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Original Article

Clinicopathological significance of signal transducer and activator of transcription 4 in bladder urothelial carcinoma tissues: a study of immunohistochemistry and bioinformatics

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Abstract: Purpose: Signal transducer and activator of transcription 4 (STAT4) has been detected in several cancers. However, the clinical significance of STAT4 in bladder urothelial carcinoma (BUC) has not been clear. Thus, the purpose of the current study was to assess the clinical value of STAT4 in BUC. Methods: The STAT4 protein expression was detected by immunohistochemistry in formalin-fixed and paraffin-embedded (FFPE) tissues in 166 patients with BUC and 56 normal bladders. The diagnostic role of STAT4 protein in BUC, as well as the relationship between STAT4 and disease progression was analyzed statistically. Next, expression of STAT4 mRNA was evaluated by microarray by using the database of the Gene Expression Omnibus (GEO). Furthermore, genomic alteration of STAT4 was analyzed from The Cancer Genome Atlas (TCGA) via the website of cBioPortal. Results: STAT4 protein expression was significantly lower in BUC (48.2%) than that in noncancerous bladder tissues (82.1%, P<0.001). The area under curve of receiver operating characteristic of STAT4 was 0.670 (95% CI=0.592-0.747, P<0.001). Spearman correlation showed that there were close negative relationships between STAT4 protein level and lymph node metastasis (r=-0.217, P=0.005) and T category (r=-0.314, P<0.001). In addition, STAT4 mRNA was significantly lower in cases with surrounding carcinoma in situ (CIS) than those without CIS (P=0.001) from GDS4456 in GSE31684. Conclusion: STAT4 might be important in the tumorigenesis and progression of BUC and could represent a promising biomarker for BUC. However, large cohort studies are urgently desired to evaluate the prognostic value of STAT4 in BUC, as well as functional experiments are required to unveil the molecular mechanism of STAT4 in BUC.

Keywords: Signal transducer and activator of transcription 4 (STAT4), bladder urothelial carcinoma (BUC), TCGA, immunohistochemistry, GEO

Introduction

Bladder cancer is one of the most common malignant tumors of the urinary system. The incidence rates are generally high in Western Europe, Northern America and Northern Africa [1]. According to the global cancer statistics, an estimation of 430,000 new bladder cancer cases and 165,100 deaths occurred in 2012 worldwide [2]. The male had an occurring rate twice to triple as compared to the female [3]. In 2016, the new cases of bladder cancer will be possible to reach 58,950 in male and 18,010 in female [4]. Bladder cancer is of great harm around the world, and early detection, accurate diagnosis and effective treatment of bladder cancer have great significance. Bladder cancers are histologically classified into urothelial carcinoma, squamous cell carcinoma, neuroendocrine tumors, lymphoid tumors, etc, [5] and bladder urothelial carcinoma (BUC) is the most common subtype.

Signal transducer and activator of transcription (STAT) family is a type of protein which is critical in the transcription of nuclear genes [6]. STAT4, a member of the STAT protein family, is located in human chromosome 2q32.3 [7]. As a vital transcription factor, STAT4 has been proved to play an essential role in T-helper type (Th1) cell

Table 1. Relationship between STAT4 expression and clinicopathological features

Clinicopathological Features		Total number (n)	Positive number (n)	Positive rate (%)	X^2	P
Tissue	Non-cancerous bladder	56	46	82.1	19.665	<0.001
	BUC	166	80	48.2		
Gender	Female	25	18	72.0	6.682	0.010
	Male	141	62	44.0		
Age	<65	90	47	52.2	1.278	0.258
	≥65	76	33	43.4		
Grade	Low (I-II)	100	54	54.0	3.397	0.065
	High (III)	66	26	39.4		
T	Ta-T1	63	43	68.3	16.366	<0.001
	T2-T4	103	37	35.9		
Diameter	<3 cm	100	54	54.0	3.397	0.065
	≥3 cm	66	26	39.4		
Tumor number	Single	157	74	47.1	0.054	0.817
	Multi	9	6	66.7		
Lymph node metastasis	No	158	80	50.6	7.819	0.005
	Yes	8	0	0		
Distant metastasis	No	161	80	49.7	0.982	0.322
	Yes	1	0	0		
Growth pattern	Papillary	129	60	46.5	0.655	0.418
	Solid	37	20	54.1		

proliferation, differentiation and the immunity of the body [8]. Hence, extensive studies have been focused on the connection between STAT4 and immune diseases [9]. Recently, the role of STAT4 has been investigated in several malignancies including hepatocellular carcinoma (HCC) [10-12], gastric cancer [13], colon and rectal cancer [14, 15], cervical cancer[16], breast cancer [17] and glioma [18]. These findings showed that STAT4 might act as a vital biomarker for diagnosis, prognosis assessment and molecularly targeted therapy in malignancies. However, as stated by the existent reports, the possible function of STAT4 in bladder cancer is still unclear. To date, there has been only one paper reporting the expression of STAT4 in bladder cancer. El-Aal et al. [19] investigated the clinical role of STAT4 in 29 cases of chronic schistosomiasis haematobium Egyptian patients who were complicated with bladder cancer by digital real-time quantitative photocytometry, and found that there existed no obvious difference in the expression of STAT4 in different pathological types or different histology grades in bilharzial bladder cancer. Since the study of El-Aal et al. [19] was performed with a specific group of patients with schistosomiasis haematobium, the function of STAT4 in bladder cancer unrelated to schistosomal infections remains unclarified. Thus, the aim of the current study was to illuminate the clinical and pathological relationship between STAT4 expression and BUC with immunohistochemistry. In addition, we also verified the role of STAT4 expression with the assistance of public data, including Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA).

Materials and methods

Patient population and clinicopathological parameters

The current study was carried out on 166 patients with BUC and 56 normal bladder formalin fixed and paraffin embedded (FFPE) tissues. BUC patients who had undergone partial or complete cystectomy were collected at the First Affiliated Hospital of Guangxi Medical University, the People's Republic of China during January 2003 to December 2007. The recruited patients comprised 141 men and 25 women (age range, 27-96 years; mean age, 61.62 years). The ages of normal bladder samples from autopsies, whose cause of death was not related with BUC or other malignancies,

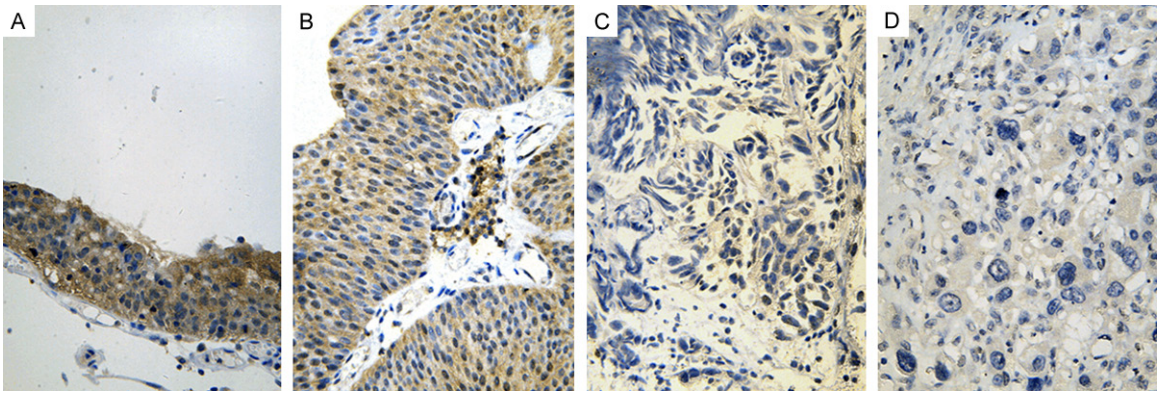

Figure 1. Protein expression of STAT4 in bladder urothelial carcinomas. The STAT4 protein signaling was located in the cytoplasm of bladder urothelial carcinoma (BUC) cells as detected by immunohistochemistry. A: Non-cancerous bladder tissue; B: BUC grade I with lymph node metastasis; C: BUC grade II with lymph node metastasis; D: BUC grade III without lymph node metastasis (400×).

were between 44 and 66 years, with a mean age of 52.43 years. Hematoxylin and eosin (H&E) method was used to stain slides and to observe tumor's differentiation level, which was subsequently divided into low or high grade [20-25]. Further, invasive status, pathologic stage (pT) [20-25], growth pattern of tumor (papillary/solid) for all BUCs were also evaluated (**Table 1**) [20-25]. None of the BUC patients in the current study had received any treatment, including prior chemotherapy, intravesical instillation or radiation therapy. The study protocol was approved by the Ethical Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent was signed by the patients and clinicians for the usage of the clinical samples for research.

Immunohistochemistry

Antibody of STAT4 was purchased from Santa Cruz Biotech Company, CA, USA (mouse monoclonal antihuman STAT4 antibody, PL-68, 1:300 dilution). The immunohistochemistry was performed as previously reported [10]. All stained tissues were diagnosed and scored by three pathological doctors independently (Wei-jia Mo, Yu-yan Pang and Gang Chen), without knowing any clinical info of patients. The percentage of positive cells per 100 cells was scored as 0 (0%), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). The intensity of immunohistochemical staining was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) [10]. Final score of each staining was achieved from the result of the positive score multiplied by the intensity score. Score more than 2 was regarded as positive expression of STAT4.

Verification of the clinical role of STAT4 mRNA by GEO

GEO is a high-throughput microarray expression database [26-28]. A literature search via GEO profile was conducted to filter microarray that studied STAT4 expression in BUC. The keywords for searching were as follows: 1) ("STAT4" OR "signal transducer and activator of transcription 4"); 2) ("bladder cancer" OR "bladder carcinoma" OR "bladder neoplasms" OR "carcinoma of bladder" OR "cancer of bladder" OR "urothelial carcinoma" OR "urothelial tumor" OR "UCC" OR "carcinoma of uroepithelium" OR "cancer of uroepithelium" OR "BUC"). The last update time for all microarrays was August 20, 2016. We sought for all related GEO profiles that explored the relationship between STAT4 and BUC and extract the data. After that, we calculated Standardized mean difference (SMD) and its 95% confidence intervals (CIs) via Stata 12.0 software (StataCorp LP, College Station, TX, USA) [29-31]. Fixed-effects model was used when heterogeneity among studies had no statistical significance (P>0.05); otherwise, random-effects model was conducted.

Further validation of the clinical role of STAT4 alterations by TCGA Dataset

To further validate the results of immunohistochemistry and GEO, cBioPortal (www.cbioportal.org) was used to assess the genetic alterations of STAT4 generated from 402 cases of

Table 2. Clinicopathological features of eligible datasets from GEO

Author	Reference Series	Related publication (PMID)	Year	Country	Dataset	Platform	Sample types	BUC (n)	Health controls (n)	Clinical parameters
Dyrskjøt L, et al.	GSE89	12469123	2003	Denmark	GDS183	GPL80: [Hu6800] Affymetrix Human Full Length HuGeneFL Array	RNA	40	0	T staging, Grade
Dyrskjøt L, et al.	GSE3167	15173019	2004	Denmark	GDS1479	GPL96: [HG-U133A] Affymetrix Human Genome U133A Array	RNA	46	14	T staging, Grade, Tissue type
Riester M, et al.	GSE31684	22228636, 24486590	2012	USA	GDS4456	GPL570: [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	RNA	93	0	T staging, Grade, Gender, Lymph node metastasis, Recurrence, Distant metastasis, Smoking state, Surrounding (CIS)
Total								179	14	

Table 3. STAT4 mRNA and clinicopathological variables in GEO

Dataset	Groups		N	Mean ± SD	T	P
GDS183	T Staging	Early	31	6.32±0.66	0.615	0.542
		Advanced	9	6.17±0.63		
	Grade	Low	6	6.21±0.67	0.334	0.741
		High	33	6.31±0.66		
GDS1479	Tissue Type	Normal	14	6.42±0.64	0.909	0.367
		BUC (In situ and invasive)	46	6.28±0.46		
	T Staging	Early	28	6.14±0.38	-3.940	<0.001
		Advanced	13	6.67±0.44		
	Grade	Low	8	6.16±0.46	0.867	0.391
		High	32	6.32±0.44		
GDS4456	T Staging	Early	27	2.41±0.46	-1.410	0.162
		Advanced	66	2.62±0.98		
	Grade	Low	6	2.23±0.00	0.951	0.344
		High	87	2.58±0.89		
	Gender	Male	68	2.52±0.83	-0.582	0.562
		Female	25	2.64±0.96		
	Lymph Node Metastasis	No	49	2.53±0.73	-0.441	0.661
		Yes	28	2.63±1.17		
	Recurrence	No	85	2.58±0.90	0.726	0.47
		Yes	8	2.34±0.32		
	Metastasis	No	35	2.59±0.88	-0.291	0.771
		Yes	58	2.54±0.86		
	Smoking	Never	18	2.67±1.04	-0.616	0.539
		Smoking	75	2.53±0.82		
	Surrounding CIS	Absence	43	2.90±1.16	-3.486	0.001
		Presence	47	2.27±0.22		

BUC from TCGA (www.cancergenome.nih.gov [32-34]) on August 20, 2016. The relevant genetic alterations encompassed gene amplification, mRNA upregulation, mRNA downregulation, protein upregulation, protein downregulation and missense mutation. The schematic of OncoPrint was produced for visualizing the alterations directly from cBioPortal in Bladder Urothelial Carcinoma (TCGA, provisional). The plots figures were generated by cBioPortal based on disease free survival (DFS) and overall survival (OS). We also analyzed the relationship between one of the alterations, mRNA level of STAT4 and histology, stages, as well as survival of BUC. Finally, the gene network of STAT4 in BUC was produced by cBioPortal.

Statistical analysis

SPSS 22.0 was applied for statistical analysis. Chi-square test was performed to compare the differences of STAT4 expression in two corresponding groups of different clinicopathological parameters. The correlation between STAT4 expression level and clinicopathological parameters was assessed by Spearman's rank correlation. Receiver operating characteristic (ROC) curves were performed to find out the diagnostic values of STAT4. Survival analysis was performed by Kaplan-Meier method, and the survival was estimated with log-rank test. *P* value <0.05 was regarded as statistically significant.

Results

Expression of STAT4 protein in BUC with immunohistochemistry

STAT4 positive expression was found in 80 out of 166 BUC patients (48.2%), notably lower than that in noncancerous bladder tissues (82.1%, 46/56, P<0.001, **Table 1**; **Figure 1**). In addition, ROC curve was conducted to explore the diagnostic value of STAT4 expression in BUC. The area under curve of low expression of STAT4 was 0.670 (95% CI=0.592-0.747,

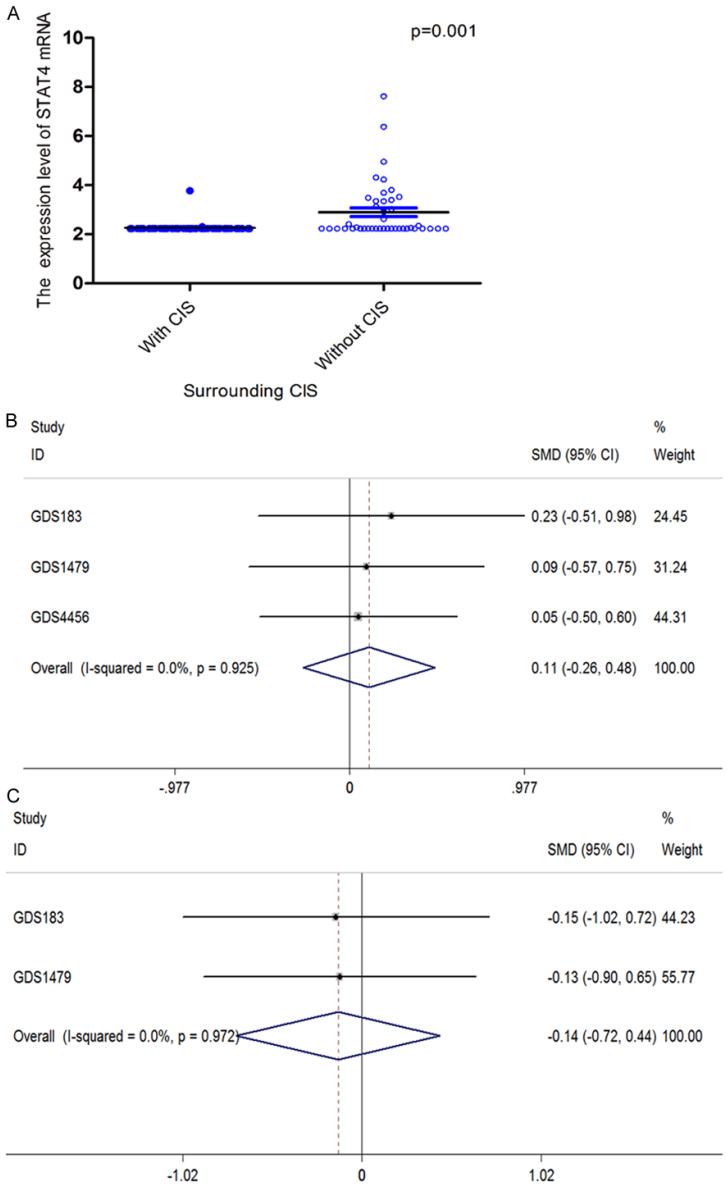

Figure 2. Clinical value of STAT4 mRNA based on GEO in patients with bladder urothelial cancer (BUC). A: The expression level of STAT4 mRNA between patients with surrounding CIS and without CIS (P=0.001, GDS4456); B: Forest plot of studies evaluating standard mean difference (SMD) of STAT4 expression between early and advanced T staging groups (a fixed-effects model). C: Forest plot of studies evaluating standard mean difference (SMD) of STAT4 expression between early low and high tumor grades (a fixed-effects model).

P<0.001) with a moderate clinical diagnostic value.

Correlation between STAT4 protein expression and clinicopathological variables of BUC

The positive ratio of Ta-T1 was 68.3% (43/63), distinctly higher than that in T2-T4 (35.9%, 37/103, P<0.001). Similarly, we found that the STAT4 expression ratio was 50.6% (80/158) in the cases with lymph node metastasis, and no STAT4 expression was noted in all eight patients with lymph node metastasis. Furthermore, the cases with lower histology grade and small size tended to have higher expression of STAT4, even the *P* value did not reach the significant level as evaluated by Chi-square test (both P=0.065, **Figure 1**; **Table 1**). The spearman correlation analysis also showed that there were close negative relationships between STAT4 protein level and lymph node metastasis (r=-0.217, P=0.005) and T category (r=-0.314, P< 0.001), which confirmed the results from Spearman's rank correlation analysis.

Verification of the clinical role of STAT4 mRNA by GEO

The characteristics of the enrolled datasets were summarized in **Table 2**, including the name of the first author, reference series, related publications, year, country, dataset, platform, sample types, numbers of samples and clinicopathological features. In total, three eligible datasets, including GDS183 (Denmark), GDS1479 (Denmark) and GDS4456 (USA) were involved in the analysis, including 179 BUC patients and 14 healthy controls. The different expression levels of STAT4 mRNA between BUC and normal tissues were analyzed. In the meantime, the association between STAT4 mRNA level and clinicopathological features were explored, including T staging, grade, gender, lymph node metastasis, recurrence, distant metastasis, smoking state and the presence or absence of surrounding carcinoma in situ (CIS).

Concerning the difference of STAT4 mRNA expression between BUC and normal controls,

Table 4. Meta-analysis of the relationship between STAT4 expression and T staging, tumor grade

Parameters	N*	Case/Control (n)*	Model	SMD (95% CI)	Heterogeneity test		Significance test
					P	I^2	P
T Staging	3*	74/100	Fixed-effect model	0.107 (-0.261, 0.475)	0.925	0%	0.568
Grade	2*	14/65	Fixed-effect model	-0.136 (-0.715, 0.443)	0.972	0%	0.644

N*: The number of the datasets included in the meta-analysis. 3*: GDS183 (Denmark), GDS1479 (Denmark) and GDS4456 (USA). 2*: GDS183 (Denmark), GDS1479 (Denmark). GDS4456 (USA) was excluded because of the standard deviation (SD) of the mean expression level of STAT4 mRNA in the group of low tumor grade was zero. Case/Control (n)*: There were 74 patients with early T staging (Ta-T1) and 100 with advanced T staging (T2-T4) in total three studies. And there were 14 patients with low grade (I-II) and 65 with high grade (III-IV) in total included two studies.

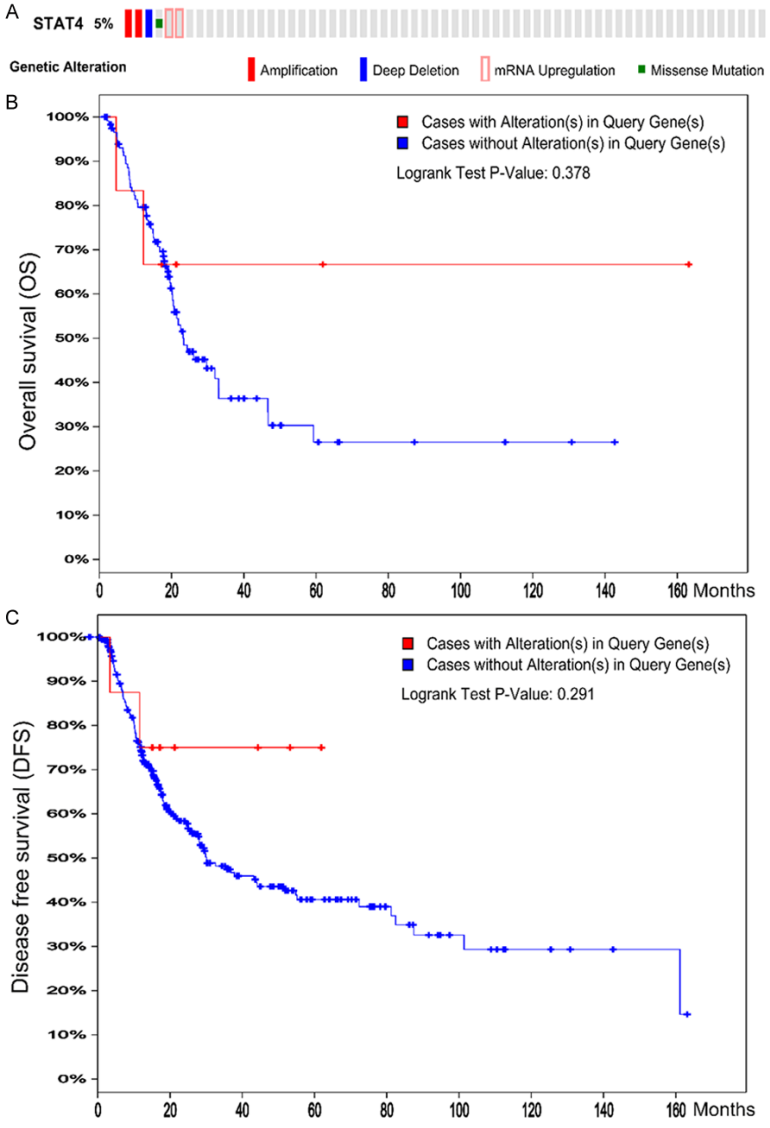

Figure 3. Clinical significance of STAT4 alteration from TCGA in patients with bladder urothelial cancer (BUC). A. The OncoPrint of genetic alterations of STAT4, including amplification, deep deletion, mRNA upregulation, and missense mutation, was exhibited in BUC by cBioPortal (www.cbioportal.org). Only part of the cases was shown representatively. B. Overall survival (OS): two cases were deceased in six cases with alterations and 58 cases were deceased in 119 cases without alterations, whose median survival time was 23.19 months (P=0.378). C. Disease free survival (DFS): one case relapsed of five cases with alterations, while 50 cases relapsed among 94 cases without alterations, whose median disease free time was 18 months (P=0.291). The survival was analyzed by Kaplan-Meier Estimate provided by cBioPortal (www.cbioportal.org).

only one microarray, Dataset GDS1479 in GSE3167 provided the relevant data, thus no meta-analysis was performed. The expression level of STAT4 mRNA in BUC was 6.28±0.46, which had no significant difference as compared to that in normal controls (6.42±0.64, P=0.367). The relationship between STAT4 mRNA expression and various clinicopathological parameters in BUC was shown in **Table 3**. We found no relative relationship between STAT4 expression and some parameters, such as gender, lymph node metastasis, recurrence, distant metastasis, smoking state (all P> 0.05) from a single microarray data (GDS4456 in GSE31684). Interestingly, the patients with surrounding CIS showed significantly lower expression of STAT4 mRNA (2.27±0.22) than those without CIS (2.90±1.16, P= 0.001, **Figure 2A**) from GDS4456 in GSE31684. For the relationship between STAT4 mRNA level and T staging, all three microarrays provided the evaluation results. Only

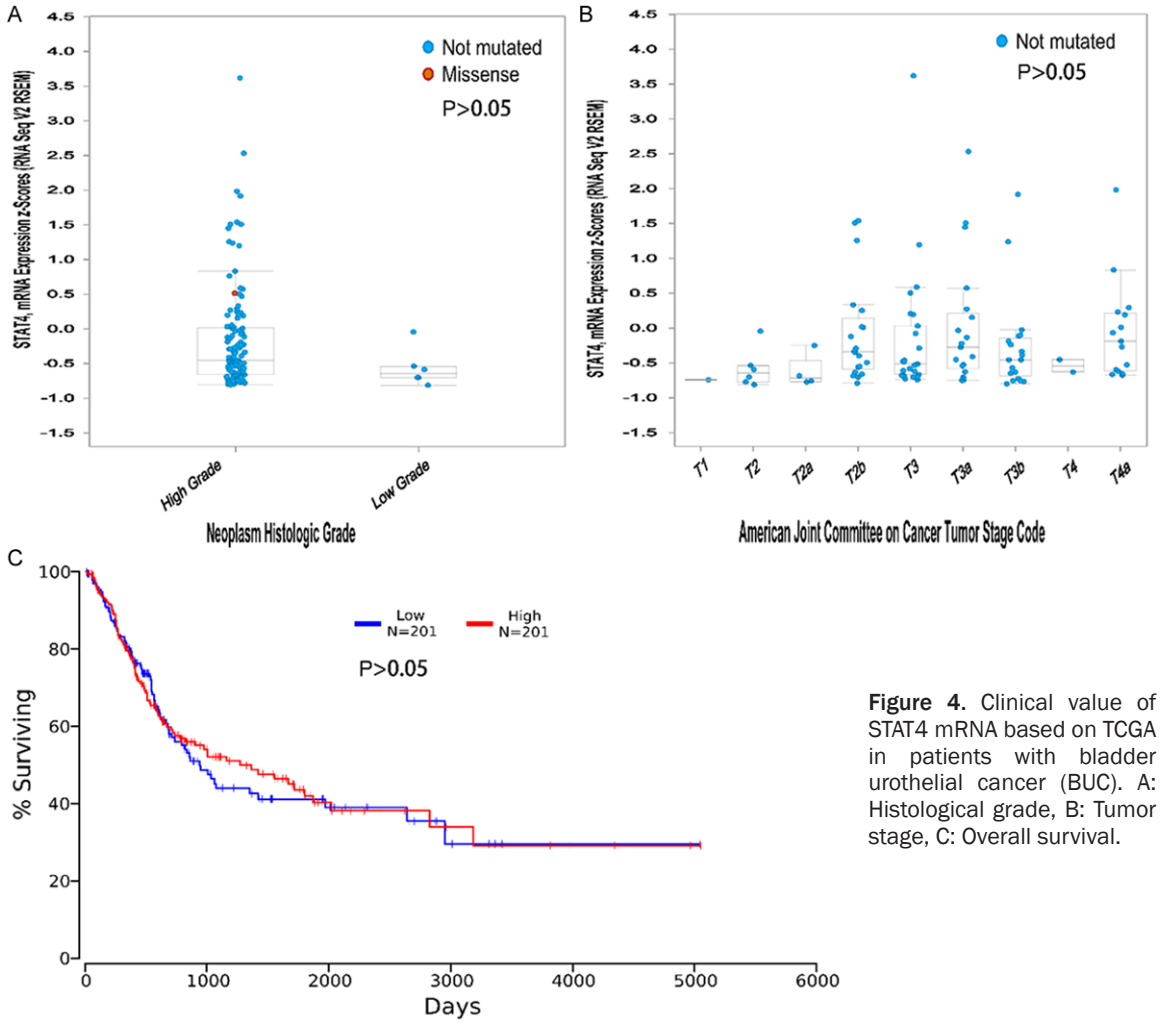

Figure 4. Clinical value of STAT4 mRNA based on TCGA in patients with bladder urothelial cancer (BUC). A: Histological grade, B: Tumor stage, C: Overall survival.

GDS1479 in GSE3167 showed a significant difference of STAT4 mRNA between early and advanced T staging (6.14±0.38 vs. 6.67±0.44, P<0.001). Then, meta-analysis to investigate the correlation between STAT4 and T Staging was performed with fixed-effect model. However, no correction was observed between STAT4 expression and T staging (SMD=0.107, 95% CI: -0.261, 0.475, P=0.568, **Table 4**; **Figure 2B**). Similarly, three microarray data also had the data to show the relationship between STAT4 mRNA and tumor grade. Neither the single study (**Table 3**) nor meta-analysis (SMD=-0.136, 95% CI: -0.715, 0.443, P=0.644, **Table 4**; **Figure 2C**) revealed that STAT4 mRNA could be related to tumor grade.

Further validation of the clinical role of STAT4 alterations by TCGA dataset

OncoPrint of STAT4 in BUC from cBioPortal showed 5% genetic alterations, including amplification, deep deletion, mRNA upregulation, and missense mutation (**Figure 3A**). Then, we wondered whether the genetic alterations had prognostic value in BUC. Unfortunately, K-M analysis showed that no correlation was observed between these genetic alterations and overall survival (OS) or disease free survival (DFS, **Figure 3B**, **3C**). Next, we selected the mRNA level of STAT4 for the further evaluation. Again, no significant correlation was noted between STAT4 mRNA level and histology, clinical stage or OS (**Figure 4A-C**). To further explore the gene network of STAT4 in BUC, cBioPortal (**Figure 5**) was used to draw the schematics.

Discussion

In the current study, we explored the clinicopathological significance of STAT4 in BUC tissues with clinical samples, as well as public databases. We found that STAT4 protein was

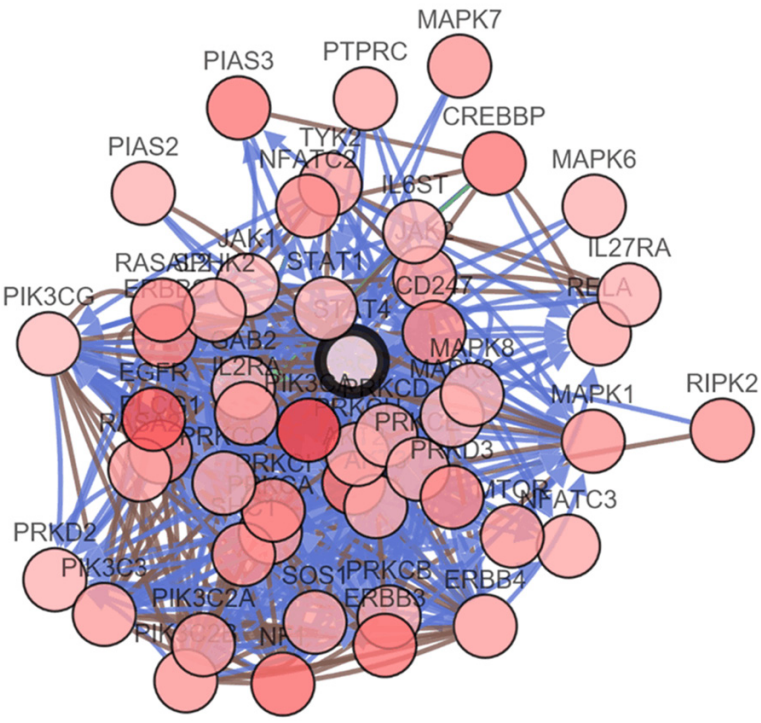

Figure 5. Gene network of STAT4 in patients with bladder urothelial cancer (BUC). The gene network in BUC for STAT4 was provided by cBioPortal (www.cbioportal.org). Circles indicated genes.

downregulated in BUC tissues as compared to non-tumorous bladder tissues. Low expression of STAT4 protein was also observed to be related to the disease deterioration as indicated by lymph node metastasis and T staging. Furthermore, GEO data showed that the STAT4 mRNA level was significantly lower than that of invasive BUC, which suggested that STAT4 might play an essential role in the tumorigenesis and progression of BUC.

As a member of the STAT family, STAT4 was identified as a crucial transcription factors mediating cytokine driven signaling [6]. The expression of STAT4 has been reported to be over-expressed in various types of malignant tumors such as gastric cancer [13], colon and rectal cancer [14, 15], cervical cancer [16], breast cancer [17] and glioma [18]. However, in HCC, the opposite lower expression of STAT4 was reported in tumor tissues by our group and other two groups [10-12], which suggested that the role and function of STAT4 may be tumor specific. The clinical role and biological function of STAT4 in BUC has not yet been fully clarified. To date, only one study has reported the expression of STAT4 in BUC tissues. El-Aal et al. [19] detected STAT4 protein in 29 cases of chronic schistosomiasis haematobium Egyptian patients complicated with bladder cancer by digital real-time quantitative photocytometry, and observed that there was no significant difference in the expression of STAT4 in different pathological types or different histology grades in bilharzial bladder cancer. Since the study of El-Aal et al. [19] was performed with a specific group of patients with schistosomiasis haematobium, the function of STAT4 in bladder cancer unrelated to schistosomal infections remains unclarified. In the current study, we for the first time, explored the clinical significance of STAT4 in BUC and found that STAT4 protein level was markedly reduced in BUC tissues, as compared to non-cancerous bladder tissues. Moreover, the AUC of STAT4 reached 0.67 to diagnose BUC. The immunohistochemical results showed that the clinical role of STAT4 in BUC was similar to the trend in HCC [10-12], and STAT4 acts more like a tumor suppressive gene. Even we did not achieve the consistent results when data of STAT4 mRNA level between BUC and non-tumor from GEO and TCGA were evaluated, one GEO profile GDS4456 showed that the tumor with surrounding CIS had lower STAT4 mRNA level than that without CIS. This result pointed out that from normal bladder tissues to CIS, the STAT4 level was reduced, suggesting STAT4 might play an essential role in the tumorigenesis of BUC.

As for the association between STAT4 and clinicopathological characteristics, higher STAT4 protein expression was detected in the groups of early tumor T stage and no lymph node metastasis, as compared to the groups with advanced disease. Furthermore, significant correlations were noted between STAT4 expression and disease deterioration, including the status of T staging and lymph node metastasis. This finding suggested that STAT4 might play an anti-oncogenic role in BUC and suppress the

deterioration of BUC. We failed to find out the relationship between STAT4 mRNA and the progression of BUC based on GEO and TCGA, probably due the small sample size. Besides, we could not assess the prognostic value of STAT4 in BUC in the current study. Therefore, the relationship between STAT4 and survival of BUC needs to be further investigated.

The mechanism of the influence of STAT4 to BUC remains largely unknown. We previously found that in HCC, knock-down of STAT4 mRNA could induce the cell proliferation and reduce the cell apoptosis in vitro. Since BUC and HCC share the similar STAT4 expression pattern, we could speculate that STAT4 may also affect tumor growth and cellular apoptosis in BUC. However, this hypothesis needs to be verified with functional experiments in the future. It remains either unclarified why STAT4 protein level is downregulated when the normal bladder epithelial tissue is transformed in cancer, as well as the cancer cells become more aggressive. Recently, Lamana A [35] reported that the presence of the rs7574865 T allele is able to enhance STAT4 mRNA transcription and protein expression. The rs7574865 T allele may enhance the signaling of molecules hinging on the STAT4 pathway based on 201 patients from PEARL (Princesa Early Arthritis Register Longitudinal) study in Spain. The rs7574865 T allele has not been reported in BUC, however, TCGA showed that some genetic alterations of STAT4 could be noted in BUC tissues, including amplification, deep deletion, and even missense mutation. The relationship between genetic alterations and STAT4 expression remains to be investigated. And the influence of SNP on STAT4 expression, as well as on the risk and progression of BUC also is required to be explored.

In summary, STAT4 might play a vital part in the tumorigenesis and progression of BUC and could represent a promising biomarker for BUC. However, large cohort studies are urgently desired to evaluate the prognostic value of STAT4 in BUC, as well as functional experiments are required to unveil the molecular mechanism of STAT4 in BUC.

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Disclosure of conflict of interest

None.

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