

## Original Article

# Stanniocalcin-1 relates to tumor recurrence and unfavorable prognosis of urothelial bladder cancer

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**Abstract:** The role of stanniocalcin 1 (STC1) was revealed in several cancers but not urothelial bladder cancer. To explore the clinical significance of STC1 in urothelial bladder cancer, we detected STC1 expression with immunoblotting in 126 samples of urothelial bladder cancer, consisting of 97 non-muscle invasive bladder cancers (NMIBC) and 29 muscle invasive bladder cancers (MIBC). Chi-square test was used to analyze the correlation between STC1 expression and clinicopathological factors. The significance of STC1 on bladder cancer recurrence and prognosis was evaluated by univariate and multivariate analysis. As the result, we demonstrated that STC1 expression was significantly associated with advanced tumor stage ( $P=0.026$ ). Additionally, STC1 expression could be identified as an independent recurrence-relative factor ( $P=0.013$ ) and unfavorable prognostic factor ( $P=0.014$ ). Moreover, we performed real-time PCR to detect and compare the mRNA levels between tumor and tumor-adjacent tissues, as well as between NMIBC and MIBC samples. Consequently, STC1 mRNA level in MIBC was significantly higher than that in NMIBC. In conclusion, STC1 expression could be identified as an independent biomarker which was related to easier recurrence and poorer prognosis in urothelial bladder cancer, pointing out the possibility of STC1 as a potential drug target in bladder cancer.

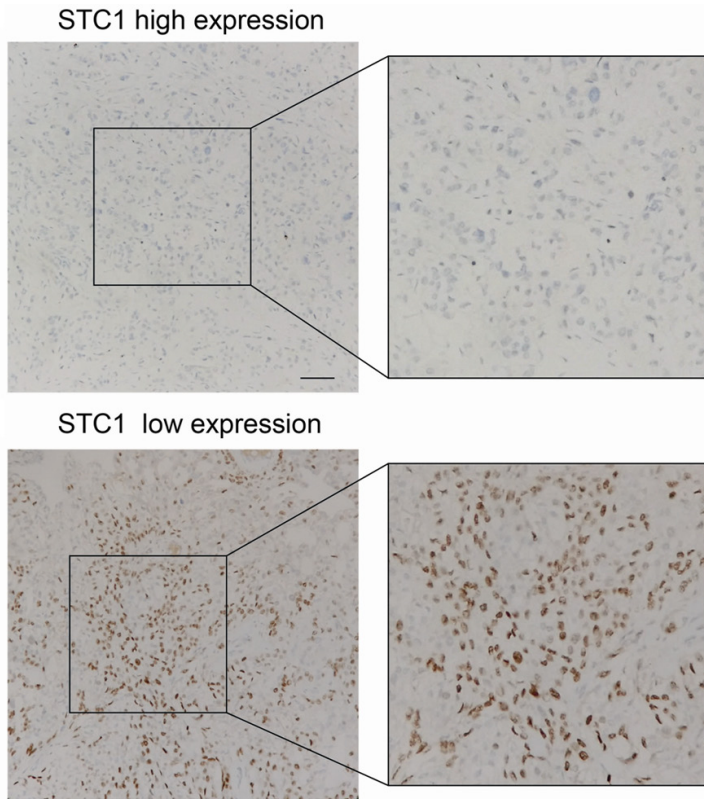
**Keywords:** Biomarker, prognosis, recurrence, stanniocalcin 1, urothelial bladder cancer

## Introduction

Bladder cancer is a kind of frequent malignancy and the fourth common cancer with approximately 380,000 new cases and 150,000 deaths per year worldwide [1]. In Europe and North America, urothelial bladder cancer accounts for more than 90% of all bladder cancers in classification of histology [2], and other histological types account for less than 10% including squamous, adenocarcinoma, micropapillary, small cell and plasmacytoid. Bladder cancers can be divided to two types with different molecular features, treating strategies and clinical outcomes, which are muscle-invasive bladder cancer (MIBC) and non-muscle invasive bladder cancer (NMIBC). Approximately 70%-80% patients with bladder cancers are NMIBC of low grade at the first diagnosis, while about 20% patients are diagnosed as MIBC [3]. NMIBC with low-grade recurs frequently but rarely progresses to muscle invasion, while

MIBC is usually clinical-silent and diagnosed with positive metastasis. Even though great advances of radical cystectomy and systemic therapy have been achieved, about 50% of patients with MIBC die from bladder cancer metastasis [4, 5]. Moreover, 50%-80% of patients with transurethral resection suffer recurrence within 2 years after the initial treatment, and 10%-25% of them may result in a high grade tumor [6, 7]. In summary, the high rate of recurrence and metastasis are the main reasons resulting in the high mortality of bladder cancer. Many efforts were made to elucidate the mechanisms of oncogenesis and find the potential drug target in bladder cancer, which resulted in many breakthrough in proteomics and genomics field, such as that recent genome-wide association studies identified bladder cancer risk-related gene variants [8]. However, the predictive or prognostic biomarkers of bladder cancer still need the support and demonstration of translational medicine.

## Expression of Stanniocalcin-1 in urothelial bladder cancer



**Figure 1.** Representative immunohistochemical figures for STC1 high- and low-expression. Scale bar: 50  $\mu$ m.

Stanniocalcin (STC) is a glycoprotein hormone first discovered in the corpuscles of Stannius in bony fish, which is proved to exist in almost all tissues and can regulate calcium homeostasis [9]. Human STC is consisted of STC1 and STC2, functioning primarily as paracrine/autocrine factors and regulating various biological functions [10]. Human STC1 homolog is a 247-amino acid protein that exists as homodimer and can be secreted into the extracellular space because of the signal peptide sequence. The study of STC is focused on its calcium-regulating functions, but emerging evidence demonstrate that STC overexpression is associated to tumor progression in kinds of cancers, including gastric cancer, lung cancer, breast cancer, colorectal cancer and esophageal squamous-cell cancer [11-14]. However, the expression level and clinicopathological significance of STC1 in bladder cancer is still unknown.

To elucidate the biological and clinicopathological significance of STC1 in bladder cancer, we detected the expression of STC1 with immunohistochemistry (IHC) in 126 cases of patients

diagnosed as urothelial bladder cancer, and analyzed the correlation between STC1 expression and other clinicopathological factors. Furthermore, we evaluated the value of STC1 as the recurrence-predictive biomarker and prognostic biomarker with univariate and multivariate analysis.

### Patients and materials

#### *Patients and follow-ups*

The primary cohort consisted 329 patients were diagnosed as bladder cancer and underwent surgical resection between 2005 to 2012 in Yidu Central Hospital of Weifang and Shandong Cancer Hospital and Institute, which contained 246 patients who underwent transurethral tumor resection and 83 patients who underwent radical total bladder cystectomy. From the primary cohort, 126 patients were enrolled into validation cohort according to criteria as follows: (1) the

histologic type is urothelial bladder cancer; (2) patients had available follow-ups and paraffin-embedded samples; (3) no history of other tumors and no neoadjuvant chemotherapy. There were 97 cases of NMIBC and 29 cases of MIBC. Besides the 126 paraffin-embedded specimens, 13 pairs of tumor tissues and adjacent tumor tissues were obtained during surgery and partially preserved in liquid nitrogen without disturbing normal pathological diagnosis. The paraffin-embedded or nitrogen-frozen specimens were obtained with the prior consent of patients and approval of the Ethics Committee of Yidu Central Hospital of Weifang and Shandong Cancer Hospital and Institute. Patients who passed away during perioperative period or had survival time less than 3 months were excluded from our cohort, and the median follow-up was 26 months.

#### *Immunohistochemical staining*

Expression of STC1 was detected by IHC with detailed method published previously [15]. The results of IHC were scored by two senior pathologists unaware of the clinical information of the

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**Table 1.** Correlation between STC1 expression and clinicopathological factors

| Factors                   | Number | STC1 expression |      | P*    |
|---------------------------|--------|-----------------|------|-------|
|                           |        | Low             | High |       |
| Gender                    |        |                 |      |       |
| Male                      | 99     | 73              | 26   | 0.462 |
| Female                    | 27     | 22              | 5    |       |
| Age                       |        |                 |      |       |
| <60                       | 43     | 33              | 10   | 0.832 |
| ≥60                       | 63     | 42              | 21   |       |
| Tumor diameter (cm)       |        |                 |      |       |
| ≤3                        | 64     | 50              | 14   | 0.537 |
| >3                        | 62     | 45              | 17   |       |
| Tumor number              |        |                 |      |       |
| Single                    | 105    | 81              | 24   | 0.404 |
| Multiple                  | 21     | 14              | 7    |       |
| pM status                 |        |                 |      |       |
| Negative                  | 116    | 89              | 27   | 0.26  |
| Positive                  | 10     | 6               | 4    |       |
| Tumour stage <sup>#</sup> |        |                 |      |       |
| Ta-T1                     | 97     | 78              | 19   | 0.026 |
| T2-T4                     | 29     | 17              | 12   |       |
| Tumour grade <sup>#</sup> |        |                 |      |       |
| Low grade                 | 51     | 41              | 10   | 0.302 |
| High grade                | 75     | 54              | 21   |       |

\*means calculated by Chi-square test; <sup>#</sup>means referring to WHO 2004 classification.

patients. The IHC score was the product of staining intensity score multiplied by the positive cell percentage score according to previous study [16]. The staining intensity was scored as weak staining (score 1), medium staining (score 2) or high staining (score 3), while positive stained cell percentage was scored as 25% (score 1), 25%-75% (score 2), 75% (score 3). The final IHC score was calculated as score(staining intensity)×score (positive cell percentage), which ranged from 1 to 9. Samples with final score ≥4 were defined as STC1 high-expression while final score <4 as STC1 low-expression.

### Cells and reagents

The human bladder cell lines TCCSUP and 5637 were originally purchased from Cell Bank of Chinese Academy of Sciences(Shanghai, China). Cell line SW780 and J82 were obtained from the American Type Culture Collection (Manassas, VA, USA). All above cell lines were

cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovineserum (Gibco, Carlsbad, CA, USA) and 1% ampicillin/streptomycin in 5% CO<sub>2</sub> resuscitation at 37°C with saturated humidity. The primary antibodies in IHC and immunoblotting were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA) without special instructions and all agents were from Sigma Corporation (St. Louis, Missouri, USA) without special instructions.

### RNA extraction and real time PCR

Total RNAs were extracted with TRIzol reagent (Invitrogen, Foster City, CA, USA) and RNeasy protect mini kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations. After quantification of extracted mRNAs, one-step real-time RT-PCR was performed with SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manual. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was applied as an internal control. The mRNA levels were expressed as normalized ratios with the average mRNA level of adjacent tumor tissues as baseline. The sequences of primers used for real-time PCR experiments were designed following previous studies and shown below [17].

STC1, forward: 5'-TGAGGCGGAGCAGAATGACT-3', reverse: 5'-CAGGTGGAGTTTCCAGGCAT-3'; GAPDH, forward: 5'-TGGAGAATGAGAGGTGGGATG-3'; reverse: 5'-GAGCTTCACGTTCTTGTATCTGT-3'.

### Immunoblotting

Expression of STC1 was detected by immunoblotting with detailed procedure described before [15]. Briefly, detected cells were lysed in the RIPA lysis buffer and centrifuged at 11,000 rpm at 4°C for 20 minutes to collect the supernatant. Concentration of supernatant containing cellular proteins were detected with BCA detection kit (Beyotime Institute of Biotechnology, Shanghai, China). Total amount of 10 µg protein was electrophoresed in a SDS-PAGE gel, then transferred to a PVDF membrane (PALL Company, USA). After incubated in corresponding primary antibody (1:1000) overnight at 4°C and in secondary antibody labeled with horseradish peroxidase for 2 hours at 37°C, membranes were finally visualized by ECL substrate (Millipore Company).

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**Table 2.** Univariate and multivariate analyses of parameters on progression-free survival in NMIBC patients

| Characters          | Univariate analysis | Multivariate analysis |       |
|---------------------|---------------------|-----------------------|-------|
|                     | P*                  | HR                    | P#    |
| Gender              |                     |                       |       |
| Male                |                     |                       |       |
| Female              | 0.926               |                       |       |
| Age                 |                     |                       |       |
| <60                 |                     |                       |       |
| ≥60                 | 0.313               |                       |       |
| Tumor diameter (cm) |                     |                       |       |
| ≤3                  |                     |                       |       |
| >3                  | 0.359               |                       |       |
| Tumor number        |                     |                       |       |
| Single              |                     | 1                     |       |
| Multiple            | 0.042               | 0.99-2.83             | 0.056 |
| Tumor grade         |                     |                       |       |
| Low grade           |                     |                       |       |
| High grade          | 0.080               |                       |       |
| STC1 expression     |                     |                       |       |
| Low                 |                     | 1                     |       |
| High                | 0.008               | 1.17-3.85             | 0.013 |

\*means calculated by Kaplan-Meier method and log-rank test.

#means calculated by Cox-regression model.

### Statistical analysis

All data were analyzed with the software SPSS 17.0. The correlations between STC1 expression and clinicopathological factors were evaluated by Chi-square test. The recurrence-free survival curves and overall survival curves were generated by Kaplan-Meier method and the difference was evaluated by log-rank test. In multivariate analysis, the Cox-regression proportional hazards model was performed to identify the independent factors. In all tests,  $P < 0.05$  was considered as statistically significant.

### Results

#### Expression of STC1 in urothelial bladder cancer tissue

Expression of STC1 was visualized by IHC. As a secreted peptide which functions mainly in a paracrine/autocrine way, STC1 was mainly observed to exist in the cell cytoplasm of urothelial bladder cancer (**Figure 1**). The validation cohort were divided into group of STC1 high expression and STC1 low expression according

to the score of IHC described in Patients and Methods. In our experiment, the STC1 high expression proportion was 24.6% (31/126).

#### Correlation between STC1 and other clinicopathological parameters

Chi-square test was used to analyze the correlation between STC1 high or low expression and other clinicopathological factors (**Table 1**). In our cohort, we demonstrated that STC1 expression was significantly associated with urothelial bladder cancer T stage. Patients with high STC1 expression appeared to have higher probability of advanced tumor stage ( $P = 0.026$ ). This result may be resulted from that STC1 could promote tumor progression such as epithelial-mesenchymal transition (EMT), which needs further experiments to verify. No other clinicopathological factors were observed to correlate with STC1 except tumor stage in our experiment.

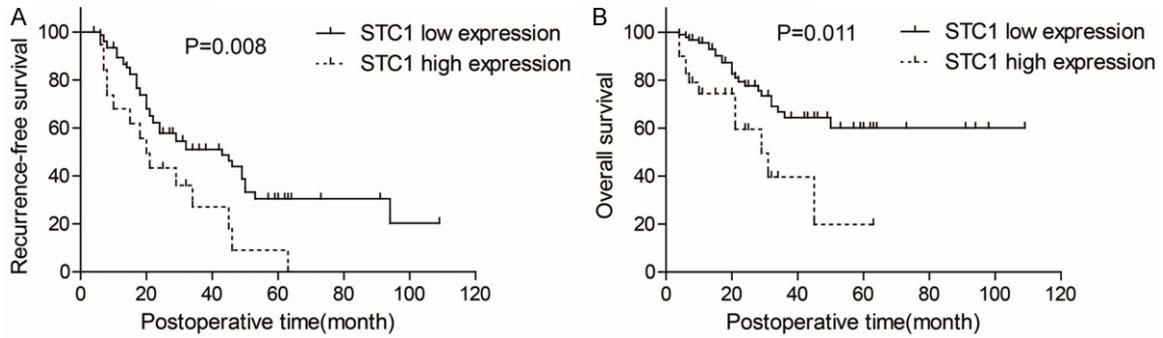
#### Value of STC1 expression in tumor recurrence

Bladder cancer is featured with high recurrence, which results in the advanced tumor progression and poorer prognosis. It was proved that more than 50% patients suffered from recurrence of bladder cancer after transurethral tumor resection in 2 years. To estimate the value of STC1 as a predictive recurrence biomarker, we used Kaplan-Meier method and Cox-regression model for univariate and multivariate analysis respectively (**Table 2**). Patients with Ta-T1 tumor stage were selected into the cohort for recurrence study and all clinicopathological factors were analyzed. In univariate analysis, tumor number and STC1 expression were identified to be associated with higher recurrence rate ( $P = 0.042$  and  $0.008$ , respectively) (**Figure 2A**). Moreover, tumor grade tended to be associated with recurrence, but this tendency was not statistically significant ( $P = 0.080$ ). Both tumor number and STC1 expression were enrolled into Cox-regression model to confirm the independent factor affecting tumor recurrence. In multivariate analysis, only STC1 was identified as recurrence-affecting factor ( $P = 0.013$ ), and tumor number was excluded because of the over high  $P$  value ( $P = 0.056$ ).

#### Prognostic value of STC1

STC1 expression was demonstrated to relate to poorer prognosis in several kinds of other

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**Figure 2.** The recurrence-free survival curves (A) and overall survival curves (B) stratified by STC1 expression were presented by Kaplan-Meier method.

**Table 3.** Prognostic value of clinicopathologic factors

| Factors                   | Univariate analysis   |        | Multivariate analysis |                |
|---------------------------|-----------------------|--------|-----------------------|----------------|
|                           | 5-year survival rate% | P*     | HR                    | P <sup>§</sup> |
| Gender                    |                       |        |                       |                |
| Male                      | 53.9                  |        |                       |                |
| Female                    | 48.6                  | 0.669  |                       |                |
| Age                       |                       |        |                       |                |
| <60                       | 57.4                  |        |                       |                |
| ≥60                       | 41.7                  | 0.804  |                       |                |
| Tumor diameter (cm)       |                       |        |                       |                |
| ≤3                        | 54.7                  |        |                       |                |
| >3                        | 54.4                  | 0.436  |                       |                |
| Tumor number              |                       |        |                       |                |
| Single                    | 61.4                  |        | 1                     |                |
| Multiple                  | 17.2                  | 0.003  | 0.80-4.92             | 0.140          |
| pM status                 |                       |        |                       |                |
| Negative                  | 55.8                  |        | 1                     |                |
| Positive                  | 0                     | <0.001 | 1.84-28.1             | 0.005          |
| Tumour stage <sup>#</sup> |                       |        |                       |                |
| Ta-T1                     | 58.0                  |        |                       |                |
| T2-T4                     | 23.6                  | 0.001  |                       |                |
| Tumour grade <sup>#</sup> |                       |        |                       |                |
| Low grade                 | 63.6                  |        | 1                     |                |
| High grade                | 43.7                  | 0.023  | 0.96-4.34             | 0.063          |
| STC1                      |                       |        |                       |                |
| Low                       | 61.0                  |        | 1                     |                |
| High                      | 47.8                  | 0.011  | 1.20-4.99             | 0.014          |

\*means calculated by Kaplan-Meier method and log-rank test. <sup>§</sup>means calculated by Cox-regression model. <sup>#</sup>means referring to WHO 2004 classification.

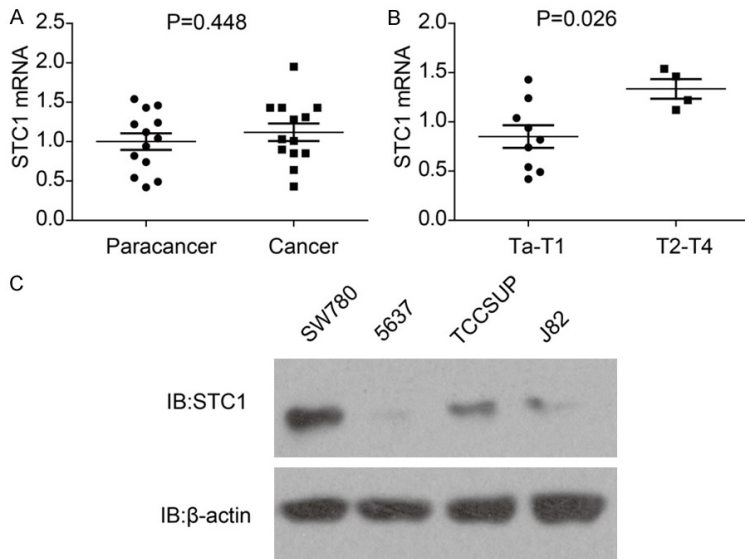
cancers such as glioma, renal cell carcinoma and esophageal squamous cell carcinoma [18-20]. We also estimated prognostic value of STC1 with univariate and multivariate analysis in our experiment (Table 3). In Kaplan-Meier method for univariate analysis, STC1 high

expression was demonstrated to be an unfavorable prognostic factor (P=0.011) (Figure 2B). Besides STC1 expression, tumor number, pM status, tumor stage and grade were also identified as prognostic factors (P=0.003, P<0.001, P=0.001, P=0.023, respectively). All above factors were selected into Cox-regression model to confirm independent prognostic factor except tumor stage because it had significant correlation to STC1 expression. In multivariate analysis, STC1 expression was identified as an independent prognostic factor (P=0.014, 95% CI=1.20-4.99). Moreover, pM status also had independent prognostic value besides STC1 expression (P=0.005, 95% CI=1.84-2.81).

### STC1 expression in fresh tissues and cell lines

We observed that STC1 expression was significantly associated with advanced T stage in Table 1. To double confirm this result, we collected 13 pairs of frozen tumor samples and adjacent para-tumor samples, including 9 cases of Ta-T1 and 4 cases of T2-T4. After purifying STC1 mRNA and real-time PCR, we compared the mRNA difference between tumor and adjacent tissues, between samples of Ta-T1

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**Figure 3.** A. The mRNA level of STC1 in the adjacent para-cancer tissue and the cancer was quantified by real-time PCR. Statistical difference between compared groups was analyzed by Student-t test and presented by mean  $\pm$  SEM. B. The mRNA level of STC1 in 13 pairs of the adjacent para-cancer tissue and the cancer was quantified by real-time PCR. Statistical difference between compared groups was analyzed by Student-t test and presented by mean  $\pm$  SEM. C. The expression of STC1 in bladder cancer cell line SW780, 5637, TCCSUP and J82 was detected by immunoblotting.

stage and samples of T2-T4 stage. In our experiment, tumor tissues had no significantly higher mRNA level than adjacent para-tumor tissues ( $P=0.448$ ), but STC1 mRNA level in samples of T2-T4 stage was remarkably higher than that in samples of Ta-T1 stage ( $P=0.026$ ), indicating that high STC1 level may promote tumor progression, resulting in advanced T stage. Moreover, STC1 expression was detected by immunoblotting in four cell lines of urothelial bladder cancer, including cell line SW780, 5637, TCCSUP and J82. The expression of STC1 was detectable in all these cell lines, with different expressive abundance. SW780 cells were proved to have the highest STC1 expression while 5637 cells had the lowest expression in our experiment (Figure 3C), which could help select appropriate cell model for STC1 cell study.

### Discussion

Bladder cancer is the fourth common cancer and the ninth leading cause of death from cancer in men, with 70,530 new cases and 14,680 cancer-related deaths in the United States in 2010 [21]. Besides a deadly threat to health, bladder cancer is also a remarkable economic burden, which encourages the study of bladder cancer. It was reported that the average cost

for each patient with bladder cancer was \$65,158 [22]. Previous studies revealed many risk factors of bladder cancer, including cigarette smoking [3], chronic urinary tract infection, chemical carcinogens for bladder like aniline dyes as well as cyclophosphamide. Urothelial bladder cancer can be divided into two subtypes originating from two different oncogenic mechanisms, which were considered to have different phenotypic variants and biological behaviors [4]. Low-grade NMIB-Cs are featured by mutations in the HRAS and fibroblast growth factor receptor 3 (FGFR3) genes, whereas high-grade MIB-Cs always have defects in the p53 and pRb tumor-suppressor pathways. Several genes were identified as important factors in these two oncogenic mechanisms.

Over the last decades, several progresses have been made in the high-throughput gene screening with microarray or mass spectrum in the fields of bladder cancer diagnosis and treatment [2, 23]. Many suspected genes or proteins were screened out with experiments in vitro but more explorations are needed to confirm these molecules as effective biomarkers. Among the candidate genes, STC1 was rarely elucidated or even mentioned though its significance was revealed in several other cancers. Up-regulation of STC1 expression was significantly associated with poorer survival in esophageal carcinoma and lymphatic metastasis in breast cancer [20, 24]. Besides expressed in tumor in situ, STC1 can also be secreted out into blood. It was reported that circulating STC1 mRNA were significantly higher in patients with non-small cell lung cancer than normal patients [12]. As to the underlying molecular mechanisms why STC1 overexpression leads to unfavorable prognosis, it is still controversial and needs more exploration. STC1 can activate signaling pathways like PI3K pathway [14], which are well-acknowledged to involve in cell proliferation and migration. However, the biological functions of STC1 vary depending on the condition and cell lines in hypoxia condition which is important in tumor cell metabolism according

to Warburg effect [25]. In previous study, STC1 could down-regulate the ERK1/2 signaling and reduced cell survival in case of oxidative stress [26]. However, STC1 expression was also proved to be promoted by hypoxia and thus associated with metastasis of early stage clear cell renal cell carcinoma [19].

In our study, we detected the expression of STC1 in 126 cases of urothelial bladder cancer and identified STC1 as an independent biomarker which could predict the recurrence and prognosis for the first time. With real-time PCR, we further demonstrated that STC1 mRNA in NMIBCs was significantly higher than that in MIBCs, indicating that STC1 may play important roles in bladder cancer progression. We hope our study could help confirm STC1 as a potential biomarker and discover a new chemical therapy for patients with urothelial bladder cancer.

### Disclosure of conflict of interest

None.

### Authors' contribution

Shufang Cai, Lanmei Liu and Dongyuan Zhu planned the project. Congzheng Wang, Hongqing Zhang, Wenbin Wu, Huanrong Yang, Xiaoqing Yang carried out experimental work. Shufang Cai, Lanmei Liu and Dongyuan Zhu wrote the paper. All authors discussed the results and commented on the manuscript. All authors read and approve the final manuscript.

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