

Urinary Protein Biomarker Panel for the Detection of Recurrent Bladder Cancer

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Abstract

Background: Up to 70% of patients with non-muscle-invasive bladder cancer (NMIBC) experience disease recurrence, making it one of the most prevalent cancers in the United States. The purpose of this study was to test the performance of a multiplex urinary biomarker assay for the monitoring of voided urine for recurrent bladder cancer.

Methods: This retrospective, multicenter study included a total of 125 subjects with a history of bladder cancer. Voided urine specimens were collected before procedure from these subjects (53 with confirmed tumor recurrence and 72 with confirmed non-tumor recurrence) for analysis. A prediction rule generated from the performance characteristics of 10 single biomarkers (IL8, MMP9, MMP10, SERPINA1, VEGFA, ANG, CA9, APOE, SERPINE1, and SDC1) was measured using ELISA. The diagnostic performance of the biomarker panel was assessed using receiver operator curves (ROC) and descriptive statistical values (e.g., sensitivity and specificity).

Results: The combination of all 10 biomarkers outperformed any single biomarker with a calculated AUROC for the diagnostic panel of 0.904 [95% confidence interval (CI), 0.853–0.956]. The multiplex assay achieved an overall sensitivity of 79% and specificity of 88% for recurrent bladder cancer and significantly outperformed the Urovysion cytogenetic assay (sensitivity 42%, specificity 94%) and voided urinary cytology (sensitivity 33%, specificity 90%).

Conclusions: A diagnostic panel of 10 urinary biomarkers that accurately detects primary bladder cancer also performs well for the detection of recurrent bladder cancer.

Impact: The identification of a reliable urine-based surveillance and detection assay would be of benefit to both patients and the healthcare system. *Cancer Epidemiol Biomarkers Prev*; 23(7); 1340–5. ©2014 AACR.

Introduction

Seventy to 80% of newly diagnosed bladder cancers are non-muscle-invasive bladder cancer (NMIBC). Although NMIBC is rarely fatal initially, there is a high rate of disease recurrence (50%–70%) and disease progression to MIBC over time (30%; ref. 1). Because of the high rate of tumor recurrence, it is estimated that 500,000 Americans suffer from bladder cancer at any one time, making it the second most prevalent cancer in the United States (2). Because of the described risks of recurrence, guidelines

recommend that patients should be monitored after initial diagnosis and treatment using a regimen that prescribes surveillance every 3 to 6 months for 4 years and at least annually beyond that. Current surveillance relies on the gold standard methods of cystoscopy and voided urine cytology (VUC), plus imaging and biopsy. There are no reliable standalone urinary biomarkers for the detection of bladder cancer recurrence currently available in the clinic. The development of an accurate, noninvasive urine-based assay would be of tremendous benefit to both patients and the healthcare system.

We have previously coupled high-throughput, discovery-based technologies (i.e., genomics and proteomics) with bioinformatics to derive diagnostic signatures that show promise for the accurate detection of primary bladder cancer in voided urine samples (3–5). Analysis of candidate protein biomarker panels in an independent patient cohorts confirmed that a panel of 10 biomarkers (IL8, MMP9, MMP10, SERPINA1, VEGFA, ANG, CA9, APOE, SERPINE1, and SDC1) was optimal for the non-invasive detection of bladder cancer (6, 7). We have subsequently reported the validation of the 10-biomarker diagnostic panel in a large cohort of patients ($n = 308$),

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which included controls with diverse urologic conditions (e.g., urolithiasis, moderate-to-severe voiding symptoms, urinary tract infection, and hematuria; ref. 8) and through analysis of samples obtained from multiple sites in the United States and in Europe ($n = 320$; Chen and colleagues, submitted for publication) in an external laboratory. In the current study, we set out to evaluate the performance of the 10 urinary biomarkers in our primary bladder cancer detection panel to detect recurrent bladder cancer in a multicenter cohort composed of patients with a history of bladder cancer undergoing routine surveillance.

Materials and Methods

Specimen and data collection

The study was approved by the Institutional Review Boards at MD Anderson Cancer Center-Orlando (Orlando, FL) and the Hospital Clinic of Barcelona (Barcelona, Spain). Banked urine samples were collected from patients presenting to the outpatient Urology clinics at the 2 institutes. From each subject just before cystoscopy, approximately 50 mL of voided urine was collected and assigned a unique identifying number before laboratory processing as previously described (4–9) and stored at -80°C before analysis. Patients with self-reported renal disease or documented renal insufficiency [glomerular filtration rate (GFR) < 60 mL/min/1.73 m²] were not selected for inclusion in the current study. The 2 tissue banks were queried for suitable specimens for analysis (i.e., samples from subjects with a history of bladder cancer who presented to clinic for routine bladder cancer surveillance), which included 147 samples. Because of inadequate volume for analysis (e.g., < 3 mL), urinary protein levels > 700 $\mu\text{g/mL}$, urinary creatinine levels < 35 mg/dL, or the absence of critical clinical data, 22 samples were excluded thus leaving us with 125 subjects, which comprised the current study cohort. Data are reported using the REMARK criteria (10). All subjects underwent cystoscopy (patients with high-grade disease underwent cystoscopy every 3 months for 2 years, then every 6 months for 2 years, and then annually, whereas patients with low-grade disease underwent cystoscopy every 6 months for 2 years and then annually). The majority of subjects had voided urine specimen sent to clinical laboratory for both voided urinary cytology (VUC; 92%) and Urovysion cytogenetic test (74%). Urovysion cytogenetic test is designed to detect aneuploidy for chromosomes 3, 7, 17, and loss of the 9p21 locus via FISH. The combined 10-urinary biomarker assay was compared with VUC and Urovysion cytogenetic test. Approximately 5% of VUC and Urovysion assays could not be interpreted because of poor quality or insufficient material. In patients with an abnormally appearing cystoscopy, abnormal VUC, and/or an abnormal Urovysion cytogenetic test, histologic examination of urothelia was performed. When cancer was confirmed histologically, tumor grade and stage were recorded. Median follow-up of the entire cohort was 18 months (range, 1–65 months).

Urinary ELISA

Levels of human interleukin-8 (IL8; Cat # ab46032, Abcam), matrix metalloproteinase-9 (MMP9; Cat # DMP900, R&D Systems Inc.), plasminogen activator inhibitor 1 (SERPINA1; Cat # EA-0207, Signosis Inc.), VEGFA (Cat # 100663, Abcam), angiogenin (ANG; Cat # CK400, CellSciences), carbonic anhydrase 9 (CA9; Cat # DCA900, R&D Systems Inc.), MMP10 (Cat # DMP1000, R&D Systems Inc.), human apolipoprotein E (APOE; Cat # KA 1031, Abnova), human A1AT (SERPINE1; Cat # ab108799, Abcam), and human Syndecan 1 (SDC1; Cat# ab46507, Abcam) were monitored in urine samples using commercial ELISA as listed above using the BioTek FLx800 Multi-Well Plate Reader (BioTek US) with the Gen5 Data Analysis Software package (BioTek US). Frozen supernatants were thawed, and ELISAs were conducted according to the manufacturer's instructions. Calibration curves were prepared using purified standards for each protein assessed. Curve fitting was accomplished by either linear or 4-parameter logistic regression following the manufacturer's instructions. Because of the unavoidable variability of voided urine with respect to total volume and time within the bladder, biomarkers were normalized to urinary creatinine for comparison (6–11). Laboratory personnel were blinded to final diagnosis.

Data analysis

We investigated the diagnostic performance of each of the 10 urinary biomarkers for the detection of recurrent bladder cancer. We used the Wilcoxon rank-sum test to determine the association between each individual biomarker and the presence of recurrent bladder cancer (yes vs. no). Each biomarkers was normalized to creatinine and cubic-root transformed. Next a prediction rule was generated = $0.223(\text{IL-8}) - 1.871(\text{MMP9}) + 2.239(\text{SERPINA1}) + 0.356(\text{ANG}) - 0.216(\text{VEGFA}) 0.330(\text{CA9}) + 0.689(\text{MMP10}) + 6.889(\text{APOE}) - 0.408(\text{SERPINE1}) + 1.021(\text{SDC1}) - 0.141$ to determine the non-parametric receiver operating characteristic (ROC) curves (12) for the combination of the 10 biomarkers by plotting the values of sensitivity against the false-positive rates ($1 - \text{specificity}$) at varying cutoff thresholds. The sensitivity, specificity, positive predictive value (PPV), and the negative predictive value (NPV) were calculated from ROC data. Statistical significance in this study was set at $P < 0.05$ and all reported P values were 2-sided. All analyses were performed using SAS software version 9.3.

Results

Pertinent information on demographics and clinicopathologic presentation of the cohort of 125 patients are presented in Table 1. Of the 125 patients with a history of bladder cancer, 53 were found to have recurrence in follow-up. Median size of recurrent tumor was 2 cm. The ability of each of the test biomarker, whether elevated or reduced, to predict the presence of recurrent bladder cancer was

Table 1. Demographic and clinicopathologic characteristics of 125-subject study cohort

Variable(s)	Recurrence		P ^a
	Yes (n = 53)	No (n = 72)	
Age, y			
Mean ± SD	71 ± 10	69 ± 12	0.651
Median (min–max)	73 (48–87)	71 (39–88)	
Sex			
Female	11 (20.8%)	11 (15.3%)	0.427
Male	42 (79.2%)	61 (84.7%)	
Race			
Other	2 (3.8%)	13 (18.1%)	0.015
White	51 (96.2%)	59 (81.9%)	
Tobacco use			
No	18 (36.0%)	20 (28.2%)	0.590
Quit	11 (22.0%)	15 (21.1%)	
Yes	21 (42.0%)	36 (50.7%)	
Unknown	3 (5.7%)	1 (1.4%)	
Urinary protein, µg/mL			
Mean ± SD	91.0 ± 206.6	135.9 ± 234.0	0.112
Median (min–max)	29.5 (0–1,077.8)	48.1 (0–1,282.9)	
Urinary creatinine, mg/dL			
Mean ± SD	57.2 ± 31.3	69.3 ± 53.3	0.458
Median (min–max)	51.1 (10.7–134.5)	55.2 (8.9–311.1)	
Initial stage			
Non-muscle-invasive (Ta = 34, T1 = 11, Tis = 4)	49 (92.5%)	49 (68.1%)	0.013
Muscle-invasive (all T2) ^b	4 (7.5%)	14 (19.4%)	
Unknown	0	9 (12.5%)	
Initial grade			
Low-grade	30 (56.6%)	21 (29.2%)	0.258
High-grade	23 (43.4%)	42 (58.3%)	
Unknown	0	9 (12.5%)	

^aP < 0.05 indicates significant imbalance between patients with recurrence and patients without recurrence.

^bSixteen subjects treated with chemotherapy and radiation therapy and 2 subjects treated with aggressive transurethral bladder tumor resection.

analyzed using nonparametric ROC analyses, according to National Cancer Institute guidelines (13). Table 2 provides AUROC and corresponding sensitivity, specificity, PPV, and NPV values for all biomarkers tested. Urinary SERPINA1 was the most accurate single biomarker with an AUROC of 0.864 [95% confidence interval (CI), 0.799–0.929], a sensitivity of 87%, specificity of 72%, PPV of 70%, and NPV of 88%. Urinary MMP10 was the second most accurate single biomarker with an AUROC of 0.837 (95% CI, 0.758–0.917), sensitivity of 77%, specificity of 82%, PPV of 76%, and NPV of 83%; and third most accurate was SDC1 with an AUROC of 0.818 (95% CI, 0.741–0.895), sensitivity of 60%, specificity of 93%, PPV of 86%, and NPV of 76%. The combination assay (all 10 biomarkers in the diagnostic panel) resulted in an AUROC of 0.904 (95% CI, 0.853–0.956), outperforming any single biomarker. The multiplex assay achieved an overall sensitivity of 79%, specificity of 88%, PPV of 82%, and NPV of 85% for bladder cancer. Table 3 compares the overall sensitivity and spec-

ificity achieved using the combined 10 urinary biomarker assays, the commercial UroVysion cytogenetic test, and VUC. Values are further segregated according to tumor grade at the time of documented bladder cancer recurrence. Overall sensitivity was highest for the 10-biomarker assay (79%), relative to the UroVysion cytogenetic test (42%), and VUC (33%). Importantly, sensitivity for the detection of low-grade tumors was markedly improved with the 10-biomarker assay (90% sensitivity) compared with UroVysion (14%) and VUC (17%). VUC and the UroVysion cytogenetic test had only slightly better overall specificity compared with the 10-biomarker assay (90%, 94%, and 88%, respectively).

Discussion

The probability of bladder cancer recurrence after initial diagnosis is associated with tumor size, stage, grade, and multifocality (14). As with primary bladder cancer, when recurrent bladder cancer is identified as NMIBC rather

Table 2. Protein biomarker performance data for the detection of recurrent bladder cancer

Biomarker	AUC	95% CI	Correctly	Correctly	Non-events	Events	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
			predicted events, n	predicted non-events, n	predicted as events, n	predicted as non-events, n				
IL8	0.774	0.685–0.862	29	69	3	24	55	96	91	74
MMP9	0.771	0.683–0.859	29	68	4	24	55	94	88	74
SERPINA1	0.864	0.799–0.929	46	52	20	7	87	72	70	88
ANG	0.804	0.726–0.882	29	68	4	24	55	94	88	74
VEGFA	0.757	0.667–0.847	30	68	4	23	57	94	88	75
CA9	0.814	0.737–0.891	32	68	4	21	60	94	89	76
MMP10	0.837	0.758–0.917	41	59	13	12	77	82	76	83
APOE	0.775	0.685–0.865	39	60	12	14	74	83	76	81
SERPINE1	0.778	0.693–0.863	29	68	4	24	55	94	88	74
SDC1	0.818	0.741–0.895	32	67	5	21	60	93	86	76
All 10 biomarkers	0.904	0.853–0.956	42	63	9	11	79	88	82	85

than MIBC or metastatic disease, the 5-year survival rate can be quite favorable, thus timely diagnosis and intervention can dramatically affect outcomes (15). Consequently, for patients with a history of bladder cancer, rigorous surveillance is well advised. The gold standard for the diagnosis of recurrent bladder cancer remains cystoscopy. While cystoscopy has good sensitivity (~75%) for the detection of bladder tumors (16), the procedure itself is invasive, uncomfortable, and costly (17–19). Complicating matters further, cystoscopy has been associated with significant patient anxiety (20) and can impact patient compliance with a strict follow-up regimen set forth by current guidelines (21). VUC (the microscopic evaluation of shed cancer cells in voided urine) is routinely used as a noninvasive adjunct test to cystoscopy but has low sensitivity particularly for the detection of low-grade tumors (~20%–40%; refs. 22, 23).

Coupled with the fact that VUC is also prone to considerable interobserver variation (24), it is understandable that this method has not emerged as a standalone test for the detection of bladder cancer. The limitations of cystoscopy and VUC underscore the continuing need to explore and validate less invasive bladder cancer detection methods that can achieve clinically acceptable levels of sensitivity and specificity.

For a urinary biomarker test to be valid for the evaluation of subjects with a history of bladder cancer, it needs to achieve high sensitivity for the early detection of bladder cancer. Acceptable sensitivity would reduce the number of unnecessary invasive cystoscopies by stratifying patients at clinical work-up. Only those patients with a positive urinary test would be deemed to be at high-risk for bladder cancer and would undergo cystoscopy to confirm or refute the presence of a cancer. With a reported sensitivity of

Table 3. Diagnostic performance of the 10-biomarker assay, Urovysion, and VUC in a cohort of patients with a history of bladder cancer

	Tumor grade at recurrence		
	Low	High	Overall
Combined 10-biomarker assay (n = 53)			
No. of detected/total cancers	25/29	15/23	42/53
Sensitivity, % (95% CI)	90 (0.786–1)	65 (0.458–0.847)	79 (0.683–0.902)
Specificity, % (95% CI)	0.88 (0.799–0.951)	0.88 (0.799–0.951)	0.88 (0.799–0.951)
Urovysion cytogenetic assay (n = 24)			
No. of detected/total cancers	1/7	9/17	10/24
Sensitivity, % (95% CI)	14 (0–0.402)	53 (0.292–0.767)	42 (0.219–0.614)
Specificity, % (95% CI)	0.938 (0.878–0.997)	0.938 (0.878–0.997)	0.938 (0.878–0.997)
Voided urine cytology (n = 45)			
No. of detected/total cancers	4/24	11/20	15/45
Sensitivity, % (95% CI)	17 (0.018–0.316)	55 (0.332–0.768)	33 (0.196–0.471)
Specificity, % (95% CI)	90 (0.834–0.971)	90 (0.834–0.971)	90 (0.834–0.971)

79% in our cohort, the combined 10-biomarker assay significantly outperformed VUC (33%) and the Urovysion assay (42%), and, if validated, approaches the level of sensitivity required for incorporation into the surveillance regimen of patients with bladder cancer.

It might be expected that a molecular assay derived to detect primary bladder cancer would not perform as well for the detection of recurrent bladder cancer. Our molecular profiling discovery studies were performed on distinct cohorts composed of patients who had confirmed bladder cancer or no disease. Those with bladder cancer had predominantly high-grade and high-stage disease, a scenario that can lead to highly sensitive assays initially, but the performance decreases as more complex cohorts with more low-grade and low-stage cases are included. Patients under surveillance for recurrent bladder cancer are more likely to present with low-stage bladder cancer. Our program of refining our candidate panels of biomarkers in a series of validation cohorts may have avoided this issue (7, 8). Continued bladder tumor recurrence after an initial diagnosis may be explained by the field-cancerization theory (25). As has also been proposed in squamous cell carcinomas of the head and neck (26), the theory suggests that secondary cancer events occur as a consequence of widespread molecular changes in previously uninvolved bladder mucosa initiated by external cancer-causing influences. This phenomenon could create latent disease or predispose the mucosa to malignant events on subsequent insult. If such widespread molecular changes were representative of established tumors, then a test that performed well for primary bladder cancer may be compromised for surveillance and detection of recurrent disease. However, in this study, this does not appear to be the situation. All of the individual biomarkers tested performed well in this surveillance cohort, and results obtained for the combined 10-biomarker urinary protein assay were similar for recurrent disease as for primary diagnosis. It may be that the analytes in the 10-biomarker assay are not impacted substantially at the expression level by the intermediate prerecurrence field-effect. The fact that a single test can detect both primary and recurrent bladder cancer is encouraging from the point of view of efficiency and standardization in a clinical setting.

Clinically, accurate noninvasive bladder cancer assays would have a clear impact on the clinical management of patients with bladder cancer. The ultimate goal is to be able to detect bladder cancer in a timely manner such that the patient can expect an improved survival as well as improved quality of life, and compliance with the recommended surveillance program is paramount. Less onerous investigation protocols would surely improve compliance, and the resulting early detection would benefit both the patient and the healthcare system. We recognize that our study has some limitations. First, the sample size ($n = 125$) is relatively small, and findings must be confirmed in a larger cohort, preferably with longer follow-up data. We are currently compiling samples from multiple centers for the next study based on

these encouraging results. In addition, this small sample size may account for the low sensitivity (52%–65%) in detecting high-grade cancers with 10-biomarker assay, VUC, or Urovysion assay. The reported sensitivities of VUC and Urovysion assay are somewhat low compared with other studies (27, 28), but this may be because these tumors are recurrent and not primary tumors. Furthermore, as there are only a small number of cases, the results for VUC and Urovysion assay, especially for low-grade tumors, may be of limited value. Second, as part of a phased methodical approach to biomarker discovery and confirmation, processed urines were retrieved from tissue banks for analysis in this study. To address this, we are investigating the performance of the selected biomarkers in urines processed via a number of different protocols, including the testing of unprocessed freshly voided urines. For clinical implementation, a molecular test needs to be cost-effective, as well as accurate, especially if that test is to be used over a long period of follow-up as in the case for bladder cancer. The detection of urinary proteins through multiplexed analyses has the potential to be relatively simple to perform and interpret and affordable. The 10-biomarker assay described here performs favorably in detecting recurrent bladder cancer, supporting the idea that a noninvasive urine test can be valuable for the surveillance of patients with a history of bladder cancer.

In this study, we were able to confirm that a diagnostic panel of 10 urinary biomarkers that accurately detects primary bladder cancer also performs well for the detection of recurrent bladder cancer. If these findings are confirmed in larger, multicenter studies, then it is conceivable that such a relatively simple, noninvasive urinary molecular assay could also be incorporated into the surveillance regimen for patients with a history of bladder cancer.

Disclosure of Potential Conflicts of Interest

C.J. Rosser is the President of Nonagen BioScience Corp. C.J. Rosser and S. Goodison have ownership interest (including patents) in Nonagen BioScience Corp. S. Goodison is an officer in Nonagen BioScience Corp. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Ross, L. Mengual
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.J. Rosser, M. Chang, Y. Dai, S. Ross, S. Goodison
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