RESEARCH ARTICLE

Significance of Suppressor of Cytokine Signaling-3 Expression in Bladder Urothelial Carcinoma in Relation to Proinflammatory Cytokines and Tumor Histopathological Grading

Hanaa Hibishy Gaballah[1]*, Noha Mohamed Shafik[1], Rania Elsayed Wasfy[2], Mohamed Osama Abou Farha[3]

Abstract

Background: Bladder cancer is among the five most common malignancies worldwide. Altered expression of suppressor of cytokine signaling -3 (SOCS-3) has been implicated in various types of human cancers; however, its role in bladder cancer is not well established. Aim: The present study was undertaken to investigate the mRNA expression of SOCS-3 in normal and cancerous bladder tissue and to explore its correlation with urinary levels of some proinflammatory cytokines, cytokeratin-18 (CK -18) and with tumor histopathological grading, in order to evaluate their role as potential diagnostic markers. Materials and Methods: SOCS3 mRNA expression levels were evaluated using quantitative real time PCR. Urinary levels of interleukins 6 and 8 were estimated by enzyme linked immunosorbent assay (ELISA). Cytokeratin-18 expression was analyzed by immuunohistochemistry then validated by ELISA. Results: SOC3 m RNA expression levels were significantly lower in high grade urothelial carcinoma (0.36±0.12) compared to low grade carcinoma (1.22±0.38) and controls (4.08±0.88), (p<0.001). However, in high grade urothelial carcinoma the urinary levels of IL-6, IL-8, total CK-18(221.33±22.84 pg/ml, 325.2±53.6 pg/ml, 466.7±57.40 U/L respectively) were significantly higher than their levels in low grade carcinoma (58.6±18.6 pg/ml, 58.3±50.2 pg/ml, 185.5±60.3 U/L respectively) and controls (50.9±23.0 pg/ml, 7.12±2.74 pg/ml, 106.7±47.3U/L respectively), (p<0.001). Conclusions: Advanced grade of urothelial bladder carcinoma is significantly associated with lowered mRNA expression of SOC3 as well as elevated urinary levels of proinflammatory cytokines and CK-18. Furthermore, our results suggested that urinary IL-8, IL-6 and CK-18 may benefit as noninvasive biomarkers for early detection as well as histopathological subtyping of urothelial carcinoma.

Keywords: Suppressor of cytokine signaling-3 - interleukins - cytokeratin -18 - urothelial carcinoma

Asian Pac J Cancer Prev, **16 (1)**, 307-314

Introduction

Bladder cancer is the second most common malignancy affecting the urinary system, with the incidence being four times higher in men than in women (Fajkovic et al., 2011). Urothelial carcinoma originates from an epithelial origin and represents approximately 90% of bladder cancers. Cigarette smoking, occupational exposures to carcinogens and chronic infection with Schistosoma haematobium have been established as risk factors for bladder cancer (Kobeissi et al., 2013).

The mammalian signal transducers and activators of transcription (STATs) family comprises seven intracytoplasmic proteins; STAT1, 2, 3, 4, 5A, 5B, and 6 , whose biological activities control critical aspects of cell growth, survival and differentiation (Santos et al., 2011). STATs activation depends on the phosphorylation of a conserved tyrosine residue (Y705) by Janus kinases (JAKs) in response to specific ligands such as cytokines (IL-6), growth factors, and extracellular signals (Quintás-Cardama et al., 2013). Dysregulation of the JAK-STAT pathway is observed in many primary human tumours (Furqan et al., 2013). Due to the disastrous biologic consequences of uncontrolled cytokine-driven STAT activation, the JAK/STAT pathway is tightly controlled by the suppressors of cytokine signaling (SOCS) proteins, namely, SOCS1- SOCS7 and the SH2 domain-containing.

As a regulator of STAT-3 activation, SOCS-3 was thought to serve as multifunctional molecular switches facilitating or suppressing neoplastic transformation depending on cellular context (Culig 2013). Altered expression of SOCS-3 protein were found to be involved in the development and progression of several malignancies such as hepatocellular (Wu et al., 2011) and renal cell carcinoma (Stofas et al., 2014). However, the existing information about the role of SOC-3 protein in bladder

[1]Medical Biochemistry Department, [2]Pathology Department, [3]Urology Department, Faculty of Medicine, Tanta University, Egypt
**For correspondence: hanaahibishy@hotmail.com*

urothelial carcinoma remains limited and inconclusive.

Growing body of evidences highlights the pivotal role of Th2 cytokines particularly Interleukin-6 (IL-6) and Interleukin-8 (IL-8) in controlling growth and differentiation of normal and malignant cells and in contributing to the evasion of immune surveillance (Töpfer et al., 2011). IL-6 is a multifunctional cytokine associated with a number of biological functions in bladder cancer (Ebadi N et al., 2014), including cell proliferation, cell transformation, inflammation, and detrusor smooth muscle contractility (Tsui et al., 2013). IL-8 is a proinflammatory CXC chemokine that modulates both the cellular transcriptome and proteome within the tumor microenvironment. It also affects the organization of the cell cytoskeleton through posttranslational regulation of regulatory proteins (Zarogoulidis et al., 2014).

Cytokeratins provide mechanical stability to tissues; their synthesis is usually maintained during malignant transformation representing one of the hallmarks of epithelium-derived tumors (Karantza, 2011). Cytokeratin 18 (CK-18), a member of the intermediate-filament gene family, is important for cellular processes such as apoptosis, mitosis, cell cycle progression, and cell signaling (Weng et al., 2012). Recently, CK-18 is attracting considerable interest as measure of cell death for monitoring of disease progression in cancer patients (Benedict et al., 2014).

Deciphering the well-orchestrated signaling pathways coordinating the bladder carcinogesis is crucial to optimizing diagnosis and treatment strategies. Therefore, we herein aimed to investigate the m RNA expression pattern of SOCS3 in normal and cancerous bladder tissue and to explore its relation to urinary levels of some proinflammatory cytokines, CK -18 and with histopathological grading, in order to evaluate their role as potential diagnostic and prognostic markers for bladder urothelial carcinoma.

Materials and Methods

This study was carried out on fifty individuals presented to the Urology department, Tanta University Hospital and its Outpatient Clinics. They included 36 male patients with age range of (46-55) and with histopathologically confirmed urothelial bladder carcinoma after transurethral resection of suspicious bladder mass. The tumors were graded according to the World Health Organization 2004 criteria (Montironi and Lopez-Beltran, 2005); pathological grades were classified to low-grade (group I) and high-grade uro¬thelial carcinoma (group II). Patients with other malignant disease, or urinary tract infection, renal insufficiency or prior chemotherapeutic or radiation therapy were excluded from the study. Also, 14 individuals with no evidence of urothelial carcinoma, gross h,ematuria, active urinary tract infection or urolithiasis who were scheduled for some endourological procedures that involved bladder visualization were representing the control group (group III).They were matched to the patients group with respect to the age, sex and smoking status.

Informed written consent was obtained from all participants. The study protocol was approved by the Local Research Ethics Committee, Tanta University and was in accordance with the principles of the Declaration of Helsinki II. All of the participants were subjected to detailed history taking, clinical examination, abdominopelvic ultrasonography, and routine laboratory investigations. Besides, a cystoscopic examination was performed for each patient, by which transurethral resection biopsies were taken from the apparent lesion, processed by standard oncological procedures, studied and graded by a specialized pathologist.

For tissue sampling, the bladder biopsies were divided into 2 portions: one portion was kept on liquid nitrogen for studying of SOCS-3 mRNA gene expression and the other one for histopathological and immunohistochemistry studies.

For histopathological study bladder tissue biopsies were kept in 10% formalin solution and embedded in paraffin. Five-μm slices were stained with hematoxylin & eosin (H.&E.) and photomicrographs were taken at 200x and 400x. All cases were histopathologically diagnosed as urothelial carcinoma.

Immunohistochemistry

Five-micrometer sections from selected tumor blocks were mounted on 3-aminopropyltriethoxysilane coated (Sigma, St. Louis, MO USA) slides and were deparaffinized in xylene, rehydrated in graded alcohols, plus rinsed in 0.05 m Tris-buffered saline. Sections were boiled in 10 mm citrate buffer for antigen retrieval, at pH 6.0. Endogenous peroxidase was blocked with aqueous 0.3% H_2O_2 for 15 min. An avidin-biotin-peroxidase method was employed as described in manufacturers' kit manual. The sections were incubated in 5% normal rabbit serum followed by one hour incubation in CK 18 (1:100, Dako). As negative control, the primary antibody was replaced by TBS, and the appropriate normal areas in the sections served as positive controls. Positive immune-reactivity was defined as more than 20% of cells staining with the proper pattern of reactivity. Immunopositivity to CK18 appeared as brown cytoplasmic staining of tumor cells.

Urine sample collection and storage

Before any therapeutic intervention, 100 mL of voided urine was obtained from each subject. Fifty milliliters of urine was used for clinical laboratory analyses as urinary cytology and urinalysis, according to standard procedures. The remaining 50 ml of urine was centrifuged at 600 ×g 4°C for 5 min. The supernatant was decanted and aliquoted, and the urinary pellet was snap frozen. Both the supernatant and pellet were stored at -80 °C prior to analysis.

Cytokine urine measurements

The levels of human IL-8 (Cat # ab46032, Abcam, Cambridge, MA, USA), were monitored in urine supernatants of all patients and controls using enzyme-linked immunosorbent assays (ELISA). Also urinary IL-6 was estimated by solid phase ELISA Quantikine IL-6 Immunoassay (Cat # HS600B, R&D Systems,

Minneapolis, MN, USA). The assays were conducted according to the manufacturer's instructions. Calibration curves were prepared using purified standards for each protein assessed.

Quantitative determination of total CK-18 in urine: The M65 ELISA kit (#Cat 10020, Peviva AB, Bromma, Sweden) was used according to the manufacturer's instructions. The M65 ELISA consists of two monoclonal antibodies (clones M5 and M6) specific for conventional epitopes on CK-18, present on both intact/uncleaved and cleaved CK-18. In brief, urine samples were first placed into 96-well plates which had been coated with a mouse M6 monoclonal antibody directed against CK-18 as solid phase catcher antibody. Following incubation for 4 h at room temperature, the plate was washed five times with phosphate buffered saline. A horseradish peroxidase conjugated M5 antibody directed against another epitope on CK-18 was then used to detect the presence and concentration of M65 (Roth et al., 2011). The absorbance at 450 nm was determined in a microplate reader within 30 min of setting up the assay.

Estimation of of SOCS-3 mRNA expression levels by real-time PCR

i) RNA extraction: Total RNA from bladder tissue samples was prepared using Qiagen RNeasy Mini Kit according to the protocol supplied by the manufacturer. RNA was eluted, its concentration was measured spectrophotometrically (280) and then stored at -80°C.

ii) cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA Synthesis kit (#K1632,Thermo Scientific Fermentas, St. Leon-Ro, Germany) according to the manufacturer's instructions. Ten µl of random hexamer primers (Roche, Mannheim, Germany) were added to 21 µl of RNA which was denatured for 5 minutes in the thermal cycler (Biometra, USA). The RNA-primer mixture was cooled to 4°C. The cDNA master mix was prepared (5 µl of first strand buffer, 10 mM of dNTPs,1 µl of RNase inhibitor, 1 µl of reverse transcriptase Superscript™ II-RT enzyme and 10 µl of DEPC treated water) according to the kit protocol and was added to each sample. The total volume of the cDNA master mix was 19 µl for each sample. This was added to 31 µl RNA-primer mixture resulting in a reaction volume of 50 µl, which was then incubated in the programmed thermal cycler one hour at 37 °C, followed by inactivation of enzymes at 95 °C for 10 minutes, and finally cooled at 4°C. The RNA was reverse transcribed into cDNA which was then stored at -20°C.

iii) Real-time quantitative PCR: One µl of the cDNA was added to a 20 µl reaction mixture of the QuantiTect SYBR-Green PCR kit (Qiagen) and 0.5 µM from the specific primer pair for human SOCS-3. This cDNA was then amplified using the Step One instrument (Applied Biosystems, USA) as follows: Initial denaturation at 95°C for 15 minutes was followed by 40 cycles with denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 20 sec. A control reaction without a DNA template was performed in parallel to detect genomic DNA contamination. Primer sequences specific for SOCS-3 were designed according to Walsh.et al., 2006; as follows: forward primer: 5'-CCCTCGCCACCTACTGAA-3' and reverse primer: 5'-TCCGACAGAGATGCTGAAGA-3. Primers for β-actin were included as an internal control: forward primer: 5'- TGGCATTGCCGACAGGATGCAGAA-3, and reverse primer: 5'CTCGTCATACTCCTGCTTGCTGAT -3'.The determination of the relative levels of gene expression was performed using the cycle threshold) △△Ct (method and normalized to the reference gene β-actin, which was not altered by the experimental conditions.

Statistical Analysis: The data were analyzed using statistical package for the social science (SPSS) version 20.0 software (SPSS Inc., Chicago, IL, USA). Quantitative data expressed as mean and standard deviation. Categorical variables were compared using Chi-square test. Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Correlations were analyzed using the Pearson test. Multiple logistic regression analysis was used to estimate the odds ratios (ORs) and 95% confidence intervals (CI) for the studied parameters. Receiver operating characteristics (ROC) analysis was used to identify the optimal threshold values of the studied parameters.

Results

A statistical comparison between the studied groups with respect to the age and laboratory biochemical findings using ANOVA test followed by Tukey's test is demonstrated in Table (1) and Figure (1). No statistically significant difference was detected between the studied groups regarding age (p>0.05). SOC-3 m RNA relative expression levels were significantly lower in high

Table 1. Demographic and Laboratory Findings of the Studied Groups

	low grade urothelial carcinoma (n=18)	High grade urothelial carcinoma (n=18)	Controls (n=14)	ANOVA F	ANOVA p value
Age (years)	49.95±3.79	51.35±2.11	53.22±3.08	0.09	0.71
SOC3 m RNA relative expression	1.22±0.38	0.36±0.12*	4.08±0.88*#	239.65	<0.001
Urinary IL-6 (pg/ml)	58.60±18.63	221.33±22.84*	50.94±23.00#	397.43	<0.001
Urinary IL-8 (pg/ml)	58.32±50.21	325.15±53.60*	7.12±2.74*#	323.84	<0.001
Urinary total CK-18 (U/L)	185.47±60.28	466.65±57.40*	106.66±47.32*#	234.33	p<0.001

*Data are presented as the mean±SD; * statistically significant at p<0.05; * as compared to low grade urothelial carcinoma group; #as compared to high grade urothelial carcinoma group; SOC3: suppressor of cytokine signaling 3., IL-6: interleukin 6., IL-8:interleukin 8., CK-18: cytokeratin 18

grade urothelial carcinoma (0.36±0.12) as compared to low grade carcinoma (1.22±0.38) and the control group (4.08±0.88), (p<0.001). However, in high grade urothelial carcinoma the urinary levels of IL-6, IL-8, total CK-18(221.33±22.84 pg/ml, 325.15±53.60 pg/ml, 466.65±57.40 U/L respectively) were significantly higher than their levels in low grade carcinoma (58.60±18.63 pg/ml, 58.32±50.21 pg/ml, 185.47±60.28 U/L respectively) and the control group (50.94±23.00 pg/ml, 7.12±2.74 pg/ml, 106.66±47.32 U/L respectively), (p<0.001).

Regarding the correlations of the studied parameters with each other in different grades of urothelial carcinoma, the mRNA relative expression levels of SOC-3 showed significant negative correlations with the urinary levels of IL-6 (r= - 0.56, p<0.01), IL-8 (r= -0.68 and total CK-18 (r= - 0.49, p<0.01) . Moreover urinary levels of IL-6 and IL-8 exhibited a significant positive correlation with each other(r= -0.61, p<0.01) and with urinary total CK-18 (r=0.59, and r= 0.47, respectively) p<0.01.None of the studied parameters showed significant correlations with age. These data are summarized in Table (2).

Furthermore, the stepwise multiple logistic regression analysis was applied to assess the association between the studied parameters as the independent or predictor variables and the urothelial bladder cancer status as the dependent variable. Of note, this regression analysis showed that lowered mRNA expression of SOC-3 (odds ratio: 0.57; 95% Confidence interval 0.12-0.76; p <0.05) followed by elevated urinary levels of IL-8 (odds ratio: 3.13; 95% Confidence interval: 2.15-8.24; p <0.05), urinary levels of total CK-18 (odds ratio: 1.49; 95% Confidence interval: 1.25-1.98; p <0.05), then IL-6 (odds ratio: 1.85; 95% Confidence interval: 1.68-2.65; p <0.05), were significantly associated with urothelial bladder cancer (Table 3). Accordingly, they might serve as potential diagnostic biomarkers for urothelial bladder carcinoma.

Receiver operating characteristics (ROC) analysis was used to assess the diagnostic and prognostic value of the urinary cytokines as well as to identify their optimal cut off values. The area under the curve can range from 0.5 to 1 and diagnostic tests that approach 1 indicate a perfect discriminator. The optimal cut off value of urinary IL-8 was 10 pg/L, the sensitivity at this cut off point was 90%, the specificity was 100%, the positive predictive value was 100% and the negative predictive value was 83.3%

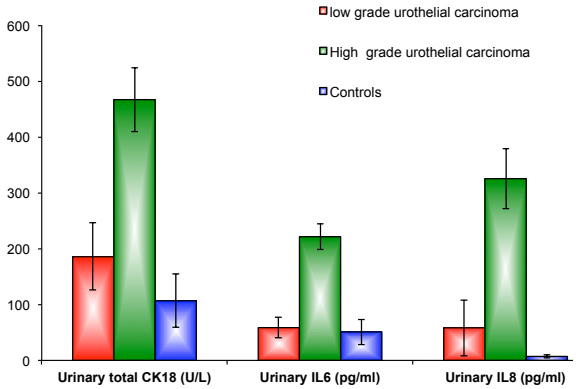

Figure 1. Urinary Levels of Total CK18,IL-6 and IL-8 in the Studied Groups. IL-6: interleukin 6., IL-8:interleukin 8., CK-18: cytokeratin 18

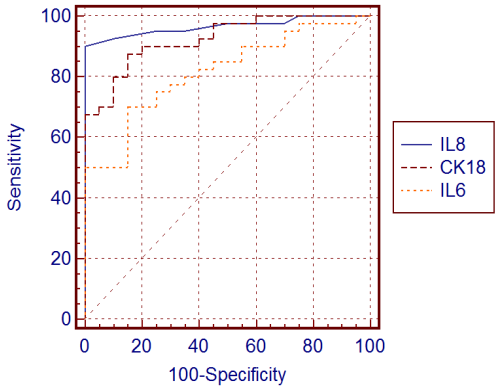

Figure 2. Receiver Operating Characteristics (ROC) Curve for Urinary IL-6, IL-8 and CK-18 in Controls and Cancer Patients Groups

Table 2. Pearson Correlation Between the Studied Parameters

Variables	SOC3 m RNA Relative Expression		Urinary IL6 (pg/ml)		Urinary IL8 (pg/ml)		Urinary total CK18 (U/L)	
	r	p value	r	p value	r	p value	r	p value
Age	0.05	0.7	0.07	0.62	0.04	0.73	0.11	0.44
SOC3 m RNA relative expression			-0.56	<0.01*	-0.68	<0.01*	-0.49	<0.01*
Urinary IL6 (pg/ml)	-0.56	<0.01*			0.61	<0.01*	0.59	<0.01*
Urinary IL8 (pg/ml)	-0.68	<0.01*	0.61	<0.01*			0.47	<0.01*
Urinary total CK18 (U/L)	-0.49	<0.01*	0.59	<0.01*	0.47	<0.01*		

*r=Pearson's correlation coefficient, P was considered significant at <0.05.; *Significant

Table 3. Logistic Regression Analysis with the Background Elimination Method Using the 3 Studied Parameters as Independent Variables, and Bladder Cancer Status as the Dependent Variable

Variables	B	S.E.	Wald	P-value	Odds ratio	95.0% C.I.for odd	
						Lower	Upper
SOC3 m RNA relative expression	-667.91	28237.65	5.56	0.001	0.57	0.12	0.76
Urinary IL6 (pg/ml)	0.31	57.25	2.02	0.045	1.85	1.68	2.65
Urinary IL8 (pg/ml)	2.92	156.25	6.1	0.003	3.13	2.15	8.24
Urinary total CK18 (U/L)	0.45	102.42	2.98	0.025	1.49	1.25	1.98

*B: regression coefficient; S.E: Standard error; CI:confidence interval; SOC3: suppressor of cytokine signaling 3; IL-6: interleukin 6; IL-8: interleukin 8; CK-18: cytokeratin 18

Table 4. Receiver Operating Characteristics (ROC) Curve for Urinary IL-6, IL-8 and CK-18 in Controls and Cancer Patients Groups

variables	ROC curve between controls and cancer patients					
	Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy
Urinary IL-6 (pg/ml)	>66.5*	70	85	90.3	58.6	0.81
Urinary IL-8 (pg/ml)	>10*	90	100	100	83.3	0.96
Urinary total CK18 (U/L)	>150*	87.5	85	92.1	77.3	0.92
Urinary IL-6 (pg/ml)	>66.5*	70	85	90.3	58.6	0.81
Urinary IL-8 (pg/ml)	>10*	90	100	100	83.3	0.96
Urinary total CK18 (U/L)	>150*	87.5	85	92.1	77.3	0.92

*PPV: positive predictive value; NPV: negative predictive value; IL-6: interleukin 6; IL-8:interleukin 8; CK-18: cytokeratin 18

Table 5. Immunohistochemical Results of Low and High Grade Urothelial Carcinoma Groups

Total CK-18 immunostaining	low grade UC		High grade UC		Total		Chi-Square	
	N	%	N	%	N	%	χ^2	P-value
Negative	8	44.4	2	11.12	10	27.78	4.15	0.033*
Positive	10	55.6	16	88.88	26	72.22		
Total	18	100	18	100	36	100		

*UC: urothelial carcinoma; CK-18: cytokeratin 18; P was considered significant at <0.05; *Significant

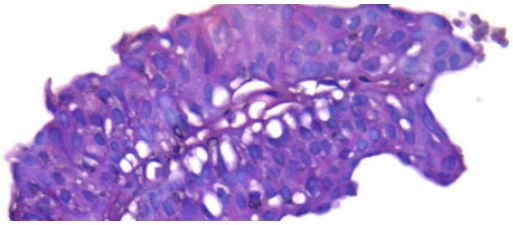

Figure 3. Papillary Low Grade Urothelial Carcinoma with Moderate Cellular Anaplasia and Pleomorphism (H&E x 200)

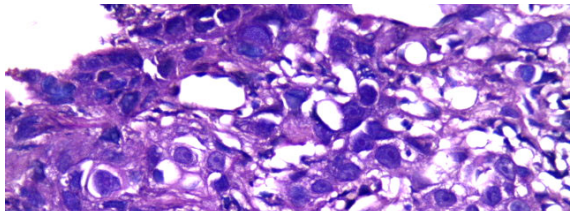

Figure 4. High Grade Urothelial Carcinoma with High Grade of Anaplasia and Pleomorphism (H&E x 200)

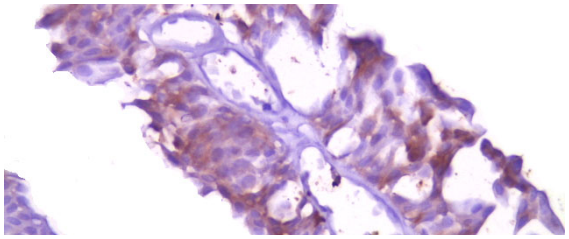

Figure 5. Papillary low Grade Urothelial Carcinoma with Diffuse Positive Cytoplasmic Stainaing of CK18 (x 400)

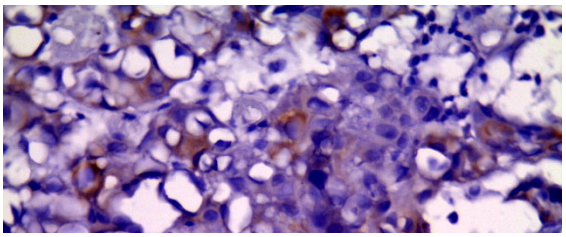

Figure 6. High Grade Urothelial Carcinoma with Diffuse Positive Cytoplasmic Staining of CK18 (x400)

and the area covered was 0. 966. Urinary IL-6 monitoring revealed a sensitivity of 70%, specificity of 85%, positive predictive value of 90.3 %, negative predictive value of 58.6%, and an accuracy of 0.815 at an optimal cut off value of 66.5pg/ml. Likewise, the cut off value of urinary total CK-18 was 150 U/L , at this point the sensitivity was 87.5%, the specificity was 85.0%, the positive predictive value was 92.1% and the negative predictive value was 77.3% and the accuracy was 0. 925. Data are summarized in Table (4) and Figure (2).

During Immunohistochemical analysis; normal areas in the sections served as positive controls showed superficially diffuse cytoplasmic staining of CK-18 in the umbrella cells. CK-18 positivity was detected by diffuse cytoplasmic staining in all tumor cells. Ten cases of low grade urothelial carcinoma were CK-18 positive and eight cases were negative. Sixteen cases of high grade urothelial carcinoma were CK-18 positive and two cases were negative. Using Chi-square test, the expression of CK-18 was significantly higher in high grade compared to low grade urothelial carcinoma (χ^2=4.15, p <0.05). These data are demonstrated in Table (5) and Figures (3-6).

Discussion

The suppressor of cytokine signaling SOCS family is characterized as endogenous negative regulators to the JAK/STAT signaling cascade which modulates various fundamental biological processes and cancer pathogenesis (Linossi et al., 2013). Altered expression of suppressor of cytokine signaling -3 (SOCS-3) has been implicated in various types of human cancers (Sasi et al., 2014); however its role in bladder urothelial carcinoma as well as its relevance to tumor histopathological grade remains controversial.

The present study revealed that SOC-3 mRNA expression levels were significantly lower in cancerous bladder tissue compared to normal bladder tissue samples. Moreover, high grade urothelial carcinoma was associated with lower SOC-3 expression than low grade carcinoma, suggesting a role for SOC-3 impaired expression in tumor

progression and cancer cell differentiation. In harmony with these findings, reduced expression of SOCS-3 mRNA had been reported in other types of cancer such as lung cancer and head and neck squamous cell carcinoma (Baltayiannis et al., 2008, Ying et al., 2010). Moreover, *in vitro* forced expression of SOCS-3 in mesothelioma and lung adenocarcinoma cell lines decreased tumor invasion by modulating expression of genes that are essential for cell proliferation, migration and invasion (Yu et al., 2009). Well in line, Sasi et al., 2010 and Wu et al., 2011 reported an inverse relationship between SOCS-3 expression and tumor stage in breast and hepatocellular carcinoma respectively, where higher expression was associated with earlier tumour stage and better clinical outcome.

Given that the constitutive JAK/STAT activation plays a pivotal role in the pathogenesis of urothelial carcinoma through regulating apoptosis, proliferation and invasion-promoting genes (Chen et al., 2008), it can be anticipated that once SOCS-3 expression is impaired and its negative regulatory effect on JAK-STAT pathway is lost, the bladder cancer cells become more sensitive to aberrant growth stimulating signals, thus promoting tumor progression. Further supporting the tumor suppressor role of SOC-3, Stahl et al., 2012 reported that SOCS-3 is an endogenous feedback inhibitor of pathologic angiogenesis acting at the converging crossroads of growth factor- and cytokine-induced vessel growth.

Noteworthy, the reported decreased SOC-3 expression in urothelial carcinoma as well as in other types of cancer can be explained by the epigenetic gene silencing through aberrant gene promoter hypermethylation which causes transcriptional downregulation of SOC-3 gene and subsequent impaired its expression (Li et al., 2012). Epigenetic dysregulation of the SOCS-3 gene may interfere with the cellular response to the complex cytokine network thus contributing to oncogenesis (Al-Jamal et al., 2014) .Well in line, demethylating agents which can restore SOC-3 expression remarkably suppressed cell proliferation and induced apoptosis of pancreatic cancer cells (Wang et al., 2014). Since SOCS-3 cytoplasmic localization is critical for mediating suppression of cytokine signaling, it seems plausible that a predominant nuclear localization might cause an impairment of its function in cancer cells that still possess detecTable SOCS-3 expression (Rossa C et al., 2012).

In contrast to these findings, Huang et al., 2009 failed to establish any significant correlations between SOCS-3 expression and invasiveness or histological grade of urothelial carcinoma. Furthermore, SOC-3 overexpression has been found to be correlated with advanced disease and poor response to treatment in other types of cancer such as lymphoma and some leukemias, representing a consequence of persistent STAT3 activation and cytokine production by tumor cells (Attia et al., 2011). This sustained STAT3 activation might be attribuTable to the failure of other negative regulatory pathways of JAK-STAT signaling as well as development of strategies to by-pass negative regulation by SOCS-3 protein (Li et al., 2011). Taken together, it is tempting to speculate that SOCS-3 plays a complex role in different types of malignancies as it may facilitate or suppress oncogenic transformation depending on cellular context which underline the need for further investigations.

On the other hand, it has been hypothesized that immune mediators in urine may serve as potential noninvasive diagnostic biomarkers for bladder cancer (Wang et al., 2012);owing to the fact that the urine of bladder cancer patients is in intimate contact with tumor cells and adjacent inflamed urothelium (Ghafouri-Fard et al., 2014). In this context, we further investigated the urinary levels of Th2 cytokines in the same cohort, where IL-6 levels were significantly increased in bladder cancer patients compared to control subjects with being significantly higher in high grade than low grade urothelial carcinoma. Concurring with this finding, Chen et al., 2013 revealed that IL-6 was overexpressed in the bladder cancer specimens compared with non-malignant tissues. In addition, they postulated that IL-6 might be related to a more malignant phenotype, as its expression was significantly increased in patients with locally advanced compared to noninvasive urothelial bladder cancer. Moreover, they provided evidences that blocking IL-6 expression resulted in significant reduction of bladder tumor growth and its invasive capability.

Along this line, the role of IL-6 in promoting bladder carcinogenesis is further underscored by the finding that IL-6, as a major activator of JAK/STAT3 signaling, enhances cell proliferation, epithelial to mesenchymal transition as well as increased angiogenesis by transcriptional activation of vascular endothelial growth factor, matrix metalloproteinase-9 and DNA methyltransferase 1 expressions (Taniguchi and Karin 2014), thus targeting IL-6 may be a promising strategy for treating bladder cancer.

Nevertheless, our data reported significantly increased urinary IL-8 levels in bladder cancer patients than controls, with being higher in high grade than low grade urothelial carcinoma. These findings are in agreement with that of Margel et al., 2011 and Urquidi et al 2012 who observed that urinary concentrations of IL-8 were significantly elevated in patients with urothelial carcinoma. Relevant to this notion, Reis et al., 2012 demonstrated that IL-8 has both mitogenic and angiogenic properties and its elevated expression was associated with enhanced bladder carcinogenicity , poor prognosis, disease recurrence and resistance to chemotherapy . Moreover, *in vitro* IL-8 signaling blockade significantly abrogated its promoting effects on cancer growth and metastasis, inhibited the expressions of matrix metalloproteinases, and decreased bladder cancer invasion (Lippitz, 2013). Collectively, these findings might provide a rationale for the therapeutic role of agents targeting Th2 cytokines in urothelial carcinoma microenvironment.

Furthermore, our data revealed a significant negative correlation between SOC-3 expression and urinary levels of IL-6 and IL-8 in the same cohort. This is in accordance with da Silva et al., 2013 who postulated that down regulation of SOC-3 expression enhances IL6/STAT proliferative signaling. Conceivably, loss of the negative feedback regulation of SOC-3 on these proinflammatory cytokines results in their pronounced secretion and pathological enhancement of carcinogenesis (Li al.

2011). Alternatively, it has been reported that IL-6 could induce the DNA methyltransferase (DMNT1) activity and mediate SOCS3 promoter hypermethylation, so maybe the reduced SOCS expression might be caused by the elevated IL-6 (Li et al., 2012).

Dysfunctional regulation of CK-18 is thought to be involved in bladder carcinogenesis by modulating intracellular signaling and operating in conjunction with various related proteins (McConkey et al., 2010). Immunohistochemical analysis of bladder tissue samples which was further validated by urinary measurement of total CK- 18 demonstrated that total CK -18 expression and urinary levels were significantly were higher in high grade compared to low grade urothelial carcinoma and control subjects .This finding is in harmony with that of Song et al., 2009 who concluded that CK -18 might serve as a useful marker for diagnosis of urothelial carcinoma of the bladder and for monitoring the state of illness . Well in line, Ramazan Sekeroğlu et al., 2002 reported that expression of CK- 18 increases in parallel with tumor grade and cell deoxyribonucleic acid content, thus it could be recognized as a marker of aggressiveness in bladder tumors. This observed correlation between CK -18 level and the grade of malignancy may represent a general loss of urothelial differentiation features in high grade tumors (Paner et al., 2014).

Recently, changes in urinary levels of total and caspase cleaved CK-18 have been used to assess the tumor cell response to Adenoviral-mediated interferon α treatment and to predict disease outcome in bladder cancer patients (Benedict et al., 2013). Of note, CK- 18 expression pattern was correlated with tumor aggressiveness, progression and poor prognosis in several types of human cancer such as renal cell (Messai et al., 2010), ovarian, colorectal and esophageal carcinomas, which might be attributed to its role in regulation of cell cycle, cell growth and apoptosis (Weng et al., 2012).

Collectively, urinary levels of IL-8, total CK-18 and IL-6 were significantly associated with urothelial bladder carcinoma; however, the diagnostic efficacy of IL-8 was superior to that of total CK-18 and IL-6 in terms of accuracy, sensitivity and specificity. Moreover, multiple logistic regression analysis underscored the significant association of the studied parameter with urothelial carcinoma development.

In conclusion, the present study demonstrated that advanced grade of urothelial bladder carcinoma was significantly associated with lowered mRNA expression of SOC-3 as well as elevated urinary levels of proinflammatory cytokines and CK-18. These findings extend our understanding of the molecular mechanisms involved in bladder carcinogenesis and lay the ground for their targeting as a therapeutic strategy for urothelial carcinoma. Furthermore, our data suggested that urinary IL-8, IL-6 and CK-18 might benefit as noninvasive biomarkers for early detection as well as histopathological subtyping of urothelial carcinoma.

Acknowledgements

The authors gratefully acknowledge Dr. Tarique N. Hasan (Pure Health, Riyadh, Saudi Arabia) for critical reading of the manuscript.

References

Al-Jamal HA, Jusoh SA, Yong AC, et al (2014). Silencing of suppressor of cytokine signaling-3 due to methylation results in phosphorylation of STAT3 in imatinib resistant BCR-ABL positive chronic myeloid leukemia cells. *Asian Pac J Cancer Prev*, **15**, 4555-61.

Attia FM, Hassan AM, El-Maraghy NN, et al (2011). Clinical significance of suppressor of cytokines signalling-3 mRNA expression from patients with non-Hodgkin lymphoma under chemotherapy. *Cancer Biomark*, **11**, 41-7.

Baltayiannis G, Baltayiannis N, Tsianos EV. (2008). Suppressors of cytokine signaling as tumor repressors. Silencing of SOCS3 facilitates tumor formation and growth in lung and liver. *J BUO*, **13**, 263-5.

Benedict WF, Fisher M, Zhang XQ, et al (2014). Use of monitoring levels of soluble forms of cytokeratin 18 in the urine of patients with superficial bladder cancer following intravesical Ad-IFNα/Syn3 treatment in a phase l study. *Cancer Gene Ther*, **21**, 91-4.

Chen CL, Cen L, Kohout J, et al (2008). Signal transducer and activator of transcription3 activation is associated with bladder cancercell growth and survival. *Mol Cancer*, **7**, 78.

Chen MF, Lin PY, Wu CF, et al (2013). IL-6 expression regulates tumorigenicity and correlates with prognosis in bladder cancer. *PLoS One*, **8**, 61901.

Culig Z (2013). Suppressors of cytokine signalling-3 and -1 in human carcinogenesis. *Front Biosci*, **1**, 277-83.

da Silva CG, Studer P, Skroch M, et al (2013). A20 promotes liver regeneration by decreasing SOCS3 expression to enhance IL-6/STAT3 proliferative signals. *Hepatology*, **57**, 2014-25.

Ebadi N, Jahed M, Mivehchi M, et al (2014). Interleukin-12 and interleukin-6 gene polymorphisms and risk of bladder cancer in the Iranian population. *Asian Pac J Cancer Prev*, **15**, 7869-73.

Fajkovic H, Halpern JA, Cha EK, et al (2011). Impact of gender on bladder cancer incidence, staging, and prognosis. *World J Urol*, **29**, 457-63.

Furqan M, Mukhi N, Lee B, et al (2013). Dysregulation of JAK-STAT pathway in hematological malignancies and JAK inhibitors for clinical application. *Biomark Res*, **1**, 5.

Ghafouri-Fard S, Nekoohesh L, Motevaseli E. (2014). Bladder cancer biomarkers, review and update. *Asian Pac J Cancer Prev*, **15**, 2395-403.

Huang WT, Yang SF, Wu CC, et al (2009). Expression of signal transducer and activator of transcription 3 and suppressor of cytokine signaling 3 in urothelial carcinoma. *Kaohsiung J Med Sci*, **25**, 640-6.

Karantza V. (2011). Keratins in health and cancer, more than mere epithelial cell markers. *Oncogene*, **30**, 127-38.

Kobeissi LH, Yassine IA, Jabbour ME, et al (2013). Urinary bladder cancer risk factors, a Lebanese case- control study. *Asian Pac J Cancer Prev*, **14**, 3205-11.

Li N, Grivennikov SI, Karin M (2011). The unholy trinity, inflammation, cytokines, and STAT3 shape the cancer microenvironment. *Cancer Cell*, **19**, 429-31.

Li Y, Deuring J, Peppelenbosch MP, et al (2012). IL-6-induced DNMT1 activity mediates SOCS3 promoter hypermethylation in ulcerative colitis-related colorectal cancer. *Carcinogenesis*, **33,** 1889-96.

Linossi EM, Babon JJ, Hilton DJ, et al (2013). Suppression of cytokine signaling, the SOCS perspective. *Cytokine Growth Factor Rev*, **24**, 241-8.

Lippitz BE. (2013). Cytokine patterns in patients with cancer, a

systematic review. *Lancet Oncol*, **14**, 218-28.
Margel D, Pevsner-Fischer M, Baniel J, et al (2011). Stress proteins and cytokines are urinary biomarkers for diagnosis and staging of bladder cancer. *Eur Urol*, **59**, 113-9.
McConkey DJ, Lee S, Choi W, et al (2010). Molecular genetics of bladder cancer, Emerging mechanisms of tumor initiation and progression. *Urol Oncol*, **28**, 429-40.
Messai Y1, Noman MZ, Derouiche A, et al (2010). Cytokeratin 18 expression pattern correlates with renal cell carcinoma progression, relationship with Snail. *Int J Oncol*, **36**, 1145-54.
Montironi R, Lopez-Beltran A. (2005). The 2004 WHO classification of bladder tumors, a summary and commentary. *Int J Surg Pathol*, **13**, 143-53.
Paner GP, Annaiah C, Gulmann C, et al (2014). Immunohistochemical evaluation of novel and traditional markers associated with urothelial differentiation in a spectrum of variants of urothelial carcinoma of the urinary bladder. *Hum Pathol*, **45**, 1473-82.
Quintás-Cardama A, Verstovsek S (2013). Molecular pathways, Jak/STAT pathway, mutations, inhibitors, and resistance. *Clin Cancer Res*, **19**, 1933-40.
Ramazan Sekeroglu M, Aydin S, Dulger H, et al (2002). Diagnostic value of cytokeratin-18 as a tumor marker in bladder cancer. *Clin Biochem*, **35**, 327-31.
Reis ST, Leite KR, Piovesan LF, et al (2012). Increased expression of MMP-9 and IL-8 are correlated with poor prognosis of Bladder Cancer. *BMC Urol*, **12**, 18.
Rossa C Jr, Sommer G, Spolidorio LC, et al (2012). Loss of expression and function of SOCS3 is an early event in HNSCC, altered subcellular localization as a possible mechanism involved in proliferation, migration and invasion. *PLoS One*, **7**, 45197.
Roth GA, Lebherz-Eichinger D, Ankersmit HJ, et al (2011). Increased total cytokeratin-18 serum and urine levels in chronic kidney disease. *Clin Chim Acta*, **412**, 713-7.
Santos CI, Costa-Pereira AP (2011). Signal transducers and activators of transcription-from cytokine signalling to cancer biology. *Biochim Biophys Acta*, **1816**, 38-49.
Sasi W, Jiang WG, Sharma A, et al (2010). Higher expression levels of SOCS 1,3,4,7 are associated with earlier tumour stage and better clinical outcome in human breast cancer. *BMC Cancer*, **10**, 178.
Sharma AK, Mokbel K. (2014). The role of suppressors of cytokine signalling in human neoplasms. *Mol Biol Int*, **2014**, 630797.
Song JW, DU LL, Zhao XW, et al (2009). Expression and clinical significance of nuclear matrix protein 22 and cytokeratin 18 in transitional cell carcinoma of the bladder. *Chinese J Oncol*, **31**, 274-7.
Stahl A, Joyal JS, Chen J, et al (2012). SOCS3 is an endogenous inhibitor of pathologic angiogenesis. *Blood*, **120**, 2925-9.
Stofas A, Levidou G, Piperi C, et al (2014). The role of CXC-chemokine receptor CXCR2 and suppressor of cytokine signaling-3 (SOCS-3) in renal cell carcinoma. *BMC Cancer*, **14**, 149.
Taniguchi K, Karin M. (2014). IL-6 and related cytokines as the critical lynchpins between inflammation and cancer. *Semin Immunol*, **26**, 54-74.
Topfer K, Kempe S, Muller N, et al (2011). Tumor evasion from T cell surveillance. *J Biomed Biotechnol*, **2011**, 918471.
Tsui KH, Wang SW, Chung LC, et al (2013). Mechanisms by which interleukin-6 attenuates cell invasion and tumorigenesis in human bladder carcinoma cells. *Biomed Res Int*, **2013**, 791212.
Urquidi V, Chang M, Dai Y, et al (2012). IL-8 as a urinary biomarker for the detection of bladder cancer. *BMC Urol*, **12**, 12.
Walsh MJ, Jonsson JR, Richardson MM, et al (2006). Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. *Gut*, **55**, 529-35.
Wang HF, Wang JS. (2012). Research progress in potential urinary markers for the early detection, diagnosis and follow-up of human bladder cancer. *Asian Pac J Cancer Prev*, **13**, 1723-6.
Wang J, Zhou H, Han Y, et al (2014). SOCS3 methylation in synergy with Reg3A overexpression promotes cell growth in pancreatic cancer. *J Mol Med (Berl)*, **92**, 1257-69.
Weng YR, Cui Y, Fang JY. (2012). Biological functions of cytokeratin 18 in cancer. *Mol Cancer Res*, **10**, 485-93.
Wu WY, Li J, Wu ZS, et al (2011). Prognostic significance of phosphorylated signal transducer and activator of transcription 3 and suppressor of cytokine signaling 3 expression in hepatocellular carcinoma. *Exp Ther Med*, **2**, 647-53.
Ying M, Li D, Yang L, et al (2010). Loss of SOCS3 expression is associated with an increased risk of recurrent disease in breast carcinoma. *J Cancer Res Clin Oncol*, **136**, 1617-26.
Yu ZB, Bai L, Qian P, et al (2009). Restoration of SOCS3 suppresses human lung adenocarcinoma cell growth by downregulating activation of Erk1/2, Akt apart from STAT3. *Cell Biol Int*, **33**, 995-1001.
Zarogoulidis P, Katsikogianni F, Tsiouda T, et al (2014). Interleukin-8 and interleukin-17 for cancer. *Cancer Invest*, **32**, 197-205.