Detection of survivin expression in bladder cancer and renal cell carcinoma using specific monoclonal antibodies

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Abstract. Survivin, which is highly expressed in the majority of tumors, but not in most normal adult tissues, has been identified to have significant clinical applications. In the present study, using survivin-specific monoclonal antibodies (mAbs), we aimed to establish methods for detecting the expression of survivin in cancer cell lines, serum samples, urine samples and cancer tissues from patients with bladder cancer (BCa) and renal cell carcinoma (RCC), and to evaluate the efficacy of survivin as a tumor marker in the surveillance of BCa and RCC. First, mAbs were labeled with horseradish peroxidase (HRP), and a sandwich enzyme-linked immunosorbent assay (ELISA) with mAbs and HRP-conjugated mAbs was developed to detect survivin expression in serum and urine samples from BCa and RCC patients, with samples from healthy controls (HCs) used for comparison. The HRP-conjugated mAbs were also used to detect survivin expression in cancer cell lines by western blotting. Survivin expression in cancer tissues from BCa patients was also evaluated by immunohistochemistry. The results showed that the sandwich ELISA was successfully established, and significantly higher expression of survivin was subsequently detected in BCa and RCC patients as compared with HCs in both urinary and serum samples ($P<0.05$), and was more pronounced in urine. The HRP-mAbs could recognize survivin in cancer cell lines. Western blotting and immunohistochemistry results confirmed survivin expression in the 5637 BCa cell line, as well as BCa tissues. In addition, the expressions of survivin in BCa tissues, urine and serum were consistent in our study. In conclusion, the sandwich ELISA successfully established in the present study was of high sensitivity and specificity in the detection of survivin expression. The results also indicated that survivin is a potential tumor marker for the surveillance of BCa and RCC.

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Introduction

Survivin, also known as baculovirus IAP repeat-containing protein 5 (BIRC5) and apoptosis inhibitor 4 (API4), is a member of the inhibitor of apoptosis protein (IAP) family (1), which all contain at least one copy of a baculovirus IAP repeat (BIR) domain, and suppress apoptosis when overexpressed in cells (2,3). Previous studies have shown that survivin participates in the suppression of apoptosis, as well as the regulation of cell division (4-6).

Survivin is a homodimer of a 16.5-kDa protein (7). Located at the tip of chromosome 17 in humans (17q25), the survivin gene has four dominant (1, 2, 3, and 4) and two hidden (2B and 3B) exons. Alternative splicing of its pre-mRNA produces splice variants, five of which are known as survivin wild-type (wt), survivin-2B, survivin-DEx3 (8), survivin-3B (9) and survivin 2α (10).

It has been demonstrated that the vast majority of tumors express Survivin mRNA and protein at high levels, whereas most normal adult tissues do not, suggesting that survivin expression is commonly associated with cancer (1,11-13). Survivin may be localized inside or outside the cell (14); inside the cell, survivin has been observed in the cytoplasm, the nucleus and the mitochondria (15-17), but it may also be released into the extracellular space through vesicles (14-18).

In previous studies, it was demonstrated that active caspase-3 and -7 co-immunoprecipitated with survivin, whereas their inactive pro-forms did not (19,20). This interaction disrupts the caspase cascade and cleavage mediated by caspases, thereby resulting in decreased apoptosis (21). In a similar manner, survivin inhibits cytochrome *c*- and caspase-8-induced DEVD (Asp-Glu-Val-Asp)-cleavage activity (21). Previous studies also revealed that survivin antisense oligonucleotides target and downregulate survivin mRNA and induce apoptosis (22,23). Survivin contains a CDE/CHR element, which is involved in cell cycle-specific regulation, implying that survivin may be involved in the cell cycle process (24). During mitosis, survivin can interact with CDK1 (24). Survivin can also interact with the cell cycle regulator CDK4, leading to CDK2/cyclin E activation and Rb phosphorylation. In a previous study, forced overexpression of

survivin resulted in an accelerated S phase and resistance to G1 arrest (25). Survivin, Borealin, INCENP and Aurora B kinase are components of the chromosomal passenger complex (CPC), which is a key regulator of chromosome segregation and cytokinesis during cell division (26,27). Knockdown of survivin expression was found to inhibit cell proliferation, arrest the cell cycle at the G2/M checkpoint and induce cellular apoptosis (28). Previous studies also showed that survivin participates in cell autophagy. The survivin inhibitor YM155 induced cell death through autophagy (26,29,30); when mRNA and protein expression levels of survivin and BCL-2 decreased, the expression levels of caspase-3, poly(ADP-ribose) polymerase (PARP), Beclin 1 and LC-3 increased (31). Survivin may also enhance DNA repair capability by upregulating Ku70 and homologous recombination (32,33).

Urinary bladder cancer (BCa) and kidney cancer are among the most frequently diagnosed cancers and are the leading causes of cancer-related death, ranking sixth and ninth, respectively, in terms of estimated new cases worldwide (34). There have been a number of reports concerning survivin as a tumor marker in the diagnosis of urothelial carcinoma, although further research and confirmation are required. Studies have shown that the serum levels of survivin protein are close to the detection limits of commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., Minneapolis, MN, USA; and Abnova, Taipei, Taiwan) (35). In the present study, using survivin-specific monoclonal antibodies (mAbs) made previously by our laboratory, we aimed to establish methods for detecting the expression of survivin in cancer cell lines, serum samples, urine samples and cancer tissues from BCa and renal cell carcinoma (RCC) patients, and to further evaluate the efficacy of survivin as a tumor marker in the surveillance of BCa and RCC.

Materials and methods

Chemical reagents. Protein-A/G Sepharose (HiTrap Protein G HP, 1 ml) was purchased from GE Healthcare Life Sciences (Little Chalfont, UK). The enhanced chemiluminescence western blotting system and bicinchoninic acid protein assay kit were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Horseradish peroxidase (HRP) (H1759) and the IgG Subclass kit were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, German). 3,3',5,5'-Tetramethylbenzidine (TMB) and ELISA stop buffer were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Phosphate-buffered saline (PBS), HRP-conjugated goat anti-mouse IgG and the immunohistochemistry detection system were purchased from ZSGB-BIO (Beijing, China). PBST (0.05% Tween-20 in PBS) was used as ELISA washing buffer, Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and TBST (0.05% Tween-20 in TBS) were used as western blotting washing buffer.

Cell lines. The lung cancer cell line A549, esophageal carcinoma cell line EC109 and human hepatoblastoma cell line HepG2 were maintained in our laboratory. The BCa cell line 5637 was purchased from the Cell Bank of the Chinese Academy of Sciences (Beijing, China). A549, EC109 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The 5637 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum.

Animals. The animal experiments were approved by the Animal Care Committee of Peking University and conformed to the guidelines of the National Institutes of Health. All efforts were made to minimize animal suffering. Balb/c mice weighing 18-22 g were purchased from the Laboratory Animal Centre of the Chinese Academy of Medical Sciences.

Human specimen collection. All human specimens were obtained from Peking University Cancer Hospital and Institute, diagnosed histopathologically, and staged according to the tumor-node-metastasis (TNM) classification released by the American Joint Committee on Cancer (AJCC, 7th edition, 2010). A total of 105 and 125 urine samples, and 122 and 208 corresponding serum samples from BC and RCC patients, respectively, were collected between March 2015 and December 2015. A total of 10 cases of formalin-fixed paraffin-embedded BCa tissue sections corresponding to the urine samples were also obtained. The healthy control (HC) groups included 131 urine samples and 198 serum samples from individuals who were health-check examinees and showed no abnormalities on laboratory examinations. On the day of collection, all urine samples were centrifuged at 3,000 rpm for 5 min, and the supernatant was acquired, aliquoted and frozen at -20˚C until detection. Each patient and healthy examinee signed an informed consent form. All study procedures were in accordance with the Helsinki Declaration and the study was approved by the Ethics Committee of Peking University Cancer Hospital and Institute.

Antibodies and standard protein. Hybridomas (A6, D8, C6, A9 and E6) were prepared previously. Culture supernatants of hybridomas were assessed for survivin expression, immunoglobulin subclass and specificity by ELISA as described below. Hybridoma cells with high signals on ELISA were injected into the abdominal cavity of Balb/c mice. mAbs from the ascites fluids of Balb/c mice were purified by protein G affinity chromatography. The titer of the purified mAb was measured using the ELISA method. Antibody concentrations were determined by measuring the absorbance at 280 nm using BSA as a protein standard. A recombinant human sequence survivin protein, MS_2-survivin, produced by our laboratory was used as a protein standard (36,37).

ELISA for the expression in hybridoma supernatants and titer of purified mAbs. Microplates (Costar; Corning Inc., Corning, NY, USA) were coated with 100 μl MS_2-survivin proteins (2.5 μg/ml) per well overnight at 4˚C, and then washed 3 times and blocked with 200 μl 5% skimmed milk for 1 h at 37˚C. After three washes, 100 μl serially diluted hybridoma supernatants (from 1:100, for the expression of mAbs) or 100 μl serially diluted purified mAbs (from 1:1,000, for the titer of mAbs) were incubated for 1 h at 37˚C. Following three washes, 100 μl HRP-conjugated goat anti-mouse IgG (1:4,000 dilution) was used as the secondary antibody. Plates were incubated for another 1 h at 37˚C, washed 3 times, and 100 μl substrate solution TMB was added. The reaction was stopped with 50 μl

stop solution for 20 min at 37˚C, and the absorbance was then measured at 450 nm using a microplate reader (model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

ELISA for the specificity and subclass of mAbs. As described for the ELISA above, microplates were coated with 100 μl 2.5 μg/ml MS_2-survivin, GST-survivin, GST-uPA, MS_2-PAI, MS_2-NSE, MS_2-MK or BSA overnight at 4˚C. Following blocking, hybridoma supernatants diluted 10-fold were added, and HRP-conjugated anti-mouse IgG was used as the secondary antibody. The subclass of the mAbs was identified in the hybridoma supernatants with the mouse mAb isotyping kit (Sigma-Aldrich; Merck KGaA).

Labeling of mAbs with HRP. Anti-survivin mAbs that produced high signals on ELISA (D8, C6, A9 and E6) were selected for labeling with HRP. mAbs were dialyzed against several changes of carbonate buffer [0.1 M sodium carbonate buffer ($NaHCO_3/Na_2CO_3$) pH 9.5] overnight at 4˚C. HRP protein was dissolved in deionized water immediately prior to use (protecting solution from light, stirring for 20 min at room temperature) at a concentration of 5 mg/ml, and dialyzed against CH_3COONa (1 mmol/l sodium acetate buffer, pH 4.4)overnight at 4˚C. mAb and HRP solutions were combined in equal quantities by gentle stirring, and incubated at room temperature for 2 h. Next, 0.1 ml NaH_4B (sodium borohydride) was added and incubated at 4˚C for 2 h. The reaction solution was dialyzed against several changes of PBS buffer (0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.4) overnight at 4˚C. After dialyzing, the reaction mixture was applied to a Sephacryl S-200 column to remove uncoupled HRP (38). The mAbs coupled with HRP were used in the subsequent experiments.

Development of a sandwich ELISA using a pair of mAbs. D8, C6, A9 and E6 (100 μl, 2.5 μg/ml) were coated on 96-well microplates overnight at 4˚C. After blocking with 200 μl 5% skimmed milk in PBS for 1 h at 37˚C and three washes with PBST, 100 μl 0.5 μg/ml MS_2-survivin was added to the corresponding wells. After washing, 1,000- and 5,000-fold diluted HRP-labeled mAbs (D8, C6, A9 and E6) were added. The plates were incubated for 1 h at 37˚C, washed 3 times and substrate solution was added. The absorbance was measured at 450 nm after the addition of stop solution. A pair of mAbs was selected to develop a sandwich ELISA system, which was evaluated according to intra-assay precision, inter-assay precision and minimum detectable dose (MDD). By replicating assays in 20 wells with 10 ng/ml survivin protein as a standard substance, the intra-assay coefficient of variation (CV) was obtained. The inter-assay CV was obtained by detecting the same concentration of survivin protein 10 times.

Detection of the survivin protein with the sandwich ELISA. Using the developed sandwich ELISA system, serum and urine samples from patients and HCs were assessed for survivin expression. Serially diluted MS_2-survivin (2,000-0.24 ng/ml) was detected as a standard, with 0 ng/ml as blank, and 500 ng/ml BSA as a negative contrast.

Western blotting. A549, EC109, HepG2 and 5637 cells were harvested, washed twice in ice-cold PBS and lysed using TPEB extraction reagent (Tiangen Biotech Co., Ltd., Beijing, China) for 30 min on ice with sonication every 10 min, after which the lysed mixture was separated by centrifugation at 14,000 x g (4˚C). The supernatants were used as cell lysates. Protein concentration was determined with a bicinchoninic acid protein assay kit. Cell lysates were boiled in lysis buffer containing 2% SDS for 10 min. MS_2-survivin fusion proteins (10 ng) or cell lysates (30 μg) were concentrated by 5% SDS-PAGE (pH 6.8) at 60 V for 30 min, fractionated by 12% SDS-PAGE (pH 8.8) at 100 V for ~2 h and transferred to nitrocellulose membranes at 200 mA for 1.5 h. Western markers (Beijing Transgen Biotech Co., Ltd., Beijing, China) were run in parallel. The blotted membranes were blocked with 5% non-fat milk in PBST and incubated overnight at 4˚C with enzyme-linked mAbs; anti-survivin mAb D8 (sc-17779; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used as a positive control. After washing, the HRP-conjugated goat anti-mouse IgG was used as the secondary antibody for D8 (Santa Cruz Biotechnology, Inc.) and incubated for 1 h at room temperature. Following three washes with PBST, bound antibodies were visualized using enhanced chemiluminescence. For normalization of the target gene, β-actin was used as an internal reference.

Immunohistochemistry. Paraffin sections of 4-μm thickness were baked for 2 h at 65˚C. Deparaffinization was performed using xylene (15 min, twice) and hydration was conducted using a series of graded ethanol (100, 95, 85 and 75%; 5 min each) to distilled water. The antigens were retrieved with pH 6.0 citrate buffer for 5 min at 125˚C in a pressure boiler. Following cooling and washing with PBST, blocking for endogenous peroxidase was performed for 10 min in 0.3% H_2O_2. After three further washes in PBST, non-specific binding was blocked with PBST containing 5% skimmed milk for 30 min at room temperature. The sections were then rinsed in PBST 3 times and incubated at 4˚C with mAbs, anti-survivin mAb D8 (Santa Cruz Biotechnology, Inc.) as a positive control, or 5% skimmed milk in PBST as negative control. Following three washes, the sections were incubated with Polymer Helper for 20 min, and then washed again 3 times prior to incubation for 30 min with polyperoxidase-anti-mouse/rabbit IgG. After a further three washes, the sections were sequentially developed in DAB solution for 5 min, counterstained in hematoxylin for 1 min, washed in tap water, rinsed in ethanol containing 1% hydrochloric acid, washed in tap water for 30 min, and dehydrated in graded ethanol (75, 85, 95 and 100%) and xylene. Coverslips were applied to the samples, which were then evaluated under light microscopy independently by two pathologists from the Department of Pathology, Peking University Cancer Hospital and Institute, without prior knowledge of the patient clinical data. The intensity of the staining was scored on a scale of no staining/negative, weak staining/(+), moderate staining/(++) and strong staining/(+++).

Statistical analysis. Statistical analysis was carried out using SPSS for Windows (version 16.0; SPSS, Inc., Chicago, IL, USA). The survivin concentrations in patients and healthy individuals were compared by Student's t-test and also assessed using the area under the receiver operating characteristic (ROC) curve (AUC). The cut-off value was determined

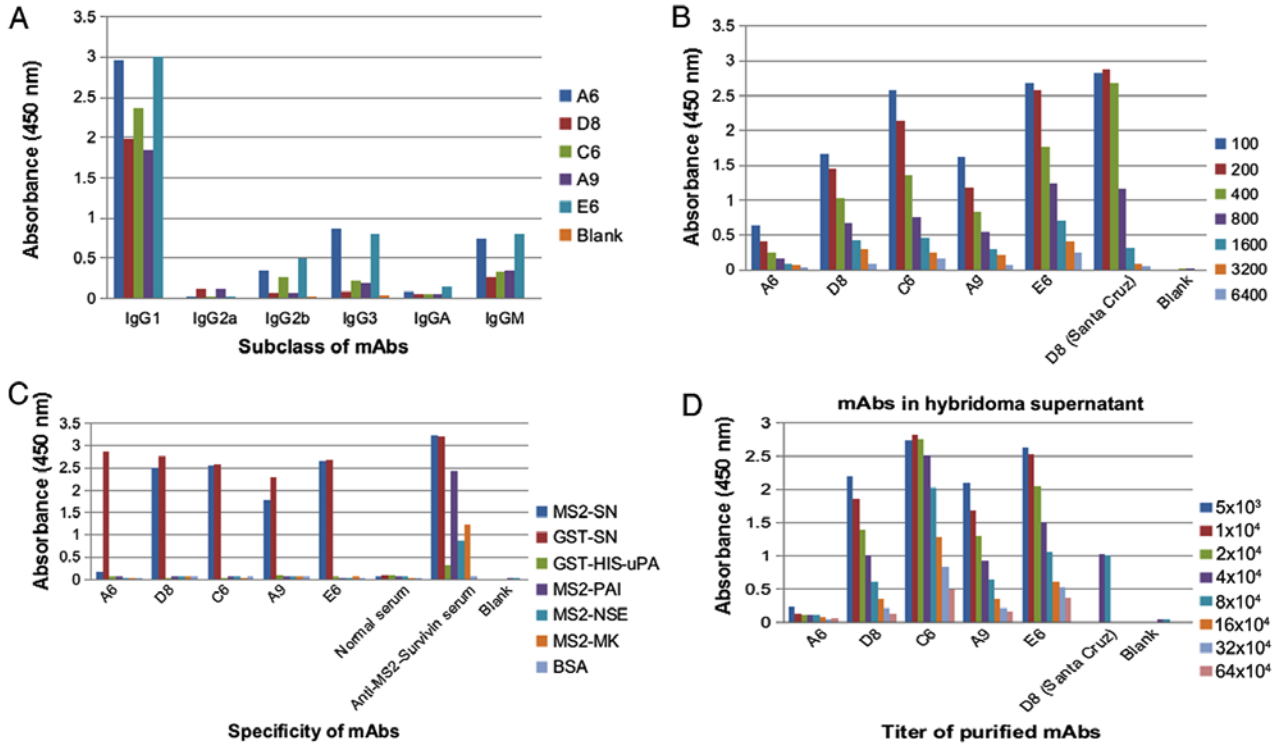

Figure 1. Characterization of survivin mAbs. ELISA for (A) the subclass of mAbs, (B) the expression of mAbs in hybridoma supernatants, (C) the specificity of mAbs and (D) the titer of purified mAbs. mAbs, monoclonal antibodies.

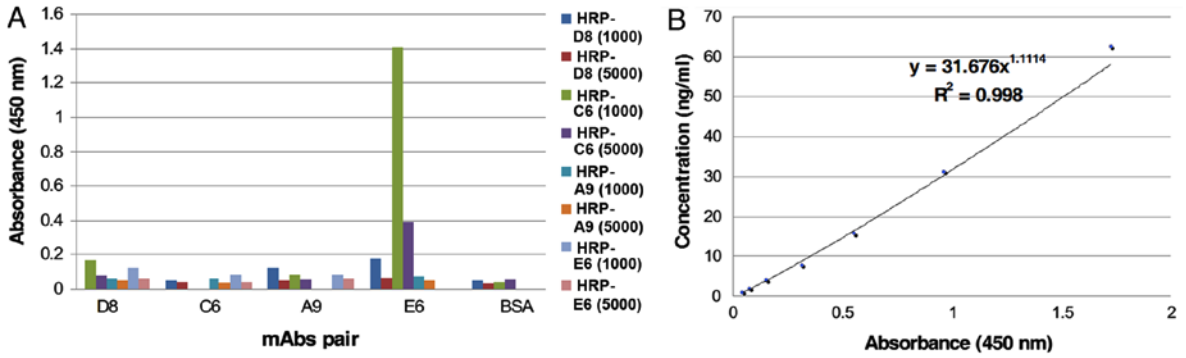

Figure 2. Development of sandwich ELISA. (A) D8, C6, and E6 were used as the coating antibodies, while the corresponding enzyme-linked mAbs HRP-D8, HRP-C6 and HRP-E6 were used as the detecting antibodies, respectively, to develop a sandwich ELISA. E6 was selected as the capturing mAb and HRP-C6 as the detecting mAb. (B) Standard curve. Serially diluted MS_2-survivin (2,000-0.98 ng/ml) was detected to establish the standard curve. mAbs, monoclonal antibodies; HRP, horseradish peroxidase.

by the optimal Youden's index (sensitivity + specificity - 1). All tests were two-sided and $P<0.05$ was set as the significance level.

Results

Expression, specificity, titer and subclass of mAbs. Hybridomas were tested for survivin subclass by indirect ELISA. Hybridomas (A6, D8, C6, A9 and E6) with high expression, specificity and antibody titer were selected for further mAb pairing. The results showed that the subclass of these mAbs was IgG1 (Fig. 1A). D8, C6, A9 and E6, which exhibited strong signals on ELISA, were chosen for subsequent mAb pairing (Fig. 1B-D).

Sandwich ELISA development and evaluation. E6 was selected as the capture mAb and HRP-C6 was selected as the detecting mAb to develop the sandwich ELISA (Fig. 2A). The intra-assay CV was 7.28% and the inter-assay CV was 9.58%, indicating that the sandwich ELISA had good reproducibility. According to the standard protein curve (Fig. 2B), the MDD of the assay was 0.98 ng/ml.

Expression levels of survivin in urine and serum samples from patients. Urine samples from 105 cases of BCa and 125 cases of RCC, as well as 122 and 208 corresponding serum samples, were assessed. The HC groups included 131 urine samples and 198 serum samples from health-check examinees who showed no abnormalities on laboratory examination results. The basic

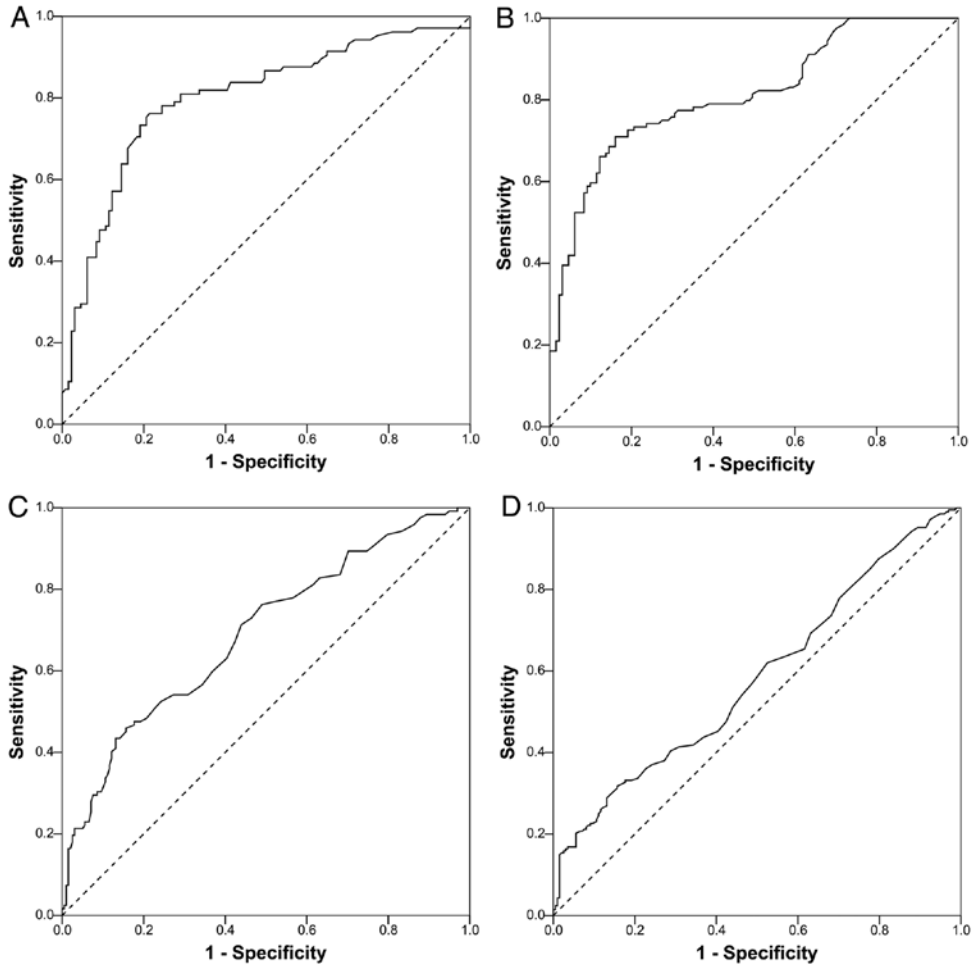

Figure 3. ROC curves based on the detection of survivin in urine and serum among cancer patients and HCs. (A) The area under the ROC curve (AUC) was 0.800 in urine from BCa patients. (B) The AUC was 0.812 in urine from RCC patients. (C) The AUC was 0.691 in serum from BCa patients. (D) The AUC was 0.600 in serum from RCC patients. ROC, receiver operating characteristic; HCs, healthy controls; BCa, bladder cancer; RCC, renal cell carcinoma.

characteristics, including the age and sex of the patients and HCs, are summarized in Table I.

In BCa and RCC patients, survivin concentrations were significantly higher compared with those in HCs in both the urinary and serum samples (P<0.05) (Table II).

ROC curves based on the detection of survivin in urine and serum samples from cancer patients and HCs are shown in Fig. 3. The AUCs were 0.800, 0.812, 0.691 and 0.600, respectively, in BCa urine, RCC urine, BCa serum and RCC serum samples. According to the optimal Youden's index, cut-off values of 8.2765 and 9.4985 ng/ml in urine samples were proposed for BCa and RCC, respectively, corresponding to sensitivity values of 76.20 and 71.00%, and specificity values of 88.60 and 84.00%. In BCa and RCC serum samples, cut-off values of 1.2385 and 1.1625 ng/ml, respectively, resulted in sensitivity values of 71.3 and 62.00%, and specificity values of 56.10 and 47.50% (Table II). The scatter plot showing the survivin concentrations in samples from BCa and RCC patients and HCs is shown in Fig. 4.

Survivin concentrations higher than the cut-off value were defined as positive. Among the 50 positive urine samples from patients with BCa, 39 (78%) of their corresponding serum samples were also positive, while in RCC, 27 serum samples (41.54%) were positive out of the 65 patients with positive urine samples. This indicated that survivin concentration in urine was fairly consistent with that in serum. No significant differences in the expression of survivin were observed between patients with primary and recurrent BCa (Tables III and V). Before and after surgery, survivin concentration also showed no significant differences in BCa or RCC patients (Tables III-VI).

Table I. Basic characteristics (age and sex) of the BCa and RCC patients and HCs.

Samples	N	Age in years [mean (range)]	Sex	
			Male	Female
Healthy urine	131	48.1679 (24-66)	108	23
Healthy serum	198	36.9141 (22-66)	69	129
BCa urine	105	61.8544 (29-84)	71	34
BCa serum	122	62.1721 (29-81)	96	26
RCC urine	124	57.0000 (24-85)	83	31
RCC serum	208	57.1394 (27-84)	132	76

BCa, bladder cancer; RCC, renal cell carcinoma; HCs, healthy controls.

Table II. Survivin level in BCa and RCC patients and HCs in both urinary and serum samples.

Samples (n)	Survivin level (mean±SD)	P-value	AUC	Cut-off value	Sensitivity	Specificity
Urine samples						
HC (131)	28.7327±75.56408					
BCa (105)	131.1819±150.13326	**<0.001**	0.800	8.2765	0.762	0.886
RCC (124)	173.4632±161.66956	**<0.001**	0.812	9.4985	0.71	0.84
Serum samples						
HC (198)	1.6221±3.45691					
BCa (122)	3.4660±8.78510	**0.009**	0.691	1.2385	0.713	0.561
RCC (208)	2.8443±7.12991	**0.028**	0.600	1.1625	0.620	0.475

P-values in bold print indicate significant difference. BCa, bladder cancer; RCC, renal cell carcinoma; HCs, healthy controls.

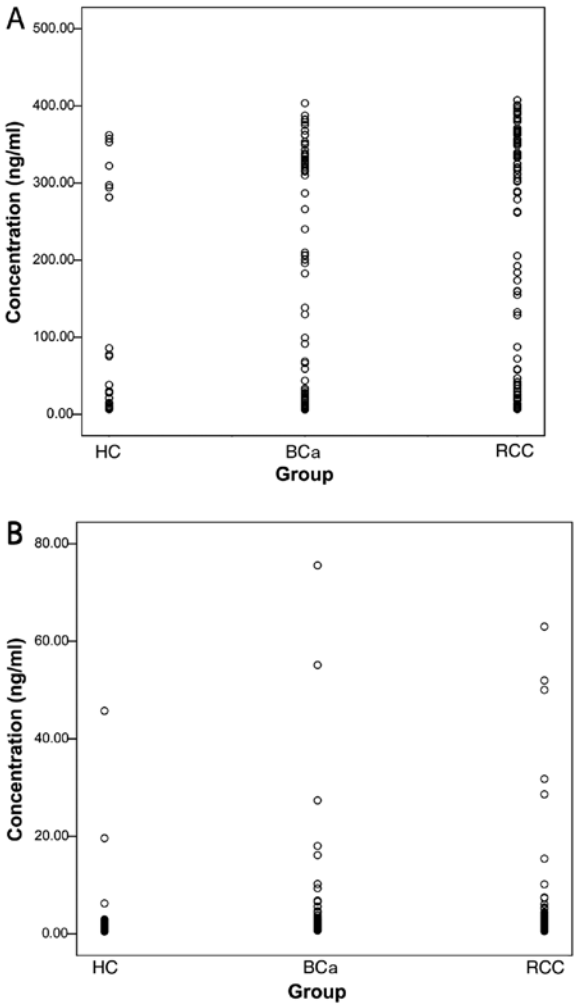

Figure 4. Scatter plots of survivin expression in BCa and RCC patients and HCs. (A) Survivin concentration in urine samples of 105 BCa patients, 125 RCC patients and 131 HC. (B) Survivin concentration in serum samples of 122 BCa patients, 208 RCC patients and 198 HC. BCa, bladder cancer; RCC, renal cell carcinoma; HCs, healthy controls.

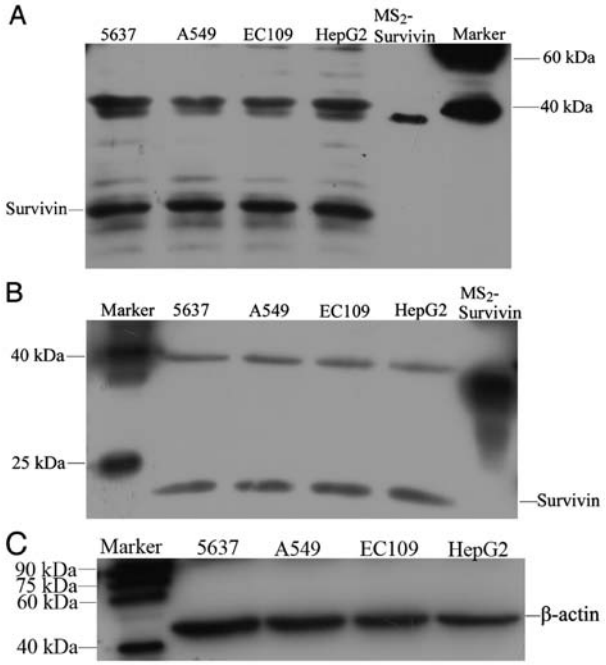

Figure 5. Western blotting of survivin expression in cancer cell lines. Expression of survivin in cancer cell lines as detected with (A) enzyme-linked mAbs (HRP-mAbs) and (B) the D8 antibody (Santa Cruz Biotechnology, Inc.). (C) β-actin was used as the internal reference. mAbs, monoclonal antibodies; HRP, horseradish peroxidas.

The associations between the expression of survivin and the clinicopathological characteristics of BCa and RCC patients were analyzed by Student's t-test. No associations were identified, except association between hypertension and the presence of survivin in the serum of RCC patients was found (P=0.012) (Tables III-VI).

In addition, previous studies have reported on the use of nuclear matrix protein 22 (NMP22) in the diagnosis of BCa (39,40). In the present study, no association between NMP22 and survivin level was found (Table III).

Expression of survivin in cancer cell lines. Western blotting was applied to determine whether survivin was expressed in cancer cell lines and whether the HRP-conjugated mAbs produced in the present study could be used to detect survivin. The western blotting results indicated that standard MS_2-survivin was detectable as a 30-kDa band, while survivin

Table III. Correlation between the level of survivin in urine and clinicopathological characteristics of the BCa patients.

Clinicopathological characteristics	n	Survivin level [mean ± SD (ng/ml)]	P-value[a]
Sex			
Male	68	147.533±150.6453	0.2308
Female	34	109.4359±150.0653	
Age (years)			
≤60	44	131.6626±158.9245	0.8543
>60	58	137.2398±145.6814	
Tumor number			
1	36	134.1215±151.2537	0.8094
≥2	29	143.405±156.5502	
Tumor size (mm)			
≤30	48	152.9562±156.843	0.5104
>30	25	127.7777±149.2811	
Primary or not			
Primary	70	139.0455±154.1364	0.775
Recurrent	27	129.2186±143.5481	
Primary			
Preoperation	29	131.741±160.0746	0.7414
Postoperation	41	144.212±151.591	
Recurrent			
Preoperation	8	181.713±144.7057	0.3015
Postoperation	17	115.344±147.2315	
Tumor grade			
G1-G2	20	151.8126±156.8784	0.5698
G3	68	129.7054±151.0077	
Tumor thrombus			
Visible	12	116.5475±126.4072	0.4722
Invisible	23	155.526±161.2349	
Nodal status			
Positive	14	102.4099±141.4549	0.4438
Negative	91	135.6084±151.6818	
Tumor stage			
<pT2	25	170.8168±167.305	0.1397
≥pT2	50	116.1421±139.9187	
TNM stage			
I-II	27	155.4603±161.8041	0.2152
III-IV	32	107.7036±130.888	
NPM22			
Positive	14	154.2549±166.2502	0.7092
Negative	29	174.1876±161.573	
Smoking status			
Yes	33	164.6349±159.3603	0.2442
No	56	125.2063±149.5392	
Hypertension			
Yes	37	151.438±160.473	0.5581
No	52	131.9741±149.1533	

[a]Analyzed by Student's t-test. BCa, bladder cancer; TNM, tumor-node-metastasis; NMP22, nuclear matrix protein 22.

Table IV. Correlation between the level of survivin in urine and the clinicopathological characteristics of the RCC patients.

Clinicopathological characteristics	n	Survivin level [mean ± SD (ng/ml)]	P-value[a]
Sex			
Male	82	178.5686±160.1717	0.7175
Female	41	167.3186±166.2319	
Age (years)			
≤60	31	144.4049±162.146	0.2272
>60	92	185.0668±161.0323	
TNM stage			
I-II	76	202.8524±165.8122	0.108
III-IV	48	202.8524±151.951	
Fuhrman grade			
I-II	55	158.7662±163.3089	0.2007
III-IV	31	205.2525±155.3374	
Histologic category			
Clear cell	89	179.8389±159.4347	0.6202
Other	29	162.6501±168.9348	
Tumor size (mm)			
≤50	54	157.3316±163.8723	0.113
>50	42	209.9445±154.5213	
Tumor thrombus			
Visible	14	135.9681±168.2002	0.2291
Invisible	71	193.1823±160.2078	
Smoking status			
Yes	35	164.2389±154.3379	0.3223
No	73	197.3422±165.3699	
Hypertension			
Yes	38	178.8563±159.3798	0.7155
No	70	190.8257±164.2466	

[a]Analyzed by Student's t-test. RCC, renal cell carcinoma; TNM, tumor-node-metastasis.

in the cell lines was observed as a 16.5-kDa band and β-actin as a 42-kDa band (Fig. 5C). 5637, A549, EC109 and HepG2 cells all expressed survivin, and the positive signals detected by the HRP-conjugated mAb (Fig. 5A) were consistent with those detected by the commercial D8 antibody (Santa Cruz Biotechnology, Inc.) (Fig. 5B). In addition, bands at 30-40 kDa were present in all of the cell lines with both mAbs, which may represent heterodimers or aggregates of survivin (Fig. 5A and B).

Survivin expression in human BCa tissue. In order to verify that survivin mAbs could identify survivin expression in human tissues, immunohistochemistry was used to detect survivin expression in BCa tissue, with the D8 antibody (Santa Cruz Biotechnology, Inc.) used as a control. Among 10 BCa samples, all samples displayed positive staining of survivin protein in the cancer cells at different expression levels using both the survivin mAbs and the D8 antibody (Santa Cruz

Table V. Corrrelation between the level of survivin in serum and the clinicopathological characteristics of the BCa patients.

Clinicopathological characteristics	n	Survivin level [mean ± SD (ng/ml)]	P-value[a]
Sex			
Male	96	3.696406±9.550458	0.5799
Female	26	2.615346±5.097275	
Age (years)			
≤60	51	2.582745±2.824331	0.2792
>60	71	4.100479±11.25758	
Tumor number			
1	46	4.352109±11.68199	0.2803
≥2	44	2.414568±2.853097	
Tumor size (mm)			
≤30	65	3.578169±9.72505	0.3181
>30	37	2.277784±2.857489	
Primary or not			
Primary	95	3.238726±9.413477	0.42
Recurrent	24	4.5705±6.482018	
Primary			
Preoperation	38	5.188211±14.65777	0.1816
Postoperation	57	1.93907±1.578393	
Tumor grade			
G1-G2	20	6.2087±16.74663	0.3343
G3	91	2.483747±3.440776	
Tumor thrombus			
Visible	19	3.250842±3.698517	0.1065
Invisible	26	1.769385±1.140501	
Nodal status			
Positive	21	2.638762±3.288208	0.223
Negative	18	1.716389±0.6870672	
Tumor grade			
<pT2	24	2.504208±5.329035	0.5181
≥pT2	70	3.5015±9.065315	
TNM stage			
I-II	61	3.486115±10.05631	0.2152
III-IV	35	2.697457±2.812385	
Smoking status			
Yes	42	2.236857±3.004294	0.2349
No	71	3.682845±9.411087	
Hypertension			
Yes	42	2.595571±4.7085	0.5244
No	73	3.406726±8.902606	

[a]Analyzed by Student's t-test. BCa, bladder cancer; TNM, tumor-node-metastasis.

Table VI. Correlation between the level of survivin in serum and the clinicopathological characteristics of the RCC patients.

Clinicopathological characteristics	n	Survivin level [mean ± SD (ng/ml)]	P-value[a]
Sex			
Male	132	3.238803±8.425232	0.2117
Female	76	2.159039±3.945889	
Age (years)			
≤50	53	3.308321±7.91286	0.5843
>50	155	2.6856±6.862163	
TNM stage			
I-II	63	3.338±9.992344	0.6743
III-IV	87	2.740908±6.052562	
Fuhrman grade			
I-II	109	3.31945±8.594069	0.08494
III-IV	56	1.84625±1.528992	
Histologic category			
Clear cell	24	2.77989±6.906926	0.7456
Other	172	3.211257±8.32561	
Tumor size (mm)			
≤50	98	3.723214±9.906343	0.1146
>50	80	2.036437±3.158853	
Tumor thrombus			
Visible	27	3.507778±9.377017	0.5324
Invisible	139	2.951094±7.665209	
Smoking status			
Yes	54	2.6185±3.955068	0.6389
No	136	3.042706±8.449693	
Hypertension			
Yes	68	1.542221±0.9502749	**0.01248**
No	128	3.605889±9.051269	

P-value in bold print indicates significant difference. [a]Analyzed by Student's t-test. RCC, renal cell carcinoma; TNM, tumor-node-metastasis.

Biotechnology, Inc.). The results revealed that survivin was distributed in the nuclei and cytoplasm of BCa cells, although predominantly in the cell nuclei. The intensity of immunostaining with the survivin mAbs was weak/(+) in 1 case (10%), moderate/(++) in 4 cases (40%), and strong/(+++) in 5 cases (50%) (Fig. 6), whereas 7 (70%) and 3 (30%) cases showed moderate/(++) and strong/(+++) staining, respectively, with the D8 antibody (Santa Cruz Biotechnology, Inc.). The corresponding urine and serum samples of the 1 weak/(+) positive BCa tissue were both negative on ELISA. In the 9 patients with cancer tissues expressing moderate or strong survivin levels, the following results were observed: the serum samples of 4 patients were not collected, while their urine samples were all positive for survivin on ELISA; in 3 of the patients, both urine and serum samples were positive on ELISA; and in the remaining 2 patients, urine samples were positive and serum samples were negative on ELISA.

Further findings suggested a positive correlation between the intensity of immunostaining and tumor grade (G1, G2, G3). Among 4 patients with tumor grade G2, the intensity of immunostaining was weak/(+) in 1 and moderate/(++) in 3;

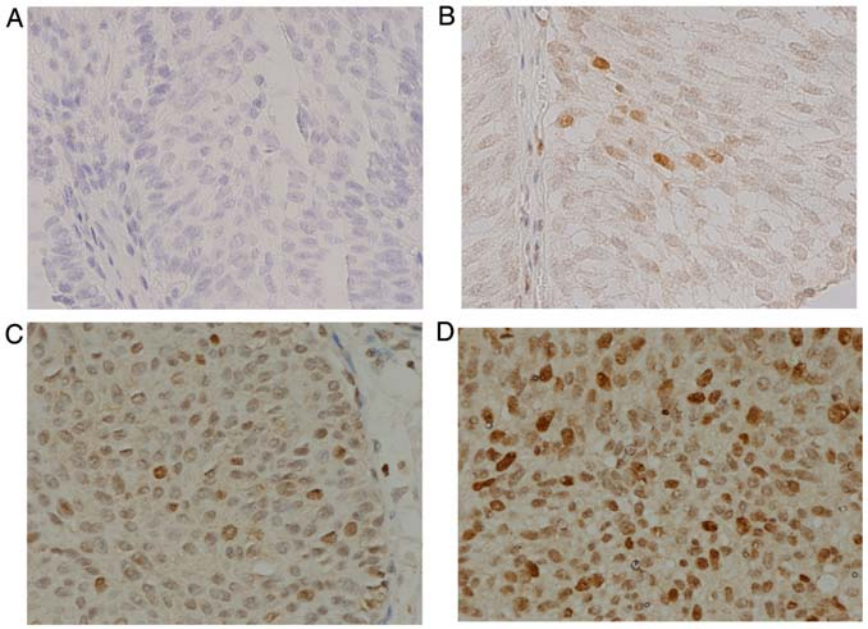

Figure 6. Immunohistochemistry images. (A) Negative control incubated with 5% skimmed milk in PBST. (B-D) BCa tissue samples showing (B) weak/(+), (C) moderate/(++) and (D) strong/(+++) expression of survivin. Magnification, x40. BCa, bladder cancer.

whereas, among 6 patients with tumor grade G3, 2 exhibited moderate/(++) and 4 exhibited strong/(+++) immunostaining.

Discussion

Survivin has been shown to have significance in clinical applications. Recent studies have demonstrated the diagnostic role of survivin in urogenital and urinary bladder cancer (41-43), and survivin overexpression may be an important prognostic factor for recurrence in certain cancers (44-46). Serum survivin levels before and during chemotherapy may serve as a predictive biomarker for the treatment response in malignant mesothelioma (47). Furthermore, studies have also shown that survivin mediates multidrug resistance and reduces apoptosis (48,49). In recent years, a number of studies have focused on targeting survivin as a therapeutic strategy, which has included the use of small-molecule inhibitors and peptidomimetics (YM155, shepherdin) (50,51), transcriptional inhibitors such as survivin antisense oligonucleotides (LY2181308, EZN-3042) (52,53), gene therapy and immunotherapy (54). Many studies have also investigated the mechanism of action of survivin. The BIR domain of survivin interferes with caspase-3 and -7 and induces inhibition of apoptosis (21). Survivin can interact with the cell cycle regulator Cdk4, leading to Cdk2/cyclin E activation and Rb phosphorylation (55). Survivin overexpression also activates NF-κB p65, which is important for the acquisition and maintenance of the oncogenic characteristics of cancer (56). In addition, the HER2-STAT3-survivin axis could serve as a predictive marker and therapeutic target to overcome radiotherapy resistance in HER2-positive breast cancer (57). However, further investigations are still required to fully elucidate the role of survivin in different types of cancers.

Previous studies have demonstrated that a detrimental feature of bladder cancer is its high recurrence rate, which necessitates frequent surveillance imaging and repetitive transurethral resections (58). In the present study, using a sandwich ELISA method developed with E6 and HRP-C6 antibodies, survivin expression in both urine and serum samples was demonstrated to be significantly higher in patients with bladder cancer or renal cell carcinoma than that noted in healthy controls, and this difference was more pronounced in urine samples. In both bladder cancer and renal cell carcinoma patients, survivin expression showed no significant differences in primary vs. recurrent cancer or before vs. after surgery. These results implicate survivin as a potential tumor marker for the diagnosis and prognosis of bladder cancer or renal cell carcinoma. In addition, hypertension is a significant risk factor for renal cell carcinoma. Several studies have shown a dose-dependent increase in renal cell carcinoma with increasing blood pressure level (59,60), and the present study demonstrated that the expression of survivin in the serum of renal cell carcinoma patients was associated with hypertension.

It has been shown that different splice variants of survivin give rise to distinct protein isoforms: survivin-2B and survivin-ΔEx3 retain anti-apoptotic activity (8); survivin-3B exerts cytoprotective functions (9); and survivin-2α is not assumed to exert any anti-apoptotic activity (10). The expression levels of the five survivin splice variants were all significantly higher in cancer tissues compared with these levels in normal tissues in previous studies (61,62). In the present study, western blotting was used to assess survivin expression in the cancer cell lines 5637, A549, EC109 and HepG2. A band at 30-40 kDa was detected using both the HRP-conjugated mAbs generated in our laboratory and the commercial antibody purchased from Santa Cruz; this band was assumed to represent heterodimers or aggregates of survivin. Previous studies have shown that, in the case of wt survivin, ~94% of wt survivin consisted of dimers containing some monomers, and the remaining 6% of wt survivin consisted of large aggregates (63). Monomers in mammalian cells can form heterodimers by binding to other proteins, such as CRM1 (63), and survivin splice variants may also heterodimerize with survivin to regulate its functions (64,65).

Previous studies have shown that survivin localization in cells is consistent with its function in cell division (nucleus) and cell viability (cytoplasm), as well as confirming the presence of different isoforms which had distinct cellular localizations (66). Immunohistochemical analysis in the present study illustrated that survivin was distributed in the nucleus and cytoplasm of bladder cancer cells, although predominantly in the cell nucleus. The expression of survivin in tissues may be consistent with that in urine and serum. Previous studies have found that the presence of nuclear survivin may be an independent biomarker for disease recurrence and overall survival in cancer patients (67,68). In post-chemoradiotherapy tissues, nuclear survivin expression disappeared completely and cytoplasmic expression increased, particularly in treatment-responsive patients (69). A positive correlation between the intensity of immunostaining and tumor grade (G1, G2, G3) was found in the present study, which further confirmed the role of survivin in tumors.

In conclusion, the sandwich ELISA established in the present study had high sensitivity and specificity for the detection of survivin expression. Survivin expression in urine and serum samples from bladder cancer and renal cell carcinoma patients was significantly higher than that in healthy controls. Western blotting of cancer cell lines with HRP-conjugated mAbs and immunohistochemistry of cancer tissues confirmed survivin expression in bladder cancer. Our study further suggests that survivin is a potential tumor marker for the surveillance of bladder cancer and renal cell carcinoma. The availability of these survivin mAbs would be of use in a wide range of studies on survivin.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

QZ conceived and designed the study. DC and JX performed the experiments. DC wrote the paper. QZ and DC reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All study procedures were in accordance with the Helsinki Declaration and the study was approved by the Ethics Committee of Peking University Cancer Hospital and Institute. The animal experiments were approved by the Animal Care Committee of Peking University and conformed to the guidelines of the National Institutes of Health.

Consent for publication

Each patient and healthy examinee provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors state that they have no competing interests.

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