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DNA methylation patterns of the SOCS1 gene in peripheral blood identifies risk loci associated with bladder cancer based on principal component analysis

Hongjun GUAN[1,2], Junhua LU[3], Mengying QU[1], Lili MENG[4], Yupeng GUO[2], Xiaoxia LI[2], Hongmei SHEN[1,*]

[1]Institute of Iodine Deficiency Disorders, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University, Harbin, China; [2]College of Public Health, Mudanjiang Medical University, Mudanjiang, China; [3]College of Nursing, Mudanjiang Medical University, Mudanjiang, China; [4]The First Clinical Medical College, Mudanjiang Medical University, Mudanjiang, China

**Correspondence: shenhm119@hrbmu.edu.cn*

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Bladder cancer (BCa) is a common carcinoma of the urinary tract, which occurs in the bladder mucosa. In recent years, people have recognized that epigenetic changes such as DNA methylation play important roles in the development of BCa but the specific mechanism is unclear. In this study, we detected the methylation rates in the SOCS1 gene of 490 subjects (including 247 patients with BCa and 243 healthy controls) using the MassARRAY EpiTYPER system. Principal component analysis (PCA) was conducted with the aim of identifying common underlying patterns that could explain the largest part of common variance in methylation across units. A logistic regression model was used to assess the relation of SOCS1 methylation patterns with factors related to BCa risk. The methylation rates varied for different CpG units and were significantly different in BCa patients compared to controls. Six principal component factors were extracted by combining initial eigenvalue, explanatory power, and Scree Plot. After adjusting for age, gender, family history of bladder cancer, smoking, and drinking, we observed that Factor 1 (OR=0.051, 95% CI: 0.015–0.178, p<0.001), Factor 2 (OR=0.146, 95% CI: 0.073–0.295, p<0.001), Factor 3 (OR=0.346, 95% CI: 0.198–0.606, p<0.001), and Factor 4 (OR=0.270, 95% CI: 0.135–0.537, p<0.001) were associated with BCa. Based on follow-up results, we found that the 1-, 3-, 5-year survival rates in the hypermethylated group were lower than in the hypomethylated group. We found that several CpG units in methylation patterns were associated with the incidence of BCa showing the important DNA methylation patterns for BCa pathogenesis. Our findings provided new insights into understanding this disease and new potential targets for therapeutic intervention for BCa patients in the future.

Key words: bladder cancer, DNA methylation, suppressor of cytokine signaling 1, principal component analysis

Bladder cancer (BCa) is a common carcinoma of the urinary tract, which occurs in the bladder mucosa, and ranks as the 12th most frequently-diagnosed cancer and 16th in terms of deaths worldwide, with an estimated 549,393 new cases diagnosed and 199,922 deaths in 2018 [1]. In 2020, there will be about 81,400 new diagnoses and 17,980 deaths in the United States [2]. According to the latest data from 2015, there were 78,100 new diagnoses of BCa and 32,100 deaths in China, and the incidence is increasing year by year [3]. BCa seriously affects the normal life of modern people, not only destroys the level of the body's metabolism but also does great harm to the urinary system [4, 5]. In the last decades, BCa has received mounting attention as a leading public health problem. Therefore, it is very important to study the pathogenesis of bladder cancer [6].

The occurrence of cancer is associated with changes in genomic levels, as well as epigenetic changes such as DNA methylation playing important roles in cancer [7–9]. In recent years, studies have found that the content of dissociated DNA in peripheral blood of tumor patients is significantly higher than that of normal people [10, 11], and the methylation of related genes in tumor tissues can also be detected [12–15]. Because peripheral blood DNA methylation can be detected before the disease, it is expected to become a new potential molecular marker for cancer diagnosis and prognosis. Compared with cystoscopy, the collection of peripheral blood samples is convenient, non-invasive, and has high patient compliance. Detection of DNA methylation in peripheral blood is beneficial to early diagnosis. DNA aberrant methylation can participate in the formation of BCa by affecting chromatin structure and expression of oncogenes or tumor suppressor genes. Nowadays, more and more studies suggested that DNA methylation, as an epigenetic change, had been confirmed to be involved in

the occurrence and development of BCa [16, 17]. Aberrant methylation of CpG islands located in the promoter region leads to inactivation by inhibiting transcription and plays a key role in tumorigenesis [18]. Therefore, DNA methylation of specific genes can be used as a biomarker to diagnose and monitor BCa.

The suppressor of cytokine signaling-1 (SOCS1) is a major inhibitor of the activity of the JAK/STAT pathway and negatively regulates the signaling of many cytokines [19, 20]. The transcriptional activity of SOCS1 is regulated by transcriptional silencing due to the methylation status [21]. We had previously reported that the methylation of SOCS1 was associated with BCa [22]. But its function and possible role in BCa pathogenesis are still unknown. In light of SOCS1's potential role in BCa risk, we explored the association between SOCS1 gene methylation and BCa, and reveal the important DNA methylation patterns for BCa pathogenesis.

Patients and methods

Study populations. Peripheral blood specimens were collected from 247 patients with BCa who were first diagnosed by pathological histology and 243 healthy controls who were the health check-up crowd. The subjects were enrolled from Tumor Hospital Affiliated to Harbin Medical University, Hongqi Hospital Affiliated to Mudanjiang Medical University, and Mudanjiang Tumor Hospital from September 2013 to March 2019. Inclusion criteria for cases: 1) at least 18 years old; 2) clearly diagnosed with BCa by postoperative pathology; 3) having a clear consciousness to complete the questionnaire; 4) with complete medical records. Exclusion criteria for cases: 1) patients with postoperative pathological diagnosis of non-bladder cancer; 2) patients who could not complete the questionnaire; 3) patients with bladder cancer complicated with other tumors. Healthy controls were selected from healthy subjects who underwent physical examinations at the above three hospitals during the same period. Meanwhile, healthy controls were without tumor. Our study was approved by the local ethics committee and all participants signed informed consent. A 5 ml peripheral blood specimen was collected from each subject and an epidemiological survey was conducted to obtain baseline characteristics of all participants. The serum was separated and stored at –80 °C for subsequent testing.

DNA extraction and methylation detection. Genomic DNA from peripheral blood specimens was extracted by using a QIAmp DNA blood mini kit according to the manufacturer's protocol (QIAGEN, Hilden, Germany). The DNA concentration and purity were measured by the NanoDrop one. According to the primer sequence, PCR reaction system and method determined in our previous study [22], we carried out experiments to detect the methylation level of CpG island in the promoter region of the SOCS1 gene. All PCR amplifications were repeated. For CpG specific analysis, discard data when the standard deviation (SD) of repeated measurements ≥5%.

Data analysis. The study database was established using Epidata (version 3.0). The continuous variables were expressed as mean and standard deviation (SD) unless noted otherwise, the categorical variables were represented by frequency unless noted otherwise. The t-test was used for the comparison of methylation rates between groups. Correlation analysis among SOCS1 CpG units was initially performed to discover the architecture of relationships among the methylation units studied. Then a principal component analysis (PCA) was carried out with the aim of identifying common underlying patterns, which could explain the largest part of common variance in methylation across units. Logistic regression models were used to assess the relation of the SOCS1 methylation patterns with factors related to BCa risk. Kaplan-Meier curve was used to discuss the median survival in both groups. A p-value <0.05 (two-tailed) was considered statistically significant. SPSS12.0 (SPSS Inc., Chicago, Illinois, USA) was applied in the data analysis.

Results

The methylated rates of the SOCS1 gene were detected in 247 BCa patients and 243 normal controls by using the MassARRAY EpiTYPER system. There was No.1 CpG island (contains 34 CpG units, the actual test coverage 28 CpG units) and No.2 CpG island (contains 62 CpG units, the actual test coverage 48 CpG units) in the promoter region of the SOCS1 gene. As shown in Figure 1 and Figure 2, the methylated rates varied for different CpG units. And compared with healthy controls, the methylation rates were significantly different in BCa patients.

Standardize the methylated data of CpG units, means and SD of methylation levels at the SOCS1 CpG units studied are shown in Table 2. We carried out a correlation analysis to identify a possible connection among CpG units with statistically significant differences in methylated rates between the case group and the control group. And then we found significant CpG units inter-correlations shown in Figure 3.

Subsequently, we conducted a PCA in order to identify common underlying components that could explain the largest part of methylation variability shared across units. In general, the Kaiser-Meyer-Olkin (KMO) test coefficient is distributed between 0 and 1. If the coefficient value is greater than 0.6, the sample is considered to meet the requirements of a reasonable data structure. According to the results of this study, the coefficient of the KMO test was 0.623, and Bartlett's sphere test rejected the null hypothesis (χ^2=437.825, p<0.001). Thus, the study data could be used for principal component extraction.

According to the criterion for factor selection were initial eigenvalue >1.0, six main methylated factors emerged with PCA (Table 1), which could explain a large part of gene methylated variance (73.3%).

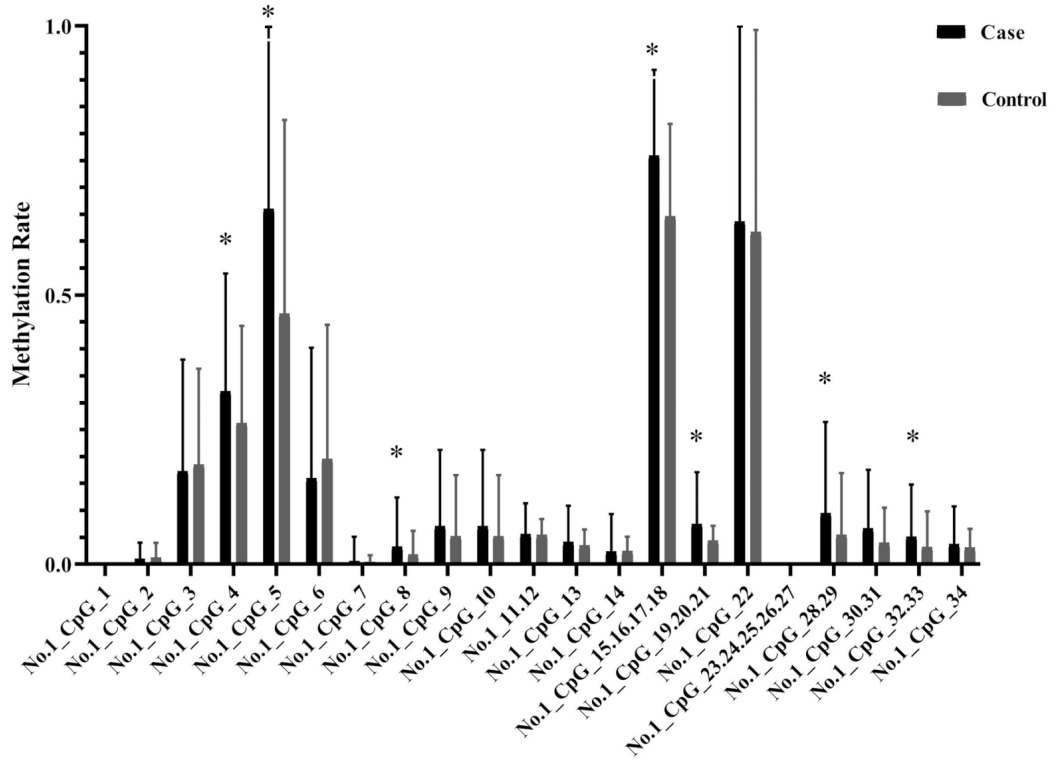

Figure 1. Comparison of CpG units methylated rates in the SOCS1 gene. (No. 1 CpG islands) The methylation level of CpG units was expressed as mean ± SD. *represented that there is a statistical difference in methylation levels of CpG units between cases and controls (p<0.05).

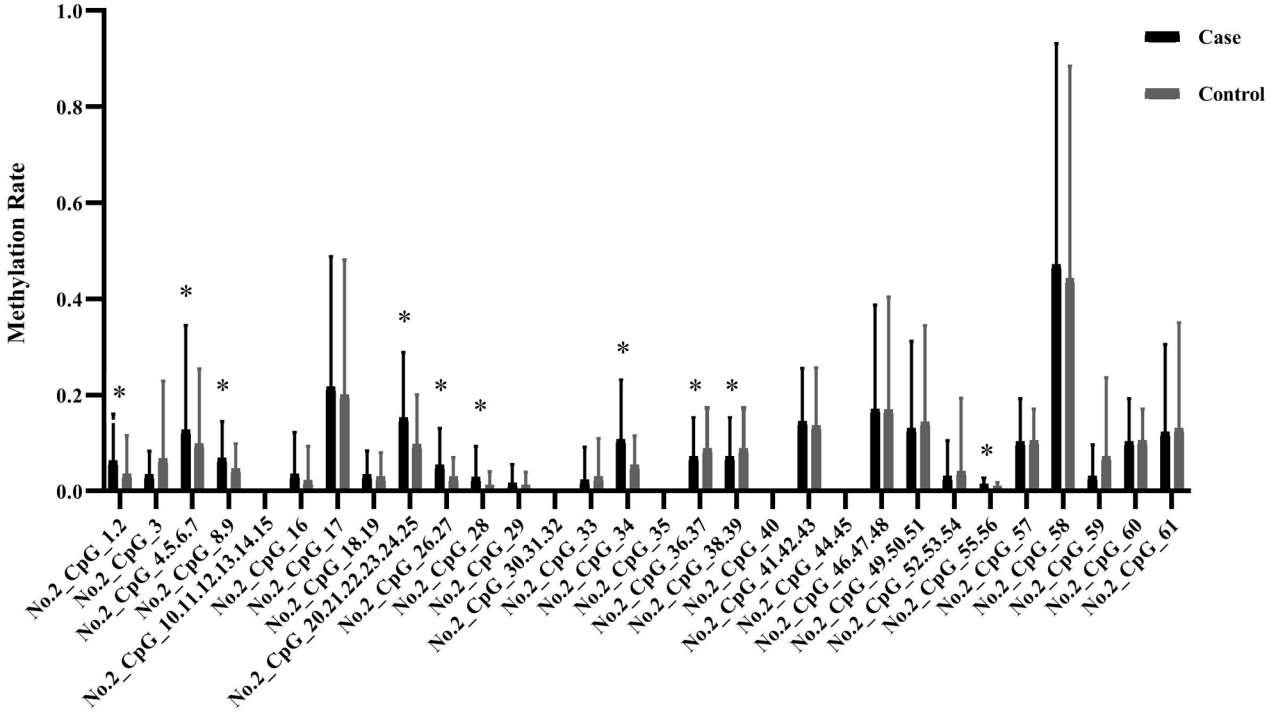

Figure 2. Comparison of CpG units methylated rates in the SOCS1 gene. (No. 2 CpG islands) The methylation level of CpG units was expressed as mean ± SD. *represented that there is a statistical difference in methylation levels of CpG units between cases and controls (p<0.05).

Table 1. Initial eigenvalues and total variance explained.

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	2.618	18.700	18.700	2.618	18.700	18.700
2	2.093	14.951	33.651	2.093	14.951	33.651
3	1.817	12.98	46.631	1.817	12.98	46.631
4	1.458	10.413	57.044	1.458	10.413	57.044
5	1.215	8.675	65.719	1.215	8.675	65.719
6	1.065	7.607	73.326	1.065	7.607	73.326
7	0.761	5.433	78.759			
8	0.719	5.133	83.892			
9	0.643	4.595	88.487			
10	0.571	4.079	92.567			
11	0.433	3.095	95.662			
12	0.344	2.454	98.116			
13	0.182	1.300	99.416			
14	0.082	0.584	100.00			

Table 2. Distribution of SOCS1 factor loadings and specific CpG unit methylation.

CpG units	Factor loading						Methylated levels		
	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	N	Mean	SD
No.1_CpG_4	0.402	–0.519	0.340	–0.219	–0.093	–0.044	486	0.292	0.203
No.1_CpG_5	0.062	–0.536	0.282	–0.066	0.245	0.627	345	0.566	0.393
No.1_CpG_8	0.134	0.152	–0.615	0.200	0.424	0.241	435	0.025	0.071
No.1_CpG_15-18	0.829	–0.354	–0.079	0.020	–0.017	0.100	487	0.704	0.175
No.1_CpG_19-21	0.917	–0.025	–0.171	0.150	0.027	0.141	444	0.059	0.072
No.1_CpG_28.29	0.393	0.406	0.433	0.325	–0.167	0.087	471	0.074	0.146
No.1_CpG_32.33	0.428	0.614	–0.423	0.115	0.155	0.065	455	0.041	0.083
No.2_CpG_1.2	0.074	0.346	–0.202	–0.749	0.151	0.579	415	0.049	0.09
No.2_CpG_4-7	0.607	0.122	0.351	0.012	–0.187	–0.373	396	0.113	0.189
No.2_CpG_8.9	–0.393	0.257	0.276	0.517	–0.141	0.223	480	0.058	0.066
No.2_CpG_20-25	0.090	0.529	0.469	0.162	0.303	0.060	387	0.123	0.123
No.2_CpG_26.27	0.126	0.446	0.541	–0.393	0.304	0.058	385	0.042	0.063
No.2_CpG_28	0.117	0.267	–0.295	–0.055	0.354	0.145	442	0.021	0.051
No.2_CpG_34	0.057	–0.31	–0.059	0.459	–0.725	–0.135	436	0.081	0.102

A scree plot was drawn according to the degree of the data variation interpreted by each principal component. In this study, the seventh principal component point was the inflection point, and the data after it tends to be flat (Figure 4). Therefore, the first six principal components could be extracted.

Six principal component factors were extracted by combining initial eigenvalue, explanatory power, and Scree Plot. Factor 1 showed high loadings of CpGs No.1_CpG_15-18, No.1_CpG_19-21, No.2_CpG_4-7. Factor 2 showed high loadings of CpGs No.1_CpG_4, No.1_CpG_32-33, No.2_CpG_20-25. Factor 3 showed high loadings of CpGs No.1_CpG_8, No.2_CpG_26-27. Factor 4 showed high loadings of CpGs No.2_CpG_1-2, No.2_CpG_8-9. Factor 5 showed high loadings of CpGs No.2_CpG_28, No.2_CpG_34. Factor 6 showed high loadings of CpGs No.1_CpG_5. As presented in Table 2.

Then, we used a logistic regression model to evaluate association between SOCS 1 methylated factors and BCa, as shown in Table 3. After adjusting for age, gender, family history of bladder cancer, smoking, and drinking, we observed that Factor 1 (OR=0.051, 95% CI: 0.015–0.178, p<0.001), Factor 2 (OR=0.146, 95% CI: 0.073–0.295, p<0.001), Factor 3 (OR=0.346, 95% CI: 0.198–0.606, p<0.001), Factor 4 (OR=0.270, 95% CI: 0.135–0.537, p<0.001) were associated with BCa. We considered that Factor 1–4 were independent risk factors for BCa.

According to the value of Factor 1–6, we looked retrospectively at the raw data. Though the distribution of Factor 1–6 varied from two groups, the distribution of factor 1 and factor 5 was statistically significant between the two groups, as shown in Table 4. Combined with the results of multiple logistic analysis, we attempted to analyze the diagnostic value of Factor 1. As shown in Figure 5, the AUC was 0.751

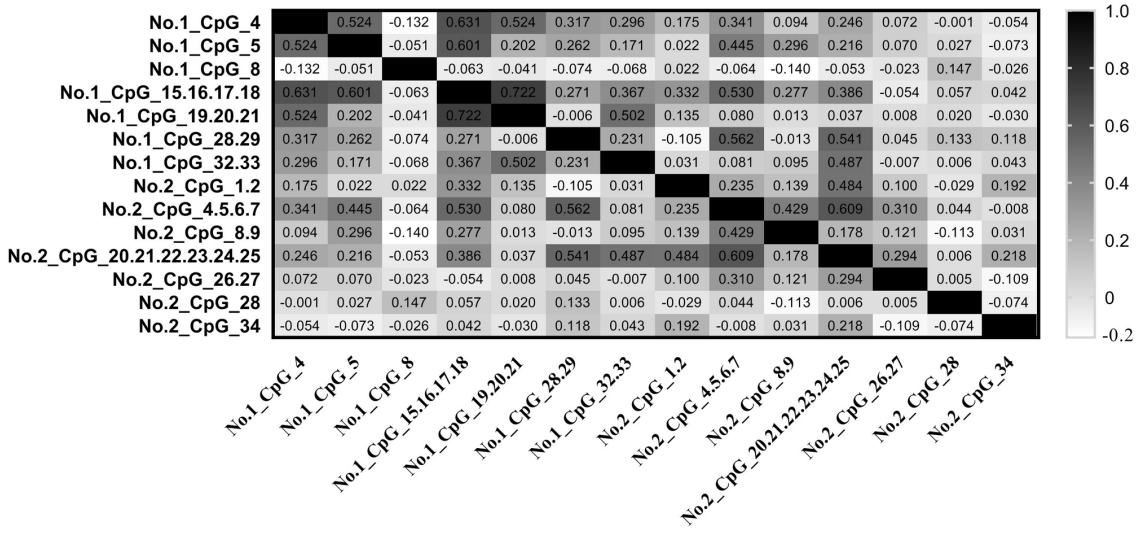

Figure 3. Correlations among the SOCS1 CpG units. Heatmap showing the SOCS1 CpG unit inter-correlations. A correlation coefficient is depicted for each CpG unit pair as color ranges from green (r=−0.40) to red (r=1.00). p-values of correlations are indicated for each CpG unit pair in the correspondent boxes.

Table 3. Multiple logistic regression between Factors and BCa.

Item	B	S.E.	Wald	df	p value	OR	OR 95% CI	
							Lower	Upper
Age	0.008	0.025	0.105	1	0.746	1.008	0.959	1.059
Gender	−0.589	0.498	1.396	1	0.237	0.555	0.209	1.474
Family history of BCa	−0.144	0.768	0.035	1	0.852	0.866	0.192	3.903
Smoking	0.384	0.453	0.720	1	0.396	1.469	0.604	3.568
Drinking	0.435	0.479	0.822	1	0.365	1.544	0.604	3.952
Factor 1	−2.98	0.639	21.763	1	<0.001	0.051	0.015	0.178
Factor 2	−1.921	0.357	28.947	1	<0.001	0.146	0.073	0.295
Factor 3	−1.061	0.286	13.794	1	<0.001	0.346	0.198	0.606
Factor 4	−1.311	0.351	13.903	1	<0.001	0.270	0.135	0.537
Constant	−0.521	2.519	0.043	1	0.836	0.594		

Note: Adjusted for age, gender, family history of BCa, smoking, and drinking.

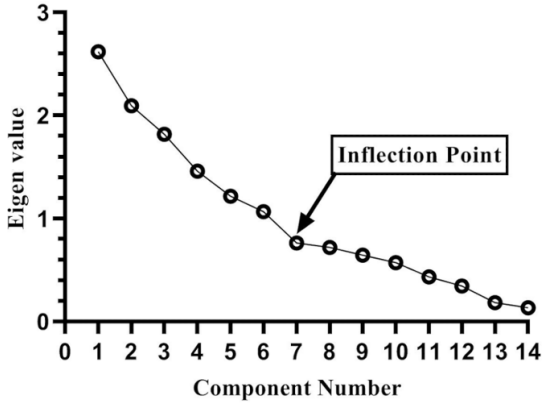

Figure 4. Scree Plot. Each principal component was a point and the number of principal components was extracted according to the location of "steep slope tends to be gentle".

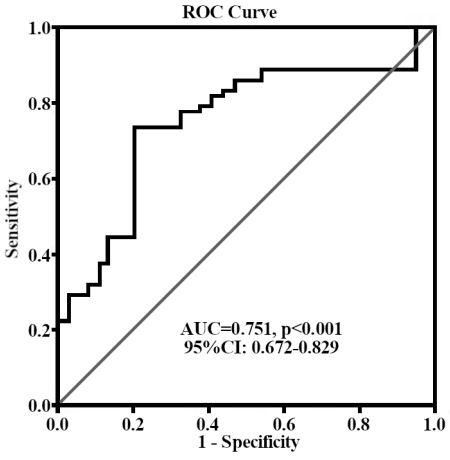

Figure 5. ROC curve for Factor 1. A ROC curve was set out to analyze the diagnostic value of Factor 1 for BCa. The area under the ROC curve (AUC) was calculated to illustrate the accuracy of the results.

(p<0.001, 95% CI: 0.672–0.829). The sensitivity was 73.6% and the specificity was 79.6%. These showed that there was good value in diagnostic for BCa.

We also followed up 143 bladder cancer patients after surgery. According to the cut-off value [22], the subjects were divided into two groups. There were 51 cases in the hypomethylation group (CpG island methylation rate <10.93%) and 92 cases (CpG island methylation rate ≥10.93%) in the hypermethylated group. A total of 3–82 months was followed up. Two patients died of other diseases (1 patient died of bloody ascites, 1 patient died of secondary infection) and 4 patients were lost to follow-up in the hypomethylated group. Three patients died of other diseases (1 patient died of abnormal liver function, 2 patients died of secondary infection) and 6 patients were lost to follow-up in the hypermethylated group. There was no difference in the distribution of deaths from other diseases and loss of follow-up between the two groups (χ^2=0.137, p=0.711). The results of the Kaplan-Meier curve showed that the median survival of the hypomethylated group was 68 months (95% CI: 53.735~82.265 months), while the median survival of the hypermethylated group was 26 months (95% CI: 11.050~40.950 months). There was statistically significance between the two groups (Log Rank (Mantel-Cox) χ^2=5.795, p=0.016) as shown in Figure 6. The 1-year, 3-year, and 5-year survival rates in the hypomethylated group were 81.3%, 75.0%, and 58.9%, respectively. However, the 1-year, 3-year, and 5-year survival rates in the hypermethylated group were 69.0%, 52.1%, and 31.5%, respectively.

As shown in Figure 7, the 1-year, 3-year, and 5-year survival rates varied from two groups, no statistical significance was found by linear-by-linear association analysis (χ^2=1.827, p-trend = 0.176). But there was a statistical significance in the 5-year survival rate between the two groups (χ^2=9.889, p=0.002).

Discussion

It is well known that DNA methylation in the promoter region is the most common epigenetic modification. It is associated with various types of human cancer development and metastasis. With the application of molecular genetics in the field of human cancer research, the importance of DNA methylation in human cancer has been gradually recognized, and the regions in the genome that may have functional significance due to inhibiting gene activity have become the focus of research. It has been revealed that epigenetic disorders of tumor suppressor genes can be used as biomarkers to predict the diagnosis and prognosis of human malignant tumors [23, 24]. Recent studies [21, 25] had shown that suppression of protein expression by aberrant methylation in the promoter region of the SOCS1 gene was associated with several types of malignancy, such as pancreatic ductal neoplasms, chronic myeloid leukemia, gastric carcinomas, etc. Besides, the association between SOCS1 gene methylation and BCa was also found in our previous research [22]. However, its methylation pattern is still unclear.

In this study, we found CpG islands investigated (No.1_CpG and No.2_CpG) in annotations of DNA methylation

Table 4. Distribution of Factor 1-6 between BCa and control groups.

Factor		Case Group	Control Group	χ^2	p-value
Factor 1	Yes	178	50	130.523	<0.001
	No	69	193		
Factor 2	Yes	105	101	0.045	0.832
	No	142	142		
Factor 3	Yes	99	85	1.359	0.244
	No	148	158		
Factor 4	Yes	128	132	0.307	0.579
	No	119	111		
Factor 5	Yes	59	33	8.532	0.003
	No	188	210		
Factor 6	Yes	32	23	0.001	0.977
	No	215	220		

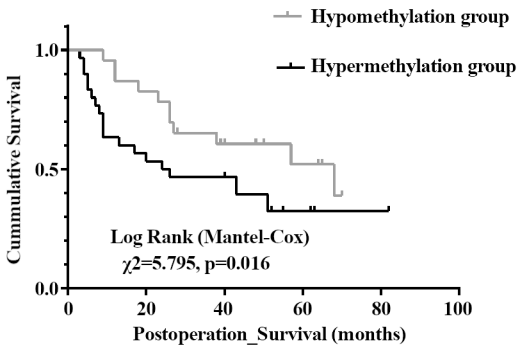

Figure 6. Survival analysis of CpG island methylation rate in the SOCS1 promoter region and bladder cancer. 143 bladder cancer patients were followed up after surgery. According to the cut-off value [22], the subjects were divided into a hypomethylated group and a hypermethylated group. Kaplan-Meier curve was used to assess the difference of median survival between the two groups. There was statistically significance between the two groups (Log Rank (Mantel-Cox) χ^2=5.795, p=0.016).

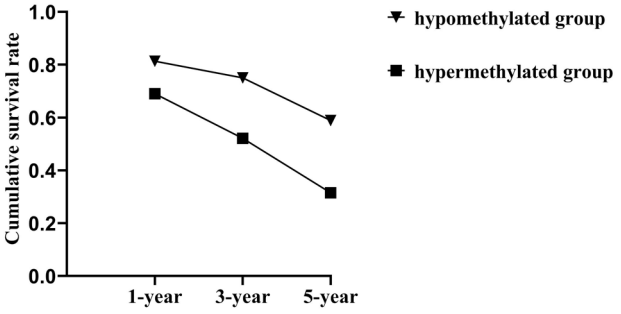

Figure 7. The 1-year, 3-year, and 5-year survival rates between the two groups. Linear-by-linear association analysis was carried out to reveal the difference of 1-year, 3-year, and 5-year survival rates between the two groups.

sites covered regions of 5000 bp upstream and 1000 bp downstream of the transcription start site (TSS) of the SOCS1 genes contained 96 CpG units. In fact, we successfully tested 76 CpG units of them. Some scholars had suggested that CpG islands associated with TSS rarely show tissue-specific methylation patterns [26, 27]. In contrast, CpG regions 2kb away from the CpG islands had highly conserved specific methylation patterns, and methylation was highly correlated with decreased gene expression [28]. Taking this into consideration, we expanded the exploration area. Based on the principal component analysis, we identified common linking patterns of CpG units investigated (Factor 1, Factor 2, Factor 3, Factor 4, Factor 5, and Factor 6). It was noted that Factor 1, Factor 2, Factor 3, Factor 4 are associated with the incidence of BCa, and it would be expected that CpG islands might display disease-specific patterns of DNA methylation.

The human SOCS1 gene is located in the 13.3p region of chromosome 16, and its expression product is a 221 amino acid protein. SOCS1, as a regulator of cytokine signal transduction, inhibits the activity of JAK2 by inhibiting the JAK/STAT signaling pathway [29, 30]. Since STAT3 is located downstream of JAK2 in the JAK/STAT signaling pathway, we speculated that the methylation of SOCS1 might affect the phosphorylation level of STAT3. Because when SOCS1 methylation occurred, IL-6 induces pSTAT3 more easily. Park et al. [31] proved that SOCS1 methylation caused gene silencing to increase the phosphorylation of STAT and promote tumor development. Inhibition of the JAK/STAT signaling pathway could lead to the activation of some cancer-promoting genes, thereby accelerating the invasion and metastasis of many solid tumor cells [32, 33]. In addition, SOCS1 could inhibit the activity of a variety of cytokines (such as IL-4, IL-6, tumor suppressor protein M, leukemia inhibitory factor, growth hormone, gamma-interferon) [34]. Cytokines and other signal transduction factors, together with SOCS1, are involved in mediating cell apoptosis and the occurrence and development of cancer [35]. Therefore, SOCS1 gene silencing caused by DNA methylation was involved in the progression of tumors and plays an important role.

Hall et al. [36] revealed that tumor stage was the only indicator of postoperative survival. Munoz et al. [37] showed that the 5-year survival rate of patients with Tis tumors, local tumors, and distant metastasis was 95.1%, 88.9%, and 16.5%, respectively. In our study, SOCS1 methylation rate was risk factor influencing the survival of BCa. A significant difference in the 5-year survival rate occurred between the hypomethylated group and the hypermethylated group. This suggests that it is important to detect the methylation level for early diagnosis and prognosis of BCa.

In conclusion, we found that several CpG units in methylation patterns were associated with the incidence of BCa and revealed the important DNA methylation patterns in the pathogenesis of BCa. Our findings provided new insights into understanding this disease and new potential targets for therapeutic intervention for BCa patients in the future. However, limited by hospital case collection systems, we only followed up patients with BCa. This was also a shortcoming of our study. Further investigations should be conducted to reveal how many healthy controls with Factor 1–4 develop BCa in 1-year, 3-year, and 5-year. And due to the limitations of geographical region and sample size, much more remains to be done to explore the role of SOCS1 in the JAK/STAT signaling and tumor pathogenesis.

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