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Original Article

Methylation of the suppressor of cytokine signaling 3 gene (SOCS3) in bladder cancer

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Abstract: Background: It has been identified consequences of dysregulation of JAK-STAT signalling, particularly in regard to JAK-STAT signalling that has been shown to have roles in the oncogenesis of several cell types. SOCS3 protein, the negative regulatory protein of JAK-STAT signaling pathway, may also plays critical regulatory roles in cancer initiation and progression. SOCS3 promoter hypermethylation has often been identified in human cancers; however, the precise role of SOCS3 in bladder cancer is unclear. Methods: The methylation status of the SOCS3 was analyzed in an age (±5 years) and sex-matched case-control study, including 112 bladder cancer cases and 118 normal controls, using the MassARRAY EpiTYPER system. Results: Methylation rate of JAK2, SOCS3 and STAT3 gene were shown to vary among different CpG island. The methylation rate of SOCS3 gene was also much higher in BCa than in normal control participants, but the methylation rate of JAK2, STAT3 gene weren't different in Bca and normal control participants. Conclusions: Our study demonstrates that promoter hypermethylation of SOCS3 gene is associated with BCa and thus, may serve as an independent prognostic biomarker.

Keywords: Blader cancer, JAK-STAT signaling pathway, DNA methylation, SOCS3

Introduction

Bladder cancer (BCa) is a frequent malignancy of the urinary tract [1, 2], the fourth most incident cancer in males and the seventh most incident in females in the world [3]. According to the International Agency for Research on Cancer (IARC) in 2012, 55,486 new BCa cases emerge and 26,820 BCa-related deaths occur in china, of which over 90% are Han Chinese [4]. In the past 10 years, bladder cancer has received increased attention as a leading public health problem. It is known that bladder cancer, similar to other types of human tumor, arises from chronic irritation and activation of oncogenes and/or inactivation of tumor suppressor genes by mutations and epigenetic modifications [5-7]. Epigenetic modifications have been recognized as important players in cancer etiology [8, 9]. DNA methylation is one of the important research content of epigenetics. Aberrant methylation of CpG islands located in promoter regions leading to their silencing play an important role in neoplasia [10-12]. Nowadays more and more studies have confirmed that DNA methylation involved into the occurrence and development of bladder cancer [13-16]. DNA methylation has not changed gene sequence, but it regulates the gene expression. DNA methylation can shut off the activity of tumor suppressor genes, demethylation induced to activation and expression of gene. Abnormal DNA methylation, including DNA hypermethylation and DNA hypomethylation, can lead to chromosome instability and transcriptional gene silencing [17]. DNA methylation has been one of the important parameters of tumor diagnosis and prognosis, particularly when the methylation silences tumor suppressor genes [18] and also become the new target for cancer treatment. Signalling from Janus kinase (JAK) and signal transducers and activators of transcription (STAT) proteins have been shown to play a significant role in various biological effects, including immune function, cell growth, differentiation and hema-

Table 1. Primer sequences for amplifying JAK2, SOCS3 and STAT3 genes

Gene	Primer sequences
JAK2	F: aggaagagagGGAGTGATTTATTTTGGTTGGTTT
	R: cagtaatacgactcactatagggagaaggctCCTAATATCTCTAAAAACCTACAACCC
SOCS3	F: aggaagagagGGAAATTTGTTGTGGGTGATTAT
	R: cagtaatacgactcactatagggagaaggctACCACACTCCTAAAAACCTAACTTC
STAT3	F: aggaagagagAGAAATAGGTGAAGGGGGTGTAG
	R: cagtaatacgactcactatagggagaaggctTATTCCTACCTCCAAAAAAACACAA

Table 2. The demographic characteristics in case groups and the control groups

Variable	Cases (n=112)		Controls (n=118)		*P* value*
	n	%	n	%	
Age					
<55	19	16.96	20	16.95	0.957
55-65	36	32.14	40	33.90	
>65	57	50.89	58	49.15	
Gender					
Male	91	81.25	91	77.12	0.441
Female	21	18.75	27	22.88	
Smoking					
Yes	49	43.75	72	61.02	0.009
No	63	56.25	46	38.98	
Drinking					
Yes	56	50.00	75	63.56	0.038
No	56	50.00	43	36.44	
Histologic classification					
Transitional cell carcinoma	104	92.86			
Squamous cell carcinoma	6	5.36			
Adenocarcinoma	2	1.78			
Grade					
I	3	2.68			
II	69	61.61			
III	40	35.71			

**P* values were calculated from chi-squared tests.

topoiesis [19]. It has also been identified consequences of dysregulation of JAK/STAT signalling, particularly in regard to JAK-STAT signalling that has been shown to have roles in the oncogenesis of several cell types [20-24]. It has been shown that aberrant hypermethylation of promoter regions in CpG islands was associated with transcriptional silencing of the genes in various cancers [25]. SOCS1 silenced by hypermethylation was found in hapatocellular carcinoma (HCC) [26], multiple myeloma [27], and hepatoblastomas [28]. However, SOCS3 protein, a critical member of SOCS family protein, silencing of SOCS3 by promoter methylation in human cancer was few reported. The present study aimed to demonstrate the correlation of methylation of the SOCS3 promoter and its transcription silencing in bladder cancer.

Materials and methods

Study populations

This study included 112 cases who first diagnosed by pathological histology and 118 cases without tumors matched age (±5 years) and sex as controls. The cases and controls were enrolled from Hongqi Hospital Affiliated to Mudanjiang Medical University, Mudanjiang Tumour Hospital from September 2013 to December 2015. All subjects are Chinese Han population, lived for more than 5 years in Mudanjiang. All subjects in this study participated in an approved protocol of Ethics Committee in Mudanjiang medical university, and signed informed consent. All individuals provided 5 ml peripheral blood specimens meanwhile completed the corresponding epidemiological investigation in order to obtain the basic characteristics for all the participants.

DNA extraction, sodium bisulfite modification, and PCR

Genomic DNA was extracted from whole blood samples using the QIAmp DNA Blood Mini Kit according to the manufacturer's protocol (QIAGEN, Hilden, Germany). The DNA concentration and purity were measured using the

Figure 1. A: CpG island methylation rate in the JAK2 gene. B: CpG island methylation rate in the STAT3 gene. C: CpG island methylation rate in the SOCS3 gene. Note: As the deeper of the colour, the methylation rate is higher.

Table 3. Methylation frequency in Bca and control groups ($\bar{x}\pm s$)

Gene	No. of CpG islands	Bca	Control	*p* value*
JAK2	29	0.0510±0.0219	0.0499±0.0314	0.871
STAT3	36	0.0531±0.0229	0.0513±0.0270	0.790
SOCS3	33	0.0556±0.0153	0.0413±0.0108	0.000

**P* values were calculated from *t* tests.

NanoDrop2000. Sodium bisulfite modification of genomic DNA was carried out using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's protocol. Bisulfite-treated genomic DNA was amplified in a 384-well plate using HotStar Taq Polymerase in a 10-μl reaction volume. PCR conditions were 94°C for 4 min followed by 45 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 60 s, with a final extension of 72°C for 3 min. Primer sequences are given in **Table 1**.

Quantitative DNA methylation analysis by MassARRAY EpiTyper

The PCR products were treated according to the standard protocol (Sequenom EpiTyper Assay) by SAP treatment and T-cleavage reaction. Reagents of SAP reaction was performed in a 384-well plate using Shrimp alkaline phosphatase (SAP) in a 2.0-μl reaction volume. SAP reaction process were 37°C for 20 min, 85°C for 5 min. T-cleavage reaction was performed in a 384-well plate using T7 RNA & DNA Polymerase in a 5.0-μl reaction volume. T-cleavage reaction conditions were 94°C for 30 s, 94°C for 5 s followed by 40 cycles of 52°C for 5 s, 5 cycles of 80°C for 5 s, with a final extension of 72°C for 3 min. And then the samples were cleaned by Resin and were dispensed to a 384 SpectroCHIP by Liquid Handler. Genomic DNA methylation levels were detected using MassARRAY EpiTYPER system (Liuhe genomics technology co., LTD, Beijing, China). The mass spectrum was collected by MassARRAY Spectrometer and analyzed by EpiTYPER.

Statistical analysis

Epidata 3.0 was used to set up databases. The Pearson's χ^2 statistics was employed to evaluate categorical variables. All continuous variables were indicated as mean ± SE unless otherwise noted, analyzed by the *t* or *F* statistics. ROC curve is used to decide the optimal model through determining the best threshold for a diagnostic and predictive test. For all the tests, statistical significance was accepted at a *P* value less than 0.05 (two-tailed). All the research data were analyzed using the SPSS12.0 statistical package (SPSS Inc., Chicago, Illinois, USA).

Results

Characteristics of the participants

The demographic characteristics of the BCa patients and controls are presented in **Table 2**. The basic personal information of these participants were analyzed, including age, gender, smoking, drinking. There were significant differences in smoking, drinking (all P<0.05) rather than age, gender between cases and controls.

Rate of methylation within Bca cases and controls

We detected the promoter methylation rate of JAK2, SOCS3 and STAT3 in 112 Bca cases and 118 normal control participants using the MassARRAY EpiTYPER system (**Figure 1A-C**).

Aberrant promoter methylation of JAK2, STAT3 and SOCS3 was detected in Bca cases and normal control participants. Additionally, the rate of JAK2 gene, SOCS3 gene promoter methylation in Bca was significantly higher than in normal control participants (**Table 3**).

And then we assessed the average CpG island methylation rate of JAK2, SOCS3 and STAT3 gene among Bca cases and normal control participants. We found that there was a significant different in the methylation rate of SOCS3 gene between Bca and normal control participants (P<0.05). In addition we neither find a significant different between Bca and normal control participants in the methylation rate of JAK2 gene (P>0.05) nor in the methylation rate of STAT3 gene (P>0.05). We also assessed the different CpG island methylation rate of JAK2, SOCS3 and STAT3 gene among Bca cases and normal control participants. Methylation rate of JAK2, SOCS3 and STAT3 gene were shown to vary among different CpG island. The methylation rate of SOCS3 gene was also much higher in Bca than in normal control participants, but the methylation rate of JAK2, STAT3 gene weren't different in Bca and normal control par-

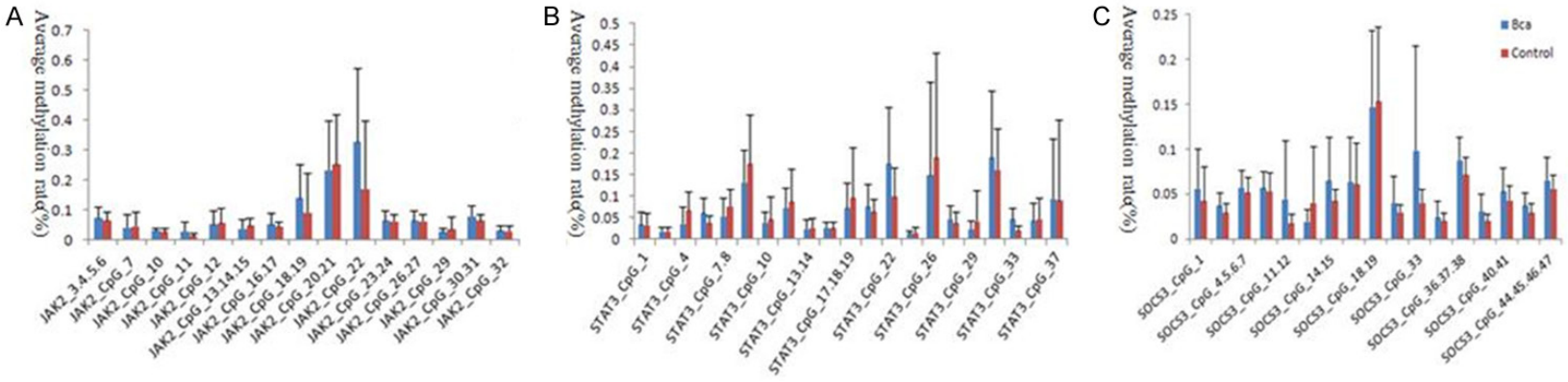

Figure 2. A: Comparison of average CpG island methelation rate in JAK gene. B: Comparison of average CpG island methelation rate in STAT3 gene. C: Comparison of average CpG island methelation rate in SOC3 gene.

Table 4. Methylation frequency of JAK2, STAT3, SOCS3 CpG islands at different grades ($\bar{x}$ ±s)

Gene	Grade			*p* value*
	I (n=3)	II (n=69)	III (n=40)	
JAK2	0.0331±0.0071	0.0483±0.0270	0.0571±0.0382	0.225
STAT3	0.0310±0.0184	0.0504±0.0301	0.0593±0.0305	0.153
SOCS3	0.0154±0.0116	0.0425±0.0256	0.0812±0.0129	0.000

**P* values were calculated from *F* tests.

ticipants (**Figure 2A-C**). After then we assessed the average CpG island methylation rates of JAK2, SOCS3 and STAT3 gene among different pathological grades of Bca. CpG island methylation of different gene was shown to vary among different grades. As shown in **Table 4**, we observed a significant increase in the methylation rate of different CpG islands of SOCS3 gene with higher pathological grades (P<0.05). A ROC curve is carried out to analyze JAK2, STAT3 and SOCS3 gene promoter CpG island methylation (**Figure 3A-C**). ROC curve is used to decide the optimal model through determining the best threshold for a diagnostic and predictive test. The larger the area under the curve (AUC) of the ROC curves, the more accurate the test is. As shown in **Figure 3**, SOCS3 gene promoter CpG sites showed high AUC of the ROC curves (SOCS3: *P*=0.002, AUC=0.746), but not JAK2 (*P*=0.265, AUC=0.585), STAT3 (*P*=0.735, AUC=0.527). These results indicate that the detection of SOCS3 gene promoter CpG methylation may be used as an early biomarker for BCa diagnosis.

Discussion

BCa is one of the most common malignant tumor in urinary system and also the second death cause in patients with urinary tract tumors [29]. It has been suggested to arise through the accumulation of multiple genetic changes involving a complex multi-step process [30]. But the precise pathogenesis and mechanisms remains unclear. It is known that DNA methylation, one of the most common epigenetic changes, frequently occurs in various types of human cancer, including bladder cancer. In recent years, numerous studies have revealed that the epigenetic dysregulation of tumor suppressor genes could serve as a biomarker to predict the diagnosis and prognosis of human malignancy [31, 32]. In the present study, we describe the blood-derived DNA aberrant methylation of JAK2, SOCS3 and STAT3 gene in BCa cases and normal control participants in China. Meanwhile, we also observe the methylation status of the CpG islands within the promoter of these genes. A number of studies have reported that aberrant methylation of SOCS3 gene in prostate cancer [33], intimal hyperplasia and restenosis [34], polycythemia vera (PV), essential thrombocythemia (ET) [35] and lung cancer [36]. Although the methylation status varied among different CpG island, we showed that frequent promoter hypermethylation of SOCS3 gene was significantly elevated in BCa compared with normal control participants.

SOCS3 protein, a member of SOCS family protein, whose expression could be induced in response to a wide range of cytokines, growth factors and hormones [37]. Many cytokines can induce the expression of SOCS3 gene to a variable extent in different cell types and tissues that signal through JAK-STAT pathway or through receptor protein tyrosine kinase (RPTK) [38]. SOCS3 protein exert function via it's Src homology 2 (SH2) domain, N-terminal region and C-terminal domain [39]. Moreover, SOCS3's Nterminus contains a kinase-inhibitory region (KIR), which can confer inhibition of JAK kinase activity [40]. The KIR of SOCS3 occludes the substrate-binding groove on JAK2 and blocks substrate association [41]. SOCS3 protein attenuate signal responses to cytokines and growth factors in target cells through these interactions. JAK-STAT signaling pathway plays a crucial role in the initiation of malignant transformation and during cancer progression [42]. SOCS3 protein, the negative regulatory protein of JAK-STAT signaling pathway, may also plays critical regulatory roles in cancer initiation and progression. In normal cells SOCS3 is normally activated by JAK-STAT signaling, which in turn inhibits JAK activity and subsequent STAT3 activation. During development process of cancer, this important negative regulatory machinery is suppressed when SOCS3 is silenced by promoter hypermethylation [36]. This may result in cells becoming more sensitive to aberrant growth stimulating signals that function through

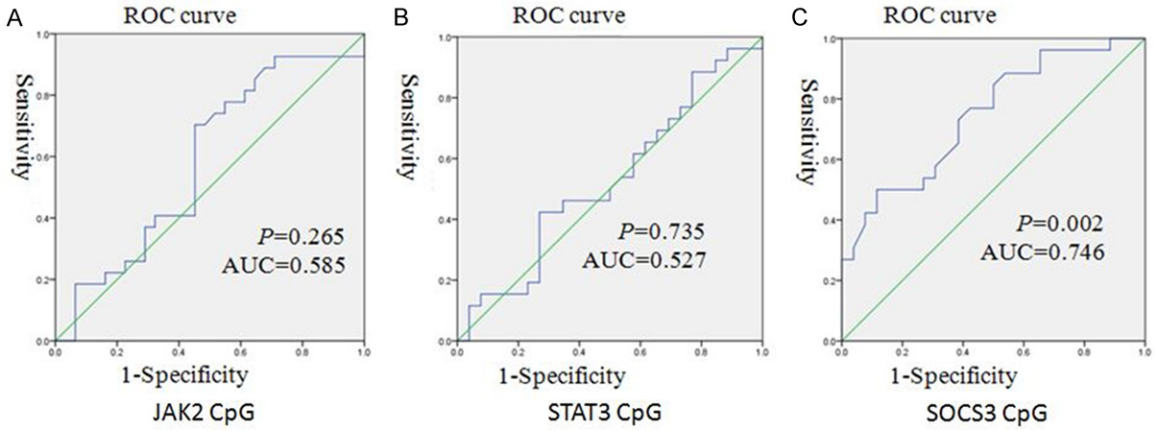

Figure 3. A: Receiver operating characteristic (ROC) curves for CpG sites in JAK2 gene promoter. B: Receiver operating characteristic (ROC) curves for CpG sites in STAT3 gene promoter. C: Receiver operating characteristic (ROC) curves for CpG sites in SOCS3 gene promoter. Note: The ROC curves plot sensitivity and 1-specificity. Areas under the curve (AUC) and *P* values were shown in the graph.

the JAK-STAT pathway, leading to cell growth and survival. He et al. [36] had demonstrated that hypermethylation of the SOCS3 promoter region also correlated with silencing of SOCS3 in breast cancer and mesothelioma cell lines. Therefore, the phenomenon of SOCS3 silencing via promoter methylation may be a common event during oncogenesis. SOCS3 gene as an important tumor suppressor gene exert function. Our results showed that the SOCS3 gene was highly methylated in BCa, which in accordance with the data of the normal control participants. Nevertheless, much work remains to be done to obtain a clearer understanding of the role of SOCS3 in JAK-STAT signaling and tumor pathogenesis.

Conclusions

In conclusion, our study demonstrates that promoter hypermethylation of SOCS3 gene is associated with BCa and thus, may serve as an independent prognostic biomarker. Despite the current study possessing enough statistical power, the sample size of our study was limit, so our findings should be confirmed in larger case control studies.

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Disclosure of conflict of interest

None.

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