

—Original—

Detection of Urine Survivin in 40 Patients with Bladder Cancer

Ikuroh Ohsawa¹, Taiji Nishimura², Yukihiro Kondo², Go Kimura², Mitsuhiro Satoh²,
Ichiro Matsuzawa², Tsutomu Hamasaki² and Shigeo Ohta¹

¹Department of Biochemistry and Cell Biology, Institute of Development and Aging Sciences,
Graduate School of Medicine, Nippon Medical School

²Department of Urology, Nippon Medical School

Abstract

Purpose: We investigated whether urine survivin, an inhibitor of the apoptosis protein, is useful for diagnosing bladder tumor.

Method: We measured urine survivin levels in 40 patients with bladder tumors and 9 healthy volunteers.

Results: The average urine survivin levels in the 40 patients and 9 healthy controls were not significantly different (3.802 ± 8.669 and 1.127 ± 1.529 , respectively ($p = 0.3646$)). However, significantly high urine survivin levels were observed in 3 of the 40 patients, but not in healthy volunteers. Urine Cyfra 21-1 was not elevated (1.3 ng/ml) in one patient with a significantly elevated urine survivin level (33.54 ng/ml), while in two patients with elevated Cyfra (320 ng/ml and 240 ng/ml), the urine survivin level was not detectable.

Conclusion: With improvements in the sensitivity of our Elisa system for urine survivin and combined use of urine Cyfra 21-1, it is possible that urine survivin will be a useful tumor marker in detecting both new-onset and recurrent bladder tumors.

(J Nippon Med Sch 2004; 71: 379–383)

Key words: survivin, bladder tumor

Introduction

Survivin, a member of the inhibitor of the apoptosis protein (IAP) family, has been shown to inhibit activation of downstream effectors of apoptosis, caspase-3 and -7, in cells exposed to apoptotic stimuli¹. Survivin is expressed during human fetal development, but is not detectable in normal adult tissues, except for the thymus and placenta. And survivin is expressed in most malignant cells, including those of lymphomas², gastric³, colonic⁴, neuroblastomas⁵, lung⁶, bladder cancers⁷, pancreatic⁸, breast⁹, and prostatic cancer¹⁰.

Smith et al. reported that survivin was detected in urine samples from all 31 patients with new or recurrent bladder cancer but was not detected in urine samples of 16 healthy volunteers¹¹. If this report can be confirmed, survivin would be a revolutionary tumor marker for bladder cancer since the sensitivity and specificity of other tumor marker for bladder cancer including CYFRA 21-1 which we previously reported¹² and that of urine cytology range from 44 to 74% and 60 to 99%¹³, respectively.

In the present study, we investigated the urine survivin levels of 40 patients with bladder tumor and 9 healthy volunteers to determine whether survivin is useful for the diagnosis of bladder tumor.

Table 1 Patient characteristics.

Variables	Patients (n)
Gender	
Male	33 (82.5)
Female	7 (17.5)
Grade	
G1	10 (25)
G2	26 (65)
G3	4 (10)
Stage	
CIS	3 (7.5)
pTa	14 (35)
pT1	19 (47.5)
≥ pT2	4 (10)
History	
New-onset	26 (65)
Recurrence	14 (35)

Number in parentheses are percentages. CIS, carcinoma in situ.

Patients and Methods

Population

We evaluated a total of 49 subjects. Group 1 included patients with diagnosis of new-onset (n = 26) or recurrent bladder cancer (n = 14). Urine specimens were obtained before the treatment. Median patient age was 69 years (range 46 to 87 years). Patient characteristics are summarized in **Table 1**.

Group 2 included healthy volunteers (n = 9). Median healthy volunteer age was 49 years (range 31 to 69 years).

Antiserum Preparation

Human survivin cDNA was obtained from neuroblastoma SK-N-SH by the reverse transcription-PCR method with a pair of primer, 5'-NNGAATTCAATCCATGGCAGCCAGCTG-3' and 5'-NNGAATTCAATGGGTGCCCGACGTTG-3' (N indicates any of the four nucleotides). On digestion with *Eco* RI, the resultant fragment was inserted into pProEx3 GST (Life Technologies, Rockville, MD, USA) and sequenced to confirm the accuracy of the full-length survivin cDNA. Overexpression and purification of the fusion protein of survivin with GST in *Escherichia coli* were performed by the

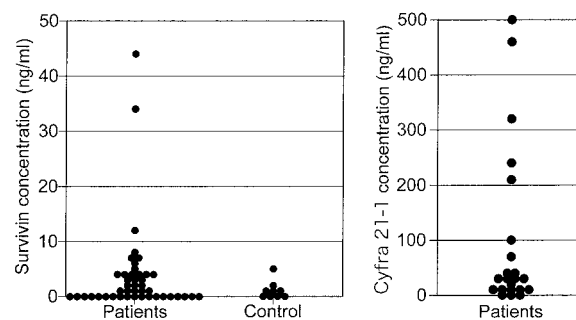


Fig. 1 Urine survivin concentrations in 40 patients with bladder cancer and 9 healthy volunteers. Urine Cyfra 21-1 levels examined in 25 of 40 patients were shown in the right column.

method described previously¹⁴. Rabbits were immunized with the purified protein in a mixture of Freund's adjuvant at a ratio of 1:1 (Life Technologies).

Tissue Processing and Immunohistochemistry

At the Department of Urology, Nippon Medical School, tumor samples were collected from patients who gave informed consent. The samples were fixed with formalin, quickly frozen and stored at -80°C until use. For immunostaining with anti-survivin antiserum, 8- μm frozen sections were stained using the Ventana NexES Staining System (Ventana, Tucson, AZ, USA) and all products without the anti-survivin antiserum needed for subsequent steps were supplied by the manufacture (Ventana). Sections were cryodissected and heated with CCl solution for 1h. After 32-min incubation at 37°C with the anti-survivin antiserum (1:250), sections were further incubated for another 10 min at 37°C with a secondary biotinylated antibody and then with avidin-peroxidase for another 10 min; 3', 3-diaminobenzidine was used as the chromogen. Slides were counterstained in Mayer hematoxylin, dehydrated, and mounted.

Survivin Elisa

First, 96-well immunoassay plates were coated with 100- μl /well affinity purified anti-survivin IgG overnight at 4°C , followed by blocking step. Plates were then incubated with 100- μl /well of test samples and standards diluted in sample buffer (PBS with 1% BSA and 0.05% Tween 20) for 1 h at 37°C .

Table 2 Characteristics of tumors from three patients with significantly elevated urine survivin levels.

cases	urine survivin level (ng/ml)	grade	stage	size (mm)	no. of tumor
1	33.54	G1=2	pTa	10	3
2	11.70	G2	pTa	30	1
3	44.46	G1	pTa	10	2

Table 3 Classification of patients with bladder cancer.

Volume	< 1 cm ³	(n=20)	versus	≥ 1 cm ³	(n=17)
Grade	grade 1	(n=10)	versus	grade 2-3	(n=27)
CIS	(+)	(n=5)	versus	(-)	(n=35)
Local stage	pTa	(n=15)	versus	≥ pT1	(n=22)
Number of tumor	1	(n=29)	versus	≥ 2	(n=8)

CIS, carcinoma in situ.

Thirty minutes after incubation with 100- μ l/well of biotinylated anti-rabbit IgG at 37°C, streptavidin-conjugated horseradish peroxidase was added for 30 min at 37°C. The peroxidase reaction was initiated by the addition of 100- μ l / well of tetramethylbenzidine/H₂O₂ and stopped after 30 min by the addition of 0.5 M H₂SO₄. Absorbance was measured at 450 nm.

Cyfra 21-1 in urine was also measured in 25 patients. The method of Elisa for measuring urine Cyfra 21-1 was previously described¹².

Statistical analysis was performed by Student's t-test.

Results

Urine survivin levels in 40 patients and 9 healthy control were 3.802 ± 8.669 and 1.127 ± 1.529 , respectively ($p=0.3646$) (**Fig. 1**).

Urine survivin levels were significantly higher in 3 of 40 patients and the characteristics of tumors in these three patients are shown in **Fig. 1** and **Table 2**. The sensitivity and specificity were 42.5% and 88.9%, respectively, with the cutoff value of urine survivin concentration at 2.5 ng/ml.

Urine survivin concentrations in relation to tumor-related factors (tumor volume, grade, CIS, local stage and tumor numbers) ;

Bladder cancer patients were classified as shown in **Table 3**, and differences between the two groups

were investigated.

Elevation of urine survivin levels was not associated with any factor, including grade ($p=0.4597$), CIS ($p=0.4751$), stage ($p=0.367$), size ($p=0.2034$), and multiplicity ($p=0.2773$) of tumor.

Urine Cyfra 21-1 level in 25 patients, in whom both urine Cyfra 21-1 and survivin levels were examined, was 88.636 ± 143.183 ng/ml (**Fig. 1**) and was correlated with urine survivin level ($p=0.0066$).

Immunohistochemical staining of surgical sections with anti-survivin serum (**Fig. 2A and B**) and normal serum (**Fig. 2C and D**) showed a survivin-positive specimen of bladder cancer in the former.

Discussion

In the initial report describing urine survivin levels in patients with bladder tumor by Smith et al¹¹, elevated urine survivin was observed in all patients with bladder tumor but not in all healthy volunteers, although elevated urine survivin was observed in 4 of 29 patients with nonneoplastic urinary tract diseases. However, the urine survivin level was not detectable in 19 of 40 patients with bladder tumor in the present study, although marked elevation of the urine survivin level was observed in 3 patients but not in healthy volunteers. The discrepant findings of Smith et al¹¹ and our study are considered due to the sensitivity of the Elisa system since methods of making the antibody

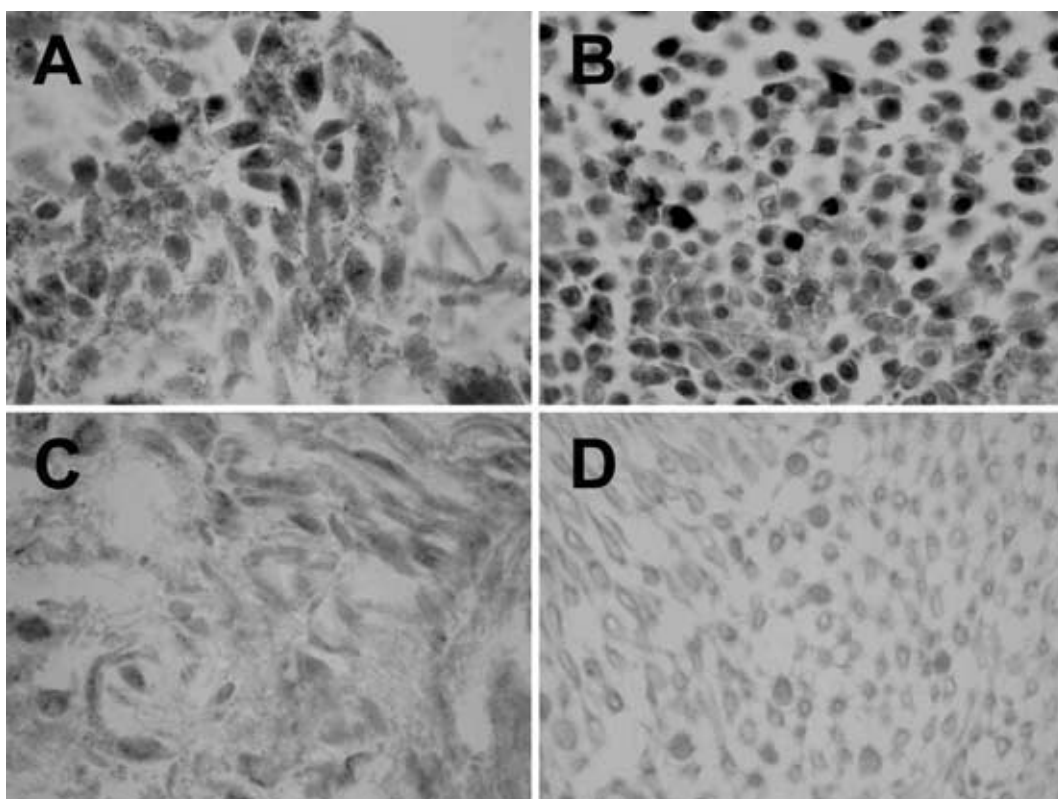


Fig. 2 Immunohistochemical staining of surgical sections staining with anti-survivin serum (A and B) and normal serum (C and D). Original magnification, $\times 4,000$. Only A and B show a survivin-positive specimen of bladder cancer.

and the sensitivity of the antibody shown in transitional cell cancer cell staining are similar. Concentration of specimen and removal of material in urine which cause adverse effect on Elisa by dialysis may increase sensitivity of our Elisa system.

Swana et al.⁷ reported positive ratio of tumor tissue survivin in immunohistochemical method was more common in higher-grade tumors, reporting findings of 100% and 65% in grades 3 and 1, respectively. They also reported that patients with CIS had considerably higher survivin scores than patients with grade 2 bladder cancer. Smith et al.¹¹ also reported weighted urine survivin score in patients with CIS was considerably higher than that in patients with grade 2 bladder cancer. However, in our present study using urine survivin we were unable to find such evidences among cis and different tumor grade.

Urine Cyfra 21-1 level was correlated with the urine survivin level ($p=0.0066$) in 25 patients in whom both urine Cyfra 21-1 and survivin levels

were examined. However, urine Cyfra 21-1 was not elevated (1.3 ng/ml) in one patient with a significantly elevated urine survivin level (33.54 ng/ml), while in two patients with significantly elevated urine Cyfra (320 ng/ml and 240 ng/ml), urine survivin was not detectable. In our previous study of urine Cyfra 21-1, the level was strongly correlated with tumor volume¹², while the urine survivin level did not correlate with tumor size in the present study. The tumor size, grade and pathological stage of two patients with with significantly elevated Cyfra and negative urine survivin were 3 cm, grade 1>2, pT1 and 5.5 cm, grade 3>2 pT1, respectively, while these of one patient with significantly elevated urine survivin level and negative urine Cyfra were 1 cm, grade 1>2, pTa. These results may reflect aforementioned correlation between tumor size and urine Cyfra level. The sensitivity of 42.5% with use of urine survivin increase to 47.5% with combination use of urine survivin and urine Cyfra 21-1.

Swana et al.⁷ also proposed usefulness of tumor

tissue survivin in expecting high risk for recurrence of bladder cancer showing their data that the mean time to first recurrence among patients with survivin-negative grade I tumor was 36 ± 16 months, as compared with 12 ± 6 months among the patients with survivin-positive grade I tumors ($P < 0.001$). Concerning the recurrence of bladder cancer, we found urine Cyfra 21-1 is useful not only in screening for bladder cancer but in detecting the recurrence.

In conclusion, with improvements in the sensitivity of our Elisa system for urine survivin and combined use of urine Cyfra 21-1, it is possible that urine survivin will be a useful tumor marker in detecting both new-onset and recurrent bladder tumors.

References

1. Shin S, Sung BJ, Cho YS, Kim HJ, Ha NC, Hwang JI, Chung CW, Jung YK, OhBH: An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and-7. *Biochemistry* 2001; 40: 1117-1123.
2. Ambrosini G, Adida C, Altieri DC: A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997; 3: 917-921.
3. Lu CD, Altieri DC, Tanigawa N: Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinoma. *Cancer Res* 1998; 58: 1808-1812.
4. Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N: Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Res* 1998; 58: 5071-5074.
5. Adida C, Berrebi D, Peuchmaur M, Reys-Mugica M, Altieri DC: Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. *Lancet* 1998; 351: 882-883.
6. Monzo M, Rosell R, Felip E, Astudillo J, Sanchez JJ, Maestre J, Martin C, Font A, Barnadas A, Abad A: A novel anti-apoptosis gene: re-expression of survivin messenger RNA as prognosis marker in non-small-cell lung cancers. *J Clin Oncol* 1999; 17: 2100-2104.
7. Swana HS, Grossman D, Anthony JN, Weiss RM, Altieri DC: Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer. *N Engl J Med* 1999; 341: 452-453.
8. Asanuma K, Moriai R, Yajima T, Yagihashi A, Yamada M, Kobayashi D, Watanabe N: Survivin as radioresistance factor in pancreatic cancer. *Jpn J Cancer Res* 2000; 91: 1204-1209.
9. Tanaka K, Iwamoto S, Gon G, Nohara T, Iwamoto M, Tanigawa N: Expression of survivin and its relationship to loss of apoptosis in breast carcinoma. *Clin Cancer Res* 2000; 6: 127-134.
10. Kishi H, Igawa M, Kikuno N, Yoshino T, Urakami S, Shiina H: Expression of the survivin gene in prostate cancer. *J Urol* 2004; 171: 1855-1869.
11. Smith SD, Wheeler MA, Plescia J, Colberg JW, Weiss RM, Altieri DC: Urine detection of survivin and diagnosis of bladder cancer. *JAMA* 2001; 285: 324-328.
12. Senga Y, Kimura G, Hattori T, Yoshida K: Clinical evaluation of soluble cytokeratin 19 fragments (Cyfra 21-1) in serum and urine of patients with bladder cancer. *Urology* 1996; 48: 703-710.
13. Ramakumar S, Bhuiyan J, Besse JA, Roberts SG, Wollan RC, Blute ML, O'Kane DJ: Comparison of screening methods in the detection of bladder cancer. *J Urol* 1999; 161: 388-394.
14. Mahotka C, Wenzel M, Soringer E, Gabbert HE, Gerharz CD: Survivin- Δ Ex 3 and survivin-2B: Two novel splice variants of the apoptosis inhibitor survivin with different antiapoptotic properties. *Cancer Res* 1999; 59: 6097-6102.

(Received, May 31, 2004)

(Accepted, August 8, 2004)