Expression of γ-Synuclein in Bladder Carcinoma: A Possible Marker for Prognosis

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Abstract. *Aim: To investigate if γ-synuclein (SNCG) could be used as a bladder cancer (BC) marker to predict prognosis of BC. Patients and Methods: Medical records of 140 patients with BC (January, 2006 to December, 2009) were retrospectively reviewed. SNCG expression level was examined by immunohistological staining. The patients' survival rate was calculated by the Kaplan–Meier method. Cox proportional regression model was used to identify independent predictors for BC. Results: Overexpression of SNCG was detected in BC tissues and the expression level of SNCG strongly positively correlated with BC recurrence. However, no correlation was found between SNCG level and tumor stage or survival rate. Conclusion: SNCG is a good marker to predict recurrence of BC, but not a reliable marker for staging or prediction of survival rate.*

Bladder cancer (BC) is one of the most frequently occurring tumors worldwide, with an estimated 430,000 new patients diagnosed annually (1, 2). The most common BC subset is urothelial carcinoma, among which approximately 80% of patients have been found to have bladder tumors that had not invaded the detrusor, termed non-muscle-invasive (NMIBC) tumors. Urothelial carcinoma of the bladder is a heterogeneous disease with a variable natural history and oncological outcome. Although the overall prognosis is good, nearly half of all cases will recur within 2 years and approximately 10-30% of cases will progress to muscle-invasive disease (MIBC) (3, 4). Due to the high recurrence and progression rate, early detection and timely intervention for BC is essential. Consequently, there is a critical need for the identification of effective biomarkers of BC to monitor recurrence, refine prognostic estimates, and predict response to treatment (5).

The synucleins (α-, β- and γ-synuclein) are a small, soluble, highly conserved group of proteins expressed mainly in neural cells (6). Synuclein α (SNCA) and synuclein β (SNCB) are ubiquitously involved in neurodegenerative disorders such as Alzheimer's and Parkinson's disease, while SNCG is primarily involved in neoplastic diseases, without involvement in neurodegenerative disease pathogenesis (7-13). SNCG was previously termed breast cancer-specific gene 1 (BCSG1), when it was first found to display stage-specific up-regulation in breast carcinoma (9). However, it is now widely suggested that expression of SNCG is highly associated with multiple human malignancies, and the expression of SNCG has been reported in gastric, pancreatic, esophagus, colon, and prostate cancer (12, 14-18). In recent years, only few studies have reported on the role of SNCG in BC (19, 20). In our study, we detected the expression of SNCG proteins by immunohistochemistry and analyzed the relationship between SNCG and the pathological characteristics of BC.

Patients and Methods

Patients and tissue samples. A total of 435 cases of patients newly diagnosed with BC were collected from the archives (between January 2006 and December 2009) of the Department of Urology, Peking Union Medical College Hospital, Chinese Academy of Medical Science. Excluding patients without complete clinical data and patients who accepted radiotherapy or chemotherapy before surgery, only 140 BC specimens and 68 corresponding non-neoplastic adjacent tissues were obtained. Specimens from patients were diagnosed

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Key Words: γ-Synuclein, recurrence, bladder carcinoma, prognosis.

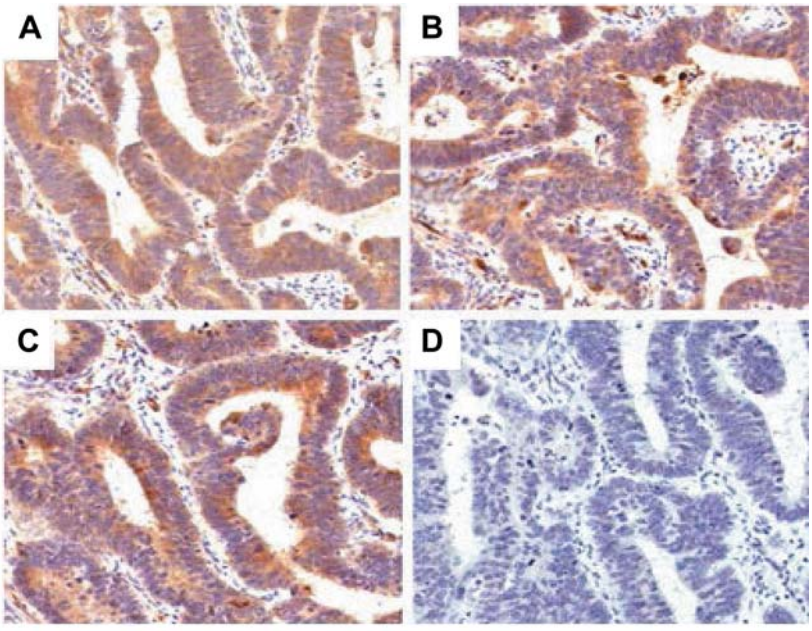

Figure 1. *Antibody to γ-synuclein (SNCG) specifically reacts with SNCG protein expressed in bladder cancer tissues but not with SNCA and SNCB. A: Anti-SNCG incubated with phosphate buffer saline; B, C: anti-SNCG incubated with GST-SNCA (B) and GST-SNCB (C) had no impact on its reaction with SNCG protein in bladder cancer tissue; D, anti-SNCG incubated with GST-SNCG did not react with the SNCG in bladder cancer tissue (original magnification ×200).*

histopathologically and staged according to the TNM International Union against Cancer classification system (21, 22). Among 140 patients with BC, 80 patients (57.1%) had tumor that did not invade the detrusor, 37 patients (26.4%) had tumor that invaded the detrusor. The median age was 63.3±14.3 years (range=17-89 years), and the median follow-up was 44.1±28.2 months (range=0-121 months). During follow-up, 17.1% (24/140) of patients died of BC and 44.3% (62/140) of patients had recurrence after surgery.

Disease-free survival (DFS) was defined as the period from the time of surgery to evidence of treatment failure (recurrence or progression). Overall survival (OS) was defined as the period of time from BC confirmation to death from any cause, or to the last follow-up. Clinicopathological information was collected from medical records of the patients. Tumor histology was confirmed independently by two pathologists. The study was approved and supervised by the Medical Ethic Committee of the Peking Union Medical College Hospital (S-K 521).

Identification of SNCG specificity in tumor cells by immunohistochemistry. The supernatent of hybridoma cells that secrete anti-SNCG was 6-fold diluted, then four aliquots of the supernatent were taken. Recombinant human GST-SNCG, GST-SNCA, and GST-SNCB were purified and characterized as previously described (23). The purification of GST–SNCA, GST–SNCB, GST–SNCG and an equal volume of phosphate buffer saline (PBS) were respectively added to the four supernatant aliquots for reaction at room temperature for 3 h. Each sample was added to the sealed bladder biopsy that come from the same wax block (SNCG protein expression-positive), and kept at 4°C overnight, then working liquid of Envision antibody was added for response for 40 min at room temperature. DAB-hydrogen peroxide was added for developing for 10 min and the reaction was terminated by rinsing in tap water. Finally, each sample was counterstained with hematoxylin and light microscopy (LaVision BioTec, Bielefeld, Germany) at ×200 was used to detect SNCG expression.

Immunohistochemistry for SNCG. All 140 BC tissues and 68 corresponding adjacent normal epithelium were analyzed by immunohistochemistry. Each tissue was embedded with paraffin and cut serially in 4-μm-thick sections. The sample was incubated with 3% hydrogen peroxidase for 10 min at room temperature and then samples were incubated with antibody to SNCG (Peking University School of oncology, Beijing, China) for 2 h at room temperature. Light microscopy (LaVision BioTec, Bielefeld, Germany) at ×200 was used to detect SNCG expression.

Evaluation of immunohistochemical staining. We used a 4-value classification method for grading the bladder samples as follows: area of staining as <10% (score 0) or >10% (score 1) of all cancer cells stained within the section; staining intensity (>10% of all cancer cells stained within the section) was graded as weak (score 1), moderate (score 2), or strong (score 3). If the score of a section was 2 or more, we defined it as SNCG-positive and if the score was less than 2, we defined it as SNCG-negative.

Statistical analysis. SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Differences in SNCG protein expression between cancer and tumor-adjacent normal epithelial tissues in the same patient were analyzed using a paired *t*-test. Pearson Chi-square test was performed for evaluating the correlation between SNCG levels and patients' clinical pathological features.

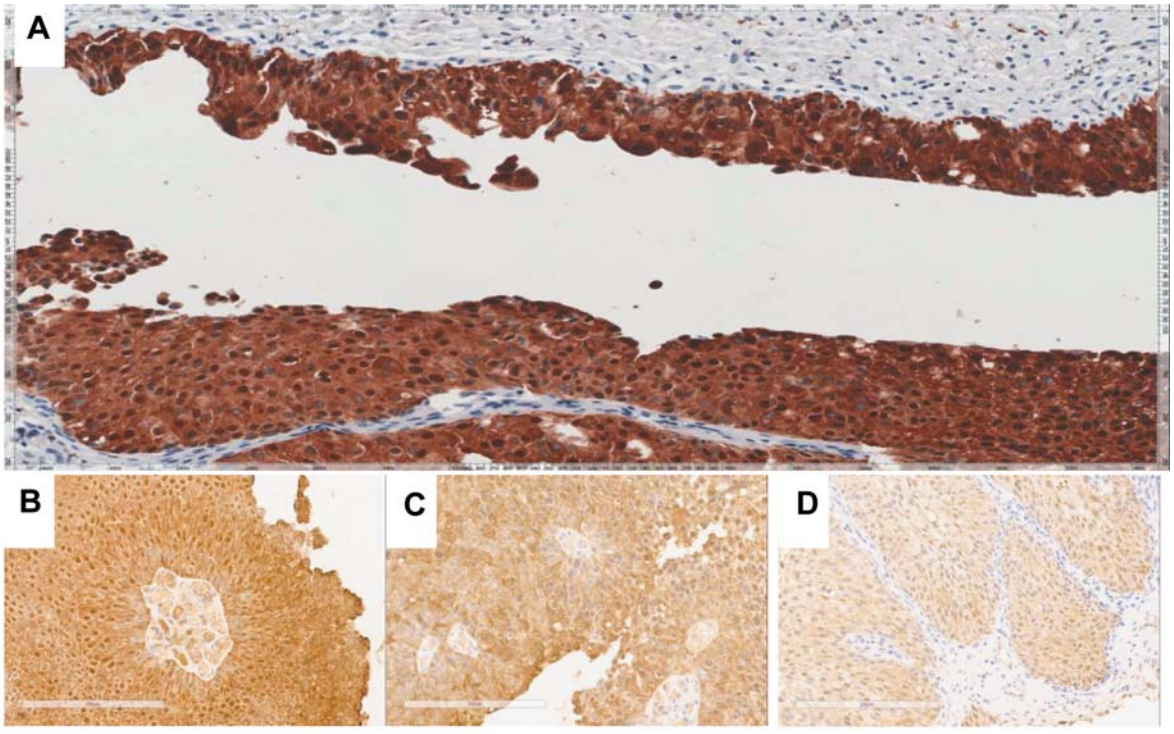

Figure 2. *Representative immunohistochemical staining for γ-synuclein (SNCG) protein in human bladder cancer tissues. A: SNCG is expressed in the cytoplasm and nucleus of bladder cancer cells but not in the adjacent normal tissues. SNCG is also expressed in bladder neuron-chords, vascular endothelial cells, and smooth muscle cells of nearly all bladder cancer specimens; B, C, and D: representative images showing strong (score 3), moderate (score 2) and weak (score 1) staining of SNCG in cancer cytoplasm (original magnification ×200).*

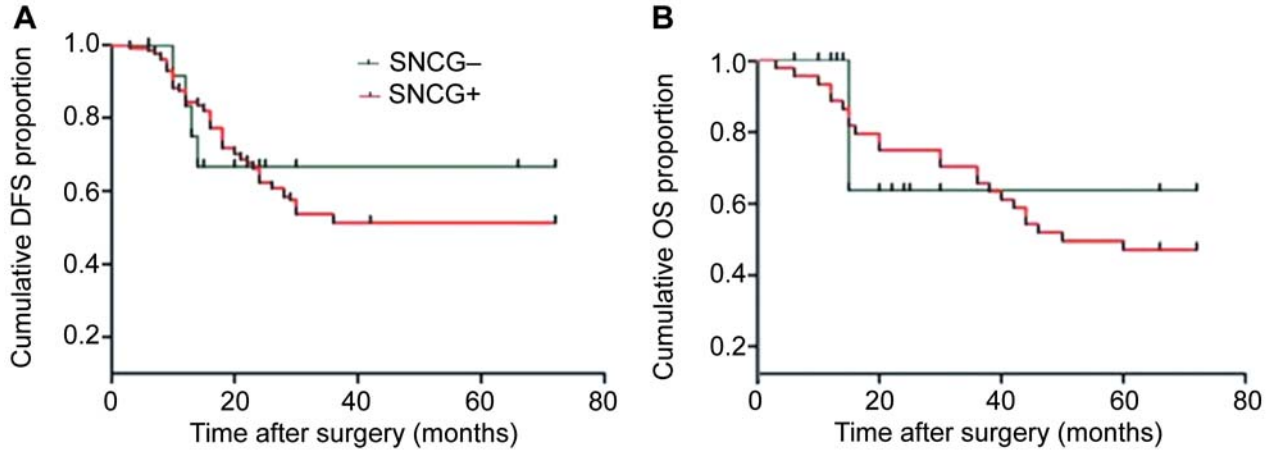

Figure 3. *Kaplan–Meier estimation of disease-free (A) and overall (B) survival. Cases with γ-synuclein (SNCG)-negative tumors (black line) versus cases with SNCG-positive (red line). Patients with SNCG-positive tumor showed no significant differences in disease-free and overall survival compared to the those with SNCG-negative tumor (p=0.821 and 0.544 by log-rank (Mantel–Cox) test, respectively).*

Results

The antibody to SNCG can be specifically used for detection of SNCG expressed in BC tissues. To test whether the antibody to SNCG can be specifically used in the detection of the SNCG expressed in BC tissue, we first made it react with excessive purification combined protein GST–SNCA, GST–SNCB and GST–SNCG, then incubated it with BC tissue biopsy. The results showed that anti-SNCG no longer reacted with the SNCG in BC tissue after it reacted with

Table I. *γ-Synuclein (SNCG) expression profile in bladder cancer tissue.*

	Cases, n	SNCG expression, n		Positive rate (%)	*p*-Value
		Negative	Positive		
Tumor-adjacent epithelium	68	68	0	0	<0.0001
Bladder cancer tissues	140	13	127	90.7	

Table II. *Correlations of γ-synuclein (SNCG) expression with clinicopathological factors and their influence on postoperative recurrence of bladder cancer.*

Characteristic		No. of cases	SNCG+ (%)	*p*-Value	No. of recurrences (%)	HR (95% CI)	*p*-Value
Gender	Male	106	96 (90.6)	0.816	43 (40.6)	1	0.079
	Female	34	31 (93.9)		19 (55.9)	0.539 (0.270-1.074)	
Age (years)	≤60	48	45 (93.8)	0.557	18 (37.5)	1	0.180
	>60	92	82 (89.1)		44 (47.8)	0.655 (0.353-1.216)	
Size (cm)	≤3	101	93 (92.1)	0.568	35 (34.7)	1	0.001
	>3	39	34 (87.2)		27 (69.2)	1.998 (1.355-2.947)	
Tumor number	Solitary	79	74 (93.7)	0.281	22 (27.8)	1	0.000
	Multiple	61	53 (86.9)		40 (65.6)	2.355 (1.589-3.489)	
Tumor stage	NMIBC	80	70 (87.5)	0.091	25 (31.2)	1	0.011
	MIBC	37	36 (97.3)		18 (48.6)	1.557 (0.906-2.675)	
	Tx	23					
Tumor grade	Low	86	77 (89.5)	0.367	34 (39.5)	1	0.110
	High	54	50 (94.3)		28 (51.9)	0.763 (0.547-1.064)	
SNCG	Negative	13	—		4 (30.8)	1	0.023
	Positive	127	—		58 (45.7)	2.044 (1.114-3.752)	

CI: Confidence interval; HR: hazard ratio.

fusion protein GST–SNCG, and also did not cross-react with other proteins, while its incubation with GST–SNCA and GST–SNCB had no impact on its reaction with SNCG protein in the cancer tissue (Figure 1), which indicates that anti-SNCG only specifically reacted with SNCG protein, but not with SNCA and SNCB.

SNCG is highly expressed in BC tissues and is related to tumor recurrence but not tumor stage. None of the 68 tumor-adjacent normal epithelia revealed SNCG-positive staining (Table I), but 127 SNCG-positive cases were detected in 140 BC tissues (90.7%). As show in Figure 2A, SNCG was specifically expressed in the cytoplasm of cancer cells, whereas no expression was observed in the adjacent normal epithelium. Figure 2B-D shows representative tissues with strong (score 3), moderate (score 2) and weak (score 1) staining of SNCG in cancer cells. We also found that SNCG was strongly expressed in bladder neuron-chords, vascular endothelial cells, and smooth muscle cells of nearly all BC specimens.

We also analyzed the associations between SNCG level and clinicopathological features in 140 patients with BC (Table II). Overall, there was no significant correlation between SNCG protein expression and gender, age, tumor size, number of tumors, tumor stage, and tumor grade. Interestingly, an association between SNCG expression and tumor stage was observed previously in many different cancer types (9, 12). The correlation between these features and tumor recurrence were also analyzed (Table II). As expected, clinicopathological features including tumor size (p=0.001), number of tumors (p<0.0001) and tumor stage (p=0.011) significantly influenced recurrence of BC, while gender, age and tumor grade did not (p>0.05). Expression of SNCG in primary tumors (p=0.023) was also significantly associated with recurrence. This was different from Zhao *et al*.'s study in which there was no correlation between the SNCG overexpression and BC recurrence (20). Sixty-two patients developed tumor recurrence during the follow-up period. While 45.7% of patients with SNCG-positive primary tumor developed tumor recurrence, only 30.8% of patients with SNCG-negative tumors developed tumor recurrence (p=0.023).

Highly expressed SNCG does not correlate with poor outcome and is not an independent prognostic indicator. In order to determine whether SNCG is a predictor of progression for BC, we correlated SNCG expression in tumors with a median follow-up of 44.1±28.2 months (range=0-121 months) after BC

surgery. We found that there was no correlation between SNCG overexpression and survival. As shown in Figure 3, it seemed that there was better prognosis in patients with SNCG-negative BC than those who were SNCG-positive for DFS and OS calculated by Kaplan-Meier analysis, but differences were not statistically significant. The DFS and OS were 78.7± 4.2 months [95% confidence interval (CI) = 70.5-86.9 months] and 80.3±4.0 months (95% CI=72.4-88.2 months) in the SNCG-negative group; for the SNCG-positive group, DFS and OS were reduced to 48.7±5.4 months (95% CI=38.1-59.3 months) and 51.1±5.2 months (95% CI=40.9-61.2 months), respectively (p=0.821 and 0.544, respectively).

We further analyzed whether SNCG was an independent prognostic indicator by performing multivariate Cox proportional hazards analysis. Univariate analyses indicated that tumor size and tumor number significantly impacted the DFS and OS of these patients (p<0.05), but the SNCG expression had no influence on both DFS and OS (p=0.186 and 0.837, respectively). In multivariate analyses, both tumor size and tumor number were independent predictors for both DFS and OS (p<0.05). The analysis identified that SNCG expression in BC tissue was not a predictor for DFS and OS.

Discussion

In this study, we first verified that antibody to SNCG could specifically be used in the detection of the SNCG expressed in BC tissue, in order to ensure the accuracy of our research. The results of our study showed that SNCG is highly expressed in BC tissue. It is no surprise that Dokun *et al*. indicated this in 2008 (19). We also found that SNCG expression was not an independent prognostic factor for patients with BC, which coincides with Zhao *et al*.'s study (20). A similar result was also observed in breast cancer (24). However, conflicting results have been reported for many other malignant tumor types, such as pancreatic, colonic, prostatic and gastric cancer (15-18). Thus, we conclude at this point in time, it would be controversial to endorse SNCG expression as an indication of worse clinical outcomes, since data suggest that it varies in different malignancies. It is important to note that multiple studies have demonstrated that SNCG expression is, in fact, an independent prognostic factor for malignancy, especially for breast cancer (13). This at least deems further studies into SNCG involvement in BC necessary. Previous studies demonstrated that SNCG expression was stage-specific in diversified cancer types, including liver, esophagus, colon, gastric, lung, cervical and breast (12). The studies of SNCG (previously termed BCSG1) in breast cancer continue and the reports about SNCG mainly focus on breast cancer (9). In Guo *et al*.'s study, SNCG expression was stage-specific in breast cancer, in advanced breast cancer, the rate of SNCG positivity was 71.4%, while for stage I/II, it was only 26.8% (13). In this study, the rate of SNCG positivity for NMIBC and MIBC were 87.5%, 97.3%, respectively. It seems that the SNCG expression was associated with the tumor stage, but no statistical significance was found (p=0.091), while in Zhao *et al*.'s study, there was a statistical significance in the difference of the SNCG expression for NMIBC and MIBC (20). This inconsistent result may due to the inconsistency of the clinical data and the limited cases involved in the study. This shows that the relationship between SNCG level and tumor stage needs further investigation.

Due to the high recurrence rate, life-long follow-up is necessary in patients with BC. The two gold-standard surveillance strategies, cystoscopy and urinary cytology, suffer limitations, such as high cost and invasiveness. This is why novel methods must be stringent for better management of BC. The tissue-based marker discussed in our current study plays an important role in the prognostication and management of many malignancies and shows promise in the management of BC. Other widely studied tissue-based markers in BC including: p53, p21, p16, retino-blastoma (Rb), and tat-interactive protein 60(TIP60), have never been used in clinical diagnosis and prediction due to impracticability (25, 26). In our study, we set out to demonstrate the role of SNCG in BC. Our results show that SNCG was overexpressed in BC tissues. Although it was not an independent factor for DFS and OS, it did predict a higher recurrence rate. Therefore, SNCG can predict the probability of recurrence in BC, which interestingly, again conflicts with Zhao *et al*.'s results (20).

In addition, according to immunohistochemical results, SNCG protein was mainly expressed in the cytoplasm of the BC cells, while hardly at all in the non-neoplastic adjacent tissues, which was consistent with Liu *et al*.'s study (27). Notably, we observed obvious SNCG staining in the nucleus and membrane in some cancer cells, and this coexisted with SNCG protein in plasma. These two cases of BC tissue samples came from two patients, both of whom experienced recurrence or metastasis after surgery. Furthermore, both died within 4 months after surgery. Due to the limited number of cases, the role and function of the SNCG expressed in the nucleus and membrane needs further research. It has been reported that SNCG localizes to spindle poles and translocates from perinuclear area to the nucleus (28, 29). We also found that SNCG was highly expressed in bladder neuron-chords, vascular endothelial cells, and smooth muscle cells, but the biochemical and cellular function is still elusive.

In conclusion, SNCG is a good marker to predict recurrence of BC, but not a reliable marker for staging or prediction of survival rate. Our study might be helpful for urologists in managing patients with BC.

Conflicts of Interest

The Authors have nothing to disclose.

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