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Prognostic and predictive values of human enhancer of filamentation-1 (HEF-1) and Sparc/osteonectin, cwcv, and kazallike domains proteoglycan 1 (SPOCK-1), in transitional cell carcinoma (TCC) of the urinary bladder: An immunohistochemical study

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ABSTRACT

Background: Transitional cell carcinoma (TCC) of the bladder forms about 90% of all bladder cancer cases. There is a need to discover novel management strategies for such cancer as it has a high incidence, high invasion, and metastasizing rates. Human enhancer of filamentation-1 (HEF-1), which is a cytoskeletal protein is an important signaling mediator of the plethora of normal cellular events: Sparc-osteonectin; cwcv & kazallike; domains-proteoglycan 1 (SPOCK-1) have many roles in normal cell cycle control, DNA repair and apoptosis. The aim of the work was to detect expression of HEF-1 and SPOCK-1 in TCC cells using immunohistochemistry, to correlate combined expression of those markers with pathological, clinical, and prognostic parameters of patients with this cancer type.

Methods: We evaluated HEF-1 and SPOCK-1 expression in sections from 40 paraffin blocks from 40 TCC patients using immunohistochemistry. Then we have correlated the combined expression of those markers with pathological, clinical, and prognostic parameters of TCC patients.

Results: The expression of HEF-1 in TCC was significantly positively associated with lower degree of differentiation and advanced stage of the cancer ($p < 0.001$), presence of distant metastasis, vascular invasion ($p = 0.031$), perineural invasion ($p = 0.004$), presence of adjacent areas of carcinoma *in situ* ($p = 0.041$), and higher mitotic index ($p = 0.003$). The expression of SPOCK-1 in TCC was significantly positively associated with lower degree of differentiation and advanced stage of the cancer ($p < 0.001$), presence of distant metastasis, vascular invasion ($p = 0.031$), perineural invasion ($p = 0.004$), presence of adjacent areas of carcinoma *in situ* ($p = 0.041$), and higher mitotic index ($p = 0.003$). Increased HEF-1 and SPOCK-1 expression was associated with poor survival rates and patients outcome ($p = 0.041$ and <0.001, respectively). A positive correlation between HEF-1 and SPOCK-1 expression in TCC was found ($p = 0.007$).

Conclusion: HEF-1 and SPOCK-1 have been considered markers of poor prognosis in patients with TCC.

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KEYWORDS

HEF-1; SPOCK-1; immunohistochemistry; survival; transitional cell carcinoma; prognosis

Introduction

Cancer bladder cancer is considered the commonest urological cancer, sixth commonest cancer in males and eighth leading cause of cancer death in men worldwide [1]. Males are affected with such type of cancer three times more than females [2]. Moreover, cancer bladder is considered the commonest urological cancer forming 90.71% and forms the third among cancers of all other organs [3]. Transitional cell carcinoma (TCC) forms 90% of bladder cancer cases [4], so it is considered the commonest among all subtypes of bladder cancer [5]. There is a need

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to discover novel management strategies for such cancer as it has high incidence, high invasion, and metastasizing rates [6]. Therefore, it will be beneficial to identify the molecular pathogenesis and biomarkers that are responsible for invasion, recurrence, and spread of TCC aiming at improving the management strategies of such cancer.

The epithelial mesenchymal transition (EMT) is the process that is extensively studied and by which cancer cells lose their epithelial criteria and transformed to mesenchymal-like cells that have more liability for invasion of the extracellular environment and metastases [7]. Several biomarkers are recently discovered to control such processes.

Human enhancer of filamentation-1 (HEF-1), which is also named neural precursor cell-expressed developmentally downregulated protein 9 (NEDD-9), is a recently detected cytoskeletal protein and a signaling mediator of plethora of normal cellular events activates, e.g., cell adhesion, apoptosis, cell cycle regulation, and even in oncogenesis [8]. The Sparc/osteonectin, cwcv, and kazallike domains proteoglycan 1 (SPOCK-1) is a protein that is primarily detected in normal human seminal vesicles [9]. SPOCK-1 has plethora of roles in control of cell-cycle, DNA repair, and apoptosis [10]; additionally, it encodes a calcium-binding glycoprotein member of the SPARC family that plays a role in protease activity modulation [11]. The role of tissue protein expression of HEF-1 and SPOCK-1 in TCC and the mechanisms by which they lead to cancer initiation, progression, invasion, and spread of TCC is not fully clarified in the previous studies.

The aim of the work was to detect expression of HEF-1 and SPOCK-1 in TCC cells using immunohistochemistry, to correlate combined expression of those markers with pathological, clinical, and prognostic parameters of patients with this cancer type.

Patients and Methods

This is a retrospective cohort study. We have included sections from 40 paraffin blocks of which were retrieved from 40 patients with TCC of the urinary bladder. The sections of all blocks collected from Pathology Department, Faculty of Medicine, Zagazig University archives in the period from May 2015 to May 2018 were assessed reevaluated, graded using World Health Organization grading system and staged using tumor node metastases (TNM) staging system by tewo senior pathologists from Pathology Department, Faculty of Medicine, Zagazig University. We have chosen all the 40 cases of TCC subtype excluding other subtypes [12,13]. We have evaluated patients' age, sex, size, grade, stage, degree of muscle invasion, presence of vascular invasion, perineural invasion, areas of nearby carcinoma *in situ*, and distant metastasis.

We have followed our patients till death in oncology departments. Our 40 patients had follow-up records for 3 years from May 2015 to May 2018. We assessed the overall survival, recurrence-free survival, and distant metastases free survival rates (DMFS) and then we have correlated all follow-up data with pathological parameters and both HEF-1 and SPOCK-1 expressions.

Immunohistochemical method

The used immunohistochemical method was the technique of streptavidin–biotin immunoperoxidase [14]. We have cut sections of paraffin blocks of all cases, the thickness of the sections were about 3–5 μm, then we had mounted sections on positively charged glass slides, de-paraffinized them in xylene, and then we had rehydrated them in ethyl alcohol of ascending grades. In the subsequent steps we have boiled sections in citrated buffer for about 20 minutes, washed them in phosphate-buffered saline. We are blocking the activity of endogenous peroxidase with H_2O_2 6% in methyl alcohol. We have incubated the slides with mouse monoclonal anti-HEF-1 antibody (clone ab18056, Abcam, Cambridge, UK; dilution 1:100), and anti-SPOCK antibody (novusbio.com/NBP1-30603; dilution 1:500) overnight. We have incubated sections with a secondary antibody, and as chromogen, we have used the diaminobenzidine substrate. Finally, we have incubated sections with Mayer's hematoxylin as a counterstain. Sections from pancreatic adenocarcinoma and normal human seminal vesicles were used as positive controls for HEF-1 and SPOCK-1, respectively, then negative control by omitting the primary antibodies and replacing them with phosphate buffered saline [15].

Evaluation of immunostaining intensity of HEF-1 proteins

We have assessed only cytoplasmic HEF-1 expression in TCC tissues and neglected any nonspecific membranous staining. We have assessed only nuclear SPOCK-1 expression in TCC tissues and neglected any nonspecific membranous or cytoplasmic staining. We have scored the immunostaining degree depending on the intensity and extent of cytoplasmic HEF-1 and SPOCK-1 stains. Stain intensity was considered: 0 (if no stain); 1 (if weak stain);

2 (if moderate stain); and 3 (if strong stain). Stain extent was considered 0, if positive cells are less than 1%; 1—if positive cells are 2%–25%; 2—if positive cells are 26%–50%; 3—if positive cells are 51%–75%; and 4—if positive cells are more than 75%. The total final HEF-1 and SPOCK-1 stain scores have been calculated by multiplying the intensity and extent where scores from 0 to 12 are achieved. For adequate statistical analysis, the cut-off value used was 4; tumors of scores more than 4 are considered with high HEF-1 and SPOCK-1 expression; while tumors of scores less than 4 are considered of low HEF-1 and SPOCK-1 expression [16,17].

Statistical analysis

Program used in statistics was SSPSS 22.0 for windows (SPSS Inc., Chicago, IL). Continuous variables expression was calculated by the mean ± standard deviation (SD) & median (range). Categorical variables expression was calculated by a number (%). Categorical variables comparison was done by using Pearson's Chi-square test. Strength of relationship between HEF-1 & SPOCK-1 and clinicopathological data was assessed by computing Spearman's correlation coefficient. We have used the Kaplan–Meier method for survival curves. Finally, we have considered a p-value of <0.05 is statistically significant in all analyzed data.

Results

We have included 40 patients, 25 males, and 15 females in this study, their demographic data are shown in Table 1.

HEF-1 expression and relations to clinicopathological data

HEF-1 expression in TCC of the urinary bladder was associated with older age of the patients ($p = 0.035$), higher tumor grade and advanced TNM stage ($p < 0.001$), more liability to the presence of distant

Table 1. Demographic data.

Characteristics	Number	%	Characteristics	Number	%
Age (year)			Vascular invasion		
Mean ± SD	58.42 ± 10.18		Absent	30	75
Median (Range)	58 (38–78)		Present	10	25
≤60	20	50			
>60	20	50			
Sex			Mitotic rate		
Male	30	75	<10	12	38
Female	10	25	>10	28	62
Multiplicity			Perineural invasion		
Absent	30	75	Absent	24	68
Present	10	25	Present	16	32
Adjacent carcinoma in situ			Distant metastases		
Absent	30	76	Absent	30	80
Present	10	24	Present	10	10
Growth pattern			Lymph node		
Solid	5	12.5	Negative	23	66
Papillary	35	88.5	Positive	17	34
Size (cm)			Stage		
Mean ± SD	4.84 ± 3.84		Stage I	8	16
Median (Range)	3 (2–12)		Stage II	12	26
≤5	25	68	Stage III	10	38
>5	15	32	Stage IV	10	20
Grade			HEF-1		
Low grade	12	34	Low	18	45
High grade	28	66	High	22	55
T			SPOCK		
Ta	5	14	Low	17	44
T1	20	48	High	23	56
T2	10	24			
T3	5	14			
Deep invasion			Outcome		
Absent	21	62	Disease free (DF)	17	34
Present	19	38	Local recurrence (LR)	23	66
			Distant metastasis (DM)	10	34
			Died	12	36

Table 2. Correlation between clinicopathological features, and HEF-1 expression in our patients.

Characteristics	All		HEF-1				*p*-value
	N = 50		Low (*N* = 18)		High (*N* = 22)		
	No.	%	No.	%	No.	%	
Age (years)							
Mean ± SD	58.42	±10.18	58.42	±11.14	54.96	±9.31	0.226•
Median (range)	58	(38–78)	64	35–75	50	40–72	
≤60	20	50	6	30	14	70	0.035‡
>60	20	50	12	60	8	40	
Sex							
Male	30	75	15	39.5	15	60.5	0.520‡
Female	10	25	3	50	7	50	
Multiplicity							
Absent	30	75	18	48.6	12	51.4	0.108‡
Present	10	25	0	00.0	10	76.9	
Adjacent carcinoma in situ							
Absent	30	76	18	65	12	35	0.041‡
Present	10	24	0	00.0	10	100	
Growth pattern							
Solid	5	12.5	1	57.1	4	42.9	0.434‡
Papillary	35	88.5	17	39.5	18	60.5	
Size (cm)							
Mean ± SD	4.74	±2.84	4.80	±3.02	4.68	±2.57	0.763•
Median (Range)	3	2–12	3	2–10	3	1–11	
≤5	25	68	10	41.2	15	58.8	0.863‡
>5	15	32	8	43.8	7	56.3	
Grade							
Low grade	12	34	10	82.4	2	17.6	<0.001‡
High grade	28	66	8	21.2	20	78.8	
T							
Ta	5	14	3	71.4	2	28.6	0.026•
T1	20	48	10	45.8	10	54.2	
T2	10	24	4	23.3	6	66.7	
T3	5	14	1	14.3	4	85.7	
Deep invasion							
Absent	21	62	16	51.6	5	48.4	0.079‡
Present	19	38	2	26.3	17	73.7	
Vascular invasion							
Absent	30	75	16	50	14	50	0.031‡
Present	10	25	2	10	8	90	
Mitotic rate							
<10	12	20	10	68.4	2	31.6	0.003‡
>10	28	70	8	25.8	20	74.2	
Perineural invasion							
Absent	12	38	5	55.9	7	44.1	0.004‡
Present	28	62	13	12.5	15	87.5	
Distant metastases							
Absent	24	68	14	50	10	50	0.031‡
Present	16	32	4	10	12	90	
Lymph node							
Negative	30	80	18	51.5	12	48.5	0.058‡
Positive	10	10	0	23.5	10	76.5	
Stage							
Stage I	7	20	3	87.5	4	12.5	0.001•
Stage II	7	20	4	46.2	3	53.8	
Stage III	10	22	5	36.8	5	63.2	
Stage IV	16	38	6	10	10	90	
SPOCK							
Low	17	45	10	59.3	7	40.7	0.007‡
High	23	55	8	21.7	15	78.3	

*Independent samples Student's t-test; •Mann Whitney *U* test; ‡Chi-square test; §Chi-square test for trend.

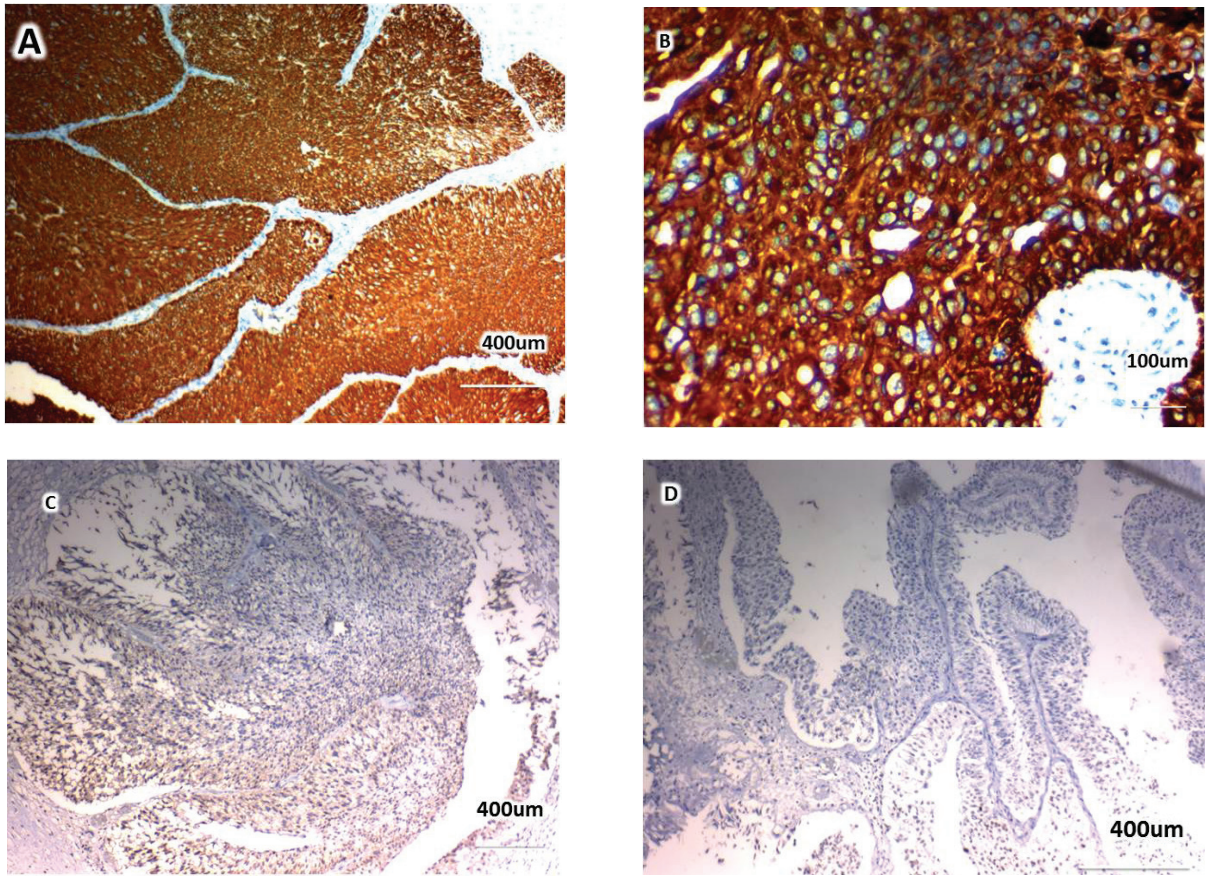

Figure 1. Immunohistochemical expression of HEF-1 (in the cytoplasm) in TCC of the urinary bladder, (A and B) high HEF-1 expression in deep muscle invasive high-grade TCC, (C and D) low HEF-1 expression in non-muscle-invasive low grade TCC. Magnification: (A) the original magnification was ×100; (B–D) the original magnification was ×400.

metastasis, higher incidence of vascular invasion ($p = 0.031$), higher incidence of perineural invasion ($p = 0.004$), higher incidence of presence of areas of adjacent carcinoma *in situ* ($p = 0.041$), deep invasion of the tumor or lymph node metastases, and higher Ki67 mitotic rate ($p = 0.003$).

There were no significant associations between HEF-1 expression, patient sex, tumor multiplicity, tumor growth pattern, whether nodular or papillary, or larger tumor size (Table 2 and Fig. 1).

SPOCK-1 immunoexpression and its correlation clinicopathological features

SPOCK-1 expression in TCC of the urinary bladder was associated with older age of the patients ($p = 0.035$), higher tumor grade and advanced TNM stage ($p < 0.001$), more liability to the presence of distant metastasis, higher incidence of vascular invasion ($p = 0.031$), higher incidence of perineural invasion ($p = 0.004$), higher incidence of presence of areas of adjacent carcinoma *in situ* ($p = 0.041$), deep invasion of the tumor or lymph node metastases, and higher Ki67 mitotic rate ($p = 0.003$).

There were no significant associations between SPOCK-1 expression, patient sex, tumor multiplicity, tumor growth pattern; whether nodular or papillary or with larger tumor size (Table 3 and Fig. 2).

The expression of both HEF-1 and SPOCK-1 was positively correlated with each other $p = 0.007$.

Survival and follow-up results

The 3-year overall survival (OS) of our patients was 52% in all cases, 44.1% and 82.4% in high and low HEF-1 expressions, respectively, and 20.5% and 82.4% in high and low SPOCK-1, respectively, the 3-year OS is inversely related to high HEF-1 immunoreactivity and high SPOCK-1 immunoreactivity ($p = 0.041$ and <0.001, respectively).

The 3-year DMFS of our cases was 65.6% in all cases, 47.6% and 100% in high and low SPOCK-1 expressions, respectively, and 31.2% and 92.7% in

Table 3. Correlation between clinicopathological features and SPOCK1 expression in our patients.

Characteristics	All		SPOCK-1				*p*-value
	N = 50		Low (*N* = 17)		High (*N* = 23)		
	No.	%	No.	%	No.	%	
Age (years)							
Mean ± SD	58.42	±10.18	55.25	±9.78	57.78	±10.62	0.387*
Median (Range)	58	38–78	50	40–75	60	35–72	
≤60	20	50	6	30	14	(70	0.297‡
>60	20	50	11	60	9	(40	
Sex							
Male	30	75	14	39.5	16	60.5	0.730‡
Female	10	25	3	50	7	50	
Multiplicity							
Absent	30	75	17	48.6	13	51.4	0.001‡
Present	10	25	0	00.0	10	76.9	
Adjacent carcinoma in situ							
Absent	30	76	17	65	13	35	<0.001‡
Present	10	24	0	00.0	10	100	
Growth pattern							
Solid	5	12.5	1	57.1	4	42.9	0.430‡
Papillary	35	88.5	16	39.5	19	60.5	
Size (cm)							
Mean ± SD	4.74	±2.84	4.80	±3.02	4.68	±2.57	0.026•
Median (Range)	3	2–12	3	2–10	3	1–11	
≤5	25	68	10	41.2	15	58.8	0.027‡
>5	15	32	7	43.8	8	56.3	
Grade							
Low grade	12	34	10	82.4	2	17.6	<0.001‡
High grade	28	66	7	21.2	21	78.8	
T							
Ta	5	14	3	71.4	2	28.6	<0.001•
T1	20	48	9	45.8	11	54.2	
T2	10	24	4	23.3	6	66.7	
T3	5	14	1	14.3	4	85.7	
Deep invasion							
Absent	21	62	15	51.6	6	48.4	<0.001‡
Present	19	38	2	26.3	17	73.7	
Vascular invasion							
Absent	30	75	15	50	15	50	<0.001‡
Present	10	25	2	10	8	90	
Mitotic rate							
<10	12	20	10	68.4	2	31.6	<0.001‡
>10	28	70	8	25.8	20	74.2	
Perineural invasion							
Absent	12	38	5	55.9	7	44.1	<0.001‡
Present	28	62	12	12.5	16	87.5	
Distant metastases							
Absent	24	68	14	50	10	50	<0.001‡
Present	16	32	3	10	13	90	
Lymph node							
Negative	30	80	17	51.5	13	48.5	<0.001‡
Positive	10	10	0	23.5	10	76.5	
Stage							
Stage I	23	66	3	87.5	4	12.5	<0.001•
Stage II	17	34	4	46.2	3	53.8	
Stage III	8	16	4	36.8	6	63.2	
Stage IV	12	26	6	10	10	90	
HEF-1							
Low	18	38	10	59.3	8	40.7	0.007‡
High	22	20	7	21.7	15	78.3	

*Independent samples student's *t*-test; •Mann Whitney *U* test; ‡Chi-square test; §Chi-square test for trend.

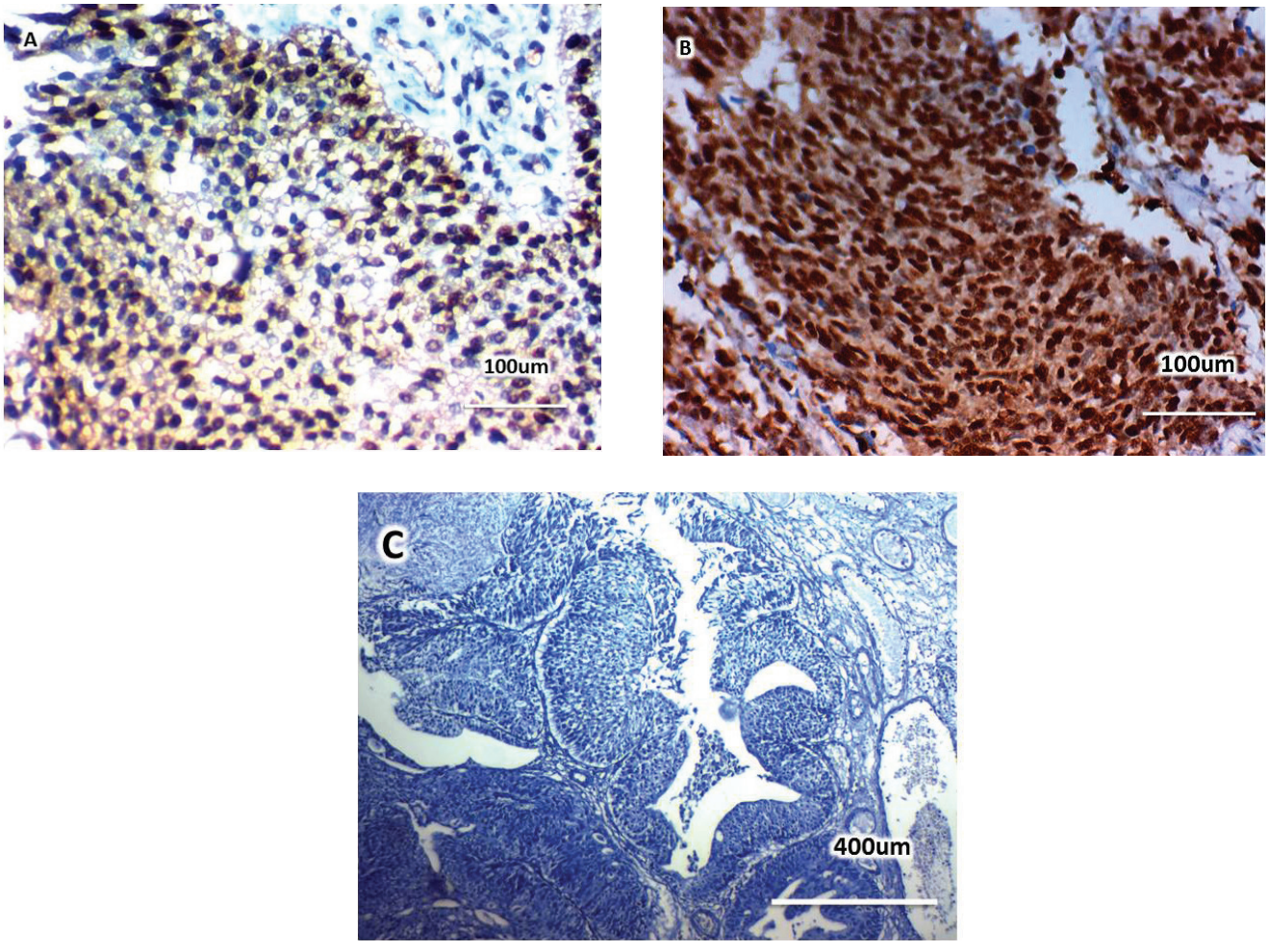

Figure 2. Immunohistochemical staining of SPOCK-1 (in the nucleus) in TCC of the urinary bladder. (A and B) High SPOCK-1 expression in deep muscle invasive, high-grade TCC. (B) Low SPOCK-1 expression in non-muscle-invasive low-grade TCC. Magnification: (A and B) the original magnification was ×400.

high and low *p*-38, respectively, and highly significant inverse relationship was found between 3-year DFS and both high HEF-1 protein expression and high SPOCK-1 protein expression ($p < 0.001$).

The high expression of both HEF-1 and SPOCK-1 was positively correlated with poor OS and DFS rates ($p = 0.041$ and <0.001, respectively) (Table 4, Figure 3).

Discussion

Cancer bladder cancer is the 13th commonest cause of malignancy-related fatality worldwide [18]. The most frequent subtype is TCC that forms about 90% of all bladder cancer cases. About one-third of cases of TCC are muscle invasive with high rate of progression and fatality [19]. The most important prognostic factors of TCC are depth of muscle invasion, status of lymph node, and presence of distant metastases [20]. It is important to identify recent prognostic and therapeutic targets to improve risk stratifications and management strategies for such common dangerous malignancy. In our study, we have assessed the expression of HEF-1 that is a coordinator of many steps of physiological and pathological vital process like cell cycle control, apoptosis, tumor invasion, and spread [21].

HEF-1 expression in TCC of the urinary bladder was associated with older age of the patients ($p = 0.035$), higher tumor grade and advanced TNM stage ($p < 0.001$), more liability to the presence of distant metastasis, higher incidence of vascular invasion ($p = 0.031$), higher incidence of perineural invasion ($p = 0.004$), higher incidence of presence of areas of adjacent carcinoma *in situ* ($p = 0.041$), deep invasion of the tumor or lymph node metastases, and higher Ki67 mitotic rate ($p = 0.003$). Moreover, we had found that the expression of HEF-1 was positively correlated with poor

Table 4. Correlation between HEF-1 and SPOCK-1 expressions and outcome of our patients.

Characteristics	All (*N* = 50)		Local recurrence		*p*-value	Distant metastasis		*p*-value	Survival		*p*-value
			No (*N* = 17)	Yes (*N* = 23)		No (*N* = 30)	Yes (*N* = 10)		Alive (*N* = 28)	Died (*N* = 12)	
HEF-1											
Mean ± SD			22.9 ± 24.7	40.5 ± 21.8	0.007‡	27.1 ± 24.2	48.8 ± 16.9	0.001‡	27.9 ± 23.9	46.2 ± 20.3	0.008‡
Median (range)			10 (3–90)	40 (5-80)		20 (3–90)	50 (20–80)		20 (3-90)	45 (5–80)	
Low	18	34%	13 (70.6%)	5 (29.4%)	<0.001§	18 (100%)	0 (0%)	<0.001§	10 (88.2%)	8 (11.8%)	0.010§
High	22	66%	5 (15.2%)	17 (84.8%)		12 (48.5%)	(51.5%)		18 (51.5%)	4 (48.5%)	
SPOCK											
Mean ± SD			35.2 ± 28.3	48.6 ± 27.4	0.071§	32.8 ± 24.6	65.8 ± 21.2	0.001§	30.4 ± 23.1	63.3 ± 18.5	<0.001§
Median (range)			20 (10–90)	60 (10–90)		20 (10–90)	70 (10–90)		20 (10–90)	70 (20–90)	
Low	17	66%	8 (42.9%)	9 (57.1%)	0.136§	15 (92.9%)	2 (7.1%)	<0.001§	16 (92.9%)	1 (7.1%)	<0.001§
High	23	44%	5 (22.7%)	18 (77.3%)		8 (31.8%)	15 (68.2%)		12 (27.3%)	11 (72.7%)	

‡Mann Whitney *U* test; §Chi-square test; •Chi-square test for trend; $p < 0.05$ is significant.

OS and DFS rates. Many other previous studies have proved results similar to ours regarding the association between HEF-1 overexpression and poor clinicopathological parameters. Zhang et al. [22] have found results similar to that in bladder cancer in addition to many other studies; cancer ovary [23], pancreas [24], gastric cancer [25], glioblastoma [26], lung cancer [27], and also in breast cancers [28]. Our results indicating that HEF-1 might be responsible for invasion and progression of TCC and other malignancies, so it could be used as a novel therapeutic target for cancer bladder and several other cancers.

Many studies have explained our results by proving that HEF-1 could be able to regulate Wnt-signaling pathways and TGF-β that had plethora of roles in cancer cell invasion and spread [29,30]. Moreover, HEF-1 might act by phosphorylation focal adhesion kinase (FAK) which is also important for motility, invasion, and spread of cancer cells as HEF-1 is a down-stream molecule of FAK [31]. Similar to our results, Sima et al. [32] found that HEF-1 overexpression activated motility and invasiveness of cervical cancer cells through a positive feedback mechanism of between FAK and HEF-1.

In addition, HEF-1 had an essential role in the induction of epithelial–EMT [27], as it has a role in E-cadherin removal of from cell junctions that subsequently leads to its degradation, decreased cancer cells adhesion and increased incidence if invasion and metastases [33].

Li et al. [34] also proved that HEF-1 has a vital role in induction of EMT in colon cancer.

Tikhmyanova et al. [33] clarified the role of HEF-1 in EMT induction by decreasing E-cadherin expression, which has confirmed our results regarding the role of HEF-1 in TCC invasion, progression, and metastasis.

To prove the role of HEF-1 expression in TCC we have evaluated its expression in addition to other marker that is incriminated in EMT induction in cancer which is SPOCK-1.

SPOCK-1 expression in TCC of the urinary bladder was associated with older age of the patients ($p = 0.035$), higher tumor grade and advanced TNM stage ($p < 0.001$), more liability to the presence of distant metastasis, higher incidence of vascular invasion ($p = 0.031$), higher incidence of perineural invasion ($p = 0.004$), higher incidence of presence of areas of adjacent carcinoma *in situ* ($p = 0.041$), deep invasion of the tumor or lymph node metastases, and higher Ki67 mitotic rate

(p = 0.003). The high expression of both HEF-1 and SPOCK-1 was positively correlated with poor OS and DFS rates 0.041. Our results proved the important prognostic role of such marker in progression of TCC.

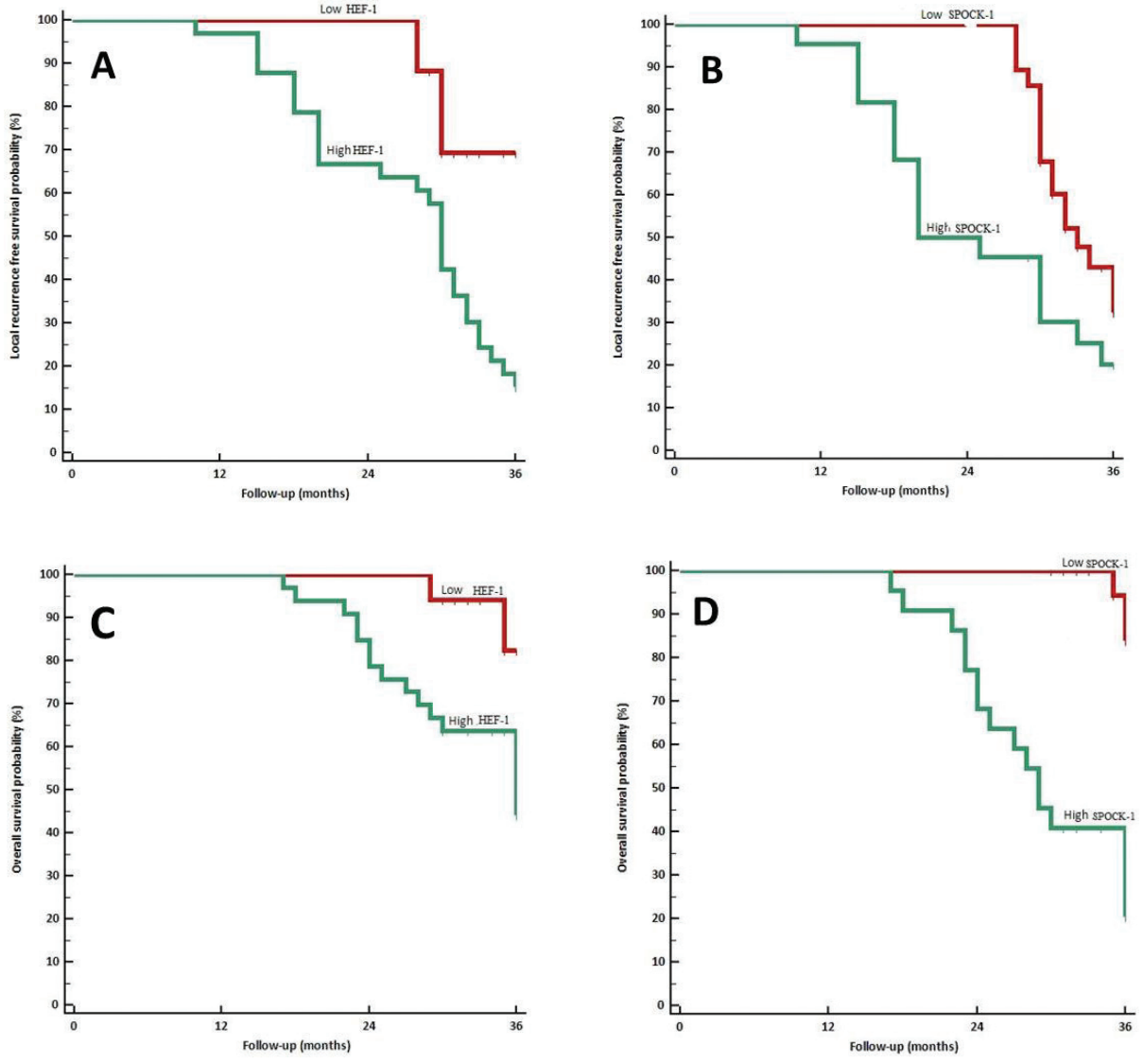

Figure 3. Kaplan-Meier plot of local recurrence free survival A& B: (A) stratified according to HEF-1, (B) stratified according to SPOCK. **Kaplan-Meier plot of overall survival C& D:** C) stratified according to HEF-1, (D) stratified according to SPOCK

Similar to our results, Ma et al. [35] and Shu et al. [36] found the same association between SPOCK-1 expression and poor prognosis of TCC & carcinoma of the gall bladder, respectively.

Similarly, *SPOCK-1 up-regulation has been detected* in lung cancer and was associated with occurrence of nodal and distant metastases, which might be explained by the SPOCK-1 role in EMT induction and increase the incidence of metastasis [37].

Many studies have proved the role of SPOCK-1 in cancer progression by EMT induction. In addition, it might be responsible for inhibition of apoptosis in cancer cells that has adverse effect by making cancer cells continue to proliferate, leading to accumulation of genetic damage, appearance of aggressive subtypes with more liability for invasion and metastases [38]. SPOCK-1 has proved to perform its anti-apoptotic effect by inhibition of the PI3K/Akt pathway that subsequently activated the caspase 9/caspase 3/ pathways, and also by inhibition of PI3K/Akt signaling which is responsible for suppression of cancer cell proliferation, motility, invasion, and EMT [36]; moreover, the association between SPOCK-1 expression and higher mitotic rate of cancer points to the relationship between

such protein and cancer cell proliferation which is promising that targeted therapies against SPOCK-1 may improve prognosis of cancer patients, decreasing cancer cell proliferation, invasion, and metastases [35].

We have found a positive association between HEF-1 and SPOCK-1 expressions in TCC patients and both markers related to poor prognosis as both are responsible for cancer cells invasion and metastases.

Summary and Conclusion

We have found in our study that both HEF-1 and SPOCK-1 are overexpressed in TCC cells, and their overexpression increased TCC cells proliferation, invasion, and metastases mainly by EMT induction, inhibition of apoptosis in cancer cells and increasing cancer cells proliferation. Therefore, both markers may be used as novel therapeutic targets for TCC.

Our conclusion is a simple observation from tissue protein expression in tumor cells and we will perform a future study which will contain other methods of assessment to strengthen such conclusions.

Recommendations

Furthermore, prospective studies on the combined tissue protein expression of both markers in a large cohort of TCC patients are needed to prove their prognostic roles and possibility of using therapeutic targets against them to improve patients' prognosis.

As the immunohistochemistry is widely used in the research study as a diagnostic and prognostic tool, however, when looking for new candidates for biomarkers, more advanced and quantitative methods need to be adopted to support the findings (e.g., qPCR for quantification of gene expression in fresh, or formalin fixed, paraffin embedded tissue). Western blot (semiquantitative method) should be used for detection of HEF-1 and SPOCK-1 expressions in fresh samples to proof the comments like "HEF-1 and SPOCK-1 are overexpressed in TCC cells, and their overexpression increased the TCC cells' proliferation, invasion, and metastases mainly by EMT induction, inhibition of apoptosis in cancer cells, and increasing cancer cells' proliferation." Those techniques we will add them in future study to prove our work and add to our results.

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