Development of a 90-Minute Integrated Noninvasive Urinary Assay for Bladder Cancer Detection

Ellen Wallace,*,† Russell Higuchi,† Malini Satya,† Leena McCann,† Mandy L. Y. Sin, Julia A. Bridge,† Huilin Wei,† Jun Zhang,† Edith Wong,† Andrew Hiar, Kathleen E. Mach, Douglas Scherr, R. Blair Egerdie, Shoichiro Ohta, Wade J. Sexton, Maxwell V. Meng, Alon Z. Weizer, Michael Woods, G. Kenneth Jansz,† Joseph Zadra, Yair Lotan,† Bernard Goldfarb and Joseph C. Liao

From Cepheid (EW, RH, MS, LM, HW, JZ, EW, AH), Sunnyvale, Department of Urology, Stanford University School of Medicine (MLYS, KEM, JCL), Stanford, Veterans Affairs Palo Alto Health Care System (MLYS, KEM, JCL), Palo Alto and University of California-San Francisco (MVM), San Francisco, California, Department of Pathology and Microbiology, University of Nebraska Medical Center (JAB), Omaha, Nebraska, Weill Cornell Medical College (DS), New York, New York, Urology Associates/Urologic Medical Research (RBE), Kitchener, Burlington (GKJ), The Male/Female Health and Research Centre (JZ), Barrie and North Bay (BG), Ontario, Canada, Kan-Etsu Hospital (SO), Saitama, Japan, Moffitt Cancer Center (WJS), Tampa, Florida, University of Michigan (AZW), Ann Arbor, Michigan, University of North Carolina (MW), Chapel Hill, North Carolina, and University of Texas Southwestern Medical Center (YL), Dallas, Texas

Purpose: Despite suboptimal sensitivity urine cytology is often performed as an adjunct to cystoscopy for bladder cancer diagnosis. We aimed to develop a noninvasive, fast molecular diagnostic test for bladder cancer detection with better sensitivity than urine cytology while maintaining adequate specificity.

Materials and Methods: Urine specimens were collected at 18 multinational sites from subjects prior to cystoscopy or tumor resection, and from healthy and other control subjects without evidence of bladder cancer. The levels of 10 urinary mRNAs were measured in a training cohort of 483 subjects and regression analysis was used to identify a 5-mRNA model to predict cancer status. The performance of the GeneXpert® Bladder Cancer Assay, an assay labeled for investigational use only to detect the 5 mRNAs *ABL1, CRH, IGF2, ANXA10* and *UPK1B*, was evaluated in an independent test cohort of 450 participants.

Results: In the independent test cohort the assay ROC curve AUC was 0.87 (95% CI 0.81–0.92). At an example cutoff point of 0.4 overall sensitivity was 73% while specificity was 90% and 77% in the hematuria and surveillance patient populations, respectively.

Conclusions: We developed a 90-minute, urine based test that is simple to perform for the detection of bladder cancer. The test can help guide physician decision making in the management of bladder cancer. Additional evaluation in a prospective study is needed to establish the clinical usefulness of this assay.

Key Words: urinary bladder neoplasms; hematuria; pathology, molecular; RNA, messenger; early detection of cancer

Abbreviations and Acronyms

ABL1 = ABL proto-oncogene 1
ANXA10 = annexin 10
BCa = bladder cancer
BCG = bacillus Calmette-Guérin
CIS = carcinoma in situ
CRH = corticotropin releasing hormone
Ct = cycle threshold
IGF2 = insulin-like growth factor 2
NPV = negative predictive value
PPV = positive predictive value
ROC = receiver operating characteristic
RT-qPCR = reverse transcriptase-quantitative polymerase chain reaction
TUR = transurethral resection
UPK1B = uroplakin 1B
UTI = urinary tract infection

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* Correspondence: Cepheid, 904 Caribbean Dr., Sunnyvale, California 94089 (telephone: 408-400-8449; e-mail: ellen.wallace@cepheid.com).
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In the United States BCa is the sixth most common cancer with approximately 79,000 new cases and 17,000 disease specific deaths predicted in 2017.[1] Most BCa cases present as nonmuscle invasive lesions that are managed by TUR but a high recurrence rate necessitates frequent surveillance.[2]

Cystoscopy, often with adjunct urine cytology, is the current standard for the diagnosis and surveillance of BCa.[3] Cytology is the only urine based test recommended for BCa diagnosis and surveillance in AUA (American Urological Association) and EAU (European Association of Urology) guidelines.[3,4] A review of 26 reports showed that the median sensitivity and specificity of cytology for BCa surveillance were 35% and 94%, respectively.[5] Of particular concern is the relatively poor 58% sensitivity of cytology for high grade BCa. Comparatively cytology is also more labor and time intensive and subject to interobserver variability.[6]

Many molecular diagnostic tests have been developed to complement or replace cytology and cystoscopy.[7] Assays that measure single protein markers such as BTA stat®, BTA TRAK® and NMP22® BladderChek® often show low sensitivity and/or specificity. More complex assays such as ImmunCyt/uCyt+ (Scimedx, Dover, New Jersey), UroVysion® Bladder Cancer Kit and Cxbladder™ are often more time-consuming and costly, and/or the result of analysis is subjective.

The GeneXpert instrument system is a multiplex polymerase chain reaction platform that integrates and automates sample preparation and target detection.[8] The versatile system allows for the development of assays that are simple to run and provide objective results in 30 to 120 minutes depending on the assay. Our goal was to develop a noninvasive, fast and simple assay to detect BCa on the GeneXpert system.

MATERIALS AND METHODS

Subjects and Specimens

Institutional review board approval was obtained from each of the 18 study sites (supplementary table, http://jurology.com/). Voided urine specimens and corresponding clinical information were collected. Subjects with gross hematuria or UTI were not excluded from analysis. Surveillance subjects were defined as those with a history of BCa. Subjects with hematuria were defined as those with gross or microscopic hematuria who were undergoing initial evaluation for BCa. Urine specimens were collected prior to office white light cystoscopy or tumor resection. In BCa positive cases cancer stage and grade were established by histopathological examination of resected tissue. The control group included healthy subjects and subjects with noncancerous urological diseases such as UTI, nephrolithiasis and ureteral stricture.

Urine Testing

Voided urine specimens were added to an equal volume of GeneXpert Urine Transport Reagent within 1 hour of specimen collection. The reagent lyses red and white blood cells present in urine but preserves urothelial cells and their RNA content, facilitating use of the test in specimens with gross hematuria or UTI.

The preserved urine samples were tested by transferring 4 ml to the GeneXpert cartridge and inserting the cartridge into the GeneXpert instrument. All reagents required for sample preparation and RT-qPCR analysis were preloaded in the cartridges by the manufacturer. In the cartridge the urine cells are filter captured and lysed by sonication. The released nucleic acid is eluted and mixed with dry RT-qPCR reagents, and the solution is transferred to the reaction tube for RT-qPCR and detection.[9]

Urine samples in the training cohort were tested with 2 separate cartridge assays to measure all 10 targets, including *ABL1*, *CRH, IGF2, ANXA10, KRT20*, *AR, PIK3CA, UPK1B, UPK2* and *MGEA5. ABL1* served as a sample adequacy control for each assay to ensure sufficient assay input. Primer and probe sets were tested with human genomic DNA to ensure that only mRNA was significantly amplified. Probes were labeled with one of 6 dye and quencher combinations, enabling multiplex detection. Each reaction contained 0.25 U/μl AptaTaq™, 0.38 U/μl M-MLV Reverse Transcriptase (Roche Custom-Biotech, Indianapolis, Indiana), 0.4 U/μl ribonuclease inhibitor (Roche), 75 mM KCl, 3.5 mM $MgCl_2$ and 20 mM tris, pH 8.5. Thermal cycling conditions were 45C for 10 minutes for reverse transcription and then 45 cycles at 95C, 60C and 72C for 5, 20 and 20 seconds, respectively.

Test cohort urines were tested with a 5-mRNA GeneXpert assay that contained primers and probes to detect *ABL1, CRH, IGF2, ANXA10* and *UPK1B*, and an internal control RNA. The concentration of the salts and enzymes was the same as in the training cohort assays but the 45C hold time for reverse transcription was extended to 15 minutes.

Statistical Analysis

Descriptive statistics were calculated for the training cohort. Ct and ΔCt, calculated as *ABL1* Ct − target Ct, were standardized by subtracting the average Ct or ΔCt and dividing by the SD of that mRNA target. Stepwise logistic regression was done on a preliminary data set to identify a reduced set of 5 mRNAs for the prediction model. The final regression model was then fit using training cohort ΔCt data for *IGF2, UPK1B, CRH* and *ANXA10*, and Ct data for *ABL1*. Model performance was assessed on the test cohort using ROC curves, AUCs and measures of diagnostic sensitivity and specificity. Analyses were performed with JMP®, version 12.

RESULTS

Initial Marker Selection

We first identified more than 100 mRNA candidate markers from the published literature and measured expression by RT-qPCR in urine specimens from