Diagnostic potential in bladder cancer of a panel of tumor markers (calreticulin, γ-synuclein, and catechol-*o*-methyltransferase) identified by proteomic analysis

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Using proteomic analysis, we previously identified calreticulin (CRT) as a potentially useful urinary marker for bladder cancer. Now, we have also identified γ-synuclein (SNCG) and a soluble isoform of catechol-*o*-methyltransferase (s-COMT) as novel candidates for tumor markers in bladder cancer, by means of proteomic analysis. In the process of establishing a superior tumor marker system, we investigated the diagnostic value of a combination assay of these three proteins. Voided urine samples were obtained from 112 bladder cancer and 230 control patients. Urinary CRT, SNCG, and s-COMT were measured as a combined marker by quantitative western blot analysis. Relative concentration of each protein was calculated and the diagnostic value of a concomitant examination of these markers was evaluated by receiver operator characteristic analysis. With the best diagnostic cutoff, the overall sensitivity of the combined markers was 76.8% (95% confidence interval, 69–81%) with a specificity of 77.4% (72–80%), while those of a single use of CRT were 71.4% and 77.8%, respectively. When evaluated in relation to tumor characteristics, such as grade, stage, size, and outcome of urinary cytology, the diagnostic capacity of the combined markers was equal to or better than that of CRT in all categories. Concomitant use of CRT, SNCG, and s-COMT had higher sensitivity for detection of bladder cancer than did single use of CRT. Our study suggests that use of this panel of markers will improve the diagnosis of bladder cancer and may allow the development of a protein microarray assay or multi-channel enzyme-linked immunosorbent assay. (Cancer Sci 2004; 95: 955–961)

Bladder cancer is a common urothelial cancer with an estimated 57,400 and 13,000 diagnosed cases per year in the United States and Japan, respectively.[1, 2] Approximately 70% of bladder cancers are superficial[3] and respond well to endoscopic transurethral resection. However, 50% to 70% of these patients experience tumor recurrence, and 10% to 15% of recurrent tumors progress to muscle invasive disease.[4] Because the propensity for local recurrence extends over the lifetime, patients with superficial bladder cancer must undergo life-long surveillance.

Cystoscopy is the mainstay in diagnosing bladder cancer. However, this procedure is unsuitable for screening of large groups because of its invasiveness and expense. In addition, follow-up cystoscopy for bladder cancer patients treated endoscopically represents a considerable part of the workload of any urological unit. Therefore, new tests with high specificity and sensitivity that are easy to perform are needed for both screening and monitoring the response to treatment of bladder cancer. Voided urine cytology (VUC) has been used in both diagnosis and follow-up of superficial bladder cancer since its first description by Papanicolaou and Marshall in 1945, but it has poor sensitivity although high specificity, particularly in well-differentiated cancer.[5] To date, several urine-based markers for bladder cancer have been identified and investigated. Bladder tumor antigen test (BTA test), BTA stat, BTA-TRAK and nuclear matrix protein-22 (NMP22) are readily available, and some other tests, e.g., telomerase, are still research tools. However, according to the most recent review,[6] these tests still are not sufficiently sensitive to be recommended for routine use. Moreover, these new markers have lower specificity than VUC, although they appear to have an advantage over VUC in terms of sensitivity. In this regard, Konety *et al*. mentioned in their recent review[7] that combined use of these new markers with VUC or the use of a panel use of markers might improve the specificity.

Recent advances in expression profiling of cancer cells by proteomic technologies, high resolution two-dimensional electrophoresis (2DE) and mass spectrometry have made it possible to identify candidate proteins as tumor markers in various cancers. In order to investigate new urine-based markers for bladder cancer, we identified 10 proteins that are increased in bladder cancer tissue using these technologies, and we reported the diagnostic value of urinary calreticulin (CRT) measurement.[8] The sensitivity and specificity were comparable with those of Food and Drug Administration (FDA)-approved urinary markers, although the assay was still a research tool in the phase of western blot analysis. To find more effective diagnostic markers for bladder cancer, we have begun to investigate the concomitant use of several markers. Among the remaining proteins, we focused on γ-synuclein (SNCG) and a soluble isoform of catechol-*o*-methyltransferase (s-COMT) as candidates for a panel of markers that have not previously been known to be present at elevated levels in bladder cancer.

SNCG, also referred to as breast cancer-specific gene 1,[9] is the third member of the neuronal protein family of synucleins,[10] which have been suggested to have important roles in tumor cell growth in human breast and ovarian carcinomas.[11–13] s-COMT is an isoform of COMT, an enzyme ubiquitously present in humans, and which mediates *O*-methylation of endogenous catecholamines and catechol estrogens. Recently, COMT polymorphism has been reported to be associated with breast cancer risk,[14, 15] and up-regulation of the COMT gene in

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Abbreviations: VUC, voided urine cytology; BTA, bladder tumor antigen; NMP22, nuclear matrix protein-22; 2DE, two-dimensional electrophoresis; CRT, calreticulin; SNCG, γ-synuclein; s-COMT, soluble isoform of catecol-o-methyltransferase; UTI, urinary tract infections; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CI, confidence interval; ROC, receiver operator characteristic.

ovarian cancer has been suggested.[16]

The aim of our present study was to investigate the diagnostic value of a panel of markers in bladder cancer. CRT was employed in this study, as well as the novel candidate tumor markers SNCG and s-COMT. We examined the urinary protein levels of these three proteins in bladder cancer and control patients by western blot analysis. Their utility as a combined marker for bladder cancer is compared with that of CRT alone.

Materials and Methods

Patients and urine samples. Voided urine samples were obtained from 112 patients with bladder cancer and 230 control patients, including benign or malignant conditions (124 with benign prostatic hyperplasia, 7 with urinary tract infections (UTI), 6 with urinary stones, 6 with microscopic hematuria without known pathology, 58 with prostate cancer, 3 with renal cell carcinoma, 10 with breast cancer, and 16 with no definitive disorders). Table 1 summarizes patient demographics and tumor characteristics. Collected urine samples were divided into 1 ml aliquots in SUMILON Non Adsorption modified tubes (Sumitomo Bakelite, Tokyo), then stored at −30°C until analysis. Urinary creatinine levels were measured for all samples using one of the aliquots. Frozen urine samples were thawed quickly and centrifuged at 18,000*g* for 5 min at 4°C. Protease inhibitors were not added. Supernatants were mixed with 4× concentrated sodium dodecyl sulfate (SDS) sample buffer [250 mmol/liter Tris-HCl (pH 6.8), 80 g/liter SDS, 400 g/liter glycerol, 40 g/liter dithiothreitol, and bromophenol blue] and then subjected to western blotting.

Written informed consent was obtained from all patients in the study in accordance with institutional guidelines.

2DE and protein identification. Proteome differential display analysis of bladder cancer tissues and healthy urothelial mucosa from patients without neoplastic disease was used to screen proteins that are increased in cancer tissue, followed by protein identification as we have described previously.[8, 17]

Briefly, total protein was applied to an immobilized pH gradient gel for isoelectric focusing, then SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 12.5% polyacrylamide gel. Protein spots were visualized by silver staining, and only spots that were clearly and reproducibly more intense in specimens from the cancer patients were analyzed. After enzymatic digestion of proteins by trypsin, matrix-assisted laser desorption ionization/time-of-flight mass spectrometric analysis was performed.

Western blot analysis. Western blot analysis was carried out on 10% SDS-PAGE for CRT and 16% SDS-PAGE for SNCG and s-COMT with 30 µl of urine mixed with 10 µl of 4× sample buffer per lane. Proteins were transferred after SDS-PAGE onto an Immobilon-P Transfer Membrane (Millipore, Bedford, MA). To block non-specific binding sites on the membrane, Super-Block Blocking Buffer in TBS (Pierce, Lockford, IL) was used. Western blotting for CRT, SNCG, and s-COMT was performed as follows.

For SNCG detection, goat anti-SNCG polyclonal antibody (E-20; Santa Cruz Biotechnology, Inc., CA) at a dilution of 1:500 was used as the first antibody. After overnight incubation, the membranes were developed with an anti-goat horseradish peroxidase-conjugated horse antibody (Rockland, Inc., Gilbertsville, PA) diluted 1:25,000. The immunoproducts were visualized with ECL-plus western blotting detection reagents (Amersham Biosciences, Piscataway, NJ) and chemiluminescence after exposure to X-ray films. For s-COMT detection, 1:10,000 rabbit anti-COMT polyclonal antibody (AB5873; Chemicon International, Inc., Temecula, CA) as the first antibody, and 1:50,000 horseradish peroxidase-conjugated anti-rabbit IgG antibody (Vector Laboratories, Inc., Burlingame, CA) as the second antibody were used. The resulting chemiluminescence was detected by a cooled ImageMaster-(Amersham Biosciences) digital charge-coupled device camera. To quantify SNCG and s-COMT, a sample from a bladder cancer patient that contained a large amount of SNCG or s-COMT was employed as a control and included in each run, because standard proteins are not commercially available. Western blot analysis for CRT was performed as we previously described using heat-

Table 1. Patient demographics and tumor characteristics

Bladder cancer (*n*=112)		Non-bladder cancer (*n*=230)	
Characteristics	*n* (%)	Characteristics	*n* (%)
Sex		Sex	
Male	89 (79)	Male	204 (89)
Female	23 (21)	Female	26 (11)
Mean age (range), years	72 (50–90)	Mean age (range), years	53 (20–88)
Grade		Neoplastic disease	
1	9 (8)	Prostate cancer	58
2	49 (44)	Renal cell carcinoma	3
3	44 (39)	Breast cancer	10
Unknown	10 (9)	Nonneoplastic disease	
Stage		Benign prostatic hyperplasia	124
Tis	12 (11)	Urinary tract infection	7
Ta	50 (44)	Urolithiasis	6
T1	22 (20)	Microscopic hematuria without known pathology	6
T2–T4	18 (16)	No definitive disorders	16
Unknown	10 (9)		
Maximum tumor diameter			
<1 cm	30 (27)		
1–3 cm	40 (36)		
>3 cm	14 (12)		
Unknown	28 (25)		
Number of tumors			
Solitary	39 (35)		
Multiple	47 (42)		
Unknown	26 (23)		

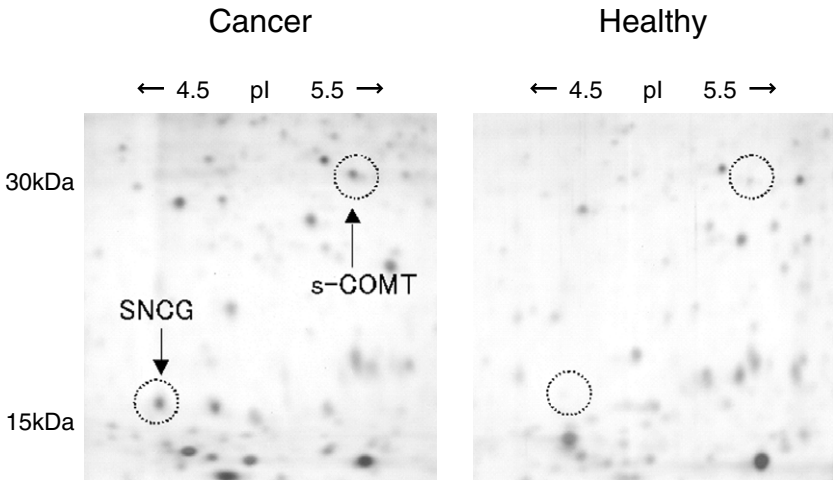

Fig. 1. Deduced silver-stained images of narrow-pH-range (pH 4.5–5.5) 2DE gels. Protein spots of SNCG and s-COMT in gels of bladder cancer tissue are shown. These spots are more intense in bladder cancer tissue than in control urothelial tissue (healthy bladder mucosa). Protein identification was done by a peptide mass fingerprinting method and verified by two-dimensional western blot analysis using specific antibodies. The theoretical molecular masses and pI's of SNCG and s-COMT are 15 kDa and 4.8 and 25 kDa and 5.1, respectively.

shocked HeLa cell extract as a standard control.[8] Washing and antibody dilution were performed with Tris-buffered saline-Tween [10 mmol/liter Tris-HCl (pH 7.6), 100 mmol/liter NaCl, 1 ml/liter Tween 20]. All tests were performed in duplicate to confirm reproducibility.

Quantification and data analysis. Visualization of immunoproducts was carried out as mentioned above. After image scanning, band quantification was performed using Scion Image software (Scion Co., Frederick, MA). The bands were considered positive when their intensities were measurable by densitometry. All the band intensities were normalized to that of the standard control (defined as 1.0 unit), then corrected according to the creatinine concentration in each sample. We performed preliminary experiments to ensure linearity of the measured protein concentrations for each of the proteins within the available analytical range.

To determine the optimal cutoff values of CRT, SNCG, and s-COMT measurements as single markers, receiver operator characteristic (ROC) curves were drawn and the areas under the curves were calculated for each test with Dr. SPSS II for Windows software (SPSS Japan, Inc., Tokyo). For the combination of markers, CRT, SNCG, and s-COMT, possible cutoff combinations of the three markers were constructed arbitrarily, because it was difficult to find a proper and practical analytical method to use in these conditions. Their diagnostic values were evaluated by plotting sensitivity against 1-specificity in an ROC space. Sensitivity, specificity, and predictive value were calculated with various cutoff combinations.

Outcomes for combined markers and VUC in individual patients were evaluated by the McNemar test. The statistical significance of differences in protein concentrations between bladder cancer and healthy urothelial tissues was assessed by applying the Mann-Whitney *U* test. A value of $P<0.05$ was considered statistically significant.

Results

Up-regulation of SNCG and s-COMT in bladder cancer tissue. Among 10 proteins that we identified by proteomic analysis,[8] SNCG (15 kDa and pI of 4.8) and s-COMT (25 kDa and pI of 5.1) were included (Fig. 1). We focused on these proteins as candidate tumor markers for bladder cancer, because the spots on silver-stained 2DE gel were more intense. In the same manner as in the previous study of CRT,[8] protein identification was verified by two-dimensional western blotting. Thereafter, high concentrations of these proteins in bladder cancer tissues were confirmed by quantitative western blot analysis using specific antibodies. SNCG and s-COMT protein levels that were normalized to β-actin concentration (defined as 1.0 unit) were examined in 22 bladder cancer and 10 healthy urothelial tissues (Fig. 2). For SNCG, the mean values±SD of the relative concentration were 0.19±0.39 (median: 0.02) in bladder cancer and 0.005±0.06 (median: 0.001) in noncancer tissue. The difference was statistically significant ($P=0.002$, Mann-Whitney *U* test). For s-COMT, the mean±SD values were 0.83±0.66 (median: 0.65) and 0.39±0.49 (median: 0.14), respectively, which were

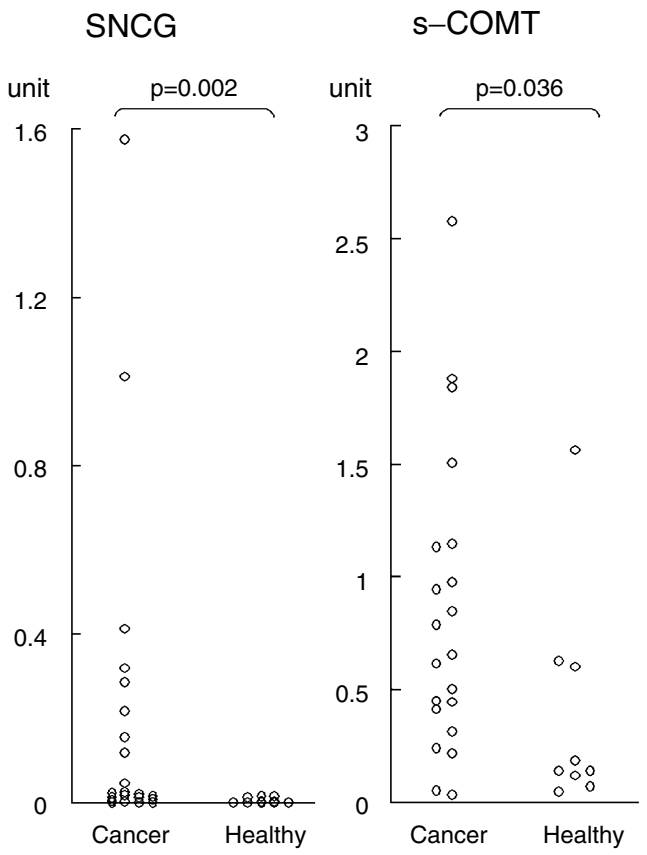

Fig. 2. Quantitative western blot analysis of SNCG and s-COMT in bladder cancer ($n=22$) and healthy urothelial ($n=10$) tissues. The ratios of SNCG or s-COMT to standard β-actin were calculated for each sample.

also significantly different (P=0.036, Mann-Whitney U test). Therefore, we employed SNCG and s-COMT in this study, in addition to CRT, as candidate urinary markers for bladder cancer.

Quantitative analysis of urinary CRT, SNCG, and s-COMT in bladder cancer and control patients. To examine the usefulness of SNCG and s-COMT as urinary markers, quantitative analysis by western blotting was performed on urine from 112 bladder cancer patients and 230 control patients. Urinary CRT measurements were also performed in this population. CRT as well as SNCG and s-COMT were detectable in urine by western blot analysis (Fig. 3). The calculated minimal detectable value of each protein was 0.01 unit. Among the urine samples of bladder cancer patients, 80 (71.4%) were positive for CRT, 45 (40.2%) for SNCG, and 41 (36.6%) for s-COMT. On the other hand, among the urine samples from control patients, 51 (22.2%) were positive for CRT, 8 (3.5%) for SNCG, and 47 (20.4%) for s-COMT.

With the best diagnostic cutoff levels determined by ROC curves (Fig. 4), the CRT (cutoff: 0.01) showed a sensitivity of 71.4% and a specificity of 77.8%. The sensitivity was comparable to those of the other available urinary markers mentioned above, and the specificity was almost equal to that noted for them, although it was of somewhat less diagnostic value than that found in our previous study.[8] For SNCG (cutoff: 0.01) and s-COMT (cutoff: 0.02), the sensitivities were 40.2% and 36.6%, respectively, and the specificities were 96.5% and 81.7%, respectively (Table 2).

As a single test, CRT showed the highest sensitivity of the three markers, which was comparable to the available urinary markers mentioned above. SNCG and s-COMT had sensitivities that are too low for clinical use, but the specificities were sufficiently high. These results suggest that concomitant use of the three markers may improve the diagnostic value with less reduction of specificity.

Concomitant examination of urinary CRT, SNCG, and s-COMT for diagnosis of bladder cancer. To study the usefulness of concomitant examination of CRT, SNCG, and s-COMT, we evaluated the diagnostic value of the combined marker compared with that of a single use of CRT. Various cutoff combinations of the

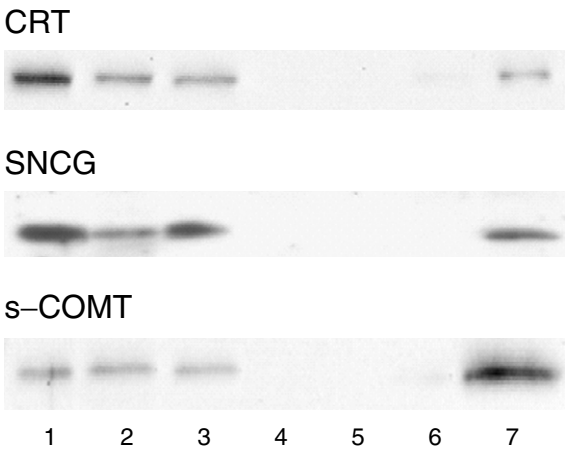

Fig. 3. Detection of urinary CRT, SNCG, and s-COMT by western blot analysis. Urine (30 μl) from patients mixed with 10 μl of 4× sample buffer was loaded in each lane. Lanes 1–3, urine from patients with bladder cancer; lanes 4–6, urine from patients without bladder cancer; lane 7, positive control (CRT: 0.2 μg of total protein extract from heat-shocked HeLa cells, SNCG and s-COMT: one sample from a bladder cancer patient that contains large amounts of these proteins).

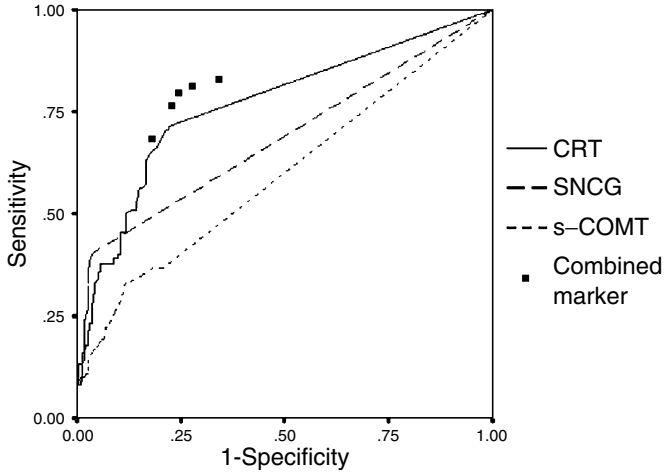

Fig. 4. ROC analysis of urinary markers. ROC curves of CRT (solid line), SNCG (hatched line), and s-COMT (dotted line) as a single test were constructed. The areas under the ROC curves were calculated at 0.8, 0.7, and 0.6, respectively. For concomitant use of CRT, SNCG, and s-COMT, the sensitivity was plotted against 1-specificity in the ROC space. Black dots indicate the results of possible cutoff conditions showed in Table 3. A shorter distance to the upper left corner of the ROC space indicates a higher diagnostic value.

Table 2. Diagnostic value of CRT, SNCG, and s-COMT as a single test with optimal cutoff levels determined by ROC analysis

Marker	Cutoff value	Sensitivity	Specificity	Positive predictive value	Negative predictive value
CRT	0.01	71.4	77.8	61.1	84.8
SNCG	0.01	40.2	96.5	84.9	76.8
s-COMT	0.02	36.6	81.7	49.4	72.6

Table 3. Diagnostic value of CRT, SNCG, and s-COMT combined marker in possible cutoff combinations[1)]

Markers/Cutoff values			Sensitivity	Specificity	Positive predictive value	Negative predictive value
CRT	SNCG	s-COMT				
0.01	0.01	0.02	79.5	67.8	54.6	87.2
0.01	0.01	0.15	77.7	75.7	60.8	87.4
0.08	**0.06**	**0.20**	**76.8**	**77.4**	**62.3**	**87.3**
0.01	0.01	0.15	74.1	77.4	61.5	86.0
0.15	0.01	0.20	70.5	80.9	64.2	84.9

The bold lettering indicates the optimal cutoff condition provided by ROC analysis.
1) Arbitrary combination of various cutoff levels of each test.

three markers were constructed arbitrarily. We defined the combined marker as positive when one or more of the individual markers were higher than their respective cutoff levels. Diagnostic values of the combined marker in various cutoff combinations are summarized in Table 3. The results evaluated in an ROC space were equal to or better than a single use of CRT in most of the cutoff combinations (Fig. 4). The combination of optimal cutoff values of each test resulted in the highest sensitivity; however, the specificity at this setting was lower than those at other settings. With the best diagnostic cutoff combination (0.08 for CRT, 0.06 for SNCG, and 0.20 for s-COMT), 86 of 112 bladder cancer patients and 52 of 230 control patients were recorded as positive. The sensitivity and specificity of the combined marker were 76.8% and 77.4%, respectively, corresponding to a 5.4% higher sensitivity and a 0.4% lower specificity compared with a single use of CRT.

The correlation between the result for the combined marker and the clinical characteristics of bladder cancer patients was also evaluated and is summarized in Table 4. The positive rates of the combined marker were equal to or higher than that of single use of CRT in every category. Notably, a considerable number of patients who are difficult to diagnose by noninvasive examination (i.e., low grade and stage, or small tumor volume) was detected by the combined marker.

These results suggest that a panel of urinary markers can be more useful than a single test for diagnosis of bladder cancer.

False-positive reactions in the 230 control patients are summarized in Table 5 according to disease category. Unfortunately, the UTI group of the control patients still had an extremely high false-positive rate (6 of 7 samples; 85.7%). When the UTI group was eliminated, the specificity was increased to 79.4% (177 negative reactions among 223 controls).

Comparison between the combined marker and established urine tests. For further evaluation of the diagnostic value of the combined marker, we compared the positive rate of the combined marker with those of the clinically available urine tests, VUC,

Table 4. Correlation between combined marker (CRT, SNCG, and s-COMT) and clinical features in bladder cancer patients

	Sensitivity		
	Concomitant use of CRT, SNCG, and s-COMT		Single use of CRT
	n	% (95% CI)	% (95% CI)
Overall	86/112	77 (69–81)	72 (64–76)
Grade			
1	4/9	44 (12–61)	33 (2–49)
2	36/49	74 (61–80)	74 (61–80)
3	37/44	84 (73–90)	84 (73–90)
Unknown	9/10	90 (71–100)	40 (10–55)
Stage			
Tis	9/12	75 (51–88)	75 (51–88)
Ta	33/50	66 (53–73)	64 (51–70)
T1	18/22	82 (66–90)	77 (56–86)
T2–T4	17/18	94 (84–100)	83 (66–92)
Unknown	9/10	90 (71–100)	70 (42–84)
Maximum tumor diameter			
<1 cm	18/30	60 (43–69)	57 (39–66)
1–3 cm	31/40	78 (65–84)	70 (56–77)
>3 cm	14/14	100	100
Unknown	23/28	82 (68–89)	75 (59–83)
Number of tumors			
Solitary	25/39	64 (49–72)	59 (44–67)
Multiple	39/47	83 (72–89)	79 (67–85)
Unknown	22/26	85 (71–92)	77 (61–85)

Table 5. False-positive rates of combined marker (CRT, SNCG, and s-COMT) in non-bladder cancer patients

	False-positives		
	Concomitant use of CRT, SNCG, and s-COMT		Single use of CRT
	n	% (95% CI)	% (95% CI)
Overall	52/230	22 (17–25)	22 (17–25)
Neoplastic disease			
Prostate cancer	13/58	22 (12–28)	19 (9–24)
Renal cell carcinoma	0/3	0	0
Breast cancer	1/10	10 (0–100)	10 (0–100)
Nonneoplastic disease			
Benign prostatic hyperplasia	25/124	20 (13–24)	21 (14–25)
Urinary tract infection	6/7	86 (60–99)	71 (33–88)
Urolithiasis	1/6	17 (0–32)	17 (0–32)
Microscopic hematuria without known pathology	2/6	33 (0–53)	33 (0–53)
No definitive disorders	4/16	25 (4–36)	31 (8–43)

Table 6. Comparison between combined marker (CRT, SNCG, and s-COMT) and urinary cytology or other commercial assays in bladder cancer patients

Urine test		Sensitivity		
		Concomitant use of CRT, SNCG, and s-COMT		Single use of CRT
	n (%)	*n*	% (95% CI)	% (95% CI)
Cytology[1)]				
Overall	105	80/105	76 (68–80)	70 (61–74)
Positive	42/105 (40)	38/42	90 (82–95)	81 (69–87)
Negative	63/105 (60)	42/63	67 (55–73)	64 (52–70)
BTA test				
Overall	15	14/15	93 (80–100)	87 (70–97)
Positive	6/15 (40)	6/6	100	100
Negative	9/15 (60)	8/9	89 (68–99)	78 (51–92)
NMP22				
Overall	22	19/22	86 (72–93)	77 (59–86)
Positive	11/22 (50)	10/11	91 (74–100)	91 (74–100)
Negative	11/22 (50)	9/11	82 (59–93)	64 (36–78)

1) Sensitivities of combined marker and urine cytology were statistically significantly different (McNemar test, $P<0.001$).

BTA test, and NMP22, in bladder cancer patients (Table 6). The positive rate of a single use of CRT was also assessed. VUC results were available in 105 bladder cancer patients. In these cases, VUC had a true-positive rate of 40% (42 of 105 cases) and a false-negative rate of 60% (63 of 105 cases). Of the 42 VUC positive cases, only 4 cases (9.5%) were not detectable with the combined marker. On the other hand, of the 63 VUC false-negative cases, the combined marker was positive in 42 cases (66.7%). The positive rate of the combined marker was still better than that of single use of CRT in both VUC-positive and negative cases. These results show that the combined marker is more useful for VUC-negative cases that are difficult to detect clinically. Although a few VUC-positive cases could not be detected by the combined marker, the positive rate was 80% (84 of 105) by combining VUC and the combined marker.

For examinations of the BTA test and NMP22, fresh urine samples of bladder cancer patients are required, especially for NMP22, which is examined using a special stock solution. Urine samples for these protein markers should not be stored frozen. We collected 15 and 22 fresh urine samples for the BTA test and NMP22 examinations, respectively. The BTA test had a true-positive rate of 40% (6 of 15 cases) and a false-negative rate of 60% (9 of 15 cases). While all 6 cases (100%) that were positive with the BTA test showed a positive reaction with the combined marker, 8 of 9 cases (88.9%) with a negative BTA test were positively detected by the combined marker. For NMP22, both the true-positive and false-negative rates were 50% (11 of 22 cases), respectively. Ten of 11 cases (90.9%) with positive NMP22 and 9 of 11 cases (81.8%) with false-negative NMP22 showed positive reactions with the combined marker. The positive rates of the combined marker in false-negative cases of both the BTA test and NMP22 were still higher than those obtained with CRT alone. We can therefore conclude that although the urine samples constituted only a small cohort, the combined marker had a considerably better capacity for bladder cancer detection in cases that were undetectable with the BTA test and NMP22.

Discussion

A number of urine-based markers have been identified and evaluated for the diagnosis of bladder cancer. Among them, the BTAtest, BTAstat, BTA-TRAK, and NMP22 are commercial assays. Glas *et al*. have presented an overview of the diagnostic value of these markers in a meta-analysis.[6)] In their report, the pooled sensitivities of the BTAtest, BTAstat, BTA-TRAK, and NMP22 test were 50%, 70%, 66%, and 67%, respectively, which are higher than that of VUC (50%), except for the BTAtest. On the other hand, these markers had lower specificities than that of VUC (94%) at 79%, 75%, 65%, 78%, respectively, especially in patients with stone disease, hematuria or urinary tract infection. In addition, the positive rates in low-grade tumors are not sufficient (range, 0 to 38% detection),[6)] though they are superior to those of VUC. These results were almost identical with those reviewed previously.[7)] Some of the newer tests that are not yet commercially available have high sensitivity and specificity, including telomerase[18)] and hyaluronidase.[19)] However, they are complex, requiring reference laboratory analysis.

Altogether, valuable urine-based markers that have sufficiently high sensitivity and specificity as single tests have not been established for both cancer screening and monitoring bladder cancer patients at low risk for tumor recurrence (grade1 pTa disease). Despite the worldwide effort to produce new urine-based markers, it seems unlikely that highly efficient urinary markers will be developed in the near future as single tests. We think that the challenging task of developing combination assays using a panel of tumor markers must be undertaken to overcome the limitations of current urinary markers.

Proteomic analysis is the most useful method to screen candidates for panel markers, and we previously identified 10 proteins that were up-regulated in bladder cancer tissue using this technology.[8)] Among those proteins, we investigated SNCG and s-COMT as novel candidates for a panel of urinary markers for bladder cancer, in addition to the already reported CRT. These two proteins have never before been evaluated for this purpose.

In this study, we showed that concomitant use of CRT, SNCG, and s-COMT improved the diagnostic value compared with use of CRT alone. In the optimal cutoff condition, determined by ROC analysis, the overall sensitivity was 76.8%, which was 5.4% higher than that of CRT at the cost of only 0.4% lower specificity. The increase in the overall sensitivity corresponds to a detection of 6 additional cases among the 112 persons in our study, and when these observations are applied to the US and Japanese populations, it is estimated that more than 3000 and 700, respectively, additional patients per year would be newly diagnosed in the two countries. Including recurrent cancer patients, more then 10,000 patients would be diagnosed non-invasively by our combined system. The significant increase of a few percent in sensitivity will greatly

help in the clinic to avoid misdiagnosis of cases. In addition, the diagnostic capacity of the combined marker was equal to or better than that of CRT in all categories, even in evaluation of the clinical characteristics of tumors. It is noteworthy that the combined marker had relatively high sensitivity in cases of low-grade (44.4%), small-volume (60%) tumors, and in cases with negative VUC (66.7%), because such cases are difficult to detect in clinical settings. The results indicate that the combined marker of CRT, SNCG, and s-COMT may be helpful in not only screening, but also monitoring of bladder cancer patients, thereby helping to indicate the required frequency of follow-up cystoscopy.

In order to produce new combined urine tests for bladder cancer that are feasible for practical use, it is necessary that the proteins have not been used before as tumor markers, because there are some proprietary limitations to the application of commercial tests in combination assays. In addition, we could not perform concomitant examinations due to differences in urine sample preparation. Therefore, we selected the present three parameters instead of BTA, or NMP22. If practicable, concomitant measurements of these three markers and/or BTA or NMP22 may provide further improvement of diagnostic value in bladder cancer. It is important to remember that VUC is still the gold standard for bladder cancer diagnosis, and we think that any new urinary testing should be used together with VUC to avoid misdiagnosis.

In conclusion, we have shown that our original concomitant use of CRT, SNCG, and s-COMT had higher sensitivity for detection of bladder cancer than CRT alone, although the sensitivity of CRT alone was comparable to that of commercial markers. The combined marker showed high sensitivity even in cases that were undetectable with VUC, BTA test and NMP22. Although more easily available methods for quantification, e.g. enzyme-linked immunosorbent assay (ELISA), should be constructed for practical use, our preliminary study suggests that a panel of markers will improve the diagnosis of bladder cancer, and it has also been shown that proteome analysis is helpful for screening marker candidates. Currently, we are evaluating whether other proteins identified by proteome analysis should be included in a panel of markers in order to obtain a higher diagnostic value, and we hope that further advances in proteomic technologies will enable detection of proteins that now are difficult to identify by 2DE, such as highly hydrophobic and extremely isometric proteins. Methods expected to be available for diagnosis of bladder cancer in the near future include a panel of markers on a protein microarray[20] and multi-channel enzyme-linked immunosorbent assays using a micro total analysis system.[21] Further comparative studies with other candidate proteins are needed to assess the utility of a panel of markers for diagnosis of bladder cancer.

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