Journal of Translational Medicine

RESEARCH

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Performance of the Oncuria-Detect bladder cancer test for evaluating patients presenting with haematuria: results from a real-world clinical setting

Ian Pagano[1], Zhen Zhang[2], Michael Luu[3], Sergei Tikhonenkov[4], Florence Le Calvez-Kelm[5], Steve Goodison[6], Toru Sakatani[3], Kaoru Murakami[3], Takashi Kobayashi[7], Patrice Avogbe[5], Howard Kim[8], Riko Lee[3], Arnaud Manel[9], Emmanuel Vian[10], Charles J. Rosser[3,8,11] and Hideki Furuya[12*]

Abstract

Background Bladder cancer is the 9th most diagnosed cancer worldwide with high incidences reported in Europe and the United States. Here, we evaluated the real-world performance of a commercially available multiplex immunoassay (Oncuria-Detect, Nonagen Bioscience Corp, Los Angeles, CA, USA) that detects bladder cancer by simultaneously measuring a panel of 10 protein biomarkers in naturally voided urine samples.

Methods We tested prospectively collected urine samples from a real-world cohort of 931 patients presenting to five US centres, one European centre and one Japanese centre with haematuria, in addition to 69 patients with either kidney or prostate cancer (disease controls). The algorithm training/refinement set comprised 617 subjects and the test set included 383 subjects. Assay results were collated with patient clinical data and a cancer diagnosis was defined by biopsy and pathology. The prevalence of bladder cancer in the study was 20%.

Results In the training set, the Oncuria-Detect assay correctly identified bladder cancer in 105 of 121 cases. In the test set, the Oncuria-Detect assay correctly identified bladder cancer in 62 of 73 cases resulting in a sensitivity of 85%, a specificity of 72%, and a negative predictive value (NPV) of 95%. The performance of Oncuria was similar for both low-grade/low-stage and high-grade/high-stage.

Conclusions The multiplex Oncuria assay identified bladder cancer with high sensitivity and NPV. Oncuria's high NPV could effectively rule out 66% of patients from requiring subsequent cystoscopy.

Keywords Biomarkers, Bladder cancer, Haematuria, Multiplex, Protein, Urinalysis

*Correspondence:
Hideki Furuya
hideki.furuya@cshs.org
Full list of author information is available at the end of the article

Background

Multiplex biomarker signatures (DNA, RNA, or protein) have emerged as powerful cancer diagnostic and predicting tools. Several molecular signature assays have been incorporated into clinical practice for managing prostate cancer [1, 2], breast cancer [3, 4], and colon cancer [5, 6]. However, no molecular signatures have been successfully incorporated into clinical practice for diagnosing and managing bladder cancer, which is the 5th most common malignancy in men in the United States and the 9th most common malignancy worldwide [7]. Bladder cancer patients typically present with haematuria and/or irritative voiding symptoms and are evaluated with cystoscopy, an excellent tool because of its low risk and ability to inspect the entire inner lining of the bladder. However, cystoscopy is highly invasive, its accuracy can be reduced by poor visualization caused by inflammatory conditions or bleeding, and flat urothelial lesions such as severe dysplasia and carcinoma in situ (CIS) may be difficult to visually distinguish from normal bladder tissue. For these reasons, non-invasive urine-based assays that can accurately identify and categorise bladder cancer status are desirable.

Over the past decade, several multiplex urine-based tests have been developed and launched into the clinic as laboratory-developed tests (LDTs). These include DNA-based tests (AssuredMDx, UroAmplitude, Early Tect BCD, Uromonitor-V2) and RNA-based tests (CxBladder, BladderCARE) [8]. Limited attention has been given to protein-based tests [9]. The Oncuria-Detect test (Nonagen Bioscience, Los Angeles, CA) is a multiplex immunoassay that analyses 10 protein biomarkers in a single voided urine sample to assess the probability of harbouring bladder cancer. An earlier validation study of Oncuria-Detect achieved 93% sensitivity, 93% specificity and 99% negative predictive value (NPV) in identifying bladder cancer among 362 patients with haematuria [10].

Real-world data include information relating to patient health status and delivery of health care routinely collected from a variety of sources [11]. There is a wealth of clinical data that is routinely collected during clinical practice during the treatment and management of patients. Real-world data typically have different quality controls compared to data collected within a more rigidly structured traditional clinical trial setting. Under certain circumstances, real-world data may be useful in helping to augment understanding of the risk–benefit profile of a diagnostic tool at various points in its development.

The primary objective of the current study was to determine the clinical performance of Oncuria-Detect for evaluating bladder cancer status in patients with haematuria (gross or microscopic). This multicentre study aims to expand upon the body of work on Oncuria by testing its sensitivity and specificity in a large real-world cohort.

Methods

Study population

The tissue repository study prospectively collected voided urine samples during routine care prior to any invasive testing. The retrospective study was performed in accordance with the Declaration of Helsinki and ethical approval was obtained by ethics committees at all study sites. All subjects provided written informed consent.

Voided urine samples were analysed from 999 non-consecutive patients identified from participating sites tumor bank who presenting to the outpatient clinics with haematuria (gross or microscopic) and without known renal insufficiency. Sixty-eight subjects were missing clinical and/or molecular data and were excluded, thus giving a total of 931 subjects at the following sites: International Agency for Research on Cancer, Lyon, France (collection dates 2015–2017; n = 31), University of Hawaii (2015–2018; n = 332), Kyoto University (2005–2010; n = 90), MD Anderson Cancer Center Orlando (2010–2013; n = 55), Mayo Clinic, Florida (2015–2016; n = 5) Cedars-Sinai Medical Center Primary Care (2019–2021; n = 45), Cedars-Sinai Medical Center Urology (2020–2021; n = 18) and University of Florida (2007–2009, n = 355) were included. All subjects underwent standard haematuria evaluation (*i.e.,* cystoscopy and imaging). Subjects whose work-up was negative (*i.e.,* no cancer noted) served as controls. Median follow-up for controls was 12 months. None of the patients had a history of bladder cancer and none of the subjects overlapped with past subjects published.

We additionally obtained 69 disease control urine samples from International Agency for Research on Cancer, Lyon, France (collection dates 2015–2017; n = 6 with prostate cancer), MD Anderson Cancer Center Orlando (2010–2013; n = 1 with renal cell carcinoma), Mayo Clinic (2015–2019; n = 23 with prostate cancer and n = 27 with renal cell carcinoma) and University of Florida (2007–2009, n = 1 with prostate cancer and n = 11 with renal cell carcinoma). The addition of a disease control group consisting of prostate cancer and kidney cancer patients is essential to assess the tumour site specificity of Oncuria-Detect in detecting primary bladder tumours. By comparing bladder cancer cases to individuals with different malignancies, we can determine whether the biomarker is uniquely associated with bladder cancer or if it is influenced by other cancers, thereby assessing its diagnostic accuracy.

Therefore, 1,000 total subjects were included in the study, 617 (121 patients with bladder cancer and 496 non-bladder cancer controls) were used for training/

locking down (training set) the diagnostic algorithm and 383 (73 patients with bladder cancer and 310 non-bladder cancer controls) for testing (test set) the locked-down algorithm with complete demographic and molecular data (Table 1). The disease control group was only included in the test set. At all study sites prior to any invasive procedure, a midstream voided urine was collected, centrifuged and decanted, with urine aliquots then immediately frozen, *i.e.,* the urine samples were collected at the very first cystoscopic evaluation.

When an abnormality was present on cystoscopy, the patient underwent a formal transurethral resection of bladder tumour (TURBT) or biopsy for histological confirmation of urothelial carcinoma, including grade and stage. Data are reported according to International Consensus Panel on Bladder Tumour Markers [12], PRoBE biomarker study design and reporting [13] and Guidelines for Reporting of Statistics for Clinical Research in Urology [14].

Sample collection, storage, and multiplex immunoassay

Oncuria-Detect is a urine test developed to identify de novo bladder cancer according to a 10-protein biomarker signature [10]. With Oncuria, 10 distinct capture bead sets (plate #1 MMP9, IL8, VEGF, CA9; plate #2 A1AT, ANG, APOE, PAI1, SDC1; plate #3 MMP10) allows the 10 target analytes to be concurrently isolated and analysed by incubation with a single urine sample. The test was performed on 300 µL of each urine specimen at a central laboratory (BioAgilityx, Cambridge, MA). Briefly, aliquots of frozen urine were passively thawed and handled on ice. The multiplex immunoassay was conducted according to manufacturer's instructions. Urine specimens were diluted twofold with assay diluent. Samples, standards and controls (50 µL) were added to the 96-well plates (Plates #1–3) in duplicate wells. A seven-point standard curve across the 5-log dynamic range was included in the current assay design. Plates were read on a Luminex® FLEXMAP 3D plate reader (Luminex Corp, Austin, TX).

Data analysis

Descriptive statistics included calculation of mean values, standard deviations (SD), percentages, and 95% confidence intervals (95% CI). Comparisons between groups were made using Kruskal–Wallis test

Table 1 Demographic and clinical-pathologic characteristics of study subjects comparing train and test sets

Training characteristic	Case N = 121	Control N = 496	p-value	Test characteristic	Case N = 73	Control N = 241	Disease control N = 69	p-value
Age			**< 0.001**	Age				**< 0.001**
Mean (SD)	69 (13)	56 (16)		Mean (SD)	72 (10)	54 (16)	68 (9)	
Median [Q1, Q3]	69 [62, 77]	58 [44, 69]		Median [Q1, Q3]	73 [67, 80]	57 [43, 65]	68 [64, 74]	
Min	20	18		Min	48	18	45	
Max	95	94		Max	90	86	86	
Sex			**< 0.001**	Sex				**< 0.001**
Male	105 (87%)	359 (72%)		Male	61 (84%)	159 (66%)	60 (87%)	
Female	16 (13%)	137 (28%)		Female	12 (16%)	82 (34%)	9 (13%)	
Race			**< 0.001**	Race				**< 0.001**
White	85 (70%)	230 (46%)		White	52 (71%)	110 (46%)	58 (84%)	
Black	1 (0.8%)	28 (5.6%)		Black	0 (0%)	15 (6.2%)	3 (4.3%)	
Asian	3 (2.5%)	136 (27%)		Asian	4 (5.5%)	69 (29%)	1 (1.4%)	
Hispanic	4 (3.3%)	16 (3.2%)		Hispanic	1 (1.4%)	7 (2.9%)	4 (5.8%)	
Pacific Islander	2 (1.7%)	29 (5.8%)		Pacific Islander	3 (4.1%)	8 (3.3%)	0	
Other	26 (21%)	57 (11%)		Other	13 (18%)	32 (13%)	3 (4.3%)	
Stage				Stage				
MIBC	32 (27%)	0		MIBC	23 (32%)	0	0	
NMIBC	85 (73%)	0		NMIBC	49 (68%)	0	0	
Unknown	4	496		Unknown	1	241	69	
Grade				Grade				
High	82 (69%)	0		High	52 (71%)	0	0	
Low	37 (31%)	0		Low	21 (29%)	0	0	
Unknown	2	496		Unknown	0	241	69	

Kruskal–Wallis rank sum test; Fisher's Exact Test

(continuous data) or Fisher's Exact Test (categorical data), as appropriate. Unlike in a past publication of Oncuria [10], the concentration of the individual biomarkers did not require log transformation.

We utilised a machine learning pipeline to evaluate various predictive models for detecting primary bladder cancer. Specifically, we explored multiple classification approaches, including logistic regression (glm), elastic net (glmnet), random forest (ranger), XGBoost (xgboost), and decision trees (rpart). Our dataset incorporated patient demographics (age, sex, race), tumor grade and stage and all 10 protein biomarkers.

The entire cohort was split approximately 2:1 into training and test groups stratifying by disease status and institutes and divided into training set (617 urine samples) and testing set (314 + 69 disease controls urine samples). Table 1 demonstrates similarities between the training and test sets. Model evaluation was conducted through cross-validation with stratified folds to ensure robustness. Each model underwent a comprehensive preprocessing workflow, including data imputation, upsampling, normalization, and categorical variable encoding. We used tenfold cross-validation to explore different hyperparameter settings—adjustable factors that affect model performance—and identified the optimal configuration, which was then applied to assess the model's performance on the test set.

We evaluated the performance across all potential cutoffs and selected the one that optimized both sensitivity and specificity. Model sensitivity and specificity were determined based on the correct classification of samples (cancer *vs.* no cancer) as confirmed by cystoscopy. The area under the ROC curve (AUROC) was used to evaluate accuracy in detecting bladder cancer and stratifying risk. The decision tree model, which integrated both clinical and molecular features, demonstrated the highest performance in terms of sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV), AUROC, and overall accuracy.

Similarly, we utilized a machine learning pipeline to evaluate various predictive models for detecting aggressive cancer (*i.e.,* high-grade and/or high-stage). Model sensitivity and specificity were determined based on the correct classification of samples (high-grade and/or high-stage *vs.* low-grade and/or low-stage) as confirmed by histopatholoigic review.

Statistical significance was defined as $p < 0.05$, with all reported p-values being two-sided. Analyses were conducted using SAS software v.9.4 (SAS Institute Inc., Cary, NC).

Results

Characteristics of the study cohort

The clinical, pathologic and demographic characteristic of the combined institutes used for training and testing comprised of 1000 subjects (194 bladder cancer, 737 non-bladder cancer and 69 diseased controls) are listed in Table 1. Median age of bladder cancer subjects was 71 years of age. Of the 194 bladder cancer subjects, 86% (166 of 194) were men and 71% (137 of 194) were white. Of all 194 bladder cancer cases, 69% (134 of 194) were classified as non-muscle invasive bladder cancer (NMIBC; stages Ta, Tis, T1), and 28% (55 of 194) were MIBC, while 30% (58 of 194) cases were reported as low-grade carcinoma and 69% (134 of 194) cases as high-grade.

Single urinary biomarkers

Urinary concentrations of 10 biomarkers were elevated in patients with bladder cancer compared with non-bladder cancer (Table 2) with statistical significance being reached for MMP9, IL8, VEGF, CA9, PAI1, APOE, A1AT, ANG and MMP10, while not being reached for SDC1. Biomarker values below or above the assay's detection limits were set to the detection limit, *i.e.,* extrapolation was not allowed.

Multiple urinary biomarker analyses

In the training set of 617 subjects, the clinical and molecular features of the diagnostic algorithm, using a relevant cutoff of 0.5, resulted in a sensitivity of 87% at a specificity of 74%, an NPV of 96% and a PPV of 44%. Subsequent testing was conducted in an independent test set of 383 samples with the same cutoff of 0.5. The test set had a sensitivity of 85% at a specificity of 72%, an NPV of 95%, and a PPV of 42% (Fig. 1a and Table 3a). Notably, the biomarker signature for aggressive disease using its own clinically relevant cutoff of 0.5, enabled discrimination of high-grade and/or high-stage from low-grade and/or low-stage disease (Fig. 1b and Table 3b), resulting in sensitivity and PPV (79 and 82%, respectively), therefore, providing additional critical data expected of a liquid biopsy to the treating physician.

Discussion

This cross-sectional multicentre study reported the Oncuria-Detect assay for detecting de novo bladder cancer in patients presenting with haematuria. The current findings demonstrate the value of performing a diagnostic test using a real-world dataset that reflects the increased variability seen in clinical practice versus in stringently controlled randomised trials in which many patient subgroups are excluded [11].

Table 2 Mean urinary (± SD) concentrations of 10 biomarkers assessed by Oncuria-Detect in study subjects

Train characteristic	Case N = 121	Control N = 496	p-value	Test characteristic	Case N = 73	Control N = 241	Disease control N = 69	p-value
MMP9			**< 0.001**	MMP9				**< 0.001**
Mean (SD)	4,606 (19,641)	907 (6,431)		Mean (SD)	5,123 (21,666)	572 (2,773)	2,916 (10,672)	
Median [Q1, Q3]	160 [11, 1,245]	11 [11, 93]		Median [Q1, Q3]	214 [18, 2,499]	11 [11, 98]	11 [11, 418]	
Min	1	0		Min	3	0	5	
Max	165,099	126,205		Max	156,738	26,043	69,146	
IL8			**< 0.001**	IL8				**< 0.001**
Mean (SD)	476 (1,224)	136 (699)		Mean (SD)	897 (2,212)	230 (950)	262 (1,540)	
Median [Q1, Q3]	33 [4, 389]	4 [1, 29]		Median [Q1, Q3]	46 [5, 538]	3 [1, 23]	7[1, 37]	
Min	0	0		Min	0	0	0	
Max	8,745	10,659		Max	13,383	8,731	12,685	
VEGF			**< 0.001**	VEGF				**< 0.001**
Mean (SD)	379 (916)	167 (322)		Mean (SD)	298 (629)	145 (281)	172 (504)	
Median [Q1, Q3]	121 [49, 278]	69 [20, 169]		Median [Q1, Q3]	103 [57, 283]	58 [12, 136]	41 [4, 124]	
Min	1	1		Min	2	1	1	
Max	6,392	2,967		Max	4,905	1,918	3,797	
CA9			**< 0.001**	CA9				**< 0.001**
Mean (SD)	45 (181)	3 (12)		Mean (SD)	23.2 (100.0)	2.4 (5.8)	8.9 (47.3)	
Median [Q1, Q3]	2 [1, 10]	1 [1]		Median [Q1, Q3]	2.4 [0.7, 7.4]	0.7 [0.6, 1.1]	0.6 [0.6, 3.2]	
Min	0	0		Min	0.2	0.1	0.1	
Max	1,349	123		Max	766.8	51.4	392.1	
SDC1			0.361	SDC1				0.632
Mean (SD)	10,691 (7,579)	9,644 (6,378)		Mean (SD)	9,996 (6,738)	9,372 (5,944)	8,309 (3,963)	
Median [Q1, Q3]	8,587 [4,935, 14,868]	8,047 [4,953, 12,910]		Median [Q1, Q3]	8,542 [4,919, 14,686]	8,136 [5,100, 12,503]	8,421 [5,347, 10,778]	
Min	897	209		Min	390	23	1,347	
Max	36,357	36,106		Max	29,502	33,767	17,806	
PAI1			**< 0.001**	PAI1				**< 0.001**
Mean (SD)	730 (3,080)	153 (1,918)		Mean (SD)	527 (1,309)	65 (203)	2,069 (16,611)	
Median [Q1, Q3]	45 [7, 177]	4 [3, 12]		Median [Q1, Q3]	55 [13, 247]	4 [3, 9]	5 [2, 16]	
Min	1	0		Min	1	0	1	
Max	29,199	42,069		Max	7,914	1,628	138,039	
APOE			**< 0.001**	APOE				**< 0.001**
Mean (SD)	13,073 (66,147)	1,482 (6,394)		Mean (SD)	5,226 (10,041)	1,180 (3,155)	4,629 (9,343)	
Median [Q1, Q3]	834 [238, 2,879]	338 [125, 958]		Median [Q1, Q3]	1,593 [353, 4,675]	298 [117, 804]	1,152 [360, 4,930]	
Min	52	3		Min	10	13	31	
Max	523,750	116,361		Max	64,295	31,875	56,423	
A1AT			**< 0.001**	A1AT				**< 0.001**
Mean (SD)	4,316,853 (24,854,132)	1,808,960 (16,609,249)		Mean (SD)	2,181,852 (9,507,126)	1,195,381 (13,152,722)	751,446 (4,664,021)	
Median [Q1, Q3]	83,334 [21,220, 258,514]	16,734 [5,348, 54,502]		Median [Q1, Q3]	102,321 [35,034, 260,601]	14,878 [4,775, 64,628]	23,238 [5,413, 106,414]	
Min	550	56		Min	56	49	863	
Max	192,712,549	191,671,537		Max	68,330,920	191,671,537	38,067,674	
ANG			**< 0.001**	ANG				**< 0.001**
Mean (SD)	3,235 (11,938)	1,051 (7,427)		Mean (SD)	4,180 (15,986)	894 (3,724)	1,394 (5,088)	
Median [Q1, Q3]	631 [86, 2,080]	118 [22, 481]		Median [Q1, Q3]	769 [167, 1,582]	87 [17, 510]	82 [10, 582]	
Min	2	0		Min	3	2	3	
Max	116,670	123,980		Max	116,670	43,371	37,306	
MMP10			**< 0.001**	MMP10				**< 0.001**
Mean (SD)	158 (652)	41 (699)		Mean (SD)	58 (187)	11 (31)	16 (41)	
Median [Q1, Q3]	8 [4, 26]	4 [4, 7]		Median [Q1, Q3]	9 [4, 26]	4 [4, 7]	4 [3, 11]	
Min	1	0		Min	2	1	1	
Max	5,356	15,569		Max	1,509	299	320	

Kruskal–Wallis rank sum test; Fisher's Exact Test

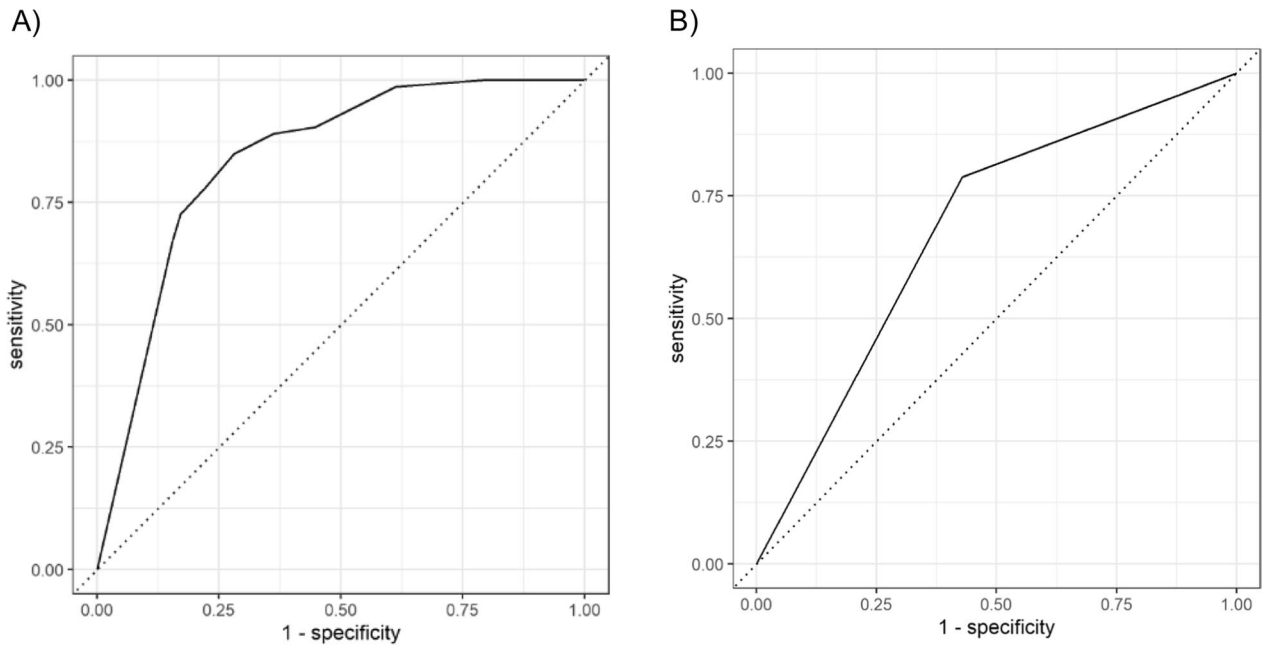

Fig. 1 Receiver operating characteristic (ROC) curves for Oncuria-Detect in (**A**) evaluating patients with haematuria for any grade/stage bladder cancer and (**B**) evaluating patients with haematuria for high-grade and/or high-stage disease

Table 3 Summary of diagnostic performance of Oncuria-Detect in (a) distinguishing cancer and (b) distinguishing high-grade and/or high-stage cancer

A									
Training Outcome	TN	FP	FN	TP	Accuracy	Sens	Spec	PPV	NPV
Overall	365	131	16	105	0.76	0.87	0.74	0.44	0.96
MIBC	379	202	2	30	0.67	0.94	0.65	0.13	0.99
NMIBC	367	161	14	71	0.71	0.84	0.7	0.31	0.96
High Grade	373	160	8	74	0.73	0.9	0.7	0.32	0.98
Low Grade	373	205	8	29	0.65	0.78	0.65	0.12	0.98
Test Outcome	TN	FP	FN	TP	Accuracy	Sens	Spec	PPV	NPV
Overall	223	87	11	62	0.74	0.85	0.72	0.42	0.95
MIBC	230	129	4	19	0.65	0.83	0.64	0.13	0.98
NMIBC	227	106	7	42	0.7	0.86	0.68	0.28	0.97
High Grade	227	104	7	45	0.71	0.87	0.69	0.3	0.97
Low Grade	230	132	4	17	0.64	0.81	0.64	0.11	0.98
B									
Training Outcome	TN	FP	FN	TP	Accuracy	Sens	Spec	PPV	NPV
Overall	25	12	24	58	0.7	0.71	0.68	0.83	0.51
Test Outcome	TN	FP	FN	TP	Accuracy	Sens	Spec	PPV	NPV
Overall	12	9	11	41	0.73	0.79	0.57	0.82	0.52

TP: true positive; TN: true negative; FP: false positive; FN: false negative; Sens: sensitivity; spec: specificity; PPV: Positive predictive value; NPV: Negative predictive value

The developmental history of the Oncuria bladder cancer test has been previously described, including initial transcriptome and proteome mining studies [15–18], selection and validation of contender biomarker analytes [19–22], to multiplex assay customization, optimization and testing [23, 24]. In this study, the Oncuria assay was used to evaluate 931 naturally micturated urine samples prospectively obtained from patients with haematuria who visited outpatient clinics at seven institutions. Specifically, after algorithm training and cut-off selection, ROC performance characteristics illustrated a favourable sensitivity 85% at a specificity of 72%, indicating considerable clinical utility in discerning cancer from non-cancer status. The selected cut-off was purposefully selected to capture the highest number of cancer-positives because missing a true-positive cancer diagnosis is ultimately more important than needing to further investigate a potential false-positive [14, 25, 26]. Considering a disease prevalence rate of 20% as estimated by the participating centres, which is on par to the prevalence in the intended population [27], the Oncuria assay's overall NPV was 95%. When evaluating haematuria, a bladder cancer assay with a high NPV is essential and can obviate the need for additional invasive cystoscopy and TURBT, both of which are associated with complications such as a urinary tract infection, bleeding, and pain. A high NPV assures the urologist that no bladder tumours are left undiagnosed. In the current study, Oncuria's high NPV could have effectively ruled out 66% of patients from requiring subsequent cystoscopy. Furthermore, for detecting high-grade and high-stage bladder cancers, Oncuria had a sensitivity and PPV of 79 and 82%, respectively, illustrating the utility associated with Oncuria's ability to rule-in who has aggressive cancer.

Bladder cancer detection currently relies upon invasive cystoscopic examination of the bladder and urine cytology. While cystoscopy has a sensitivity of 85–90% for detecting papillary tumours and 67% for CIS, it may miss up to 20% of small, low-grade tumours [28]. The Oncuria assay had an overall sensitivity of 85% with an associated high NPV for low-grade and low stage tumours, 98 and 97%, respectively. Cytology requires that the tumour sheds exfoliated cells into the urine for microscopic analysis, and that these cells are captured and visualised. Because not all bladder tumours shed cells, the sensitivity of cytology has frequently come into question [29]. In one analysis, cytology showed an overall sensitivity of 48% for identifying bladder cancer, but only 16% sensitivity for detecting low-grade tumours [30]. Oncuria demonstrated superior overall sensitivity than both cystoscopy and cytology in identifying bladder cancer, with the most obvious comparative benefit being Oncuria's ability to reliably and non-invasively identify low-grade tumours.

In this study, Oncuria's overall specificity of 72% for identifying bladder cancer is lower than previously reported (93%) [10]; this is likely due, at least in part, to the use of real-world samples collected across multiple sites and time frames. Although clinical trials remain the definitive approach for elucidating causal relationships, large amounts of real-world evidence, by definition non-randomised and unblinded, accumulate during the clinical employment of treatments and diagnostics and may prove to be useful. Importantly, these real-world data often comprise subjects with conditions and vulnerabilities (*e.g.*, multimorbidity, etc.) that may exclude them from participation in randomised trials. For example, though we purposefully excluded patients with a known history of renal insufficiency, those without a formal diagnosis may have been included. We know from past reports that in the face of renal insufficiency, patients spill abundant amounts of proteins into the urine, which can adversely affect the performance of Oncuria [31].

Protein-based urine assays have significant advantages over its DNA- or RNA-based counterparts for bladder cancer detection. First, no target amplification is needed for protein analysis, whereas PCR amplification used by some nucleic acid tests can increase assay costs, time-inputs, and complexity. Second, secreted proteins are generally stable molecules, especially compared to mRNA transcript and noncoding microRNA targets [32]. Third, while protein biomarkers are secreted from living cells, DNA targets for mutation, methylation, and copy number analysis depend on tumour cell shedding, tumour cell apoptosis or cell death to release these biomarkers. Thus, nucleic acid analytes may be present in urine in low and variable amounts, especially in early disease with a limited tumour burden.

Identifying multiple-protein urinary signatures of bladder cancer may improve testing accuracy compared to evaluating single proteins [9]. Current FDA-approved protein-based urine bladder cancer assays are single-plex tests that evaluate urinary levels of either bladder tumour antigen (BTA; *e.g.*, BTA-Stat® and BTA-Trak®) or nuclear matrix protein 22 (NMP22; *e.g.*, NMP22 BladderChek®) [33]. Both BTA and NMP22 assays have difficulty in identifying low-grade bladder cancer (BTA sensitivity = 36%; NMP22 sensitivity = 25%), and both analytes may be elevated in non-neoplastic inflammatory states. A newer single-plex protein-based assay, ADxBladder®, measures levels of mini chromosome maintenance 5 (MCM5) protein, a marker of cell proliferation, also has a low 50% sensitivity (95%CI: 0.36–0.64) for detecting low-grade tumours (79% for high-grade tumours), an AUC

of 0.75, and an NPV of 96.4% [34]. Importantly, neither BTA, NMP22, nor MCM5 is uniquely expressed by bladder cancer cells [33]. Multiplex protein analysis offers improved diagnostic accuracy by simultaneously evaluating urine levels of multiple biomarkers that together yield a bladder cancer signature that can identify and characterise disease states. The multiplex Oncuria assay simultaneously evaluates urinary levels of 10 protein biomarkers and automatically calculates a bladder cancer risk score using a weighted algorithm that additionally considers age, gender, and race [35]. The multiplex Oncuria assay's current overall sensitivity for identifying bladder cancer, 85%, is higher than the sensitivity achievable with contemporary single-plex protein tests.

While some may contend that Oncuria-Detect's performance in the evaluation of patients with haematuria (sensitivity 85%, specificity 72%) may be inadequate for clinical implementation, it is essential to consider the specific clinical utility and intended role of the test in this context. Again, these patients with haematuria may have a positive evaluation for cancer ranging from 5–25%. Therefore, we perform 75–95% negative cystoscopies in evaluating them. If we could reliably rule out 66% and reduce these negative cystoscopies to 9–29%, then we obviously are subjecting fewer patients to negative cystoscopy, relieving backlog in our cystoscopy scheduling and saving the US healthcare system up to $379 M/year (average $667 for cystoscopy: CPT 52000 [36]. Therefore, if a patient presents with haematuria and a urine sample demonstrates a positive Oncuria-Detect finding this would then give the treating urologist a greater sense of certainty that immediate cystoscopy is in order as we know that delays in evaluating bladder cancer patients can have a profound effect on survival [37]. Barriers to the implementation of Oncuria-Detect include special handling of the urine samples, resistance of commercial payers to reimburse, and the inability to be incorporated into practice guidelines.

A primary strength of the present investigation is that it is one of the largest real-world studies conducted to date for testing a urinary biomarker panel for detecting *de novo* bladder cancer. Utilizing multicentre real-world data diminishes the potential impact of selection bias and expands generalizability of study findings. The multiplex proteomics approach represents the application of state-of-the-art technology in efforts to optimise diagnostic performance. Study limitations include the lack of detailed information on the extent of haematuria (*i.e.*, microscopic versus gross) for which participants initially sought treatment, lack of detailed information on the tumour stage (*i.e.,* Ta, T1, etc.) and the potential for selection bias in patient recruitment (*i.e.,* patients with complex medical history may have been omitted). Potential confounding influences that were not controlled for include sample preparation technique and storage conditions, but this would tend to worsen the test performance based on previous work [38]. Also, information on clinical variables such as tobacco use, tumour size, and tumour multiplicity were not available for all samples. However, we believe that these potential confounders represent real-life conditions that may exist during ordinary clinical evaluation. Current findings are generalizable across adults in the French, Japanese and US populations enrolled in this study. These cross-sectional data will be validated in ongoing multicentre, international prospective clinical trials (NCT 03193528 and NCT 03193541) evaluating subjects with gross and microscopic haematuria.

Conclusions

This large cross-sectional real-world multicentre study of the Oncuria-Detect urine test demonstrated clinically relevant assay sensitivity and negative predictive values when evaluating patients with haematuria for bladder cancer. This urine-based bladder cancer diagnostic test holds promise for non-invasively evaluating bladder cancer risk in an outpatient setting.

Abbreviations

NPV	Negative predictive value
CIS	Carcinoma in situ
LDT	Laboratory-developed test
TURBT	Transurethral resection of bladder tumor
PRoBE	Prospective specimen collection, retrospective blinded evaluation
A1AT	SerpinA1
ANG	Angiogenin
APOE	Apolipoprotein
CA9	Carbonic anhydrase 9
IL8	Interleukin 8
MMP9	Matrix metalloproteinase 9
MMP10	Matrix metalloproteinase 10
PAI1	Plasminogen activator inhibitor 1/SerpinE1
SDC1	Syndecan 1
VEGF	Vascular endothelial growth factor
SD	Standard deviation
AUCROC	Area under the ROC curve
PPV	Positive predictive value
MCM5	Mini chromosome maintenance 5

Acknowledgements

We are thankful to Matthew Silverman PhD (Biomedical Publishing Solutions; Tallahassee, FL) for scientific and technical editing. This work was supported in part by the Biostatistics Shared Resource of Cedars-Sinai Cancer Center.

Disclaimer

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Author contributions

Study concept and design—Zhang, Furuya, Rosser. Acquisition of samples and data—Tikhonenkov, Le Calvez-Kelm, Goodison, Sakatani, Murakami, Avogbe, Kim, Kobayashi, Lee, Manel, Vian. Data analysis and interpretation—Zhang, Pagano, Luu. Drafting of manuscript—Furuya. Critical revision of the

manuscript for important technical content—all authors. Obtaining funding—Rosser. Administrative technical and material support—Rosser, Furuya. Supervision—Rosser.

Funding
This work was supported by research grants UH3 CA271377 (CJR), R01 CA277810 (HF/CJR), R01 CA1988887 (CJR) and U54 CA274375-01 (HF/CJR). In addition, the study was sponsored by Nonagen Bioscience. They were involved in the design of the study and allowed access to the proprietary Oncuria kits.

Availability of data and materials
Reasonable requests for data will be made available for review.

Declarations

Ethics approval and consent to participate
Cedars Sinai Local ethics review board approved. Subject gave written consent.

Consent for publication
Not applicable.

Competing interests
Dr. Charles Rosser is an officer of Nonagen Bioscience. All other authors declare that they have no competing interests.

Author details
[1]Population Sciences in the Pacific Program, University of Hawaii Cancer Center, Honolulu, HI, USA. [2]Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD, USA. [3]Cedars-Sinai Medical Center, Samuel Oschin Comprehensive Cancer Institute, Los Angeles, CA, USA. [4]Translational and Clinical Program, University of Hawaii Cancer Center, Honolulu, HI, USA. [5]International Agency for Research On Cancer, Lyon, France. [6]Quantitative Health Sciences, Mayo Clinic Florida, Jacksonville, FL, USA. [7]Department of Urology, Kyoto University, Kyoto, Japan. [8]Department of Urology, Cedars Sinai Medical Center, Los Angeles, CA, USA. [9]Le Creusot Hospital, Le Creusot, France. [10]Urology Department, Protestant Clinic of Lyon, Lyon, France. [11]Nonagen Bioscience Corp, Los Angeles, CA, USA. [12]Department of Biomedical Science, Cedars-Sinai Medical Center, 110 N. George Burns Road, Davis 2025, Los Angeles, CA 90048, USA.

Received: 6 May 2025 Accepted: 9 June 2025
Published online: 18 June 2025

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