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Application of a multiplex urinalysis test for the prediction of intravesical BCG treatment response: A pilot study

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Abstract.
BACKGROUND: Intravesical Bacillus Calmette-Guerin (BCG), a live attenuated tuberculosis vaccine that acts as a non-specific immune system stimulant, is the most effective adjuvant treatment for patients with intermediate or high-risk non-muscle-invasive bladder cancer (NMIBC). However, to date, there are no reliable tests that are predictive of BCG treatment response. In this study, we evaluated the performance of Oncuria™, a bladder cancer detection test, to predict response to intravesical BCG.
METHODS: Oncuria™ data was evaluated in voided urine samples obtained from a prospectively collected cohort of 64 subjects with intermediate or high risk NMIBC prior to treatment with intravesical BCG. The Oncuria™ test, which measures 10 cancer-associated biomarkers was performed in an independent clinical laboratory. The ability of the test to identify those patients in whom BCG is ineffective against tumor recurrence was tested. Predictive models were derived using supervised learning and cross-validation analyses. Model performance was assessed using ROC curves.
RESULTS: Pre-treatment urinary concentrations of MMP9, VEGFA, CA9, SDC1, PAI1, APOE, A1AT, ANG and MMP10 were increased in patients who developed disease recurrence. A combinatorial predictive model of treatment outcome achieved an AUROC 0.89 [95% CI: 0.80–0.99], outperforming any single biomarker, with a test sensitivity of 81.8% and a specificity of 84.9%. Hazard ratio analysis revealed that patients with higher urinary levels of ANG, CA9 and MMP10 had a significantly higher risk of disease recurrence.
CONCLUSIONS: Monitoring the urinary levels of a cancer-associated biomarker panel enabled the discrimination of patients who did not respond to intravesical BCG therapy. With further study, the multiplex Oncuria™ test may be applicable for the clinical evaluation of bladder cancer patients considering intravesical BCG treatment.

Keywords: Biomarkers, bladder cancer, multiplex, protein, BCG

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1. Introduction

Up to 60% of non-muscle invasive bladder cancer (NMIBC) cases that are treated by transurethral resection (TUR) will experience disease recurrence. Guidelines for NMIBC management include the recommendation for post-TUR intravesical instillation therapy [1,2]. Intravesical Bacillus Calmette-Guerin (BCG), a live attenuated tuberculosis vaccine that acts as a non-specific immune system stimulant, has proven to be the most successful adjuvant treatment to date, assisting in the eradication of residual disease, reducing recurrence rates, and decreasing disease progression to muscle-invasive bladder cancer (MIBC) [1,2]. However, despite considerable success, as many as 30% of patients will develop tumor recurrence and up to 15% can progress despite BCG therapy [3,4]. Failure to intervene with definitive radical cystectomy prior to progression to MIBC is associated with a significant reduction in long-term survival probability [5,6]. Thus, early identification of patients suited for bladder preservation with BCG treatment or for radical cystectomy is essential. At this time, the decision to preserve the bladder or to perform a cystectomy depends on models based on clinicopathological parameters [7,8], but these tools have limited accuracy for predicting disease recurrence or progression [9,10]. Furthermore, there is currently no established evaluation test available for the prediction of patient response to intravesical BCG.

Previously, we have identified a panel of protein biomarkers that are associated with bladder cancer [11–14], and we have developed a multiplex immunoassay for the automated detection of the analyte panel in voided urine [15–17]. The test has been validated for non-invasive diagnosis, but in this study, we tested the potential clinical utility of the multiplex test for the prediction of BCG treatment response in a small prospective cohort.

2. Patients and methods

2.1. Patients, specimens and data collection

Patients with intermediate and high risk NMIBC (Tis, Ta or T1) [18] were previously recruited and reported in a clinical trial in which intravesical BCG was administered [19]. Briefly, spontaneous voided urine samples were collected prior to BCG treatment. All urine samples were centrifuged at 1,500 g for 10 min, and cell-free urine samples were stored at -80°C prior to analysis. After the initiation of intravesical BCG treatment, all participants were followed up with periodic cystoscopic medical examination. The endpoint of interest in each patient was disease recurrence, defined as a newly identified bladder tumor after a previous negative follow-up cystoscopy. Patients with noted abnormality on cystoscopy, underwent cystoscopy with bladder biopsy or transurethral resection of a bladder tumor (TURBT) followed by pathological interpretation.

2.2. Multiplex immunoassay

The concentrations of the 10 proteins (A1AT, APOE, ANG, CA9, IL8, MMP9, MMP10, PAI1, SDC1 and VEGFA) were monitored using an analytically validated multiplex bead-based immunoassay (Oncuria^{TM}) from R&D Systems Inc. (Minneapolis, MN) [15–17]. Urine samples were passively thawed on ice, centrifuged for 10 minutes $\times$ 1,000 g and handled on ice prior to diluting 2-fold with R&D Assay Diluent. Samples, standards and controls (50 μl) were added to the 96 well plate in duplicate. The multiplex immunoassay was conducted according to the manufacturer's instructions. A seven-point standard curve across the dynamic range of the assays was included in the current assay design. Plates were read on the Luminex® 200 plate reader (Luminex Corp, Austin, TX). Calibration curves were generated along with optimal fit in conjunction with Akaike's information criteria (AIC) values [20].

2.3. Data analysis

Wilcoxon rank sum tests were used to determine the association between each biomarker and bladder cancer recurrence. Nonparametric receiver operating characteristic (ROC) curves were generated to plot assay sensitivity against the false-positive rate (1-specificity). The relative ability of each biomarker to predict bladder cancer recurrence was evaluated by calculating the area under the curve (AUC), and AUCs were compared by chi-square test. The sensitivity and specificity of each biomarker individually and in combination were estimated at the optimal cutoff value defined by the Youden index [21]. To assess the independent association between biomarkers and bladder cancer recurrence, we used logistic regression analysis with recurrence status (yes vs. no) as the response variable and biomarker concentrations as explanatory variables. Multivariate analysis using Cox proportional hazards models for recurrence was performed to evaluate the influences of each biomarker on disease-specific survival. The

all-subset method was used to evaluate the predictive value of each possible combination of biomarkers, and the Bayesian information criterion (BIC) was used to compare models. The BIC, a widely used criterion in model selection, balances the model likelihood and the number of biomarkers included in the model [22]. The Bootstrap method (using 1000 Bootstrap samples) was used [23] to select the most efficient and stable predictive model. 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 (SAS Institute Inc., Cary, NC).

3. Results

The study population was comprised of 64 subjects with NMIBC who were scheduled to be treated with intravesical BCG. Among the 64 study subjects in this application, all 64 had high-grade disease with 23 with intermediate risk disease (i.e., ALL Ta high-grade $<$ 3 cm) and the remaining 41 high risk disease (i.e., 29 with high-grade T1, 7 with high-grade Ta $>$ 3 cm and 5 with CIS). The mean age of subjects was 65.8 $\pm$ 11.3 years, 78.1% of the subjects were men, 85.9% were Caucasian, and 54.7% of the subjects presented with Tis/Ta disease while 45.3% of the subjects presented with T1 disease (Table 1). Of the total 64 subjects, 11 (15.6%) were found to have post-treatment bladder cancer recurrence on follow-up. Median time to recurrence was 6 months (range 1–17 months). The recurrences were noted to be non-muscle invasive bladder cancer (NMIBC; stages Ta, Tis, T1) high-grade in 81.8% ($n = 9$), and muscle invasive bladder cancer (MIBC; stage $\geqslant$ T2) high-grade in 18.2% ($n = 2$). Of these 11 subjects, two were noted to have a second recurrence.

For each of the 64 clinical samples, we reported the mean $\pm$ SD and range of each biomarker, along with the percentage of samples in which the biomarker was detectable (Table 2). Each individual biomarker was detectable in $>$ 90% of the samples, except for A1AT (detected in 58% of samples). Increased urinary concentrations of MMP9 ($P = 0.53$), VEFGA ($P = 0.20$), CA9 ($P = 0.19$), SDC1 ($P = 0.23$), PAI1 ($P = 0.19$), ApoE ($P = 0.21$), A1AT ($P = 0.04$), ANG ($P = 0.02$) and MMP10 ($P = 0.15$) were observed in subjects with disease recurrence. The urinary concentration of IL8 was unchanged.

Table 3 provides AUC data for each individual biomarker and for the combination of all 10 biomarkers in the Oncuria™ test. Using optimal cutoff values defined by the Youden index from this cohort, the 10-biomarker model resulted in an AUC of 0.8971 (95% confidence interval, 0.8000–0.9942), with a sensitivity value of 81.8% and a specificity value of 84.9% for the prediction of disease recurrence (Fig. 1). Patients with higher urinary levels of CA9 (HR: 3.44, 95% CI: 1.21–9.76; $P = 0.02$), ANG (HR: 42.89, 95% CI: 3.06–602.10; $P = 0.005$) and MMP10 (HR: 3.86, 95% CI: 1.06–14.05; $P = 0.04$) had a significantly higher risk of disease recurrence. Increased levels of PAI1 (HR: 3.54, 95% CI: 0.87–14.43; $P = 0.08$), APOE (HR: 4.64, 95% CI: 0.91–23.59; $P = 0.06$) and A1AT (HR: 3.72, 95% CI: 0.94–14.83; $P = 0.06$) approached significance (Table 4).

Table 1
Demographic and clinical-pathologic characteristics of study cohorts

		No recurrence ($N = 53$)		Recurrence ($N = 11$)		
Variable	Value	n	%	n	%	P
Age	18–54	9	17.0	2	18.2	0.37
	55–64	16	30.2	4	36.4	
	65–74	17	32.1	1	9.1	
	75+	11	20.8	4	36.4	
Sex	Female	10	18.9	4	36.4	0.22
	Male	43	81.1	7	63.6	
Race	White	46	86.8	9	81.8	0.67
	Other	7	13.2	2	18.2	
Stage	Ta	26	49.1	4	36.4	0.21
	Tis	5	9.4	0	0.0	
	T1	22	41.5	7	63.6	
Cytology	Negative	24	45.3	2	18.2	0.10
	Positive	25	47.2	6	54.5	
	Missing	4	7.5	3	27.3	

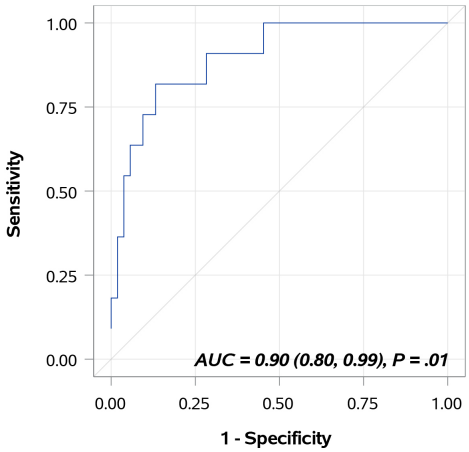

Fig. 1. Diagnostic performance of bladder cancer-associated molecular signature. The areas under the curves was 0.8971 (95% confidence interval, 0.8000–0.9942), with a sensitivity value of 81.8% and a specificity value of 84.9% for the prediction of disease recurrence.

Table 2
Mean urinary (± SD) concentrations of 10 biomarkers assessed by Oncuria™

Biomarker pg/mL	Detectable %	No recurrence ($N = 53$)				Recurrence ($N = 11$)					
		Mean	SD	Min	Max	Mean	SD	Min	Max	P	Cutoff
MMP-9	92	10,873	12,604	292	60,355	13,986	14,885	653	39,466	0.53	$>$ 6,051
CXCL8/IL-8	100	425.8	373.6	6.4	1,508.1	417.2	395.4	39.4	1,322.3	0.95	$<$ 254.4
VEGF-A	100	209.9	114.6	40.6	496.7	327.6	279.7	86.0	900.5	0.20	$>$ 209.3
IX/CA9	91	2.07	3.46	0.07	20.81	6.87	11.31	0.32	32.32	0.19	$>$ 1.55
Syndecan-1	100	6,580	2,860	1,256	14,433	7,911	3,294	3,341	12,176	0.23	$>$ 6,562
Serpin E1/PAI-1	100	448.7	439.5	35.7	1,990.0	955.7	1,196.0	78.4	3,827.0	0.19	$>$ 378.0
ApoE	100	8,714	9,513	676	53,748	12,925	9,763	2,555	34,151	0.21	$>$ 7,664
Serpin A1	58	771,434	716,725	38,750	1,940,791	1,257,290	645,741	66,035	1,631,774	**0.04**	$>$ 629,949
Angiogenin	100	2,284	1,323	24	6,718	4,179	2,339	1,185	8,603	**0.02**	$>$ 2,683
MMP-10	95	236.6	286.5	7.5	1,341.3	405.9	346.5	84.7	1,059.6	0.15	$>$ 192.1

Table 3
Performance of the Oncuria™ test for the prediction of BCG treatment response

Biomarker	Area under the curve	95% confidence interval	No. of correctly predicted events	No. of correctly predicted nonevents	No. of nonevents predicted as events	No. of events predicted as nonevents	Sensitivity	Specificity	PPV	NPV
A1AT	0.6364	(0.4606, 0.8121)	9	30	23	2	0.818	0.566	0.281	0.938
ANG	0.7444	(0.5700, 0.9189)	7	43	10	4	0.636	0.811	0.412	0.915
APOE	0.6724	(0.4985, 0.8463)	9	27	26	2	0.818	0.509	0.257	0.931
CA9	0.6878	(0.5203, 0.8553)	7	37	16	4	0.636	0.698	0.304	0.902
IL8	0.5146	(0.3146, 0.7146)	4	41	12	7	0.364	0.774	0.250	0.854
MMP9	0.542	(0.3469, 0.7371)	10	14	39	1	0.909	0.264	0.204	0.933
MMP10	0.7238	(0.5787, 0.8690)	8	36	17	3	0.727	0.679	0.320	0.923
PAI1	0.6261	(0.4306, 0.8215)	7	35	18	4	0.636	0.660	0.280	0.897
SDC1	0.6106	(0.3927, 0.8286)	6	47	6	5	0.545	0.887	0.500	0.904
VEGFA	0.5935	(0.3904, 0.7965)	3	52	1	8	0.273	0.981	0.750	0.867
10-biomarker combination	0.8971	(0.8000, 0.9942)	9	45	8	2	0.818	0.849	0.529	0.957

Table 4
Hazard ratio of tumor recurrence based on molecular signature

Biomarker (log10 pg/ml)	HR	LCL	UCL	P-value
MMP-9	1.36	0.48	3.84	0.56
CXCL8/IL-8	0.97	0.31	3.03	0.96
VEGF-A	7.38	0.68	80.43	0.10
IX/CA9	3.44	1.21	9.76	**0.02**
Syndecan-1	6.82	0.29	159.23	0.23
Serpin E1/PAI-1	3.54	0.87	14.43	0.08
ApoE	4.64	0.91	23.59	0.06
Serpin A1	3.72	0.94	14.83	0.06
Angiogenin	42.89	3.06	602.10	**0.005**
MMP-10	3.86	1.06	14.05	**0.04**

4. Discussion

A comprehensive review of the literature regarding potential predictive biomarkers of BCG response reveals limited results. In 2003, investigators reported that the failure to detect urinary IL-2 during a BCG induction course correlated with the time to disease recurrence and progression [24]. Similarly, Kamat and others developed the CyPRIT assay and noted that an increase in nine cytokines, including IL-2, induced after a BCG treatment correlated with disease recurrence, and that combinatorial changes in the cytokine panel could best predict disease recurrence [19]. Monitoring cytokine profile changes is promising as a test of immune response once BCG treatment is initiated and may guide BCG treatment frequency or continuance in the individual.

Previous studies have assessed the ability of the diagnostic FISH test (UroVysionTM, Abbott Molecular Inc.) to predict whether patients with NMIBC would incur disease recurrence or progression after BCG treatment. UroVysion testing was applied at the beginning and the end of the BCG induction cycle (typically involving 6 weekly instillations), and urine cytology and cystoscopy were performed six weeks after cycle completion. In a multivariate analysis, the presence of high-grade disease and a positive UroVysion test after BCG initiation were significant predictors of disease recurrence [25,26]. Again, in these studies, the urine-based results were only informative after the initiation of BCG treatment, and so are not predictive of BCG outcome

Table 5
Annotated urine-based bladder cancer associated diagnostic

Full name	Abbreviation	Ascribed function	Location	Interacts with other members of signature
Interleukin 8	IL8	Chemoattractant & angiogenesis	Extracellular	MMP9, SDC1
Angiogenin	ANG	Angiogenesis	Extracellular, nucleus	None
Vascular endothelial growth factor A	VEGFA	Angiogenesis	Extracellular, cytoplasm	None
Matrix metallopeptidase 9	MMP9	Breakdown of extracellular matrix	Extracellular	IL8, MMP10
Matrix metallopeptidase 10	MMP10	Breakdown of extracellular matrix	Extracellular	MMP9
Serpin peptidase inhibitor	SERPINA1	Serine protease inhibitor	Extracellular	None
Serpin peptidase inhibitor	SERPINE1	Serine endopeptidase inhibitor	Extracellular, plasma membrane	None
Carbonic anhydrase IX	CA9	Catalyze the reversible hydration of carbon dioxide	Plasma membrane	None
Apolipoprotein E	APOE	Lipoprotein catabolism and metabolism	Extracellular, plasma membrane, cytoplasm	None
Syndecan 1	SDC1	Cell binding, cell signaling, cytoskeletal organization	Plasma membrane, cytoplasm	IL8

prior to the first cycle of instillation. Therefore, the information would not be available to guide the clinical management of the individual patient regarding the decision to embark on an intravesical BCG treatment regimen. In a small prospective study, Lotan and others noted a positive FISH test prior to BCG to be associated with recurrence and progression, hazard ratio HR 2.59 (95% CI 1.42–4.73) [27].

Here, we evaluated the performance of the diagnostic Oncuria™ test to predict the response to BCG therapy, *prior* to treatment initiation. In this pilot study, we modified the established diagnostic algorithm, which weights biomarker values to produce a risk score, to the fit the predictive scenario. The 10 biomarkers that comprise the Oncuria™ test were reliably detected in almost all of the 64 urine samples, and the levels of 9 of the biomarkers were elevated in pre-treatment urine samples from subjects who had a subsequent bladder cancer recurrence after the completion of BCG treatment. As shown in diagnostic applications, it is the combination of the biomarker values through a weighted analytical algorithm that provides a model with sufficient predictive power for potential clinical application. In this case, the test shows promise for the evaluation of patients with respect to the decision to undergo BCG treatment.

The biomarkers that compose the bladder cancer diagnostic signature have a varied range of reported functions including angiogenesis, breakdown of extracellular matrix, serine protein inhibitor, catalyze the reversible hydration of carbon dioxide, lipoprotein metabolism and cell binding/signaling (Table 5) with the two primary functional groups being extracellular matrix remodeling (MMP9 and MMP10) and angiogenesis (IL8, VEGFA and ANG). Angiogenesis, the development of new blood vessels from existing blood vessels, is essential for normal growth and development of tissues and organs. A balance of pro-angiogenic factors and anti-angiogenic factors tightly controls this process [28–30]. However in solid tumors, the balance may favor pro-angiogenic factors, ensuring nutrients are provided to the rapidly dividing cancer cells, thus allowing the support of the abnormal growth seen in tumors [31]. Recent studies also suggest ANG and PAI1 can breakdown the extracellular matrix [32,33], allowing cancer cells to invade and metastasize [34]. The extent of tumor vascularization differs between malignancies, and has been shown to correlate directly with metastatic potential [35].

We recognize that the study has several limitations. First, as a pilot study, with only 64 total subjects the study is small and underpowered. Second, defining truly independent disease recurrence and distinguishing new lesions from regrowth from a previous tumor resection site can be challenging. In future studies, we would propose noting the location of the primary tumor, as well as the location and timing of the recurrence, realizing that a second tumor within the previous tumor bed at 3 months follow-up may not necessarily be due to a lack of BCG response, while recurrences outside of the tumor bed may be more indicative of BCG failure. The evaluation of the test in a larger, prospective cohort with more information on lesion characteristics before and after BCG treatment may determine whether the urinary biomarker profile indicates residual disease or a field change in the urothelial or immune landscape which may be predictive of the response to intravesical BCG.

The development of an accurate and robust test that can predict BCG treatment response would benefit both

patients and healthcare systems. The identification of predictive molecular signatures has the potential to better tailor treatment regimens for individual patients, avoiding potentially significant delays in clinical management. In this pilot study, the multiplex Oncuria™ test achieved encouraging predictive performance. The test uses established technology facilitating uptake in clinical laboratories once validated for defined applications. Additional studies are underway to evaluate the potential added value of the test in clinical decision making.

Ethics approval and consent to participate

MD Anderson Cancer Center local ethics review board approved.

Consent for publication

Not applicable.

Availability of data and materials

Reasonable requests for data will be made available for review.

Competing interests

Dr. Charles Rosser is an officer of Nonagen Bioscience. No financial or commercial conflicts of interest were declared by other co-authors.

Conflict of interest

CJR is an officer of Nonagen Bioscience Corporation.

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Author contributions

Conception, interpretation or analysis of data: KM.
Interpretation or analysis of data, revision for important intellectual content: AK.
Interpretation or analysis of data: IP, RC, YS.
Revision for important intellectual content: AG and SG.
Conception, supervision, preparation of the manuscript: CJR and HF.

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