Expression of Signal Transducer and Activator of Transcription 3 and Suppressor of Cytokine Signaling 3 in Urothelial Carcinoma

Wan-Ting Huang,[1,3] Sheau-Fang Yang,[1,2] Chun-Chieh Wu,[1,2] Wan-Tzu Chen,[1] Ya-Chun Huang,[2] Yue-Chiu Su,[1] and Chee-Yin Chai[1,2]

[1]Department of Pathology, Kaohsiung Medical University Hospital; [2]Department of Pathology, Faculty of Medicine, and [3]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.

In this study, we investigated the expression of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) Tyr705 and suppressor of cytokine signaling 3 (SOCS3) in urothelial carcinoma (UC). p-STAT3 (Tyr705) and SOCS3 were analyzed by immunohistochemistry using tissue microarray and Western blotting. Our results showed that p-STAT3 (Tyr705) was frequently detected in high-grade and infiltrating UC. However, there was no difference in p-STAT3 (Tyr705) staining between UC of the upper and lower urinary tracts. In addition, there was no significant correlation between expression of SOCS3 and histological differentiation and invasiveness of UC. These findings suggest that overexpression of p-STAT3 (Tyr705) occurs in UC, and that pathways other than SOCS3 may contribute to its activation in this cancer.

Key Words: immunohistochemical staining, p-STAT3, SOCS3, tissue microarray, urothelial carcinoma

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Urothelial carcinoma (UC) originates from the urothelium and accounts for more than 90% of bladder cancers in Western countries. Upper urinary tract UC is rare, however, accounting for only 5% of cases of this disease [1]. Many studies have demonstrated a preponderance of high-grade and advanced-stage disease when the upper urinary tract is affected, compared with the lower urinary tract [1–5], though the mechanisms giving rise to this difference remain unclear. The Janus family of tyrosine kinases (JAK) and the signal transducer and activator of transcription (STAT) family function in diverse signal transduction pathways that are involved in cell proliferation, differentiation, survival and apoptosis [6]. The results of numerous studies have supported a role for STAT proteins, especially STAT3, in oncogenesis [7]. STAT3 is a cytoplasmic latent transcription factor that is persistently tyrosine-phosphorylated by JAK. It can directly or indirectly up-regulate the expression of genes required for proliferation and survival of tumor cells. STAT3 is persistently activated in many human cancer cell lines and in a wide variety of hematologic and epithelial malignancies, including breast, head and neck, lung, ovarian [7–15] and prostate [12] cancers. Suppressors of cytokine signaling (SOCS) have been proposed as negative regulators of cytokine signaling. They have distinct mechanisms of inhibiting the JAK-STAT pathway as part of a classic feedback loop [16,17]. The SOCS family consists of eight members,

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Address correspondence and reprint requests to: Dr Chee-Yin Chai, Department of Pathology, Kaohsiung Medical University Hospital, 100 Tzyou 1st Road, Kaohsiung 807, Taiwan.
E-mail: cychai@kmu.edu.tw

including CIS and SOCS1 through to SOCS7. A previous study found that deletion of the SOCS3 gene promoted the activation of STAT3 in hepatocytes [18]. However, the relationship between STAT3 and SOCS3 in UC is still poorly understood. In this study, we investigated the relationships between p-STAT3 (Tyr705) and SOCS3 expression and clinicopathological features in 174 patients presenting with UC, including 51 cases with urinary bladder tumors and 123 with renal pelvis and ureter tumors.

Materials and Methods

Surgical specimens

UC tissue specimens were obtained between 1991 and 2000 from the Department of Surgery at Kaohsiung Medical University Hospital by biopsy, cystectomy or nephrectomy. One hundred and seventy-four cases (80 males and 94 females; mean age, 63.5 years; age range, 21–81 years) were identified. None of the patients had received any previous treatment, including chemotherapy or radiotherapy. The available fresh tissue samples were stored at –140°C for Western blotting analysis, and the residual specimens were routinely fixed in 10% buffered formalin and embedded in paraffin. Several 5-μm-thick sections were cut from each tissue specimen. One section from each specimen was stained with hematoxylin and eosin for conventional light microscopic analysis, and histopathological diagnosis was confirmed by two independent pathologists (W.T.H. and C.Y.C.).

Clinicopathologic parameters, including gender, age, tumor location (upper *vs.* lower urinary tract), tumor invasiveness (noninvasive *vs.* infiltrating) and histological grade (low grade *vs.* high grade) were recorded prospectively (Table 1). Tumor invasiveness and grade were defined according to the WHO/International Society of Urologic Pathology 2004.

Tissue microarray construction

The tissue microarray (TMA) was constructed using formalin-fixed and paraffin-embedded UC tissue samples. Slides containing a representative area of tumor were selected and marked with colored ink. For each case, one core of tumor (2.0 mm in diameter) was transferred from the selected areas to the recipient block. Serial 5-μm sections of the TMA block were cut and stained with hematoxylin and eosin to verify that the cores adequately represented diagnostic areas.

Immunohistochemistry of TMA sections

Immunohistochemistry was performed on 5-μm tissue sections from the TMA blocks. The sections were placed on slides, deparaffinized, rehydrated, and then pretreated by autoclave for antigen retrieval.

Table 1. Correlations between expression of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) Tyr705 and suppressor of cytokine signaling 3 (SOCS3), and clinicopathologic characteristics in urothelial carcinoma

Characteristics	p-STAT3 expression		*p*	SOCS3 expression		*p*
	Low, *n* (%)	High, *n* (%)		Low, *n* (%)	High, *n* (%)	
Gender			0.474*			0.277*
Male	24 (30.0)	56 (70.0)		15 (18.8)	65 (81.2)	
Female	33 (35.1)	61 (64.9)		12 (12.8)	82 (87.2)	
Age (yr)			0.308*			0.937*
≤67	26 (29.2)	63 (70.8)		14 (15.7)	75 (84.3)	
>67	31 (36.5)	54 (63.5)		13 (15.3)	72 (84.7)	
Location			0.416*			0.337*
Upper	38 (30.9)	85 (69.1)		17 (13.8)	106 (86.2)	
Lower	19 (37.3)	32 (62.7)		10 (19.6)	41 (80.4)	
Tumor invasiveness			0.038*			0.065†
Noninvasive	20 (45.5)	24 (54.5)		3 (6.8)	41 (93.2)	
Infiltrating	37 (28.5)	93 (71.5)		24 (18.5)	106 (81.5)	
Histological grade			0.000*			0.376†
Low	17 (68.0)	8 (32.0)		2 (8.0)	23 (92.0)	
High	40 (26.8)	109 (73.2)		25 (16.8)	124 (83.2)	

*χ^2 test; †Fisher's exact test.

Immunohistochemistry was performed using the following antibodies and dilutions: p-STAT3 (Tyr705) (Cell Signaling Technology, Beverly, MA, USA) at dilution 1:75, and SOCS3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at dilution 1:75. The DAKO REAL EnVision Detection System (DAKO, Glostrup, Denmark) was also used. Finally, tissue sections were counterstained with Mayer's hematoxylin. Negative controls were obtained by replacing the primary antibody with non-immune serum.

Scoring of antibody staining

The TMA slides were evaluated twice at different times by two investigators blinded to the clinical characteristics and survival data. The results for nuclear p-STAT3 (Tyr705) staining were assigned based on the intensity grade [19]. The intensity grade represented the average nuclear staining intensity of positive tumor cells (grade 1: minimal staining; grade 2: weak staining; grade 3: moderate staining; grade 4: strong staining). Cytoplasm immunostaining intensity results were also divided into four grades for SOCS3 (grade 1: minimal staining; grade 2: weak staining; grade 3: moderate staining; grade 4: strong staining). The rare cases with discordant scores were re-evaluated and scored on the basis of consensual opinion.

Western blot analysis

All fresh tissue samples were washed in phosphate-buffered saline (PBS) and extracted in PBS containing 50 mM/L Tris-HCl (pH 7.4), 150 mM/L NaCl, 10 g/L Triton X-100, 1 g/L sodium dodecyl sulfate (SDS), 1 mM/L EDTA, 1 mM/L AEBSF, and 20 μg/L leupeptin. Equal amounts of protein, as determined by Bradford assay (Bio-Rad, Hercules, CA, USA), were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting onto a polyvinylidene-difluoride membrane. The membranes were blocked with 5% non-fat dried milk in Tris-buffered Saline (pH 7.4) with Tween-20, and then incubated with either anti-phospho-STAT3 (Tyr705) (1:1,000 dilution; Cell Signaling Technology) or SOCS3 rabbit polyclonal primary antibody (Santa Cruz Biotechnology Inc.). Secondary antibody was conjugated with horseradish peroxide (Santa Cruz Biotechnology Inc.). Protein bands were detected by chemiluminescence (Amersham, Buckinghamshire, UK). The anti-GADPH rabbit polyclonal antibody (Cell Signaling Technology) was used as an internal control.

Statistical analysis

The intensities of p-STAT3 nuclear staining and SOCS3 cytoplasmic staining were further classified into low expression (grade 1/2) and high expression (grade 3/4) before statistical evaluation. The correlations between p-STAT3 (Tyr705) expression and SOCS3 expression, and histopathological grade and invasiveness of UC were measured using χ^2 or Fisher's exact tests. All analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). A p value of < 0.05 was considered statistically significant.

RESULTS

Expression profiles of p-STAT3 (Tyr705) and SOCS3 in UC

p-STAT3 (Tyr705) staining was predominantly detected in the nucleus of neoplastic urothelial cells, and cytoplasmic staining was minimal (Figures 1A and 1B). The expression of p-STAT3 in normal urothelium was negative (Figure 1A insert). High-grade UC exhibited a higher p-STAT3 (Tyr705) intensity grade than low-grade UC ($p < 0.0001$). Comparing infiltrating and noninvasive UC, 20 out of 44 noninvasive UCs showed a low-intensity p-STAT3 (Tyr705) grade, while 93 out of 130 infiltrating UCs revealed a high-intensity grade. There was a significant correlation between p-STAT3 (Tyr705) intensity grade and invasiveness ($p = 0.038$) (Table 1). The p-STAT3 (Tyr705) intensity grades were similar in tumors of the upper and lower urinary tracts ($p = 0.416$) (Table 1). In addition, SOCS3 staining was diffuse in the cytoplasm of the urothelial cells (Figures 1C and 1D). There was no correlation between grade and invasiveness and SOCS3 expression ($p = 0.376$ and $p = 0.065$, respectively) (Table 1). The SOCS3 intensity grades were similar in the upper and lower urinary tracts ($p = 0.337$) (Table 1). The clinical characteristics are shown in Table 1. There were no statistically significant correlations between p-STAT3 (Tyr705) or SOCS3 expression and either gender or age.

Correlation between p-STAT3 (Tyr705) expression and SOCS3 expression in UC

Comparing p-STAT3 (Tyr705) expression and SOCS3 expression, it can be seen that 10 out of the 57 tumors with low-grade SOCS3 expression also showed

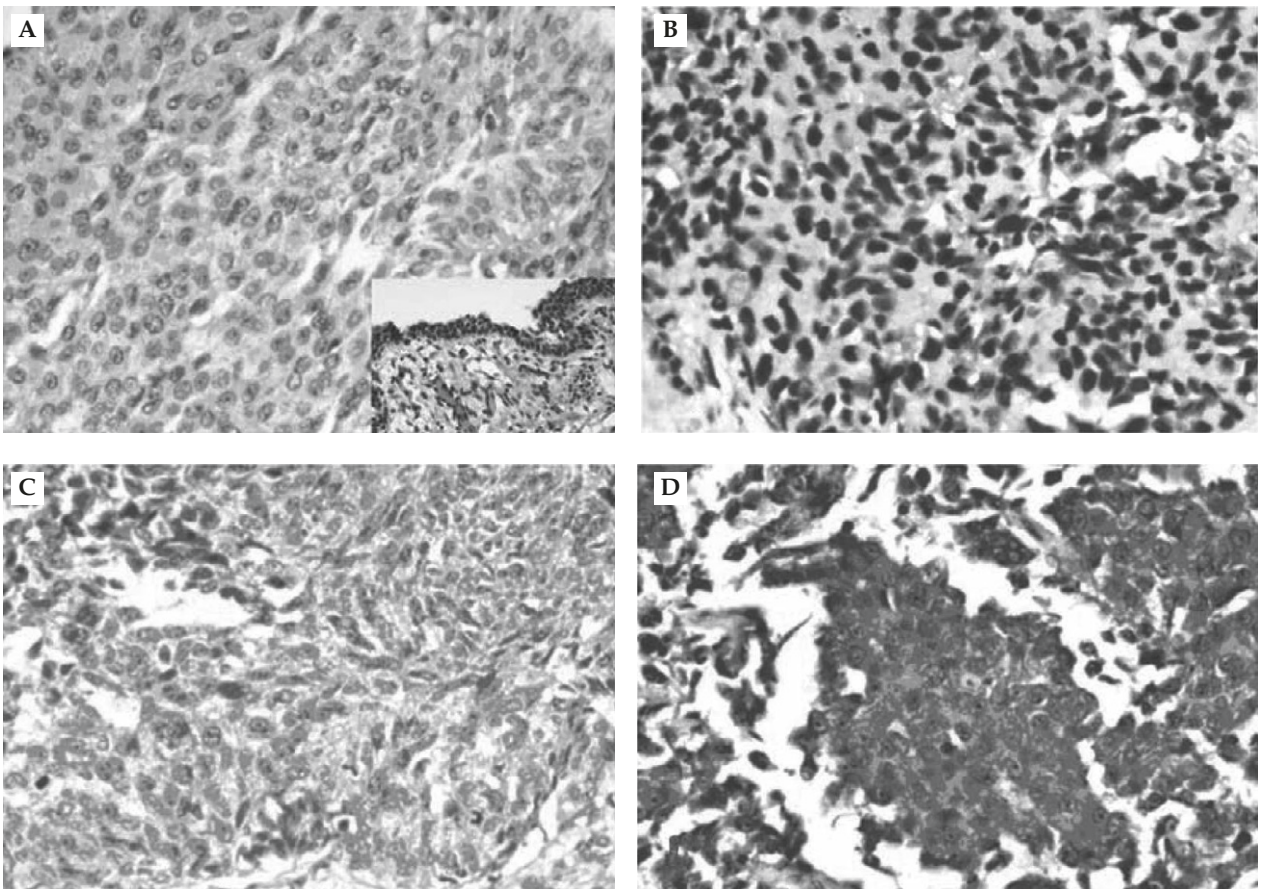

Figure 1. *Immunohistochemical staining of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) Tyr705 and cytokine signaling 3 (SOCS3) in urothelial carcinomas. Nuclear staining of p-STAT3 (Tyr705): (A) low intensity grade (insert in A is normal urothelium); (B) high intensity grade. Cytoplasmic staining of SOCS3: (C) low intensity grade; (D) high intensity grade. Original magnification, 400×.*

low-grade expression of p-STAT3 (Tyr705), and 100 out of 117 tumors with high-grade p-STAT3 expression also demonstrated high-grade SOCS3 expression. There was no significant correlation between p-STAT3 (Tyr705) and SOCS3 expression ($p = 0.606$) (Table 2).

Western blotting

Western blot analysis of p-STAT3 (Tyr705) and SOCS3 protein expression showed markedly elevated p-STAT3 (Tyr705) expression in high-grade UC. p-STAT3 (Tyr705) expression was low in low-grade UC. However, there was no correlation between SOCS3 protein expression and tumor grade (Figure 2). The intensities of p-STAT3 (Tyr705) expression measured by densitometer were 0.45 in low-grade UC and 116.65 in high-grade UC. The relative intensities of SOCS3 expression in low- and high-grade UCs were 618.84 and 615.40, respectively.

Table 2. Correlations between the expression of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) Tyr705 and suppressor of cytokine signaling 3 (SOCS3) in urothelial carcinoma

SOCS3 expression	p-STAT3 expression, *n* (%)		*p**
	Low	High	
Low	10 (17.5)	47 (82.5)	0.606
High	17 (14.5)	100 (85.5)	

*χ^2 test.

DISCUSSION

STAT3 is ubiquitously expressed in most tissues. Ligand-dependent activation of STAT3 is a transient process in normal cells, lasting for several minutes to several hours. In many cancerous cell lines and tumors, however, STAT3 protein is persistently

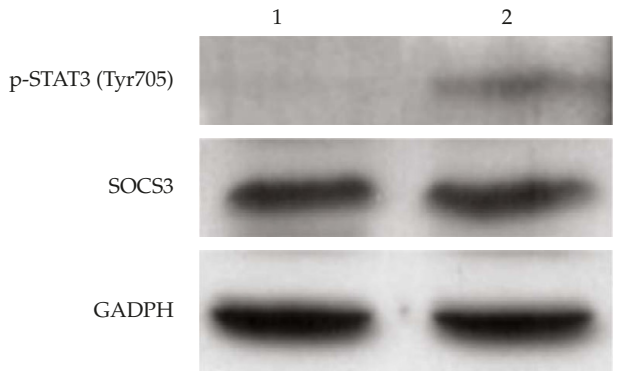

Figure 2. *Western blotting of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) Tyr705 and cytokine signaling 3 (SOCS3) protein in low-grade and high-grade UCs. p-STAT3 (Tyr705) expression was low and high in low- and high-grade UCs, respectively. Lane 1=low-grade UC; lane 2=high-grade UC. However, the expression of SOCS3 was similar in low- and high-grade UCs. GADPH protein expression was used as an internal control.*

tyrosine-phosphorylated or activated. The abnormal, persistently activated STAT3 protein can mediate transformation [20]. Thus, STAT3 is capable of regulating cell proliferation and survival [21]. Numerous studies have investigated the clinical and biological significances of p-STAT3 (Tyr705) in human malignancies, and STAT3 has been implicated in the invasiveness and metastasis of certain types of cancers, such as ovarian cancer [22] and renal cell carcinoma [23]. However, to the best of our knowledge, there has been no previous report on p-STAT3 (Tyr705) expression in UC. In the current study, we investigated whether infiltrating UC had higher p-STAT3 expression than noninvasive UC, and determined that high-grade UC expressed higher levels of p-STAT3 than low-grade UC. Taken together, the expression level of p-STAT3 (Tyr705) was closely associated with the invasiveness and degree of differentiation of UC. Although the clinical features of upper urinary tract UC are distinct from those in the urinary bladder, no conspicuous differences in p-STAT3 (Tyr705) expression were found between tumors from the upper and lower urinary tracts.

SOCS protein, which is stimulated by several cytokines, such as growth hormone, prolactin, interleukins and insulin, is a negative feedback regulator that inhibits the JAK/STAT pathway [24,25] and promotes their degradation via the proteasome pathway. Some reports have indicated that SOCS3 plays an essential role as a negative regulator in STAT activation [16]. Nevertheless, control of the physiological processes of SOCS3 has been found to differ between species and tissues, possibly due to differences between *in vitro* and *in vivo* experiments [26]. In this study, we assessed SOCS3 protein expression in UCs of the upper and lower urinary tracts. However, there were no significant correlations between SOCS3 expression and invasiveness or histological grade of UC. We speculated that p-STAT3 (Tyr705) expression would be negatively associated with SOCS3 expression. However, no significant correlation between p-STAT3 (Tyr705) and SOCS3 expression was detected in our investigation.

More than 90% of UC occurs in the urinary bladder in western countries. Nevertheless, the occurrence of upper UC is uncommonly high in Taiwan [27]. Owing to its infrequency, few studies have investigated the molecular indicators of UC in the upper urinary tract, such as p53 mutation [28], epidermal growth factor receptor or ErbB2 [29]. The conclusions have been controversial to date, because of the limited number of cases. In this study, 123 upper UCs and 51 bladder UCs were analyzed. The p-STAT3 (Tyr705) intensity grade was significantly associated with histological grade and invasiveness of UC. However, there was no significant difference in p-STAT3 (Tyr705) expression between the upper and lower urinary tracts. Our results indicate that altered p-STAT3 (Tyr705) expression in UC is correlated with histological grade and invasiveness. Some reports have suggested that STAT3 activation is suppressed by SOCS3 protein, but there was no significant correlation between p-STAT3 (Tyr705) and SOCS3 in our study. We therefore suggest that other pathways may contribute to the activation of STAT protein and that p-STAT3 (Tyr705) may have developed strategies to overcome negative regulation by SOCS3 protein.

In conclusion, we suggest that increased phosphorylation of STAT3 may be associated with invasiveness and degree of histological differentiation in UC. However, no association between p-STAT3 (Tyr705) and SOCS3 was detected in our study.

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轉錄信號傳導子和激活子 3 及細胞素信號傳導抑制因子 3 在泌尿道上皮癌之表現

黃琬婷[1,3] 楊曉芳[1,2] 吳俊杰[2] 陳婉姿[2] 黃雅君[3] 蘇月秋[1] 蔡志仁[1,2]

[1]高雄醫學大學附設醫院 病理科

[2]高雄醫學大學醫學院 病理學科

[3]基礎醫學研究所

訊息傳導子和轉錄激活子 3 (**signal transducer and activator of transcription 3 , STAT3**)，在人類的數種癌症中，已證明酪氨酸 705 有持續被磷酸化 (**phosphorylation**) 的現象，也因此 **STAT3** 被認為是一種致癌基因。而細胞素傳導抑制因子 (**suppressor of cytokine signaling 3, SOCS3**) 則被認為可阻止 **STAT3** 的磷酸化，扮演負向調控 (**negative control**) **STAT3** 的角色。本研究的目的是探討泌尿道上皮癌的 **p-STAT3** (**Tyr705**) 和 **SOCS3** 表現與泌尿道上皮癌之分化程度及侵襲性的關係。結果顯示，**p-STAT3** (**Tyr705**) 在分化較差及具侵襲性的泌尿道上皮癌中，的確出現較高的表現強度以及染出比例，而 **SOCS3** 並未出現顯著相關。

關鍵詞：免疫組織化學染色，訊息傳導子和轉錄激活子 3 ，細胞素信號傳導抑制因子，泌尿道上皮癌

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通訊作者：蔡志仁醫師

高雄醫學大學附設醫院 病理科

高雄市 **807** 三民區十全一路 **100** 號