

Interplay between CKS2, N-cadherin, and PD-ECGF in Non-Schistosomal and Schistosomal-Associated Bladder Cancer: A Prospective Comparative Study in the Egyptian Population

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Abstract

Background: The current study investigated the possible role of the cell cycle regulator, cyclin-dependent kinases regulatory subunit-2; the angiogenic factor, platelet-derived endothelial cell growth factor; and the cell adhesive molecule, neural cadherin, as prognostic factors in schistosomal and non-schistosomal-associated urothelial carcinomas. We also investigated the possible correlation between cyclin-dependent kinases regulatory subunit-2, platelet-derived endothelial cell growth factor, neural cadherin, tumor grade, and stage.

Methods: The study included 40 patients with primary bladder cancer (25 non-schistosomal-associated and 15 schistosomal-associated) who had no prior anticancer treatment. Tumor specimens were collected at the time of the transurethral resection. The vivid portions of the resected tumors were subjected to routine pathological examinations to determine stage, grade, and schistosomal infestation. Control bladder tissues (5 cases) were obtained by cystoscopic biopsies from patients who underwent transurethral resection of the prostate. Platelet-derived endothelial cell growth factor and neural cadherin protein expressions were evaluated by immunohistochemical staining. RNA was extracted, reverse transcribed, and amplified by PCR using cyclin-dependent kinases regulatory subunit-2 specific primers. Relative expression was detected by a comparison of the expression in normal and tumor samples relative to GAPDH (housekeeping) gene expression.

Results: We detected a positive correlation between neural cadherin and platelet-derived endothelial cell growth factor proteins in schistosomal-associated and non-schistosomal-associated bladder cancer. Significant overexpression of relative cyclin-dependent kinases regulatory subunit-2 gene, neural cadherin, and platelet-derived endothelial cell growth factor proteins were detected in invasive stages and higher grades of bladder cancer. Differential expressions of neural cadherin and platelet-derived endothelial cell growth factor proteins, tumor recurrence, and tumor progression were detected between schistosomal-associated and non-schistosomal-associated bladder cancer.

Conclusion: Cyclin-dependent kinases regulatory subunit-2, neural cadherin, and platelet-derived endothelial cell growth factor may be used as biomarkers for predicting bladder cancer outcome and aid in selecting patients for more aggressive treatments.

Keywords: Bladder cancer, CKS2, N-cadherin, Platelet-derived endothelial cell growth factor, Schistosomal infestation

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Introduction

Cancer of the urinary bladder is one of the most common human malignancies. It is an aggressive epithelial tumor with a high rate of early systemic dissemination.^{1,2} The most common bladder cancer (BC) is urothelial carcinoma (UC), which accounts for 90% of cases. Most urothelial neoplasms are low grade non-muscle-invasive papillary tumors, which tend to be multifocal and recur but have a relatively good prognosis. High grade muscle-invasive tumors are less common and have a much poorer prognosis.³

In about 70% of patients with non-muscle-invasive BC, tumors recur. Some of these patients will eventually show progression towards muscle-invasive cancer. One of the important focuses in BC research is the prediction of tumor recurrence and tumor progression. Conventional prognostic factors do not accurately predict the clinical outcome of many patients with BC, so additional effective biomarkers are required to explain the variability of outcome in patients with BC.⁴

In Egypt, carcinoma of the bladder currently ranks first in males.⁵ This high frequency of BC in Egypt is due to an endemic infection by *Schistosoma haematobium*, which contributes to defining a characteristic pathology - schistosomal-associated BC.⁶ Despite the marked decrease in prevalence of endemic schistosomiasis over the last 2 decades, Egypt still suffers from the toll of the previously high prevalence of this disease. It can be anticipated that in the near future there will be a marked decrease in schistosomiasis associated BC in Egypt as a sequel to schistosomiasis control. The potential risk is the rise in incidence of BC related to other risk factors.⁷

The molecular mechanisms of BC development and progression are complicated. They likely involve the interaction of tumor suppressor genes, oncogenes, growth factors, adhesion molecules, and angiogenic factors that lead a normal transitional cell to acquire the malignant phenotype.⁸ During the last two decades, a better understanding of the molecular mechanisms involved in carcinogenesis and tumor progression has provided numerous molecular markers of BC

that have potential diagnostic and prognostic value.⁹

Cell cycle regulatory proteins control proliferation and cell cycle progression in healthy, non-malignant cells. Neoplasms are characterized by an uncontrolled cell growth. Loss of cell cycle control seems to be an early sign of malignant transformation and cancer progression. Several alterations of genes and protein products of cell cycle regulation are identified in bladder tumors and appear to be associated with development of transitional cell carcinoma (TCC).¹⁰ Cyclin-dependent serine/threonine protein kinase (CDC28) belongs to a family of small 9-18 kilodalton (kDa) proteins with two human homologues; cyclin-dependent kinases regulatory subunit 1 & 2 (CKS1 and CKS2) modulate the

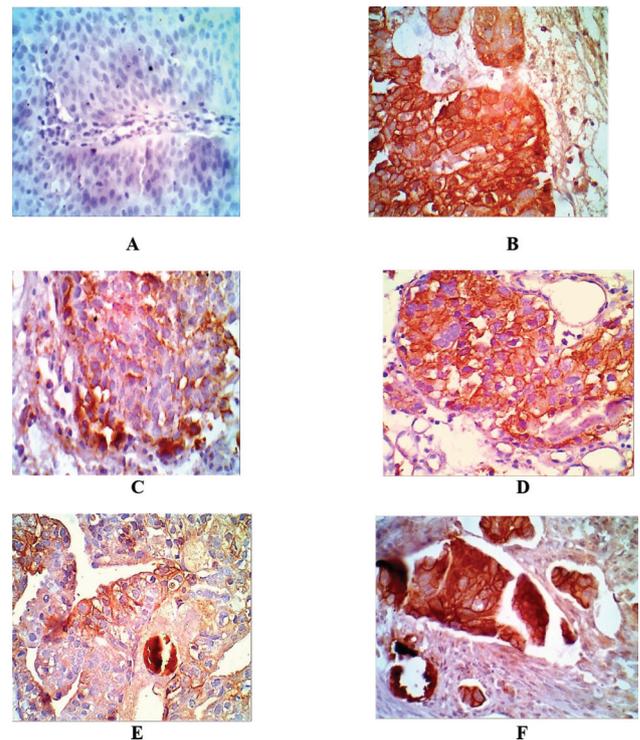


Figure 1. N-cadherin immunostaining (400×).

A: Low grade (pTa) non-schistosomal non-muscle-invasive UC showing negative membranous immunostaining; B: High grade (pT1) non-schistosomal non-muscle-invasive UC showing strong membranous immunostaining; C: Low grade non-schistosomal muscle-invasive UC showing moderate membranous immunostaining; D: High grade non-schistosomal muscle-invasive UC showing strong membranous immunostaining; E: High grade schistosomal-associated non-muscle-invasive (pT1) UC showing moderate membranous immunostaining; F: High grade schistosomal-associated muscle-invasive UC showing strong membranous immunostaining.

levels and/or activities of the cyclin/cyclin-dependent kinase complexes and are involved in cell cycle control.¹¹ *CKS2* is important in controlling the first metaphase-anaphase transition of mammalian meiosis.^{12,13} Gene expression analysis studies of several different tumor cells have shown that *CKS2* is one of the most frequently overexpressed proteins in cancer cells. Overexpression of this protein may be deleterious to cell integrity and possibly a part of the process of malignant transformation.¹⁴

Alteration of cadherin expression is associated with the loss of cellular differentiation, acquisition of an invasive phenotype, and poor prognosis in many cancer types. It has received significant consideration as a potential indicator of the metastatic phenotype in a variety of epithelial and non-epithelial malignancies.^{15,16} Neural cadherins (N-cadherins) are calcium-dependent cell adhesion molecules that mediate cell-cell adhesion, modulate cell migration, and tumor invasiveness.¹⁷ N-cadherins are associated with the invasive phenotypes in prostate, melanoma, and breast cancer.¹⁸⁻²⁰ There is a growing body of evidence which suggests that alterations in the adhesion properties of neoplastic cells may play a pivotal role in the development and progression of BC.²¹ N-cadherin is an important player in tumor development and, therefore, a potential target for novel therapeutic approaches.²²

Bladder cancer, like all solid malignancies, is dependent on angiogenesis to grow progressively and metastasize efficiently.²³ Induction of angiogenesis is mediated by a variety of molecules released by tumor cells as well as host stromal cells, one of which is the platelet-derived endothelial cell growth factor (PD-ECGF). Platelet-derived endothelial cell growth factor has been found to induce endothelial cell migration *in vitro* and angiogenesis *in vivo*. According to research, this growth factor is identical to thymidine phosphorylase (TP), an enzyme involved in pyrimidine nucleoside metabolism.^{24,25} Platelet-derived endothelial cell growth factor upregulates in several cancers and is correlated with tumor growth, induction of angiogenesis,

and metastasis. Therefore, high TP levels are most likely associated with a poor prognosis.²⁶⁻²⁸

While the status of individual molecular markers does not add sufficient value to outcome prediction in patients with advanced UC of the bladder, combinations of molecular markers may improve molecular staging, prognostication, and possibly prediction of response to therapy.²⁹ The ability to predict, at the first biopsy, whether a BC shift to progression is probable will facilitate the selection of appropriate treatment modalities and improve prognosis of patients with this cancer.³⁰⁻³²

The present study intended to study the interplay between *CKS2*, N-cadherin and PD-ECGF in UC non-schistosomal- and

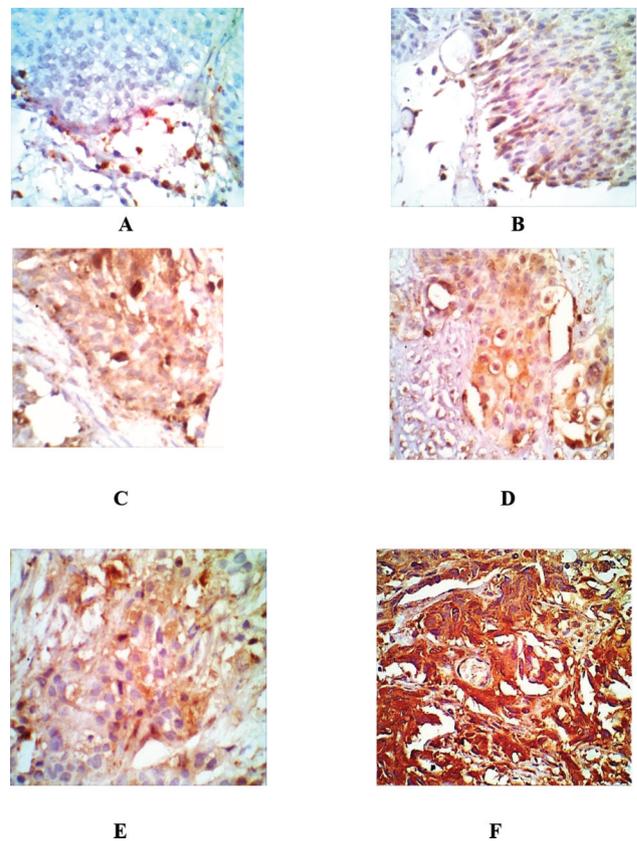


Figure 2. PD-ECGF immunostaining (400 \times).

A: Low grade (pT1) non-schistosomal non-muscle-invasive UC showing negative staining against a positive internal control in the inflammatory cells; B: Low grade (pT1) non-schistosomal non-muscle-invasive UC showing weak cytoplasmic immunostaining; C: Low grade non-schistosomal muscle-invasive UC showing moderate cytoplasmic and nuclear staining; D: High grade non-schistosomal muscle-invasive UC showing moderate cytoplasmic and nuclear immunostaining; E: High grade schistosomal-associated muscle-invasive UC showing moderate cytoplasmic immunostaining; F: High grade schistosomal-associated muscle-invasive UC showing strong cytoplasmic immunostaining.

schistosomal-associated tumors, and to correlate them to tumor stage, tumor progression, and recurrence.

Patients and Methods

Patients

This prospective study included 45 cystoscopic biopsies randomly selected from cases that presented to the Urology Department of the Faculty of Medicine, Alexandria University from 2009 to 2012. The sample size was based on a previously published work. We intended to include cases at early stages with reasonable representation of schistosomal cases which have become rare due to intensive eradication programs in Egypt. All subjects were recruited according to the ethical rules approved by the Ethics Committee of the Medical Research Institute (MRI) based on the Belmont Report.³³ The cases were subdivided as follows: 40 primary and recurrent urothelial carcinomas [UC; includes both transitional cell carcinoma (TCC) and squamous CC] of the urinary bladder that included 25 non-schistosomal cases and 15 schistosomal-associated cases. Non-schistosomal cases ranged in age from 34 to 79 years (mean \pm SD: 59.96 \pm 11.08 years) with a male to female ratio of 4:1. The 15 schistosomal-associated cases ranged in age from 47 to 70 years (mean \pm SD: 57.80 \pm 6.78 years) with a male to female ratio of 4:1. The control group consisted of 5 tumor-free urinary bladder biopsies collected from patients who underwent transurethral resection of the prostate (TURP).

Clinical diagnosis, treatment, and clinical follow up (range: 10-45 months; mean 32 months) were performed as described by El-Abd et al.³⁴ Samples for the progression group were selected by two criteria: (a) superficial tumors with no prior muscle-invasive tumors and (b) subsequent progression to a higher-stage tumor with invasion into the bladder muscle as verified by microscopy. We defined recurrence as any evidence of a tumor in a retained bladder at least 3 months after treatment. Disease progression was defined as a recurrent tumor that had a more advanced stage or higher grade than the primary tumor.³⁵

Methods

We subdivided the collected specimens into two parts - one fixed immediately in 10% buffered formalin for preparation of paraffin embedded tissue sections and the other stored at -80°C until its use in reverse transcriptase-polymerase chain reaction (RT-PCR).

Pathological investigations

Hematoxylin (Genzyme, England) and eosin (Chematec, UK; H&E) stained urinary bladder carcinoma sections (5 μ m) were examined by a pathologist to determine tumor type, size, grade,³⁶ stage,³⁷ and schistosomal infestation in the tumor or adjacent non-neoplastic bladder tissues. Masson trichrome staining was used to differentiate muscle fibers from collagen in muscle-invasive tumors.³⁸

Immunohistochemistry

Paraffin sections (5 μ m) were mounted on coated slides and stained for N-cadherin (mouse monoclonal antibody: clone 5D5; ab98952, Abcam, UK, dilution: 1:200) and PD-ECGF (pre-diluted mouse monoclonal antibody: clone SPM322; ab17806, Abcam, UK) using the streptavidin-biotin-immunoperoxidase system.³⁹ Epitope retrieval was performed before incubation with the primary antibody by microwave heating in citrate buffer (pH 6). The peroxidase-antiperoxidase technique was used with diaminobenzidine (Dako) as the chromogen. Sections were counterstained lightly with hematoxylin, cleared in xylene, and mounted on coverslips with DPX (Distrene-80, a plasticizer, and xylene).

Normal urothelium was the negative control for N-cadherin. The negative control for PD-ECGF was carried out by omitting the primary antibody. Breast carcinoma was the positive control to adjust for scoring of PD-ECGF. Normal urothelium samples were also used as controls.

Positive immunoreactivity for N-cadherin was appreciated when membranous staining was detected. In few cases, a cytoplasmic staining was observed which was not considered to be staining for a functional protein. The immunore-

Table 1. Comparison between schistosomal- and non-schistosomal-associated UC groups according to histopathological tumor stage and tumor grade

Tumor stage Tumor grade	Non-schistosomal UC [N (%)]			Schistosomal UC [N (%)]				
	Low grade	High grade	Total	Low grade	High grade	Total		
Non-muscle invasive	7 (58.3)	5 (41.7)	12 (100)	2 (40)	3 (60.0)	5 (100.0)		
pTa	1 (8.3)	0 (0)	1 (8.3)	0 (0)	0 (0.0)	0 (0.0)		
pT1	6 (50)	5 (41.7)	11 (91.7)	2 (40)	3 (60.0)	5 (100.0)		
Muscle invasive	2 (15.4)	11 (84.6)	13 (100)	1 (10)	9 (90.0)	10 (100.0)		
Total	9 (36)	16 (64)	25 (100)	3 (20)	12 (80.0)	15 (100.0)		

UC: Urothelial carcinoma; N: Number

activity for PD-ECGF was in both the cytoplasm and nuclei of malignant cells, as well as in stromal cells. Staining intensity and density of tumor cells were scored according to morphological criteria after inclusion of appropriate controls.

For N-cadherin and PD-ECGF staining the epithelial reactivity was semi-quantified taking into consideration the percentage of positive epithelial cells and the intensity of the reaction. Immunoreactivity (nuclear or cytoplasmic) was quantified as the percentage of positive cells in relation to the total number of tumor cells encountered in 10 representative high power fields that covered the entire section and averaging the results.

Molecular studies

Tissue samples (0.2-0.3 g) were homogenized and total ribonucleic acid (RNA) was extracted using a QIAamp RNA Mini Kit (Qiagen) according to the manufacturer's instructions.⁴⁰ The quantification and purity of the prepared RNA was examined by a spectrophotometer (Unicam; Helios Delta, England) at wavelengths of 260 nm and 280 nm and by agarose gel electrophoresis. Full-length complementary deoxyribonucleic acid (cDNA) prepared from

total RNA (5 µg) by the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany) and random hexamer primer according to the manufacturer's protocol.⁴¹ Then, cDNA (~ 250 ng/µl) was amplified using DreamTaq™ Green PCR Master Mix (2X) polymerase (Fermentas, Germany) and the sequence specific primers (2.5 pmol/µl of each forward and backward primer) for *CKS2* (5'-CATGAGCCAGAACCACATATTC-3' and 5'-CAGCTCATGCACAGGTATGG-3'). In order to verify the integrity of the extracted total RNA, all samples were additionally assayed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a house keeping internal control gene (5'-CAAGGTCATCCATGACAACCTTTG-3', and 5'-GTCCACCAC CCTGTTGCTGTAG-3') (Metabion International AG, Deutschland). Positive and negative controls were included as appropriate. The amplification protocol included an initial denaturation step for 10 min at 95°C followed by 40 cycles (95°C for 15 s, 60°C for 1 min, and 72°C for 1 min), and a final extension at 72°C for 10 min. The amplified products, *CKS2* (183 bp) and GAPDH (496 bp) were separated by agarose gel electrophoresis (3%), stained with ethidium bromide, and visualized by using a UV Trans-illuminator 2000 (Bio-Rad Laboratories,

Table 2. Relative *CKS2* gene expression with tumor stage and grade in all UC groups.

Parameters	Relative <i>CKS2</i> gene expression		P-value
	Number (%)	Median	
Tumor stage			
Non-muscle invasive (N = 17)	5 (29.4)	0.0	<0.001*
Muscle-invasive (N = 23)	19 (82.6)	1.50	
Tumor grade			
Low (N = 12)	2 (16.7)	0.0	<0.001*
High (N = 28)	22 (78.6)	1.50	

P: P-value for Mann Whitney test; *: Statistically significant at $P \leq 0.05$; N: Number of cases; UC: Urothelial carcinoma.

Table 3. Relation between qualitative *CKS2* gene expression with tumor stage and grade in schistosomal and non-schistosomal-associated UC groups.

Groups	Parameters	Qualitative <i>CKS2</i> gene expression		FEp
		Negative cases N (%)	Positive cases N (%)	
Non-schistosomal UC				
	Tumor stage			
	Non-muscle invasive (N = 12)	9 (75)	3 (25)	0.005*
	Muscle-invasive (N = 13)	2 (15.4)	11 (84.6)	
	Tumor grade			
	Low (N = 9)	7 (77.8)	2 (22.2)	0.017*
	High (N = 16)	4 (25)	12 (75)	
Schistosomal UC				
	Tumor stage			
	Non-muscle invasive (N = 5)	3 (60)	2 (40)	0.251
	Muscle-invasive (N = 10)	2 (20)	8 (80)	
	Tumor grade			
	Low (N = 3)	3 (100)	0 (0)	0.022*
	High (N = 12)	2 (16.7)	10 (83.3)	

FEp: P-value for Fisher's exact test; *: Statistically significant at $P \leq 0.05$; N: Number of cases; UC: Urothelial carcinoma

Italy). Photos were documented with a digital camera (HP photo Smart 735, resolution: 5.2 MP, Germany). The relative expression level⁴² of *CKS2* relative to the GAPDH expression ratio in both normal and tumor samples was quantified using the Scion image program for Windows.

Statistical analysis

Data were analyzed by Predictive Analytics Software (PASW Statistics 18). Qualitative data were described using numbers and percents. We determined associations between categorical

variables using the chi-square test. When more than 20% of the cells had an expected count less than 5, correction for chi-square was conducted with Fisher's exact test or Monte Carlo correction.

Quantitative data were described by median, minimum and maximum, as well as mean and standard deviation. The distributions of quantitative variables were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilks tests. We used the D'Agostino test if there was a conflict between the two previous tests. If the results indicated normal data distribution,

Table 4. Relation between relative *CKS2* gene expression with tumor stage and grade in schistosomal- and non-schistosomal-associated UC groups.

Groups	Parameters	Relative <i>CKS2</i> gene expression		P-value
		Number of positive cases	Median	
Non-schistosomal UC				
	Tumor stage			
	Non-muscle invasive (N = 12)	3	0.0	<0.001*
	Muscle-invasive (N = 13)	11	1.50	
	Tumor grade			
	Low (N = 9)	2	0.0	0.004*
	High (N = 16)	12	1.50	
Schistosomal UC				
	Tumor stage			
	Non-muscle invasive (N = 5)	2	0.0	0.022*
	Muscle-invasive (N = 10)	8	1.38	
	Tumor grade			
	Low (N = 3)	0	0.0	0.025*
	High (N = 12)	10	1.13	

P: P-value for Mann Whitney test; *: Statistically significant at $P \leq 0.05$; UC: Urothelial carcinoma

Table 5. Relation between N-cadherin staining index with tumor stage and tumor grade in all cases of UC groups.

Parameters	N-cadherin staining index		P-value
	Number of positive cases (%)	Median	
Tumor stage			
Non-muscle invasive (N = 17)	7 (41.2)	0.0	0.001*
Muscle-invasive (N = 23)	18 (78.3)	4.0	
Tumor grade			
Low (N = 12)	4 (33.3)	0.0	0.002*
High (N = 28)	21 (75)	4.0	

P: P-value for Mann Whitney test; *: Statistically significant at $P \leq 0.05$; UC: Urothelial carcinoma

parametric tests were applied. However, if the data were abnormally distributed, non-parametric tests were used.

For normally distributed data, comparison between two independent populations was done using the independent t-test. For abnormally distributed data, the Mann-Whitney test for data distribution that significantly deviated from the normal was used to analyze two independent populations. Significance test results were quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

Results

We observed no significant differences between the schistosomal and non-schistosomal groups in terms of age ($P=0.500$) and sex ($P=1.000$), with a male predominance in both groups (male to female ratio: 4:1).

Both non-schistosomal-associated and

schistosomal-associated UC displayed similar histopathological features (Table 1). There were no significant differences between the two groups in terms of tumor stage ($P=0.364$) and grade ($P=0.477$).

Cyclin-dependent kinases regulatory subunit 2 (CKS2) gene expression

There was no *CKS2* mRNA detected in normal bladder tissues. However, the *CKS2* gene expressed in 60% (24/40) of UC cases (14 non-schistosomal and 10 schistosomal). *CKS2* gene also expressed in 29.4% (5/17) of non-muscle-invasive tumors (3 non-schistosomal and 2 schistosomal), and in 82.6% (19/23) of muscle-invasive tumors (11 non-schistosomal and 8 schistosomal; Table 2). *CKS2* expressed in 16.7% (2/12) of low grade tumors, all of which were non-schistosomal and in 78.6% (22/28) of high grade tumors (12 non-schistosomal and 10 schistosomal;

Table 6. Relation between N-cadherin staining index to tumor stage and grade in schistosomal- and non-schistosomal-associated UC groups.

Groups	Parameters	N-cadherin		P-value
		Number of positive cases (%)	Median	
Non-schistosomal UC				
Tumor stage				
	Non-muscle invasive (N = 12)	5(41.7)	0.0	0.011*
	Muscle-invasive (N = 13)	10(77)	4.0	
Tumor grade				
	Low (N = 9)	3(33.3)	0.0	0.016*
	High (N = 16)	12(75)	4.0	
Schistosomal UC				
Tumor stage				
	Non-muscle invasive (N = 5)	2(40)	0.0	0.032*
	Muscle-invasive (N = 10)	8(80)	4.0	
Tumor grade				
	Low (N = 3)	1(33.3)	0.0	0.074
	High (N = 12)	9(75)	4.0	

P: P-value for Mann Whitney test; *: Statistically significant at $P \leq 0.05$; UC: Urothelial carcinoma

Table 7. Relation between PD-ECGF staining index with tumor stage and grade in UC cases.

Parameters	PD-ECGF		P-value
	Number of positive cases (%)	Median	
Tumor stage			
Non-muscle invasive (N =17)	7 (41.2)	0.0	0.002*
Muscle-invasive (N =23)	18 (78.3)	4.0	
Tumor grade			
Low (N =12)	4 (33.3)	0.0	0.002*
High (N =28)	21 (75)	4.0	

P: P-value for Mann Whitney test; *: Statistically significant at $P \leq 0.05$; UC: Urothelial carcinoma

Table 2). *CKS2* relative gene expression significantly upregulated in muscle-invasive and high grade UC tumors ($P < 0.001$; Table 2).

In non-schistosomal-associated UC cases, qualitative *CKS2* gene expression significantly differed with both tumor stage ($P = 0.005$) and grade ($P = 0.017$; Table 3), which was the same as relative *CKS2* gene expression for tumor stage ($P < 0.001$) and grade ($P = 0.004$; Table 4). In the schistosomal-associated UC tumors qualitative *CKS2* gene expression significantly differed only with tumor grade ($P = 0.022$; Table 3). Relative *CKS2* gene expression revealed significant differences with both tumor stage ($P = 0.022$) and grade ($P = 0.025$; Table 4).

Relative *CKS2* gene expression significantly upregulated in tumor stage for the non-schistosomal ($P < 0.001$) and schistosomal ($P = 0.022$) groups as well as in the high-grade tumors for the non-schistosomal ($P = 0.004$) and

schistosomal ($P = 0.025$; Table 4) groups. Qualitative ($P = 0.422$) and relative ($P = 0.861$) expression levels of *CKS2* did not significantly differ with schistosomal infestation.

Neural cadherin (N-cadherin)

Normal bladder tissues had no detectable N-cadherin expression. We detected N-cadherin expression in 62.5% (25/40) of UC cases, 15 non-schistosomal and 10 schistosomal. N-cadherin staining index significantly increased in muscle-invasive tumors compared to non-muscle-invasive tumors ($P = 0.001$; Table 5; Figure 1). Low grade UC had a significantly lower N-cadherin staining index compared to the high-grade tumors ($P = 0.002$; Table 5; Figure 1).

A significant relation existed between the staining index and both tumor stage and tumor grade in both schistosomal- and non-schistosomal-associated UC ($P = 0.032$ & $P = 0.011$; respectively)

Table 8. Relation between PD-ECGF staining index with tumor stage and grade in schistosomal- and non-schistosomal-associated UC groups.

Group	Parameters	PD-ECGF		P-value
		Number of positive cases (%)	Median	
Non-schistosomal UC				
Tumor stage				
	Non-muscle invasive (N =12)	5 (42)	0.0	0.014*
	Muscle-invasive (N =13)	10 (77)	4.0	
Tumor grade				
	Low (N = 9)	3 (33.3)	0.0	0.016*
	High (N =16)	12 (75)	4.0	
Schistosomal UC				
Tumor stage				
	Non-muscle invasive (N = 5)	2 (40)	0.0	0.075
	Muscle-invasive (N =10)	8 (80)	6.50	
Tumor grade				
	Low (N = 3)	1 (33.3)	0.0	0.099
	High (N =12)	9 (75)	5.0	

P: P-value for Mann Whitney test; *: Statistically significant at $P \leq 0.05$; UC: Urothelial carcinoma

Table 9. Correlation between N-cadherin and PD-ECGF immunohistochemical distribution in schistosomal- and non-schistosomal-associated UC groups

Groups	PD-ECGF	N-cadherin				Total	MCPc
		Negative No (%)	Positive				
			Weak No (%)	Moderate No (%)	Strong No (%)		
Non-schistosomal UC							
	Negative	10 (100)	0 (0)	0 (0)	0 (0)	10	
	Positive						
	Weak	0 (0)	1 (50)	1 (50)	0 (0)	2	
	Moderate	0 (0)	2 (25)	4 (50)	2 (25)	8	<0.001*
	Strong	0 (0)	0 (0)	1(20)	4 (80)	5	
	rs (p)	0.923* (<0.001)					
Schistosomal UC							
	Negative	5 (100)	0 (0)	0 (0)	0 (0)	5	
	Positive						
	Weak	0 (0)	0 (0)	1 (100)	0 (0)	1	
	Moderate	0 (0)	2 (66.7)	0 (0)	1 (33.3)	3	<0.001*
	Strong	0 (0)	0 (0)	4 (66.7)	2 (33.3)	6	
	rs (p)	0.831* (<0.001)					

MCPc: *P*-value for Monte Carlo test; rs: Spearman coefficient; *: Statistically significant at $P \leq 0.05$; UC: Urothelial carcinoma

(Table 6). Also, we observed a significant positive relation between N-cadherin expression and higher grade in the non-schistosomal group ($P=0.016$), while it was insignificant in the schistosomal group ($P=0.074$; Table 6).

Platelet-derived endothelial cell growth factor (PD-ECGF)

We detected positive immunostaining for PD-ECGF in the cytoplasm, nucleus or both of examined cells. Immunoreactivity was also present in inflammatory and stromal cells. We included the appropriate positive (breast carcinoma) and negative (carried out by omitting the primary antibodies) controls in each staining round. PD-ECGF immunostaining was negative in normal bladder tissues. We observed positive staining in 62.5% (25/40) of UC (15 non-schistosomal and

10 schistosomal). The PD-ECGF staining index significantly increased in muscle-invasive tumors compared to non-muscle-invasive tumors ($P=0.002$; Table 7; Figure 2). Low grade UC had a significantly lower PD-ECGF staining index compared to high grade tumors ($P=0.002$; Table 7; Figure 2).

We observed a significant positive relation between PD-ECGF staining and tumor stage in the non-schistosomal group ($P=0.014$); this relation was absent in the schistosomal group ($P=0.075$; Table 8). We observed the same significant positive relation between the PD-ECGF staining index and tumor grade in the non-schistosomal group ($P=0.016$). This finding was insignificant for schistosomal tumors ($P=0.099$; Table 8).

Correlation between neural cadherin (N-cadherin)

Table 10. Relation between relative CKS2 gene expression, N-cadherin, and PD-ECGF with tumor recurrence and tumor progression in all UC cases.

Parameters	Relative CKS2 gene expression		N-cadherin staining		PD-ECGF staining	
	Median	<i>P</i> -value	Median	<i>P</i> -value	Median	<i>P</i> -value
Tumor recurrence						
Negative (N = 8)	0.0		0.0		0.0	
Positive (N = 9)	0.10	0.017*	2.0	0.002*	3.0	0.002*
Tumor progression						
Negative (N = 13)	0.0		0.0		0.0	
Positive (N = 4)	0.32	<0.001*	2.50	0.065	4.50	0.041*

P: *P*-value for Mann Whitney test; *: Statistically significant at $P \leq 0.05$; UC: Urothelial carcinoma

Table 11. Relation between relative *CKS2* gene expression, N-cadherin, and PD-ECGF staining with tumor recurrence and tumor progression in schistosomal and non-schistosomal-associated UC groups.

Parameters	Relative <i>CKS2</i> gene expression		N-cadherin staining		PD-ECGF staining	
	Median	<i>P</i> -value	Median	<i>P</i> -value	Median	<i>P</i> -value
Non-schistosomal UC						
Tumor recurrence						
Negative (N = 6)	0.0	0.059	0.0	0.007*	0.0	0.007*
Positive (N = 6)	0.05		2.50		3.0	
Tumor progression						
Negative (N = 10)	0.0	0.005*	0.0	0.055	0.0	0.030*
Positive (N = 2)	0.33		4.0		4.50	
Schistosomal UC						
Tumor recurrence						
Negative (N = 2)	0.0	0.197	0.0	0.197	0.0	0.197
Positive (N = 3)	0.15		1.0		3.0	
Tumor progression						
Negative (N = 3)	0.0	0.053	0.0	0.519	0.0	0.519
Positive (N = 2)	0.30		1.50		3.0	

P: *P*-value for Mann Whitney test; *: Statistically significant at $P \leq 0.05$; UC: Urothelial carcinoma

and platelet-derived endothelial cell growth factor (PD-ECGF) immunostaining index

There was a significant direct correlation between N-cadherin and the PD-ECGF immunostaining index ($r=0.831$; $P<0.001$; Table 9).

Recurrence and progression

We observed recurrence in 52.9% (9/17) of non-muscle-invasive tumors (6 non-schistosomal and 3 schistosomal) within 3-36 months. Four out of the recurrent five high grade tumors (2 non-schistosomal and 2 schistosomal) progressed to the muscle-invasive stage.

We detected *CKS2* gene expression in 55.6% (5/9) of recurrent tumors (3 non-schistosomal and 2 schistosomal) from which four cases (2 non-schistosomal and 2 schistosomal) progressed to the muscle-invasive stage. A significant relation existed between relative *CKS2* gene expression and both tumor recurrence ($P=0.017$) and tumor progression ($P<0.001$; Table 10). Although there was greater *CKS2* gene expression in the recurrent non-schistosomal ($P=0.059$) and schistosomal cases ($P=0.197$) this was not a statistically significant finding (Table 11). We detected *CKS2* gene expression in the 4 recurrent high grade non-muscle-invasive tumors that progressed to the muscle-invasive stage with a significant relation between relative gene expression and

tumor progression in the non-schistosomal group ($P=0.005$; Table 11).

There were 7 out of 9 (77.8%) recurrent tumors that expressed N-cadherin, 5 non-schistosomal (2 low grade and 3 high grade tumors) and 2 schistosomal (one low grade and one high grade tumor). Of these, 3 of the recurrent N-cadherin positive cases progressed to the muscle-invasive stage (2 non-schistosomal and one schistosomal). We observed a positive relation between N-cadherin staining and tumor recurrence ($P=0.002$). However, its expression was insignificant with tumor progression ($P=0.065$; Table 10). A positive relation existed between N-cadherin staining and tumor recurrence in the non-schistosomal group ($P=0.007$; Table 11). Although N-cadherin expression in patients that had a shift to muscle-invasive cancer was higher ($P=0.055$) than in those without muscle invasion ($P=0.519$), this was not statistically significant in both groups (Table 11).

PD-ECGF immunostaining was found in 77.8% (7/9) of recurrent tumors, 5 non-schistosomal (2 low grade and 3 high grade tumors) and 2 schistosomal (one low grade and one high grade tumor). Of these, 3 of the recurrent PD-ECGF positive cases progressed to the muscle-invasive stage (2 non-schistosomal and one schistosomal). We observed a positive relation

between PD-ECGF immunohistochemical expression and tumor recurrence ($P=0.002$) as well as tumor progression ($P=0.041$; Table 10). There was a positive relation between PD-ECGF expression and both tumor recurrence ($P=0.007$) and tumor progression ($P=0.030$) in the non-schistosomal group (Table 11).

Discussion

The current study was the first to investigate the relation between *CKS2*, N-cadherin, and PD-ECGF expression in BC. It documented a positive correlation between N-cadherin, and PD-ECGF proteins in schistosomal- and non-schistosomal-associated BC. It revealed over expressions of relative *CKS2* gene, N-cadherin, and PD-ECGF proteins in the invasive stages and higher grades of BC. We detected differential expression of N-cadherin, PD-ECGF proteins, tumor recurrence, and tumor progression between schistosomal-associated and non-schistosomal-associated BC.

Similarly, comparative studies between schistosomal-associated and non-schistosomal-associated tumors showed distinct biochemical and molecular profiles of tumor development and progression. It was suggested that variations might reflect different etiologies and/or risk factors, different pathological and genetic mechanisms that might have relevant clinical and therapeutic implications.^{6,43-50} On the other hand, other studies showed that most of the detected genetic imbalances in schistosomal-associated BC were repeatedly reported in non-schistosomal BC, which has suggested that the cytogenetic profiles of chemical and schistosomal-induced carcinomas are largely similar.⁵¹⁻⁵³

In this study, *CKS2* gene expression was only detectable in bladder tumors. Microarray analysis and/or real-time RT-PCR studies⁵⁴⁻⁵⁶ showed significant upregulation of *CKS2* gene expression in BC compared to normal bladder tissue and thus suggested the aberrant *CKS2* gene expression as a diagnostic biomarker for BC initiation.

Significant elevated relative *CKS2* gene expression is detected in invasive stages and higher grades. Similarly, Kawakami et al.,⁵⁴

showed a unique association between *CKS2* gene expression and tumor stage, which suggested that this gene could be a biomarker for staging of BC. Mezzasoma et al.,⁵⁵ studied the sediments of bladder washings and found that the *CKS2* transcript level quantified by RT-PCR significantly up-regulated in superficial BC, which remained almost unchanged in the invasive ones, thus suggesting a possible involvement of this gene in the early events during tumor development. The discrepancy between this study and ours might be due to the different methodologies, the use of different gene sequences, and the different analyzed specimens. In fact, the results that emerged from tissue analysis were not always paralleled by the same significance in other samples.

Overexpression of *CKS2* has been reported to be associated with poor differentiation features and high aggressiveness in several tumors including meningioma, hepatocellular carcinoma, esophageal carcinoma, gastric cancer, colorectal cancer and laryngeal cancer.⁵⁷⁻⁶²

In the current work, *CKS2* gene expression significantly related to tumor recurrence and tumor progression. On the other hand, we observed a significant relation between *CKS2* gene expression and tumor progression in the non-schistosomal group, whereas in the schistosomal group there was no significant relation with progression noted. This might be due to the smaller number of cases in the schistosomal group. A microarray study by Kawakami et al.⁵⁴ confirmed the association of *CKS2* gene expression with tumor progression and clinical outcomes. The researchers found that the difference in the *CKS2* expression level between invasive BC and normal bladder tissue was greater than between superficial BC and normal bladder tissue, which suggested that *CKS2* expression might influence BC progression via cell cycle advancement. It was suggested that deregulations of cell cycle genes were necessary for urothelial transformation.⁶³

The involvement of cadherins in the regulation of diverse cellular processes, particularly in the progression, invasion, and metastasis of

transitional cell cancer of the bladder, prompted many researchers to study their expression patterns and association with invasive phenotypes of cancer cells. In our study, N-cadherin expression expressed in 62.5% of BC specimens but not in any of the controls. Although Rieger-Christ et al.⁶⁴ detected N-cadherin expression in 39% of tumors. Both Rieger-Christ et al.⁶⁴ and Lascombe et al.⁶⁵ showed that N-cadherin did not express in normal urothelium but was observed only in neoplastic cases. Increased expression of N-cadherin has been reported in bladder tumors,⁶⁶⁻⁶⁸ UC of the upper urinary tract,⁶⁹ renal cell carcinomas,⁷⁰ and prostate carcinoma.^{18,71-74}

In the present study, we detected significantly increased expression of N-cadherin in muscle-invasive compared to non-muscle-invasive tumors in both non-schistosomal and schistosomal tumors. Lascombe et al.⁶⁵ recorded membranous N-cadherin immunostaining in 14% of pT1 tumors and 60% of pT2-T3 tumors. A review by Bryan and Tselepis⁶⁸ of cadherin expression in BC stated that over 80% of Ta/T1 tumors lacked N-cadherin expression. However, over 60% of advanced muscle-invasive BC expressed N-cadherin. On the other hand, in a more extensive microarray study conducted by Baumgart et al.,⁷⁵ the authors recorded N-cadherin expression in only 8.2% of bladder tumors. Expression of N-cadherin was observed to be focal in nature throughout the tissue microarray and its expression was recorded approximately equally in the superficial and invasive tumor groups. The discrepancy between our results and this study might be related to the limited specimens as the researchers used core biopsies.

The current study showed a significant positive relation between N-cadherin expression and tumor grade. Similarly, Cui et al.⁷⁶ reported higher expression of N-cadherin in the poorly differentiated cases of BC. On the other hand, Lascombe et al.⁶⁵ and Baumgart et al.⁷⁵ showed that expression of N-cadherin had no significant association with BC grade. The discrepancy between these findings might be explained by using different immunostaining methods, antigen

retrieval, and interpretation of the immunostaining reactivity.

Upon stratification of non-schistosomal and schistosomal tumors, we detected a significant positive relationship between N-cadherin staining index and high grade only in the non-schistosomal group. The insignificant relation between the expression of N-cadherin and BC grade in schistosomal tumors might be due to the low number of cases in this group attributed to schistosomal eradication programs.

There was a positive relation between N-cadherin expression and tumor recurrence in the present study. We observed an insignificantly higher N-cadherin expression in patients with progressive cancer. However, upon stratification of both studied groups, any positive relation between N-cadherin expression and tumor recurrence existed only in the non-schistosomal group. There was no significant relation noted between N-cadherin expression and tumor progression in both groups.

Muramaki et al.⁷⁷ investigated the impact of N-cadherin expression in non-muscle-invasive BC on the probability of intravesical recurrence and recorded positive expression in 41.7% from which 68.8% of these developed intravesical recurrence. They suggested that N-cadherin expression was the most powerful independent predictor for intravesical recurrence following TUR on multivariate analysis. Lascombe et al.⁶⁵ reported that N-cadherin expression correlated with a significant decrease in survival without progression. The authors analyzed N-cadherin mRNA expression by RT-PCR analysis and evaluated protein expression by immunohistochemistry in non-muscle-invasive and muscle-invasive bladder tumors. The authors found that superficial tumors which expressed N-cadherin could recur more frequently or progress to a more invasive stage compared with those that did not express N-cadherin. The authors found that N-cadherin expression was a prognostic marker of progression for pT1 tumors, which identified this adhesion molecule as a predictive factor of pT1 tumor progression. This confirmed

the relevance of cell adhesion molecule expression to the clinical and biological behavior of superficial bladder tumors. In the current study, we found a significant relation between N-cadherin expression with tumor recurrence in the 17 non-muscle-invasive cases and in the non-schistosomal non-muscle-invasive tumors, but we did not observe any significant relation with progression. The discrepancy between our work and the Lascombe et al.⁶⁵ study might be explained using additional RT-PCR for N-cadherin detection and different staining methodologies. The small sample size in the current work might also be a factor.

Several investigators report that tumor angiogenesis is important for continued tumor growth and progression. It has been suggested that angiogenic capacity is an early marker of preneoplastic and neoplastic lesions.⁷⁸⁻⁸⁰ In the present work PD-ECGF immunostaining was negative in normal urothelium and positive in 62.5% (25/40) of the examined tumor specimens. Some studies showed higher PD-ECGF expression in bladder TCC compared to normal bladder mucosa.⁸¹⁻⁸³

In the present study, we detected a significant positive relation between PD-ECGF staining index and tumor stage. O'Brien et al.^{84,85} and Arima et al.⁸⁶ observed higher PD-ECGF expression in invasive bladder tumors compared to superficial tumors. They suggested that the angiogenic pathway associated with PD-ECGF expression and the molecular genetic changes of chromosome 17p might be deeply involved in the development of invasive cancer. Sawase et al.⁸⁷ and Li et al.⁸² found significantly higher expression of PD-ECGF/TP in T1 BC compared to Ta BC. These results suggested that submucosal infiltration of cancer cells might be the first step in inducing the expression of PD-ECGF/TP. In the present study, the single pTa included tumor was negative. Previous reports on TCCs found a correlation between angiogenesis, tumor stage, and lymph node metastasis.⁸⁸⁻⁹⁰ Tanaka et al.⁹¹ found that PD-ECGF/TP expression correlated significantly with depth of invasion and lymphatic invasion.

All positive cases in both non-schistosomal and schistosomal tumors showed moderate to strong PD-ECGF immunostaining reaction, with a significant positive relation between PD-ECGF expression and tumor stage in the non-schistosomal group ($P=0.014$), while in the schistosomal group this finding did not reach statistical significance ($P=0.075$). This might be due to the smaller number of cases. Also, the presence of schistosomal infection might be a factor.

In the current study, we detected a significant positive relation between PD-ECGF staining index and tumor grade. Many studies on BC that used RT-PCR and immunohistochemistry showed that increased PD-ECGF/TP expression correlated to high tumor grade.^{81,82,85-87,92-97}

We found a significant positive relation between PD-ECGF staining index and high grade in the non-schistosomal group ($P=0.016$). On the other hand, although we found a higher expression of PD-ECGF in high grade schistosomal tumors, this was not statistically significant ($P=0.099$).

In the present study, we detected a positive relation between PD-ECGF immunohistochemical expression and tumor recurrence as well as tumor progression. Tumors that presented with PD-ECGF immunoreactivity progressed more rapidly compared with tumors that did not express this marker.

The non-schistosomal group showed a significant relation between PD-ECGF expression and both tumor recurrence and tumor progression. This was an insignificant finding in the schistosomal group. Li et al.^{82,95} showed higher PD-ECGF/TP expression in cases which recurred after transurethral resection (TUR). Aoki et al.⁹⁷ suggested that PD-ECGF/TP expression might be a prognostic marker for predicting recurrence in primary superficial BC that included both pTa and pT1. O'Brien et al.⁸⁵ suggested that PD-ECGF/TP facilitated the transition from carcinoma *in situ* to invasive cancer.

Mizutani et al.⁹² and Sawase⁸⁷ reported that elevated PD-ECGF/TP expression correlated with stage progression. Mizutani et al.⁹² reported that

pTa bladder carcinoma patients with low PDECGF/TP expression had a longer tumor-free interval compared with those with high PDECGF/TP expression in a 3-year follow-up. These findings suggested that PDECGF/TP might play an important role in the establishment and growth of BC and might be a poor prognostic factor. Also, a study by Kubota et al.⁹³ showed that among the invasive grade 3 bladder tumors, the mean activity of PDECGF/TP seemed to increase with the depth of invasion. They suggested that PDECGF/TP might be involved in facilitating the progression of high-grade BC to invasive disease.

A comparison of non-schistosomal-associated and schistosomal-associated UC showed no significant differences between both groups regarding *CKS2* gene expression, N-cadherin, and PD-ECGF immunohistochemical expression. The absence of significant differences might reflect the proposal of changing patterns and histopathological profile of BC over the past 26 years. These changes were indicative of changes in exposures related to BC induction such as increased cigarette smoking and chemical exposures.⁹⁸ Through the years, schistosomal-associated BC was believed to be a unique disease entity different from urothelial cancer. As carcinogenesis is a highly complex process that results from the accumulation of many genetic and epigenetic changes which lead to alterations in cell proliferation and regulation process, confirmation of their minute differences or similarities are extremely difficult. In BC, many of these carcinogenic cascades have not been fully documented despite researchers' efforts. The control of schistosomiasis and subsequent decrease in the intensity of infestation showed feature changes that approached non-schistosomal urothelial tumors. However, schistosomal-associated BC still presents in more advanced stages than schistosomal non-associated urothelial cancer. Numerous data have proven that, upon applying the same treatment protocol and management care, a stage-by-stage comparison of the treatment end results were found to be similar

in BC patients with different etiologies.⁴⁶

We observed a strong correlation between the immunohistochemical expression of both N-cadherin and PD-ECGF in both groups. Kelland⁹⁹ reported that N-cadherin played a role in the maintenance of tumor vasculature and tumor angiogenesis. Hence, abnormal activation of N-cadherin, would be a subsequent event which promoted angiogenesis and tumor progression.^{100,101}

Conclusion

CKS2, N-cadherin, and PD-ECGF may be used as biomarkers to predict BC outcome and aid in selecting patients for more aggressive treatments.

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Conflict of Interest

No conflict of interest is declared.

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