

Identification by Proteomic Analysis of Calreticulin as a Marker for Bladder Cancer and Evaluation of the Diagnostic Accuracy of Its Detection in Urine

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Background: New methods for detection of bladder cancer are needed because cystoscopy is both invasive and expensive and urine cytology has low sensitivity. We screened proteins as tumor markers for bladder cancer by proteomic analysis of cancerous and healthy tissues and investigated the diagnostic accuracy of one such marker in urine.

Methods: Three specimens of bladder cancer and healthy urothelium, respectively, were used for proteome differential display using narrow-pH-range two-dimensional electrophoresis. To evaluate the presence of calreticulin (CRT) as detected by Western blotting, we obtained 22 cancerous and 10 noncancerous surgical specimens from transurethral resection or radical cystectomy. To evaluate urinary CRT, we collected 70 and 181 urine samples from patients with and without bladder cancer, respectively. Anti-CRT COOH-terminus antibody was used to detect CRT in tissue and urine.

Results: Proteomic analysis revealed increased CRT (55 kDa; pI 4.3) in cancer tissue. Quantitative Western blot analysis showed that CRT was increased in cancer tissue ($P = 0.0003$). Urinary CRT had a sensitivity of 73% (95% confidence interval, 62–83%) at a specificity of 86% (80–91%) for bladder cancer in the samples tested.

Conclusions: Proteomic analysis is useful in searching for candidate proteins as biomarkers and led to the

identification of urinary CRT. The diagnostic accuracy of urinary CRT for bladder cancer appears comparable to that of Food and Drug Administration-cleared urinary markers, but further studies are needed to determine its diagnostic role.

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Bladder cancer is the second most common urologic cancer, with 57 400 newly diagnosed cases and 12 500 resulting deaths in the US annually (1). Approximately 70% of all newly diagnosed bladder cancers are well- or moderately differentiated superficial papillary tumors (2), with recurrence taking place after transurethral resection in the majority of cases. The remaining 30% of bladder cancers are muscle invasive, and radical cystectomy is the standard treatment when no metastases are found at the time of initial diagnosis. However, many alternative techniques to spare the bladder have been developed even for muscle-invasive cancers (3).

High-quality detection methods are needed not only for initial diagnoses but also in surveillance for recurrent tumors. Although cystoscopy provides accurate diagnosis, it is invasive, uncomfortable, and costly and is unsuitable for screening of large groups. Voided urine cytology remains the gold standard as a noninvasive diagnostic method, but its sensitivity is relatively low, particularly in well-differentiated cancer (4). Recent reports have described the application of urine-based markers, including bladder tumor antigen (BTA),⁶ nuclear matrix protein-22 (NMP22), and the urinary bladder cancer (UBC) test

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⁶ Nonstandard abbreviations: BTA, bladder tumor antigen; NMP22, nuclear matrix protein-22; UBC, urinary bladder cancer test; 2DE, two-dimensional electrophoresis; CRT, calreticulin; TCC, transitional cell carcinoma; UTI, urinary tract infection; SDS, sodium dodecyl sulfate; and CI, confidence interval.

(5, 6). These markers appear to have an advantage over urine cytology in terms of sensitivity, especially for detecting low-grade tumors, although they are not likely to replace cytology because of the higher false-positive rates.

Recent advances in expression profiling of cancer cells by proteomic technologies, high-resolution 2-dimensional electrophoresis (2DE), and mass spectrometry have made it possible to identify candidate proteins as tumor markers in various cancers. In bladder cancer, Celis et al. (7) performed proteome analysis by extensive 2DE and developed a comprehensive 2DE database for bladder cancer that includes profiles of both transitional and squamous cell carcinoma. They also developed a database of excreted proteins found in the urine of patients with bladder cancer. Through these studies they discovered a potential tumor marker, psoriasin, excreted in the urine of patients with squamous cell bladder carcinoma (8). We have also performed proteome differential display analysis of bladder cancer and healthy urothelium to establish good-quality urine-based markers and have identified proteins that are increased in bladder cancer tissue.

Calreticulin (CRT), an endoplasmic reticulum chaperone, is 1 of these 10 candidate proteins. We detected increased CRT concentrations in bladder tumors and excretion of a large amount of CRT in the urine of bladder cancer patients. CRT is a ubiquitous protein that was first identified as a Ca^{2+} -binding protein in skeletal muscle sarcoplasmic reticulum (9). Later it was found in non-muscle endoplasmic reticulum membrane as well as in muscle cells, and cDNA and genes encoding CRT have been isolated from several vertebrates and invertebrates as well as from higher plants (10). Participation of CRT has been implicated in many cellular functions, including Ca^{2+} storage and signaling, lectin-like chaperoning, regulation of gene expression, cell adhesion, and autoimmunity. CRT also has the role of stress protein, and its production can be induced by a variety of pathophysiological stresses, heat shock, and amino acid deprivation (11, 12). Increased production of CRT has been reported in several cancerous tissues, such as breast, liver, and prostate cancer (13–17). Although increases in CRT in tumor cells and proliferating cells are well known, details of the mechanisms of these increases are as yet undetermined. In tumor cells, it seems that increased CRT production is caused by cellular stress rather than as a consequence of cell proliferation (18).

There has been no published report of increased CRT production in bladder cancer. Here we describe the increased production of CRT in bladder cancer tissue, its detection in urine of bladder cancer patients, and its potential usefulness as a urinary tumor marker.

Materials and Methods

PATIENTS AND SAMPLES

Three specimens each of transitional cell carcinoma (TCC) and healthy urothelial mucosa were used for screening of tumor-specific proteins by proteome analysis. TCC tissues

were obtained from patients who had undergone transurethral resection ($n = 2$) or radical cystectomy ($n = 1$). Healthy urothelial mucosae were obtained from non-TCC patients who had undergone retropubic prostatectomy for large benign prostatic hyperplasia ($n = 2$) or radical nephrectomy for small renal cell carcinoma ($n = 1$). Fresh tissues were stored immediately at -80°C until sample preparation.

For one-dimensional Western blot analysis to confirm the presence of CRT, 22 TCC specimens from patients who had undergone transurethral resection of a bladder tumor or radical cystectomy were used. As controls, 10 samples of nonneoplastic mucosa obtained from cystectomized bladders were used.

Voided urine samples were obtained from 70 patients with bladder cancer and 181 patients with benign or malignant conditions [96 with benign prostatic hyperplasia, 8 with urinary tract infections (UTIs), 6 with urinary stones, 6 with microscopic hematuria without known pathology, 51 with prostate cancer, 4 with renal cell carcinoma, and 10 with breast cancer]. Table 1 summarizes the patient demographics and tumor characteristics. Collected urine samples were divided into 1-mL aliquots in SUMILON Non Adsorption modified tubes (Sumitomo Bakelite) and then stored at -30°C until analysis.

Processing of urine was performed as follows. Frozen urine samples were thawed quickly and centrifuged at 18 000g for 5 min at 4°C . Protease inhibitors were not added. Supernatants were mixed with 4 \times concentrated sodium dodecyl sulfate (SDS) sample buffer [250 mmol/L Tris-HCl (pH 6.8), 80 g/L SDS, 400 g/L glycerol, 40 g/L dithiothreitol, and bromphenol blue] and then applied for Western blotting.

Surgical specimens and urine samples were collected after informed consent was provided by patients according to institutional guidelines.

2DE

To screen for proteins that are increased in cancer tissue, we used proteome differential display analysis of bladder cancer tissue and healthy urothelial mucosae from patients without neoplastic diseases.

For protein extraction, ~ 100 mg of tissue was homogenized with a micropestle in 700 μL of lysis buffer containing 8 mol/L urea, 20 g/L CHAPS, 10 g/L dithiothreitol, 5 g/L Pharmalyte 3–10 (Amersham Biosciences), 100 g/L glycerol, and 10 mL/L Protease Inhibitor Cocktails (Nacalai tesque), then centrifuged at 18 000g for 10 min at 4°C . The supernatant was used as the sample. Protein concentration was determined by a modified Bradford protocol using Protein Assay CBB Solution (Nacalai tesque) with bovine serum albumin as the calibrator. To perform zoom gel analysis, we used narrow-range immobilized pH gradient strips (18 cm), with pH ranges of 4.0–5.0, 4.5–5.5, 5.0–6.0, 5.5–6.7, and 6.0–9.0 (Amersham Biosciences) (19). Isoelectric focusing was carried out with IPGphor (Amersham Biosciences) ac-

Table 1. Patient demographics and tumor characteristics.

Bladder cancer patients (n = 70)		Non-bladder cancer patients (n = 181)	
Characteristics	n (%)	Characteristics	n (%)
Sex		Sex	
Male	59 (84)	Male	159 (88)
Female	11 (16)	Female	22 (12)
Mean age (range), years	72 (50–88)	Mean age (range), years	66 (20–88)
Grade		Neoplastic disease	
1	5 (7)	Prostate cancer	51
2	33 (47)	Renal cell carcinoma	4
3	32 (46)	Breast cancer	10
Stage		Nonneoplastic disease	
Tis	11 (16)	Benign prostatic hyperplasia	96
Ta	37 (53)	Urinary tract infection	8
T1	10 (14)	Urolithiasis	6
T2–T4	12 (17)	Microscopic hematuria without known pathology	6
Maximum tumor diameter			
<1 cm	27 (38)		
1–3 cm	21 (30)		
>3 cm	11 (16)		
Unknown	11 (16)		
Number of tumors			
Solitary	23 (33)		
Multiple	36 (51)		
Unknown	11 (16)		
Cytology			
Negative	40 (57)		
Positive	30 (43)		

cording to the manufacturer's instructions. For the analytical run, 120 μ g of total protein was applied to an immobilized pH gradient gel. Preparative gels were loaded with 1 mg of total protein. After isoelectric focusing, the strip gels were equilibrated for 30 min at room temperature in 50 mmol/L Tris-HCl (pH 6.8) containing 6 mol/L urea, 20 g/L dithiothreitol, 10 g/L SDS, and 300 g/L glycerol. SDS-polyacrylamide gel electrophoresis was performed at 20 mA/gel with 12.5% polyacrylamide gels without stacking gels (22 cm \times 20 cm \times 1 mm). The running buffer consisted of 25 mmol/L Tris, 192 mmol/L glycine, and 1 g/L SDS. Protein spots were visualized by silver staining, and gel images were scanned by a flatbed scanner.

Primary gel images were processed with PDQUEST, Ver. 5.1 (pdi Inc.), and protein spots were detected by use of the default autodetection function for silver stain gels. Only spots that were clearly and reproducibly more intense in specimens from all of the cancer patients as judged by visual inspection of the silver-stained gels were analyzed. In this preliminary study we did not estimate quantitative variations.

PROTEIN IDENTIFICATION

Enzymatic digestion of proteins by trypsin (Promega) was performed according to the method of Gharahdaghi et al. (20) with partial modification. Matrix-assisted laser de-

sorption/ionization time-of-flight mass spectrometric analysis was performed on a Voyager RP mass spectrometer (Applied Biosystems) operated in the delayed extraction and reflector mode (21). Peptide mixtures were analyzed with a saturated solution of α -cyano-4-hydroxycinnamic acid in 500 mL/L acetonitrile containing 1 g/L trifluoroacetic acid. Mass measurements were made after internal calibration using the monoisotopic masses of autolysis trypsin fragment ions at m/z 842.51 and 2211.10, which gave mass accuracies better than ± 50 ppm.

For amino acid sequencing, proteins in 2DE gels were electroblotted on Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories) with 6-aminohexanoic acid buffers. The spots visualized by Coomassie Brilliant Blue R-250 staining were excised and applied to the protein sequencer.

WESTERN BLOT ANALYSIS

For two-dimensional Western blotting, proteins were transferred after 2DE to an Immobilon-P Transfer Membrane (Millipore). The membrane was incubated overnight at 4 $^{\circ}$ C in SuperBlock Blocking Buffer in Tris-buffered saline (Pierce) to block the nonspecific binding site. As first antibodies, we used a 1:20 000 dilution of anti-CRT COOH-terminus polyclonal antibody (StressGen Biotechnologies) or a 1:10 000 dilution of anti-CRT monoclonal antibody (FMC75; StressGen Biotechnolo-

gies), and the membranes were incubated for 1 h at room temperature. After a 1-h incubation at room temperature with the second antibodies, the immunoproteins were visualized with ECL Western blotting detection reagents (Amersham Biosciences) and chemiluminescence by exposure to x-ray films. Each wash and antibody dilution was performed with Tris-buffered saline-Tween [10 mmol/L Tris-HCl (pH 7.6), 100 mmol/L NaCl, 1 mL/L Tween 20].

To quantify CRT from TCC and control tissue, we loaded 1 μ g of total protein from each lysate on a 10% SDS-polyacrylamide gel and performed Western blot analysis with anti-COOH-terminus antibody as the first antibody. As a control, we used 1 μ g of total protein from heat-shocked HeLa cell extract (StressGen Biotechnologies) for every gel. We performed preliminary experiments to determine that the appropriate amount of tissue protein was applied to each lane to avoid saturation of band intensity. In addition, analyses were performed in duplicate to obtain accurate results. Visualization of immunoproteins was carried out as mentioned above. After image scanning, band quantification was performed with Scion Image software (Scion Corporation).

To detect urinary CRT we also performed Western blot analysis with anti-COOH-terminus antibody and 10% SDS-polyacrylamide gels. The immunoproteins were visualized with ECL Plus Western blotting detection reagents (Amersham Biosciences), and chemiluminescence was detected by a cooled ImageMaster-CL (Amersham Biosciences) digital charge-coupled device camera. As a control, 0.2 μ g of total protein from heat-shocked HeLa cell extract was used for every gel. The bands were considered positive when their intensities were >10% of the control CRT band by quantification with Scion Image software. To monitor reproducibility, we performed tests in duplicate, and results for all urine samples were similar in both analyses. In no case did qualitative judgment conflict in duplicate urinary CRT tests.

IMMUNOPRECIPITATION

To search for variant forms of CRT that bind to anti-COOH-terminus antibody, we performed immunoprecipitation. Bladder cancer tissue was homogenized in buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 10 mL/L Triton X-100, 5 g/L deoxycholate, 1 g/L SDS, and 10 mL/L Protease Inhibitor Cocktails. Tissue lysate was incubated at 4 °C for 30 min with anti-COOH-terminus antibody-conjugated protein A Sepharose beads (Amersham Biosciences). Normal rabbit IgG-conjugated protein A Sepharose beads were used as a control. The reaction mixtures were washed five times with lysis buffer without the protease inhibitors. The antigens were then eluted with SDS sample buffers for Western blotting.

IMMUNOHISTOCHEMISTRY

Paraffin-embedded sections (4 μ m thick) were made from cancerous and noncancerous tissues obtained from pa-

tients with bladder and ureteral cancer. After deparaffinization and rehydration, the slides were treated with microwave heating in 10 mmol/L citrate buffer (pH 6.0) for 10 min. Anti-COOH-terminus antibody was applied at a 1:100 dilution, and the slides were incubated for 60 min at room temperature. Peroxidase-labeled amino-acid-polymer-conjugated anti-mouse and anti-rabbit IgG (Nichirei) was used as a secondary antibody. Diaminobenzidine was used as a substrate and then counterstained with hematoxylin.

STATISTICAL ANALYSIS

We compared the mean CRT band intensities obtained from Western blotting of bladder cancer and healthy tissue with the Mann-Whitney *U*-test. Outcomes for urinary CRT and urine cytology in individual patients were evaluated by the McNemar test. A *P* value <0.05 was considered to indicate statistical significance, and all tests were two-tailed. All statistical analyses were performed on a personal computer with the statistical package SPSS 11.0J for Windows (SPSS Japan).

Results

PROTEOME DIFFERENTIAL DISPLAY

Although visual comparison of 2DE gels of TCC and noncancerous urothelium showed similar expression profiles, 15 protein spots (U-1 to U-15) were more intense in TCC samples (Fig. 1). We could identify 10 of the proteins by use of a peptide mass fingerprinting method (data not shown). One spot among them, with an apparent mass of 55 kDa and pI of 4.3, was identified as CRT (spot U-2 in Fig. 1A). From NH₂-terminal amino acid sequencing, 10 amino acids were sequenced (EPAVYFKEQF), and they were identical to residues 1–10 of mature human CRT according to the sequence homology search.

TWO ISOFORMS OF CRT IN 2DE GELS

To confirm the presence of isoforms of CRT, we performed two-dimensional Western blotting with two different antibodies: monoclonal antibody FMC75, which was produced against recombinant human CRT; and a polyclonal antibody that was produced using synthesized peptides of the human CRT COOH terminus (amino acids 388–400) as an immunogen. On Western blots with anti-COOH-terminus antibody, only one of the two spots was visualized, whereas both spots became visible on blots incubated with FMC75 (Fig. 2). One was the same as the 55-kDa (pI 4.3) spot, and the other had an apparent molecular mass of 40 kDa and pI of 4.5. This lower-molecular-mass spot had the same NH₂-terminal amino acid sequence as amino acids 1–10 of mature human CRT as shown by amino acid sequencing. We therefore believe that the higher-molecular-mass spot was the full-length form and the other spot was a cleaved form that is truncated elsewhere in the COOH domain. Production of the full-length CRT in cancer tissue was increased compared with in healthy tissue, but the spots for cleaved

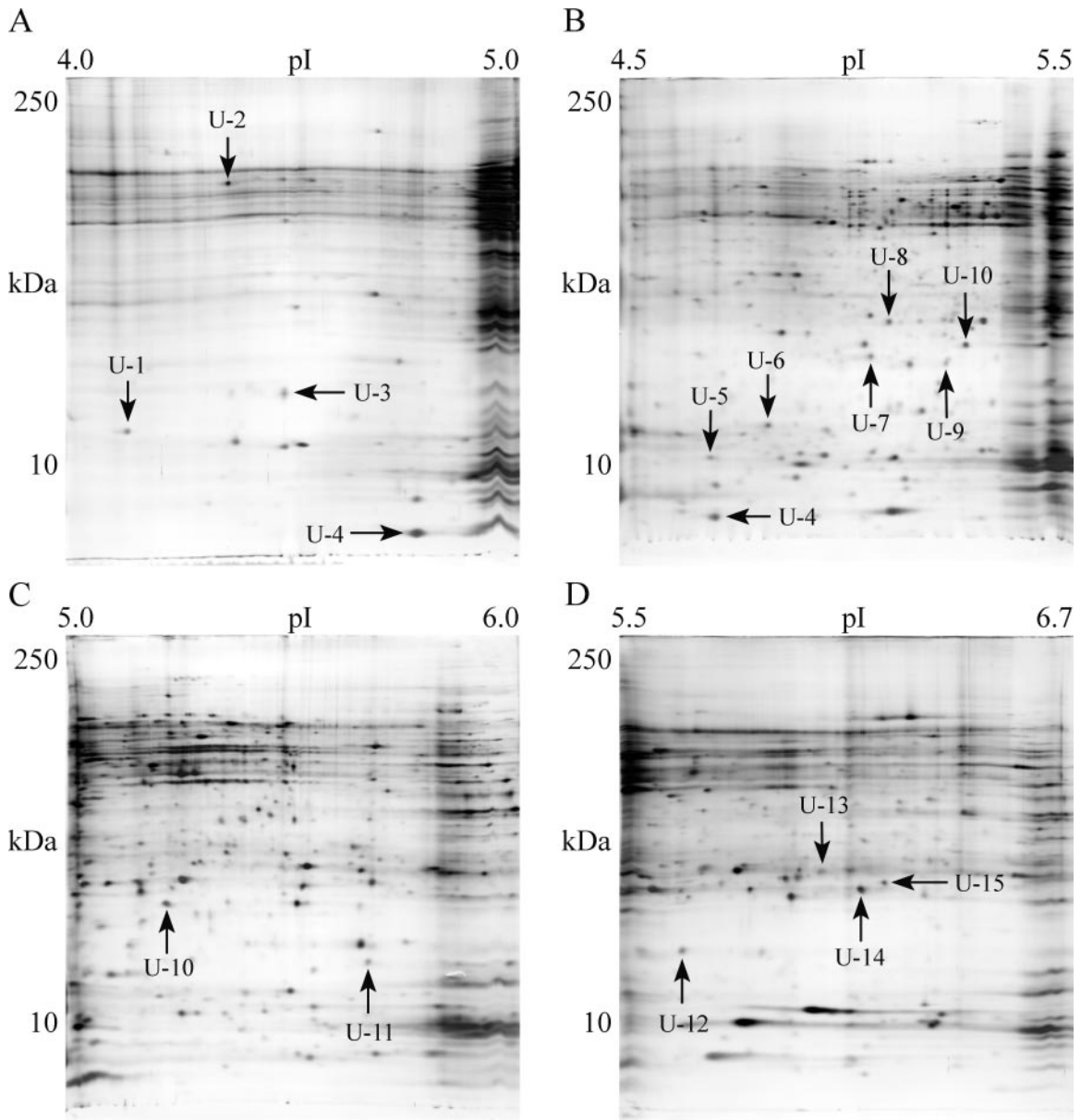


Fig. 1. Silver-stained images of analytical narrow-pH-range 2DE gels of proteins from bladder cancer.

(A), pH 4.0–5.0; (B), pH 4.5–5.5; (C), pH 5.0–6.0; (D), pH 5.5–6.7. Arrows indicate spots (U-1 to U-15) containing higher amounts of protein. Spot U-2, ~55 kDa and pI 4.3, was confirmed to be CRT by a peptide mass fingerprinting method and NH_2 -terminal amino acid sequencing.

CRT in cancerous and healthy urothelium had intensities that were similar and were reproducible on all silver-stained 2DE gels.

Subsequently we tried to confirm whether anti-COOH-terminus antibody binds to molecules other than full-length CRT and performed immunoprecipitations (Fig. 3). The Western blot of the immunoprecipitate extracted from cancer tissue revealed only one band, and we concluded that anti-COOH-terminus antibody binds specifically to full-length CRT of ~55 kDa. We therefore judged that full-length CRT recognized by anti-COOH-terminus antibody is appropriate as a tumor marker.

QUANTITATIVE ANALYSIS IN CANCEROUS AND HEALTHY TISSUE

To validate the 2DE finding of increased production of full-length CRT in bladder cancer tissue, we performed quantitative Western blot analysis using anti-COOH-terminus antibody. We compared CRT band intensities for 22 cancerous with 10 noncancerous tissues. For band quantification, we defined the CRT band derived from a total of 1 μg of heat-shocked HeLa cell extract as 1.0 unit/ μg of protein. The mean (SD) concentrations in cancerous and healthy tissue were 1.0 (0.4) and 0.4 (0.3) units/ μg of protein, respectively (Mann-Whitney *U*-test,

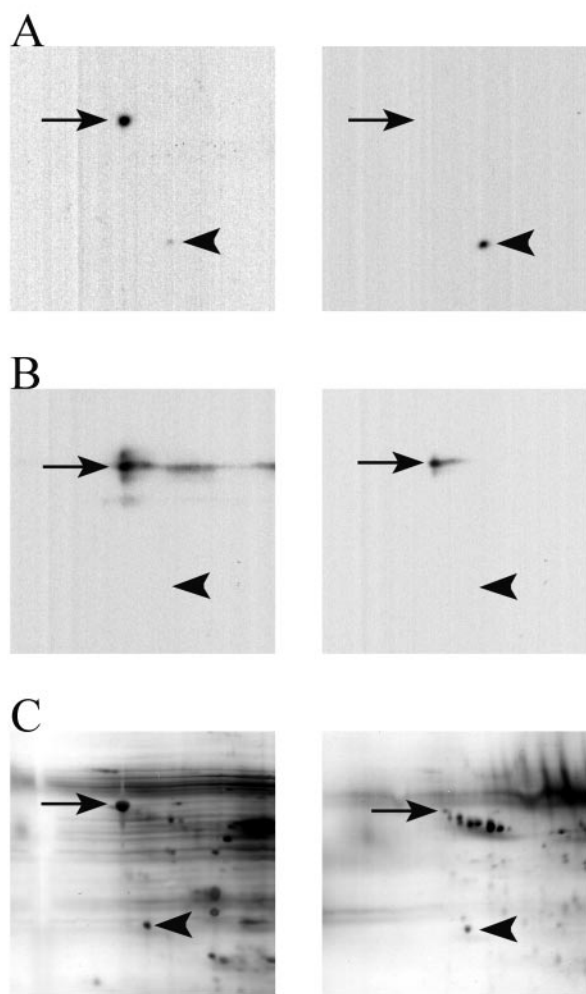


Fig. 2. Close-up sections of two-dimensional Western blot images obtained with two different antibodies, FMC75 (A) and anti-COOH-terminus antibody (B), and proteins in silver-stained pH 4–7 gels (C).

Left panels are bladder cancer and right panels are healthy urothelium. Arrows indicate full-length CRT (55 kDa; pI 4.3), and arrowheads indicate cleaved CRT (40 kDa; pI 4.5). These two CRT forms have the same NH₂-terminal amino acid sequence (EPAVYFKEQF), but cleaved CRT is considered to lack the COOH terminus because of no immunoreactivity for anti-COOH-terminus antibody.

$P = 0.0003$; Fig. 4). Among these tissue samples were six pairs of cancerous and noncancerous specimens obtained from the bladders of patients who had undergone radical cystectomy. CRT concentrations were higher in all cancer tissues compared with the corresponding healthy urothelium.

QUALITATIVE ANALYSIS OF URINARY CRT

To confirm whether CRT can be detected in the urine of patients with bladder cancer, we performed Western blot studies. Among 70 urine samples obtained from bladder cancer patients, 51 [73%; 95% confidence interval (CI), 62–83%] showed a positive band corresponding to full-length CRT in Western blots (Fig. 5). Fortunately, degradative bands were not observed, although we did not use protease inhibitors.

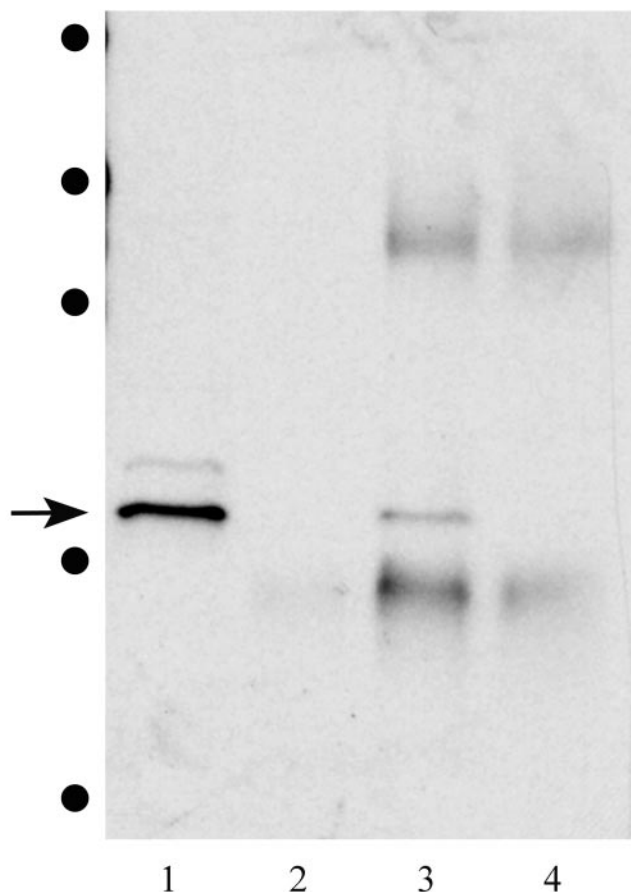


Fig. 3. Western blot with FMC75 antibody.

Arrow indicates 55-kDa full-length CRT. Lane 1, total cell lysate; lane 2, extraction from protein A Sepharose beads that did not bind antibody; lane 3, immunoprecipitant eluted from beads binding anti-COOH-terminus antibody; lane 4, immunoprecipitant extracted from beads binding normal rabbit IgG. ● indicates approximate molecular masses of 160, 105, 75, 50, and 35 kDa.

The correlation between the qualitative results for urinary CRT and the clinical characteristics of bladder cancer patients is summarized in Table 2. It should be noted that the population in which diagnosis of bladder cancer is difficult by noninvasive examination (i.e., low grade and stage, small tumor volume, or negative by urine cytology) had high positive rates for urinary CRT.

On the other hand, 32 (18%) of 181 urine samples from patients without bladder cancer revealed a positive reaction (Table 3). According to disease category, the UTI group had an extremely high positive rate (7 of 8 samples; 88%). When the UTI group was eliminated, specificity was 86% (95% CI, 80–91%), with 148 negative reactions among 173 samples.

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To confirm full-length CRT expression in healthy and cancerous urothelium, we applied anti-COOH-terminus antibody to paraffin-embedded sections. Noncancerous specimens showed moderate staining in the epithelium (Fig. 6A). In tumor specimens, CRT concentrations were

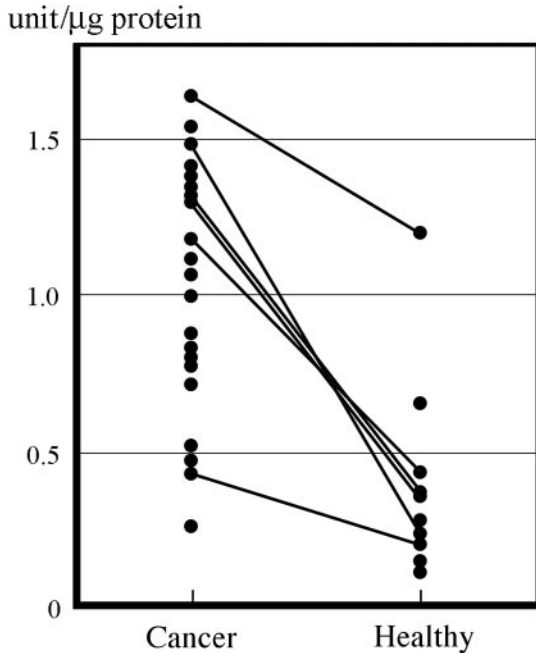


Fig. 4. Quantitative Western blot analysis of cancerous and healthy tissues using anti-COOH-terminus antibody. Lines show six pairs of cancerous and healthy specimens obtained from the bladders of patients who had undergone radical cystectomy.

much higher in cancer cells than in healthy urothelial cells (Fig. 6, B and C). At $\times 400$ magnification, CRT appeared to be localized in the cytoplasm of the tumor cells (Fig. 6D).

Discussion

Previously we investigated the urothelium-specific proteins, uroplakins, and their clinical application (22–25). We could detect cancer cells in the peripheral blood of urothelial cancer patients by nested reverse transcription-PCR and suggested molecular staging for micrometastasis (22–24). We also produced anti-human uroplakin Ia anti-

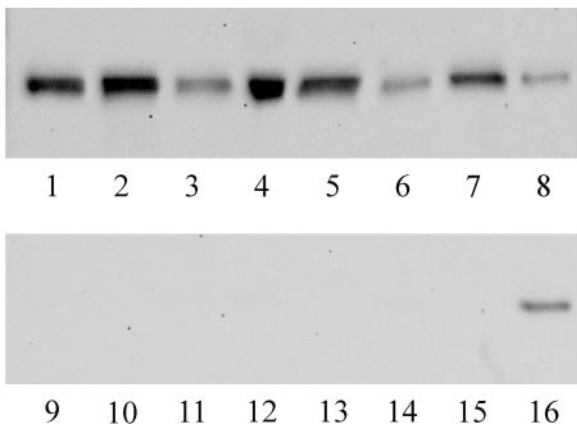


Fig. 5. Detection of urinary CRT by Western blot analysis. Urine (30 μ L) from patients mixed with 10 μ L of 4 \times sample buffer was loaded in each lane. Lanes 1–7, urine from patients with bladder cancer; lanes 9–15, urine from patients without bladder cancer; lanes 8 and 16, extract from heat-shocked HeLa cells (0.2 μ g of total protein) as a positive control.

Table 2. Correlation between urinary CRT and clinical features of bladder cancer patients.

	Sensitivity	
	n	% (95% CI)
Overall	51/70	73 (62–79)
Grade		
1	2/5	40 (0–83)
2	25/33	76 (61–90)
3	24/32	75 (60–90)
Stage		
Tis	7/11	64 (35–92)
Ta	26/37	70 (56–85)
T1	8/10	80 (55–100)
T2–4	10/12	83 (62–100)
Maximum tumor diameter		
<1 cm	18/27	67 (49–84)
1–3 cm	15/21	71 (52–91)
>3 cm	11/11	100
Unknown	7/11	64 (35–92)
Number of tumors		
One	14/23	61 (41–81)
Multiple	30/36	83 (71–96)
Unknown	7/11	64 (35–92)
Cytology ^a		
Negative	28/40	70 (56–84)
Positive	23/30	77 (62–92)

^a Sensitivities of urinary CRT and of voided urine cytology were statistically different (McNemar test, $P = 0.001$).

body and showed its usefulness as a specific histologic marker because of its stable expression even in metastatic lesions (25). However, in spite of much effort we could not establish a diagnostic method using uroplakins in sera or urine from patients. We therefore screened candidate molecules other than the uroplakins for diagnosis of urothelial cancer.

To search for candidates as new tumor markers, we performed proteomic analysis of bladder cancer. Proteomics, which evolved from recent improvements in the

Table 3. Results of urinary CRT analysis and false-positive rates for non-bladder cancer patients.^a

	False positives	
	n	% (95% CI)
Neoplastic disease		
Prostate cancer	7/51	14 (4–23)
Renal cell carcinoma	0/4	0
Breast cancer	1/10	10 (0–35)
Nonneoplastic disease		
Benign prostatic hyperplasia	14/96	15 (8–22)
UTI	7/8	88 (65–100)
Urolithiasis	1/6	17 (0–46)
Microscopic hematuria without known pathology	2/6	33 (0–71)

^a Specificity is 86% (95% CI, 80–91%), with 148 negative reactions among 173 samples (UTIs were eliminated).

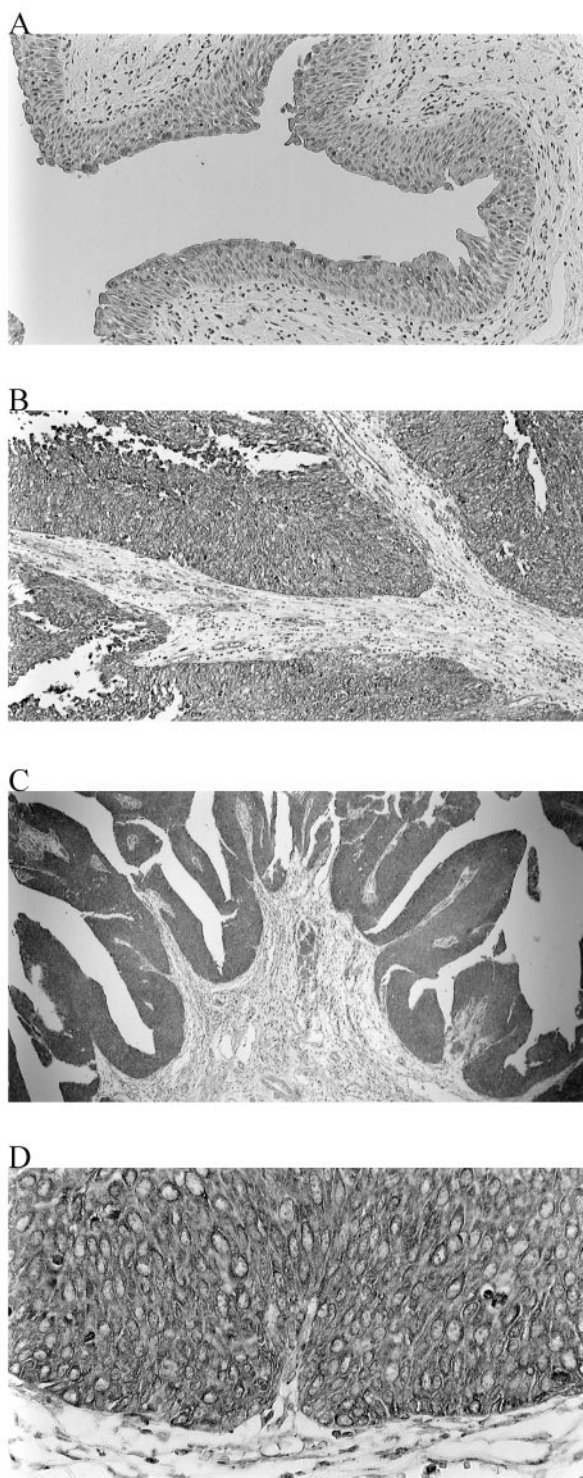


Fig. 6. Immunohistochemical study of CRT expression in urothelial carcinoma and healthy urothelial tissues.

Tissue samples were examined for full-length CRT expression using anti-COOH-terminus antibody. (A), healthy ureteral tissue. CRT expression is found predominantly in epithelial cells. Reduced from $\times 200$. (B), grade 2 ureteral cancer tissue. CRT is increased in tumor cells compared with healthy epithelium. Reduced from $\times 100$. (C and D), grade 2 bladder cancer tissue. High concentrations of CRT are present. Immunoprotein confined to the cytoplasm is observed in tumor cells. Reduced from $\times 40$ (C) and $\times 400$ (D).

techniques of protein analysis and advances in bioinformatics, is an emerging area of research in the postgenomic era. This approach is very useful for examination of protein production in individual diseases, and it may allow identification of new disease-associated diagnostic markers or targets for drug therapy. Protein profiling of healthy and malignant epithelium obtained by laser capture microdissection could provide more accurate information (26), although we could not use this method because we lacked the instrumentation.

Our experiments have produced two important findings. The first is that increased production of CRT in bladder cancer tissue was confirmed by proteome profiling by 2DE. Increased production of CRT in malignant tumors in other organs has been the subject of several reports (13–17), but increased production of CRT in bladder cancer has not been described. Celis et al. (27) mentioned that some molecular chaperones, including CRT, were increased in primary cultures derived from low-grade superficial bladder TCC. Furthermore, we detected two isoforms of CRT, and full-length CRT was more useful than cleaved CRT for distinguishing bladder cancer from healthy tissue.

The second finding is that we elucidated the presence of CRT in the urine of bladder cancer patients. We speculate that direct contact between urine and bladder tumor cells yields high concentrations of urinary CRT. According to recent findings, there is considerable evidence that CRT is present outside the cells (28). Perhaps viable cancer cells secrete CRT into urine. Certainly the presence of CRT is not specific to bladder cancer; therefore, future studies should be directed toward determining its presence in various tumors. Although we found CRT in the urine of patients with other urologic cancers adjacent to the urinary tract, including renal cell carcinoma and prostate cancer, the positive rates were lower than in bladder cancer. Two respective groups have reported higher concentrations of CRT in breast cancer tissues than in healthy mammary tissues, using 2DE proteome differential display as we did in bladder cancer (13, 14). We therefore studied breast cancer as a representative tumor that reveals increased production of CRT. Although the number of breast cancer patients was small, the positive rate (10%) for urinary CRT was much lower than in the patients with bladder cancer (73%). This may support our speculation concerning the origin of urinary CRT. To establish diagnostic specificity, we will test urine from patients with many different kinds of cancer in the future.

Voided urine cytology is the gold standard as a non-invasive method for diagnosing bladder cancer (4). However, it lacks the sensitivity to detect low-grade tumors and is interpreter dependent (5). There thus is a need to develop methods to improve the detection of bladder cancer. Recently, several urinary diagnostic markers for bladder cancer have been identified, including NMP22, BTA, telomerase, fibrinogen degradation products, Lewis

X antigen, cytokeratins, survivin, and BLCA-4 (5, 29–32). Currently, BTA, BTA stat, NMP22, and fibrinogen degradation products have been cleared by the US Food and Drug Administration as diagnostic markers. The sensitivities and specificities, respectively, of these ancillary detection methods reported by various authors have ranges of 28–79% and 40–96% for BTA, 57–83% and 68–90% for BTA stat, 48–84% and 64–97% for NMP22, and 48–81% and 75–91% for fibrinogen degradation products (29). In the present study the estimated sensitivity and specificity of urinary CRT were 73% and 85%, respectively, at least equal to these tests. When a quantitative urinary CRT assay is developed, its diagnostic value might exceed that of existing diagnostic tests.

The problem of almost all urine-based tests is low sensitivity for low-grade, low-stage, or small-volume tumors (33). Recent studies reported that BTA stat and NMP22 detected, respectively, 23–53% and 18–82% of grade 1 tumors. Similarly, sensitivities for stage Ta tumors were 48–60% and 40–83%, respectively (34–39). In our study, urinary CRT detected 40% (95% CI, 0–83%) of grade 1 and 70% (56–85%) of Ta tumors; therefore, its diagnostic value can be considered comparable to these established tests. It is noteworthy that urinary CRT detected 70% (56–84%) of cytology-negative cancers.

Some authors have mentioned that BTA stat and NMP22 are not reliable for urine samples from patients with several pathologic conditions, including gross hematuria, UTI, urolithiasis, bowel reconstruction of the urinary tract, and other genitourinary cancers (40–43). Although NMP22 does not exist in red blood cells, hematuria significantly affected the urinary NMP22 concentration (42). Red blood cells do not have CRT (44); in our study there was no correlation with urinary CRT and red blood cell counts in urine (data not shown).

UTIs affected urinary CRT and gave high false-positive rates; patients with UTIs should therefore be excluded when administering the urinary CRT test. CRT testing should be used in such patients only after administration of adequate antibacterial therapy, similar to the exclusion from prostate-specific antigen studies of patients with prostatitis. For most physicians, it is relatively easy to diagnose UTIs by clinical symptoms (e.g., pain on urination, cloudy urine, frequent urination, and fever) and urine sediment. In increasing the accuracy of CRT testing, however, it is important to establish criteria to easily identify or rule out patients with subclinical UTIs.

Not only the current clinically available urinary markers but also our urinary CRT test have limitations and cannot replace cystoscopy when used as a single test. We identified 10 candidates as tumor markers for bladder cancer. At present we are evaluating the potential diagnostic usefulness of the remaining proteins other than CRT for bladder cancer. We are therefore planning a project to develop a highly sensitive and highly specific urine test based on a combination of our tumor marker

candidates that could be useful even in random testing of urine samples under various conditions.

In conclusion, we have shown that proteomic analysis is very useful to screen disease-associated proteins, although proteome differential display analysis was performed in a small cohort. Moreover, we verified that CRT is increased in bladder cancer tissue and found high concentrations of CRT in urine from bladder cancer patients. The diagnostic value of urinary CRT in our study was comparable to that of previously established urinary markers. Further prospective and comparative studies are needed to assess the promising diagnostic role of urinary CRT.

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