary, CIS cases show significantly higher parameters of hnRNP-K expression compared to the other groups ($p < 0.001$). SCC cases showed higher hnRNP-K percentage and score compared to UC ($p < 0.001$) (Table 3).

Table 4. Difference in Expression of Cyclin D1 and HnRNA Parameters in Urothelial Carcinoma (UC) in Relation to Tumor Grade, Muscle Invasion, Papillary Pattern and Bilharzial Association

Parameter (n)		Cyclin D1			HnRNA		
		percent	intensity	score	percent	intensity	score
Tumor Grade							
High grade (26)	Mean	35.1	1.81	1.84	80.75	2.51	202.13
	S.D	29.41	0.83	0.91	10.85	0.5	46.55
Low Grade (40)	Mean	50.62	2	2.37	25.12	1.5	45
	S.D.	32.5	0.63	0.71	15.49	0.52	36.15
p value (t-test)		N.S	N.S	<0.05	<0.00	<0.00	<0.00
Muscle invasion							
Negative (42)	Mean	69.29	2.86	2.71	43.33	1.83	98.33
	S.D	20.46	0.36	0.47	29.88	0.7	90.87
Positive (24)	Mean	36.88	1.63	2.06	79.35	2.44	190.8
	S.D.	26.01	0.49	0.67	10.92	0.49	36.45
p value (t-test)		< 0.001	< 0.001	< 0.01	<0.00	<0.00	<0.00
Papillary pattern							
Non-papillary (38)	Mean	37.33	1.6	2.13	82.5	2.6	213.12
	S.D	27.85	0.49	0.73	13.17	0.49	47.35
Papillary (28)	Mean	64.38	2.75	2.5	47.86	2.01	115.71
	S.D.	20.89	0.45	0.52	29.42	0.77	92.23
p value (t-test)		< 0.01	< 0.001	< 0.05	<0.00	<0.00	<0.00
Bilharzial Association							
Negative (54)	Mean	46.59	2.05	2.27	66.05	2.32	163.42
	S.D	29.19	0.75	0.69	27.54	0.66	81.39
Positive (12)	Mean	50.76	2.11	2.09	78.35	2.2	171.87
	S.D.	0.46	0.19	0.01	5.37	0.42	30.26
p value (t-test)		N.S.	N.S.	N.S.	<0.05	N.S.	N.S.

S.D, standard deviation

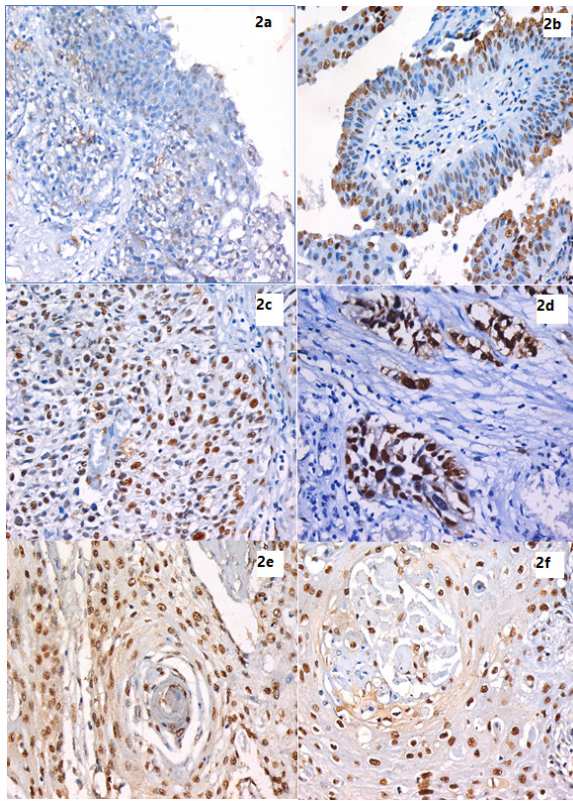


Figure 2. IHC Using Anti-hnRNP-K Monoclonal Antibody and DAB in Bladder Tissue Sections Expressed as Brown Nuclear Staining. 2a. Full-thickness urothelium in case of chronic cystitis showing mild positive nuclear expression of hnRNP-K scattered through all layers. (IHC stain for hnRNP-K, X400). 2b. A case of Low grade superficial papillary UC showing mild positive nuclear expression of hnRNP-K mostly in the superficial layer. (IHC stain for hnRNP-K, X200). 2c. High grade papillary UC showing higher hnRNP-K expression than previous low grade superficial tumor. (IHC stain for hnRNP-K, X400). 2d. High grade non-papillary, muscle invasive UC showing marked hnRNP-K expression. (IHC stain for hnRNP-K, X400). 2e. A case of Bilharzial-associated SCC, showing high hnRNP-K expression. (IHC stain for hnRNP-K, X400). 2f. A case of non-bilharzial-associated SCC, showing high hnRNP-K expression. (IHC stain for hnRNP-K, X400).

UC cases with high grade, muscle invasion and non-papillary pattern showed high significant difference in all parameters of hnRNP-K expression compared to low grade, non-muscle invasive and papillary counterparts ($p < 0.001$) (Table 4). UC associated with bilharziasis showed significantly higher percentage of hnRNP-K expression ($p < 0.05$) and non-significant higher expression score ($p > 0.6$) compared to non-bilharzial ones, however, they showed non-significant lower hnRNP-K intensity of expression compared to non-bilharzial cancers ($p > 0.05$).

Discussion

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). Nonetheless, early diagnosis is critically important to alleviate morbidity and mortality rate, associated with recurrent disease and to meliorate the chances of a prosperous outcome (Ibrahim et al., 2014).

Overexpression of the cyclin D1 gene has been reported in many preneoplastic lesions and human tumors including bladder transitional cell carcinoma (Arber et al., 1996; Oya et al., 1998). Ohtsubo and Roberts (1993) and Quelle et al., (1993) found that alteration in cyclin D1 expression is an early event in bladder tumorigenesis. Cyclin D1 protein expression has been correlated with both poor prognosis (Lopez-Beltran et al., 2004a) and good prognosis (Lopez-Beltran et al., 2004b).

In the present study, cases of chronic cystitis and SCC showed respectively negative and lower scores of cyclin D1 expression compared to cases of UC. This is in accordance with Kopparapu et al., (2013) who stated that cyclin D1 protein expression is higher in UC versus the adjacent non-malignant bladder tissues.

Our results showed also that cyclin D1 expression significantly correlates with low-grade, low-stage and papillary tumor growth. This goes with Levidou et al., (2010); Kopparapu et al., (2013) and Khabaz et al., (2016) who found that high level of cyclin D1 immunoreactivity was more frequent in low grade and NMIBC, while high grade and advanced stage tumors, MIBC tumors and tumors with vascular invasion and lymph node involvement showed lower cyclin D1 score levels. The negative correlation of cyclin D1 expression with tumor grade and stage is in alignment with the experimental data indicating that cyclin D1 expression in urothelial tissue supports not only cell proliferation, but also cell differentiation and raises the hypothesis that cyclin D1 overexpression may play an important role in the early T-categories of bladder tumorigenesis, promoting the acquisition of autonomous growth properties (Proctor et al., 1991).

Nucleus is the ordinary location of cyclin D1 protein in normal cells. A report by Fristrup and colleagues showed that high cyclin D1 expression is predominantly nuclear in tumor cells from NMIBC (Ta/T1) UC, and that this nuclear cyclin D1 expression is associated with poor patient outcome (Fristrup et al., 2012). Controversially, Kopparapu et al., (2013) reported that absence of nuclear cyclin D1 expression in tumor cells was significantly associated with MIBC. Thus, cyclin D1 may have some value as a predictive marker for a sub-group of patients with UC.

No significant difference in cyclin D1 expression was achieved between bilharzial and non-bilharzial associated urothelial carcinoma in our study. This was in agreement with Hammam et al., (2009) who reported that cyclin D1 expression in non-bilharzial and bilharzial associated cancer was almost similar.

Aberrant overexpression of hnRNP-K has been reported in various cancers including those of the colon (Carpenter et al., 2006), lung (Tang et al., 2014), renal (Otoshi et al., 2015) and pancreas (Zhou et al., 2012). Several studies have concluded that hnRNP-K has been robustly involved in the development and the progression

of cancer through various biological events including transcriptional regulation, translational control chromatin remodeling, and cellular signal transduction (Lu and Gao, 2016). However, to our knowledge, there is no other studies examined the behavior and expression of hnRNP-K in bilharzial-associated bladder cancer.

Our study detected that, all SCC (100%) and most UC (76.5%) cases show significantly high hnRNP-K expression score compared to cystitis cases. This goes with findings of Guo et al., (2012) who reported high hnRNP-K expression in hepatocellular carcinoma (HCC) tissue compared to the cirrhosis.

CIS shows the highest hnRNP-K expression; a finding that could indicate a critical role of this protein in initiation of bladder carcinogenesis.

We also examined the correlation between hnRNP-K expression and pathological grade and stage in bladder cancer specimens. The expression level of hnRNP-K was positively significantly correlated with higher grade and muscle invasive tumors. This goes with Chen et al., (2017) who reported higher hnRNP-K expression in poorly differentiated and muscle invasive bladder tumors as compared to well-differentiated and non-invasive tumors. This also can parallel findings in studies performed on other organs. Guo et al., (2012) found higher hnRNP-K expression in late compared to early HCC. Additionally, Ayham et al., (2009) found a significant decrease in nuclear hnRNP-K expression levels in primary colon tumor compared to colon cancer with lymph node metastasis. Ootshi et al., (2015) found a significant positive correlation between hnRNP-K staining score and tumor aggressiveness in renal cell carcinoma.

We also observed that the non-papillary bladder cancers exhibited significantly higher value of all parameters of hnRNP-K expression compared to the papillary variants. These findings may suggest that the relative score of hnRNP-K expression proportionate to the degree of invasiveness and aggressiveness of the tumor.

Interestingly, we found that bilharzial-associated bladder cancer showed higher percentage and score of hnRNP-K expression, but lower hnRNP-K expression intensity compared to non-bilharzial associated cases, however, these correlations were statistically insignificant. This is the first study that tries to evaluate the relation between bilharzial associated bladder cancer and expression of hnRNP-K.

In conclusion, the immunohistochemical findings of this study elucidated a possible contribution of cyclin D1 and hnRNP-K expression in the initiation and progression of urinary bladder carcinoma. The lower expression of cyclin D1 and hnRNP-K in cystitis lesions and its higher expression in bladder cancer lesions indicates that changes in these proteins levels represent early events during the development of bladder cancer. In addition, the patterns of cyclin D1 and hnRNP-K expression with tumor grade and stage suggests the involvement of these proteins in bladder cancer progression variably, so, both of them can be used in predicting progression of urinary bladder carcinoma and to differentiate between UC and SCC in high grade tumors. The possible role of both markers in immunotherapy deserves supplementary studies.

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