Expression profile of *SPACA5*/*Spaca5* in spermatogenesis and transitional cell carcinoma of the bladder

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Abstract. The majority of bladder cancer-associated mortalities are due to transitional cell carcinoma (TCC), which is the most prevalent and chemoresistant malignancy of the bladder. Sperm acrosome associated 5 (SPACA5)/Spaca5 is a sperm acrosome-associated, c-type lysozyme-like protein that has been recently identified, and has been designated as an attractive candidate antigen for cancer testis. In the present study, the expression profile of *SPACA5*/*Spaca5* was analyzed in spermatogenesis and TCC of the bladder using diverse molecular and cellular biology methods. Using reverse transcription-polymerase chain reaction (RT-PCR) to analyze the multi-tissue distribution and temporal expression of *SPACA5*/*Spaca5*, the *SPACA5*/*Spaca5* gene was determined to be generally not expressed in normal tissue, with the exception of the testis, and it could be detected at a low level on day 20 after birth in mouse testes and at a higher level on day 28. Immunohistochemistry staining revealed that the SPACA5/Spaca5 protein was exclusively observed in the elongated spermatid of the normal testes, and was ectopically expressed in the cytoplasm of TCC, while it was not expressed in normal bladder tissues. The frequency of *SPACA5* messenger RNA was detected in 45% of TCC (9/20) by RT-quantitative PCR. Furthermore, SPACA5 protein was more frequently detected in high-grade than in low-grade tumors (61.54 vs. 30.00%, P=0.035). Accordingly, high SPACA5 staining scores were observed to be significantly associated with high-grade tumors (n=65, R=0.279, P=0.027). Collectively, our findings indicated that *SPACA5*/*Spaca5* may be important in male spermatogenesis and may be used as a potential target for specific immunotherapy in patients suffering from TCC.

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Introduction

Bladder cancer is the most common malignancy of the urinary tract, ranking the first in incidence in China and the fourth in Western countries (1). At initial diagnosis, ~95% of bladder cancers are determined to be transitional cell carcinoma (TCC), which results in significant morbidity and mortality (2). Among them, ~70% are superficial (3), and >50% of these cancers recur after surgery, with 15-20% of the recurrences progressing to a more invasive form (4). This high rate of disease recurrence requires lifelong surveillance. Currently, the most standard diagnostic approaches for identification and monitoring of recurrence and progression of bladder cancer are cystoscopy (5) and urine cytology (6). However, the application of these approaches is limited in routine clinical practice due to the invasive nature, expensive cost and inconvenience of cystoscopy, and the low sensitivity of urine cytology (7). Therefore, there is a requirement for alternative approaches and therapeutic targets for better management of bladder TCC patients.

Therapeutic approaches to bladder cancer are often limited to radiation therapy and chemotherapy, with poor overall clinical outcome (8). Recently, immunotherapy has emerged as a promising treatment modality due to its low side effects and high specificity, which results in an improved quality of life for patients (9). Cancer testis (CT) antigens are named after their pattern of expression, as they are abundantly detected in various types of cancers, including melanoma, lung cancer, bladder cancer, liver cancer and breast cancer (10), but generally not in normal tissue, with the exception of the testis (11-13). Furthermore, CT antigens are not expected to induce autoimmune damage to any normal tissues due to the testes being immune-privileged (14). Due to these unique expression patterns, CT antigens have been the focus of attention as potential targets in immunotherapy for cancer (15).

Sperm acrosome associated 5 (SPACA5), also named lysozyme-like (LYZL) 5, encodes a putative protein of

159 amino acids that has been mapped to p11.23 of the X chromosome (16). SPACA5 is a sperm acrosome-associated, lysozyme-like protein that was first identified by searching for transcript clusters that map to multiple locations on the chromosome, followed by *in silico* analysis of its gene expression profile (16). Of the ~100 CT genes or gene families identified thus far, ~30 belong to multigene families on the X chromosome (17). To date, a number of CT antigens, including melanoma-associated antigen (MAGE)-A1 (18), MAGE-A3 (19,20), MAGE-A9 (4), MAGE-A12 (18), cutaneous T-cell-lymphoma-associated antigen (cTAGE-1) (18), cTAGE-2 (18), cancer/testis antigen 2 (21) and New York esophageal squamous cell carcinoma 1 (NY-ESO-1) (21-23), have been confirmed to express a prolific and specific profile in TCC, providing a possibility of early detection, antigen-specific immunotherapy and polyvalent vaccination. In a previous study, *SPACA5* messenger RNA (mRNA) expression was detected mainly in the testes and was designated as an attractive candidate CT antigen (16). Following a Basic Local Alignment Search Tool analysis, it was determined that mouse *Spaca5* is 81% identical to human *SPACA5*. However, to the best of our knowledge, little is known regarding the properties and function of *Spaca5* in mouse testes, and *SPACA5* has not been studied in TCC in the literature. The current study is therefore designed to investigate the temporal and spacial expression of *SPACA5*/*Spaca5* in mouse and human testes in order to explore the possible correlation between the expression of the *SPACA5* antigen and the clinicopathological characteristics of patients with TCC, and to evaluate whether *SPACA5* could be applied as a specific immunotherapy target in these patients.

Materials and methods

Ethics statements. Human tissues were collected between 2010 and 2013 under the approval of the Ethics Committee of Shenzhen Second People's Hospital (Shenzhen, China), and informed consent forms were signed by the patients. The present study was approved by the Institutional Review Board of Shenzhen Second People's Hospital.

Animals and patients samples. A total of 30 female and 10 male C57BL/6J mice (18-22 g, 6-weeks-old) were acquired from the Laboratory Animal Center of Southern Medical University (Guangzhou, China) and were maintained in a humidity and temperature-controlled room. All animals had free access to standard water and mouse chow. Female and male mice (3:1) were allowed to mate naturally, and the day of birth was termed day 1. Testes from C57BL/6J mice were collected on days 9 (n=10), 14 (n=8), 18 (n=5), 20 (n=5), 22 (n=5), 25 (n=3), 28 (n=3), 30 (n=3) and 35 (n=3), as well as at 6 weeks (n=2). Other tissues containing brain, heart, liver, lung, spleen, skeletal muscle, esophagus, stomach, small intestine, bladder, epididymis, kidney and pancreas were also collected from adult mice (n=2).

The TCC specimens and adjacent normal bladder tissues were retrospectively recruited from 65 TCC patients (aged 35-80 years) at the Clinical Data and Specimen Bank of Biological Resources of Major Diseases in Shenzhen (Shenzhen, China). All patients provided informed consent to participate in the experiments prior to using their tissues. None of the TCC cases have received any therapy, including chemotherapy, radiotherapy or other treatment, prior to surgery. Pathologic grading (G1-G4) and staging (Tis, T_a and T_1-T_4) were determined according to the 2004 World Health Organization bladder tumor classification criteria (24) by two experienced pathologists (with >10 years of clinical experience) under optical microscopes. In addition, total RNA from human normal tissues, including brain, heart, spleen, lung, thymus, skeletal muscle, stomach, small intestine, testis, bladder, kidney, liver, placenta, thyroid gland, prostate, ovary and colon, was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Upon surgical resection, all specimens were initially stored in RNAlater® (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and immediately frozen at -80˚C in liquid nitrogen until further analysis.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from the tissue samples was extracted with the RNeasy Plus Mini kit (Qiagen GmbH, Hilden, UK). RT-PCR was conducted to analyze and confirm the expression of the *SPACA5*/*Spaca5* genes. Total RNA (2 μg) was reverse transcribed into complementary DNA in a total volume of 10 μl with MuLV Reverse Transcriptase (Thermo Fisher Scientific, Inc.). Forward and reverse oligonucleotide primers specific to *SPACA5*/*Spaca5*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* and *Gapdh* were designed using Primer Express 3.0 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) and were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primer sequences for each gene analyzed were summarized in Table I. The genes *GAPDH* and *Gapdh* were used as the internal controls.

The following PCR conditions were used: 95˚C for 3 min; 40 cycles of 95˚C for 30 sec, 65˚C for 30 sec and 72˚C for 30 sec; and 72˚C for 5 min. The PCR products were subjected to electrophoresis by 1.5% agarose gels and monitored under ultraviolet light. Each sample was analyzed in triplicate. Relative levels of *SPACA5*/*Spaca5* mRNA expression were normalized to *GAPDH*/*Gapdh* mRNA expression.

RT-quantitative (q) PCR. *RT-qPCR* analysis was performed with a SYBR-Green Chemistry kit (Qiagen GmbH) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.), with each sample analyzed in triplicate. The following qPCR conditions were used: 95˚C for 3 min; 40 cycles of 95˚C for 30 sec, 65˚C for 30 sec and 72˚C for 30 sec; and 72˚C for 5 min, followed by a typical dissociation stage. *GAPDH* was used as a reference gene in parallel (Table I). The results were presented as the levels of expression following normalization to *GAPDH* using the $2^{-\Delta\Delta Cq}$ method (25).

Immunohistochemistry. Sections of 5-μm thickness were sectioned from the selected paraffin blocks, mounted on silane-coated slides and dried at 37˚C overnight. The sections were deparaffinized using xylene, rehydrated through descending grades of alcohol to distilled water and incubated in 3% H_2O_2 solution for 10 min to quench endogenous peroxidase activity. Subsequently, the slides were heated for

Table I. Primers for reverse transcription-polymerase chain reaction analysis.

Genes	Primer sequence (5'-3')	Annealing temperature (˚C)	Product size (bp)
SPACA5	F: GGCAGCAGTGAATATGGCATT	60	188
	R: CTCCAAGAAGTCCAGGCAGAA	60	
GAPDH	F: AGAAGGCTGGGGCTCATTTG	60	258
	R: AGGGGCCATCCACAGTCTTC	60	
Spaca5	F: GCAGCATTGTGGTAGTGATCTT	60	308
	R: GCGGTTGAGCAGGTCATTAC	60	
Gapdh	F: CCGGGGCTGGCATTGCTCTC	60	150
	R: GTCCTTGCTGGGGTGGGTGGTC	60	

SPACA5, sperm acrosome associated 5; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

10 min in a microwave oven for antigen retrieval and washed three times in phosphate-buffered saline (PBS). Upon nonspecific binding blocking with 5% normal goat serum (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) for 20 min at room temperature, sections were incubated with a primary rabbit polyclonal anti-SPACA5/Spaca5 antibody (dilution 1:1,000; cat. no. ab111474; Abcam, Cambridge, UK) overnight at 4˚C. Following three washes in PBS, the sections were incubated for 1 h at 37˚C with biotin-conjugated goat anti-rabbit secondary antibody (dilution 1:5,000; cat. no. KIT-9710; Fuzhou Maixin Biotech Co., Ltd.). After three washes in PBS, the sections were incubated at room temperature for 10 min with streptavidin-anti-biotin peroxidase. Following three washes in PBS, the sections were incubated at room temperature for 5 min with 3,3'-diaminobenzidine, which generated a brown color at the site of peroxidase activity. Sections were then washed (3x5 min) in deionized water, and the nuclei were counterstained with hematoxylin. Finally, the sections were dehydrated and mounted. In the negative control slides, the primary antibody was replaced by normal immunoglobulin G (IgG) (dilution 1:500; cat. no. ab172730; Abcam). The results were observed under a microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. All statistical tests were performed with SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). The correlations between SPACA5 expression and clinical parameters, including gender, tumor stage, tumor grade and staining score, were evaluated by Pearson's χ^2 test. All analysis were two-tailed, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Tissue distribution of SPACA5/Spaca5 mRNA in humans and mice. The tissue expression pattern of *Spaca5* was evaluated by RT-PCR in 14 different mouse tissues, including brain, heart, liver, lung, spleen, skeletal muscle, esophagus, stomach, small intestine, bladder, testis, epididymis, kidney and pancreas. The gene was specifically expressed in testis and not in the other normal tissues (Fig. 1A). Having certificated the unique distribution of *Spaca5* in mouse normal tissues, the expression pattern of *SPACA5* was subsequently investigated in different human tissues to decide whether *SPACA5* possesses CT antigen expression characteristics. RT-PCR consisting of 40 amplification cycles was performed on total RNA isolated from 17 human normal tissues. *SPACA5* mRNA was not expressed in any of the normal tissues with the exception of testes (Fig. 1B), which parallels the results of multi-tissue RT-PCR in mice. Thus, the expression pattern

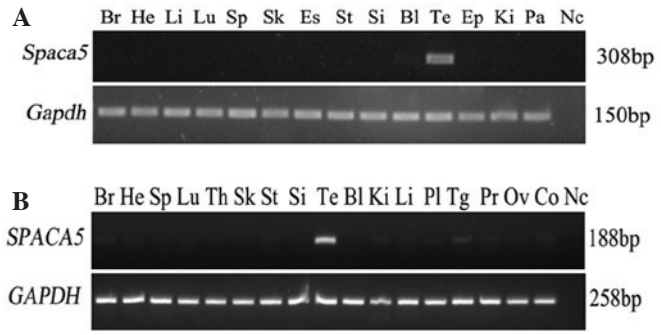

Figure 1. Tissue distribution of *SPACA5*/*Spaca5* mRNA in humans and mice. (A) Distribution of *Spaca5* mRNA in 14 diverse mouse tissues. Expression of *Spaca5* was detected only in the testis. *Gapdh* gene expression was used as an internal control. (B) Expression pattern of *SPACA5* mRNA in 17 different human tissues. The gene was not detected in any of the normal tissues with the exception of the testis. *GAPDH* gene expression was used as an internal control. Br, brain; He, heart; Li, liver; Lu, lung; Sp, spleen; Sk, skeletal; Es, esophagus; St, stomach; Si, small intestine; Bl, bladder; Te, testis; Ep, epididymis; Ki, kidney; Pa, pancreas; Th, thymus; Pl, placenta; Tg, thyroid gland; Pr, prostate; Ov, ovary; Co, colon; Nc, negative control; *Spaca5*, sperm acrosome associated 5; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA.

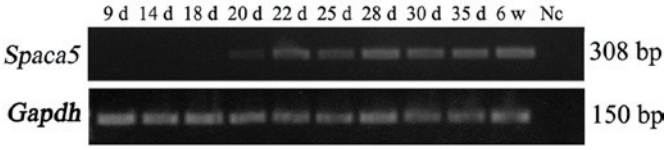

Figure 2. Temporal expression analysis of *Spaca5* during testis development. Mouse *Spaca5* messenger RNA was not detected in mouse testes on days 9, 14 or 18, and was expressed at a low level on day 20. The expression of *Spaca5* increased gradually from day 20 to day 28 and was stabilized after day 28. *Gapdh* gene expression was used as an internal control. *SPACA5*, sperm acrosome associated 5; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; Nc, negative control.

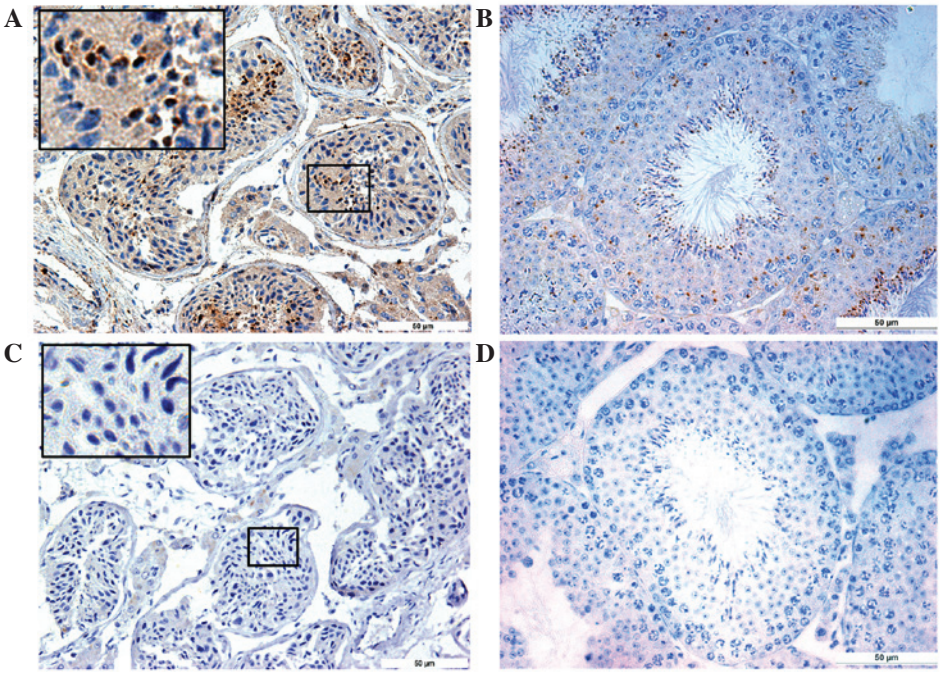

Figure 3. Immunohistochemical analysis of SPACA5/Spaca5 protein expression in normal human and mouse testes (magnification, x200). (A) SPACA5 protein was predominantly located in the elongated spermatid in normal human testes. (B) Spaca5 protein was predominantly located in the elongated spermatid in normal mouse testes. No staining was observed in (C) normal human testes and (D) normal mouse testes when the anti-SPACA5/Spaca5 antibody was replaced by normal immunoglobulin G. SPACA5, sperm acrosome associated 5.

of *SPACA5/Spaca5*, similar to that of other CT antigens, is restricted to testis (11-13).

Temporal expression analysis of Spaca5 during testis development. To further examine the temporal expression of *Spaca5*, a time-course study was performed during testis development. Our results revealed that *Spaca5* mRNA was not detected on day 9 through day 18 in mice testes, but a low expression level of *Spaca5* could be detected on day 20, when late pachytene spermatocytes appeared (26). Its expression increased gradually from day 20 to day 28, when round spermatids developed (Fig. 2). The current study indicated that the expression profile of *Spaca5* is developmental-stage specific.

Localization of SPACA5/Spaca5 protein in normal human and mouse testes by immunostaining. In the normal testes, all stages of spermatogenic cells were present in the seminiferous epithelia. As shown in Fig. 3A and B, SPACA5/Spaca5 protein was mainly located in elongated spermatids, with no background signal in Leydig cells or basal membranes. No staining was observed in the tissue sections where the anti-SPACA5/Spaca5 antibody was replaced by normal IgG (Fig. 3C and D).

RT-qPCR analysis of SPACA5 transcripts in TCC. RT-qPCR was used to determine whether low copy numbers of *SPACA5* transcripts could be detected in certain normal tissues, and to compare the levels of transcript expression in tumor cells with those of normal tissues. Analysis of 20 paired TCC specimens and adjacent normal bladder tissues revealed that *SPACA5* was ectopically expressed in TCC tissues, while it was not

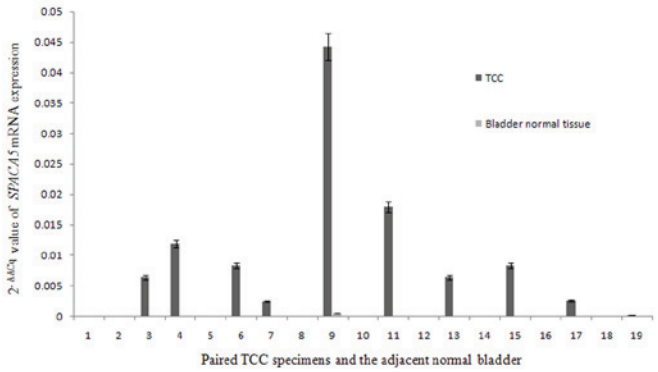

Figure 4. Reverse transcription-quantitative polymerase chain reaction analysis of mRNA expression in 20 pairs of human TCC specimens and adjacent normal bladder tissues. *SPACA5* mRNA expression is shown as the $2^{-\Delta\Delta Cq}$ value. mRNa, messenger RNA; TCC, transitional cell carcinoma; *SPACA5*, sperm acrosome associated 5.

expressed in normal bladder tissues (Fig. 4). The frequency of *SPACA5* expression in TCC was 45% (9/20). Together with the findings of *SPACA5* expression in normal tissues, which revealed that *SPACA5* expression is restricted to the testis, our results indicate that *SPACA5* is a novel CT antigen in TCC.

SPACA5 protein expression in TCC is confirmed by immunohistochemistry. To examine whether *SPACA5* mRNA was translated into protein in TCC cells, paraffin sections were prepared. Specimens from a group (n=65) of TCC patients and normal bladder tissue were investigated for SPACA5 protein expression by immunohistochemical analysis. SPACA5 protein was specifically detected in TCC cells but not in normal bladder tissue. Positive cytoplasmic staining in TCC cells was detectable,

Table II. Correlation between SPACA5 protein expression in TCC and clinicopathological variables.

Characteristics	Patients, n (%)	SPACA5 protein$^+$, n (%)	P-value
All cases	65 (100.00)	25 (38.46)	
Gender			
Male	51 (78.46)	21 (41.18)	0.390
Female	14 (21.54)	4 (28.57)	
Tumor grade			
Low (G1+G2)	50 (76.92)	15 (30.00)	0.035
High (G3+G4)	13 (20.00)	8 (61.54)	
Unknown	2 (3.08)	2 (100.00)	
Tumor stage			
NMIBC (Tis, T_a, T_1)	19 (29.23)	10 (52.63)	0.121
MIBC (T_2, T_3, T_4)	41 (63.08)	13 (31.71)	
Unknown	5 (7.69)	2 (40.00)	

The numbers in the table represent the total cases of each cancer type, while the numbers in brackets represent the percentage of immunohistochemically positive cases of each cancer type. NMIBC, non-muscle-invasive bladder cancer; MIBC, muscle-invasive bladder cancer; TCC, transitional cell carcinoma; SPACA5, sperm acrosome associated 5.

Table III. Sample classification based on anti-SPACA5 staining score.

Score	Positive tumor cells (%)	Grade	Number of cases (%)
0-3	0-100	Total	65 (100.0)
		Low	50 (76.9)
		High	13 (20.0)
		Undefined	2 (3.1)
0	0	Total	40/65 (61.5)
		Low	35/50 (70.0)
		High	5/13 (38.5)
1	<10	Total	11/65 (16.9)
		Low	7/50 (14.0)
		High	3/13 (23.1)
		Undefined	1/2 (50.0)
2	10-50	Total	9/65 (13.8)
		Low	6/50 (12.0)
		High	3/13 (23.1)
3	>50	Total	5/65 (7.7)
		Low	2/50 (4.0)
		High	2/13 (15.4)
		Undefined	1/2 (50.0)

Grade/score correlation, R=0.279, P=0.027. SPACA5, sperm acrosome associated 5.

with weak to strong intensity (Fig. 5). SPACA5 protein was detected in 25 of 65 (38.46%) tumor specimens (Table II).

Correlation between SPACA5 protein expression and clinicopathological variables in TCC. Patients with primary bladder cancer stage are categorized into two groups with different performance characteristics: Non-muscle-invasive bladder cancer (NMIBC) or muscle-invasive bladder cancer (MIBC) (27). At initial diagnosis, NMIBC (which consist of stages T_a, Tis and T_1) accounts for 75-80% of bladder cancer patients, whereas the remaining 20-25% of primary tumors are already MIBC (which consists of stages T_2, T_3 and T_4) or metastatic at the time of initial presentation (21). TCC comprises nearly 95% of all primary malignant tumors of the bladder, showing a broad biological spectrum ranging from superficial to invasive tumors (28). Tumor grade is based on cellular dysplasia and architectural abnormalities in tumor tissue, and is usually employed to categorize TCC in terms of malignant potential (29). High-grade (G3) TCC progresses to muscle invasion more frequently than low-grade (G1 and G2) tumors (30). Although G1 and G2 tumors often recur, they are less likely to become invasive (30).

In the present study, the frequency of SPACA5 protein expression in TCC tumors was 38.46% (25/65). SPACA5 expression was not significantly associated with gender (males vs. females, P=0.390) or tumor stage (NMIBC vs. MIBC, P=0.121), but the frequency of SPACA5 expression was significantly higher in high-grade (G3+G4) than in low-grade (G1+G2) (61.54 vs. 30.00%, P=0.035) tumors (Table II).

The intensity of staining was semiquantitatively evaluated (score 0, 1, 2 and 3), and the association with clinicopathological features was assessed (Table III): Staining detectable in <10% of tumor cells was defined as score 1; staining detectable in 10-50% of tumor cells was defined as score 2; and staining detectable in >50% of tumor cells was defined as score 3. Score 0 was attributed to negative samples. The high SPACA5 staining scores were observed to be significantly associated with high tumor grade (n=65, R=0.279, P=0.027) (Table III).

Discussion

C-type lysozyme is expressed in the majority of species, and due to its ability to act on microbial membranes, it is considered to be important in innate immune defense (31). In the human male reproductive system, four c-type lysozyme genes (*LYZL2, LYZL3*/*SPACA3, LYZL4* and *LYZL6*) have been identified, which are highly expressed in the testes or epididymis (32,33). Mouse *Lyzl3*/*Spaca3 and Lyzl4* can block sperm-egg binding or fusion in the hamster oocyte penetration assay, which indicates their possible role in fertilization (34,35). *SPACA3* is a novel CT antigen in hematological malignancies, and is immunogenic in cancer-bearing patients *in vivo* and a target for tumor immunotherapy (36). Among the SPACA family, only SPACA5 and SPACA3 have strong amino acid homology, containing conserved c-lysozyme-like domains (37). *SPACA5* is also a c-type lysozyme gene named *LYZL5*; therefore, we presume that *SPACA5*/*Spaca5* participates in male spermatogenesis and is a CT antigen in TCC. However, the expression pattern of *SPACA5*/*Spaca5* and its association with

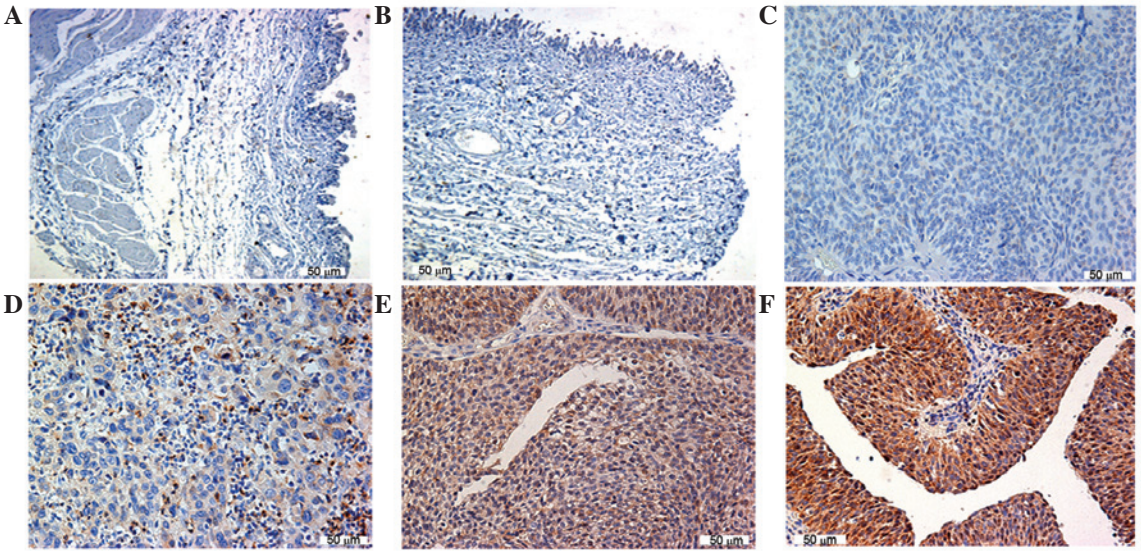

Figure 5. Immunohistochemical staining of SPACA5 protein in TCC tissues (magnification, x100). Panels refer to representative sections showing positive cytoplasmic staining. (A and B) Negative SPACA5 staining in normal bladder tissue. (C) Negative SPACA5 staining in TCC tissue. (D) Weak positive cytoplasmic staining in TCC tissue. (E) Moderate positive cytoplasmic staining in TCC tissue. (F) Strong positive cytoplasmic staining in TCC tissue. TCC, transitional cell carcinoma; SPACA5, sperm acrosome associated 5.

clinicopathological characteristics with TCC are not clear. In the present study, we report a unique expression pattern of *SPACA5*/*Spaca5* in humans and mice, and determine whether *SPACA5* is a potential CT antigen in TCC.

Spermatogenesis is a complicated biological event that includes the mitotic proliferation of spermatogonia, the meiotic division of spermatocytes and the morphogenic differentiation of spermatids to mature spermatozoa (38). In previous studies, it has been reported that different CT antigens are involved in different stages of spermatogenesis. Certain CT antigens are expressed exclusively in one stage, including synaptonemal complex protein 1, which is expressed in the synapsed regions of meiotic prophase spermatocytes only, and sperm protein 17, which is restricted to the mature spermatozoa (39,40). Other CT antigens such as NY-ESO-1 and preferentially expressed antigen in melanoma-like 1 (PRAMEL1) are expressed predominantly in several stages of spermatogenesis. NY-ESO-1 is strongly expressed in spermatogonia and in primary spermatocytes (41), whereas PRAMEL1 is expressed in spermatocytes through elongated spermatids (42). In the present study, RT-PCR was performed to detect the expression profile of *Spaca5* in different developmental stages of mouse testis. It has been reported that, in C57BL/6J mice, primitive type A spermatogonia occurs at postnatal days 4-5; type A and type B spermatogonia occur at day 9; preleptotene and leptotene spermatocytes appear at day 14; zygotene spermatocytes are detected at day 18; pachytene spermatocytes are present at day 20; round spermatids production and elongated spermatid formation occur at day 28-35; and normal postpubertal spermatogenesis occurs at week 6 (26). Similar to the *PRAMEL1* gene, we confirmed that *Spaca5* is broadly expressed in different types of germ cells during spermatogenesis. *Spaca5* mRNA was expressed at a weak level in mouse testes on day 20, and its expression was increased after day 20 and remained stably at a high level after day 28. According to the aforementioned developmental stages of mouse testis, we propose that *Spaca5* may participate in the generation of round and elongated spermatids.

Having demonstrated the expression of *Spaca5* in mouse spermatogenesis, *SPACA5*/*Spaca5* expression characteristics were then investigated in different tissues to determine whether *SPACA5* possesses CT antigen expression characteristics. RT-PCR consisting of 40 amplification cycles was performed on total RNA obtained from 17 and 14 normal tissues in humans and mice, respectively. The results of multi-tissue RT-PCR confirmed that, similar to other CT antigens, the expression of *SPACA5*/*Spaca5* is restricted to the testis, which provides evidence supporting the testis-specific expression of *SPACA5*/*Spaca5*, and makes it a potentially useful target for tumor-specific immunotherapy.

To examine whether *SPACA5* mRNA was also present in TCC cells, 20 paired bladder cancer specimens and adjacent normal bladder tissues were investigated by RT-qPCR. *SPACA5* mRNA was expressed in 45.00% of bladder TCC specimens, but not in the adjacent normal tissues. This predominant *SPACA5* gene expression in bladder TCC patients was similar to other known potential CT antigens. For example, the mRNA expression of the well-characterized CT antigen NY-ESO-1 was detected in 45.10% of bladder TCC specimens (18), and MAGE-A10 expression was detected in 32.43% of bladder carcinomas (43).

The specificity of SPACA5 protein expression in normal and TCC tissues was then validated using an immunohistochemical assay. The results revealed that SPACA5 protein is expressed in TCC tissues, but not in normal bladder tissue. Specimens from a group (n=65) of TCC tissues, of which 13 were high-grade tumors, 50 were low-grade tumors and 2 were unknown. The present study is the first report to determine that SPACA5 protein is exclusively present in the cytoplasm of TCC cells, and to demonstrate an association between SPACA5 expression and tumor grade, since 15 of 50 (30.00%) grade 1 and grade 2

(G1+G2), and 8 of 13 (61.54%) grade 3 and grade 4 (G3+G4) tumors were SPACA5-protein positive. In addition, specific staining was observed more frequently in high-grade compared with low-grade tumors (61.54 vs. 30.00%, P=0.035). In previous studies on urothelial carcinoma of the bladder, Sharma *et al* documented that NY-ESO-1 was highly expressed in high-grade carcinoma, forming the basis of a vaccine clinical trial in which the NY-ESO-1 protein was used as an adjuvant treatment following complete resection of urothelial carcinoma (22). Consequently, the significant correlation between specific staining and high tumor grade of TCC suggests that SPACA5 protein expression may be a characteristic of aggressive TCC, and may pave the way for successful immunotherapy with high efficiency. However, 52.63% of superficial tumors expressed SPACA5 protein, while 31.71% of invasive tumors expressed SPACA5 protein. Since this protein was not observed in normal bladder tissue, these results make *SPACA5* a highly relevant target for superficial bladder, which suggests its potential role in early bladder tumorigenesis. By contrast, no significant association between SPACA5 expression and tumor stage was observed (P=0.121). In addition, no gender differences in the expression of SPACA5 in TCC were observed. Together with the findings of *SPACA5*/*Spaca5* testicular-specific expression, our results suggest that *SPACA5* is a potential CT antigen in TCC.

In conclusion, the present study provided the first evidence that *SPACA5*/*Spaca5* is important in spermatogenesis and also possesses several features of CT antigens. In addition, SPACA5 expression is more abundant in high-grade TCC than low-grade TCC, which indicates its aggressive role in tumorigenesis. Thus, *SPACA5* could be a potential target for specific immunotherapy in patients suffering from TCC. However, large prospective studies are required to confirm our preliminary findings.

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