

**Title:** Deep Sequencing of Urinary RNAs for Bladder Cancer Molecular Diagnostics

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## **Translational Relevance**

While bladder cancer can be managed by transurethral resection in most patients, the high recurrence rate necessitates lifetime cystoscopic surveillance. The need for frequent invasive surveillance contributes to the high cost of treatment for bladder cancer. A urine test with sufficient accuracy to prioritize high-risk patients to undergo timely cystoscopy and reduce procedural frequency for low-risk patients has remained elusive. This study focused on identifying bladder cancer-specific urinary mRNA markers by sequencing RNA extracted from urine sediment using RNA-seq. A model based on gene expression of a 3-marker panel was constructed. Validation of the model demonstrated a similar sensitivity for high grade and an improved sensitivity for low grade cancer compared to other urine tests suggesting the high translational potential of our panel as majority of bladder cancer patients present with low grade disease. Moreover, serial testing with our panel following 6 patients was consistent with cystoscopic and pathologic results indicating our urine test may serve as a complement to cystoscopy.

## Abstract

**Purpose.** The majority of bladder cancer patients present with localized disease and are managed by transurethral resection. However, the high rate of recurrence necessitates lifetime cystoscopic surveillance. Developing a sensitive and specific urine-based test would significantly improve bladder cancer screening, detection, and surveillance.

**Experimental Design.** RNA-seq was used for biomarker discovery to directly assess the gene expression profile of exfoliated urothelial cells in urine derived from bladder cancer patients (n=13) and controls (n=10). Eight bladder cancer specific and 3 reference genes identified by RNA-seq were quantitated by qPCR in a training cohort of 102 urine samples. A diagnostic model based on the training cohort was constructed using multiple logistic regression. The model was further validated in an independent cohort of 101 urines.

**Results.** 418 genes were found to be differentially expressed between bladder cancer and controls. Validation of a subset of these genes was used to construct an equation for computing a probability of bladder cancer score ( $P_{BC}$ ) based on expression of 3-markers (*ROBO1*, *WNT5A*, and *CDC42BPB*). Setting  $P_{BC}=0.45$  as the cutoff for a positive test, urine testing using the 3-marker panel had overall 88% sensitivity and 92% specificity in the training cohort. The accuracy of the 3-marker panel in the independent validation cohort yielded an area under the curve of 0.87 and overall 83% sensitivity and 89% specificity.

**Conclusions.** Urine-based molecular diagnostics using this 3-marker signature could provide a valuable adjunct to cystoscopy and may lead to a reduction of unnecessary procedures for bladder cancer diagnosis.

## Introduction

Bladder cancer is the fifth most common cancer with about 74,000 new cases and 16,000 disease-specific deaths in 2015 in the United States (1). The majority of cases are non-muscle invasive bladder cancer (NMIBC) at diagnosis and are primarily managed with transurethral resection (TUR). With a recurrence rate of up to ~70% at 5 years, bladder cancer requires lifelong cystoscopic surveillance (2). Due to the invasiveness of cystoscopy, there are strong interests to develop non-invasive urine-based diagnostics. A reliable urine test could improve surveillance strategies by prioritizing high-risk patients to undergo cystoscopy and biopsy, while reducing procedural frequency in low-risk patients. Despite inadequate diagnostic sensitivity for low grade (LG) and high grade (HG) cancer at ~20% and ~80%, respectively, urine cytology is commonly performed due to its high specificity (>95%) and positive predictive value (3). Other FDA-approved urine tests including single-biomarker immunoassays, fluorescent immunohistochemistry, and fluorescence *in-situ* hybridization (4,5) are available. However, these tests have not been widely adopted due to insufficient diagnostic performance (6).

Emerging bladder cancer molecular diagnostics have focused on development of multi-biomarker panels ranging from 2 to 18 targets (7-11). Most biomarker discovery efforts have depended on microarray-based screening of the bulk mass of tumor tissues. However, challenges of lower specificity than cytology and low sensitivity for LG tumors have hindered adoption of these test (8,12). To identify biomarkers for urine-based molecular diagnostics, exfoliated urothelial cells may be a better starting material given the continuous contact of bladder tumors with urine (13). RNA sequencing (RNA-seq) is a next generation sequencing technology that offers unbiased identification of known and novel transcripts, single base-pair resolution, high sensitivity and high specificity, broad dynamic range of over 8000-fold for gene expression quantification and ability to detect rare and low-abundance genes (14).

In this study, we applied RNA-seq as a discovery tool for bladder cancer-specific urinary RNA markers. Deep sequencing of RNA extracted from urine sediments from bladder cancer patients and controls resulted in an average of 100 million sequencing reads per sample. Genes selected based on the RNA-seq analysis were evaluated using quantitative real-time polymerase chain reaction (qPCR) in a training cohort. This data was used to select a 3-marker panel consisting of two cancer-specific genes (*ROBO1*, *WNT5A*) and one reference gene (*CDC42BPB*). The diagnostic accuracy of the 3-marker panel was evaluated in an independent patient cohort and compared favorably to urine cytology.

## Methods

### Study design

The study protocol was approved by the Stanford University Institutional Review Board and Veterans Affairs Palo Alto Health Care System (VAPAHCS) Research and Development Committee. All patients were recruited from VAPAHCS. The study was divided into 3 parts: 1) biomarker discovery, 2) construction of the diagnostic model, and 3) validation of the diagnostic model (Figure 1). For each part, urine samples were collected from bladder cancer and control subjects. Patients of both genders  $\geq 18$  years old were eligible for enrollment. Patients with other urological cancer were excluded. For biomarker discovery, urine samples were collected from 23 subjects (13 bladder cancer and 10 controls) for RNA-seq. To construct the diagnostic model, expression of candidate genes identified by RNA-seq was analyzed in urinary RNA extracts from a training cohort of 102 urines samples (50 bladder cancer and 52 controls) using qPCR. The 3-marker diagnostic panel was then validated in 101 urine samples (47 bladder cancer and 54 controls) to determine assay diagnostic sensitivity and specificity. The diagnostic performance of the 3-marker panel was also compared to urine cytology collected per routine clinical care in a subset of samples.

### Patient population and samples

The “bladder cancer-evaluation” group included patients with no prior history of bladder cancer who were undergoing urological work-up, primarily for hematuria. The “bladder cancer-surveillance” group included patients with prior history of bladder cancer undergoing routine surveillance. The “control” group included patients with non-neoplastic urological diseases and healthy volunteers  $\geq 35$  years old. Urine was collected prior to cystoscopy or tumor resection for bladder cancer-evaluation and surveillance groups and mid-day for the control group. Urine samples were categorized as cancer or benign based on corresponding tissue histopathology from TUR or cystoscopic biopsy when available. For urine samples without a matching tissue sample from bladder cancer evaluation or surveillance patients, diagnosis was based on cystoscopic findings. Urine samples from patients with non-neoplastic urological diseases (e.g. kidney stones) and healthy control groups that did not undergo cystoscopy were presumed negative for bladder cancer based on clinical history (Table 1). Cytology results were considered positive when reported as suspicious or malignant, and negative when reported as atypical or negative.

### **Urine sample preparation**

For RNA-seq, urine samples (10 - 750 ml) were processed within two hours of collection. Urine sediment was collected by centrifugation for 15 minutes at 500xg and pellets were washed 3 times with PBS. Washed urine sediment was depleted of red and white blood cells (RBCs and WBCs). RBCs were selectively lysed by addition of 1 ml of 10-fold diluted RBC lysis solution (Miltenyi Biotec). Remaining cells were collected by centrifugation at 300xg for 5 minutes and cell pellets were washed 3 times with PBS. To deplete WBCs, cells were incubated for 15 minutes at 4°C with 80 µl of magnetic-activated cell sorting (MACS) buffer (PBS, 0.5% bovine serum albumin, and 2 mM EDTA) and 20 µl of anti-CD45 magnetic micro-beads. Then 1 ml of MACS buffer was added and cells collected by centrifuged at 300xg for 15 minutes at 4°C. The cells were re-suspended in 500 µl MACS buffer and applied to a MACS LD column (Miltenyi Biotec). The column was washed twice with 1 ml MACS buffer and the total effluent was collected. For RNA extraction, urothelial cells were collected by centrifugation and resuspended in 1 ml TRIzol® (Invitrogen) and stored at -80°C. Total RNA from the urothelial cells was extracted with TRIzol® reagent followed by DNA degradation with RQ1 RNase-free DNase (Promega) then purification on RNeasy® MinElute Cleanup columns (Qiagen) according to the manufacturer's instructions. An Agilent 2100 Bioanalyzer and RNA Pico chips were used for total RNA quantification and qualification analysis. RNA concentration and RNA integrity number (RIN) were determined for each sample.

### **Library preparation and RNA-seq**

cDNAs were synthesized from samples with total RNA  $\geq 6$  ng in 12 µl of nuclease-free water using the Ovation RNA Seq System V2 kit (NuGEN Technologies) according to manufacturer's instructions. cDNAs were fragmented with S-Series Focused-ultrasonicator (Covaris). To enrich for cDNAs >300 bases in length, cDNAs were size fractionated by incubating with 0.8 volume of Agencourt AMPure XP beads (Beckman Coulter) for 10 minutes followed by bead separation on 96S super magnet plate (Alpaque) for 10 minutes. Beads were then washed three times with 80% ethanol and air-dried for 15 minutes on the magnetic plate. cDNA products were eluted with 102 µl of RNase-free water and quantity was measured by spectrophotometry (NanoDrop). Barcoded sequencing libraries were prepared using a NEBNext® Ultra<sup>II</sup> DNA Library Prep Kit for Illumina® (New England Biolabs) and cDNA libraries were enriched with the Agencourt AMPure XP beads (Beckman Coulter) as described above and eluted with 30 µl of buffer EB (Qiagen). Sequencing libraries were paired-end

sequenced with reads of 100 bases long on the Illumina HiSeq 2000 at Stanford Stem Cell Institute Genome Center.

### **RNA-seq gene expression analysis and candidate selection**

RNA-seq reads were mapped to the human genome (GRCh38) using TopHat. Mapped reads were assembled and gene expression analysis was performed using Cufflinks software tools. The sequence fragments were normalized to take into account both gene length and mapped reads for each sample, to measure the relative abundance of genes based on fragments per kilobase of exon per million fragments mapped (FPKM). Standard differential analysis based on the FPKM values was performed to compare gene expression profiles of control, bladder cancer, HG, and LG using Cuffdiff software to identify and prioritize cancer-specific genes by the fold-change of genes with a false discovery rate (q-value)  $\leq 0.05$ . To select against candidate markers also highly expressed in blood cells, the gene expression profiles of potential candidate genes in blood cells were examined using gene expression commons, an open platform for absolute gene expression profiling in the human hematopoietic system (15).

### **qPCR gene expression analysis**

For qPCR analysis, total urine sediments were collected and RNA was extracted, purified and quantitated as described above, but without blood cell depletion. cDNAs for all samples were generated using the Ovation RNA Seq System V2 kit (NuGEN Technologies) according to manufacturer's instructions, and in 4 samples (1 LG, 1 HG, and 2 controls in the training cohort) cDNA synthesis was also carried out with High-Capacity RNA-cDNA kit (Applied Biosystems) for comparison. cDNAs were enriched for > 300 base fragments with the AxyPrepMag™PCR Clean-up bead solution (AxyPrep™) and bead separation on 96S super magnet plate (Alpaque), eluted and quantitated as described above for RNA-seq analysis. cDNA products were amplified in single reactions using TaqMan® Gene Expression Assays (Applied Biosystems). The TaqMan® primers and probes were selected to span an exon-exon junction without detecting genomic DNA. qPCR reactions were performed in triplicate. For each reaction, 10 ng cDNA in 9  $\mu$ l was mixed with 10  $\mu$ l 2X TaqMan® Gene Expression Master Mix (Applied Biosystems) and 1  $\mu$ l 20X TaqMan® Gene Expression Assay solution in a final volume of 20  $\mu$ l and amplified in an ABI Prism® 7900 HT Sequence Detection System (Applied Biosystems). Reactions were heated to 50°C for 2 minutes and 95°C for 10 minutes before being cycled 40 times at 95°C for 15 seconds and 60°C for 1 minute. qPCR results were processed with SDS

2.4 and RQ manager software packages (Applied Biosystems). An automated threshold and baseline were used to determine the cycle threshold value ( $C_t$ ). The mean of the triplicate measurements of  $C_t$  was used for data analysis. For genes with undetermined  $C_t$  values,  $C_t$  value of 45 was assigned. Samples with  $C_t \geq 37$  for 2 of 3 reference genes (*QRICH1*, *CDC42BPB*, and *DNMBP*) in the training cohort and the 1 reference gene (*CDC42BPB*) in the validation cohort were excluded from analysis due to insufficient RNA quantity or quality.

### Statistical analysis

For initial diagnostic model construction, 21 markers were tested with 29 urine samples. The relative expression level of cancer genes was evaluated as the geometric average of the  $C_t$  of 5 reference genes –  $C_t$  of the cancer gene ( $\Delta C_t$ ). The initial panel was narrowed to 11 markers (8 cancer and 3 reference) for testing of an additional 73 urine samples. The  $C_t$  values of the 11-marker panel were used for statistical analysis with JMP Pro 12 (SAS Institute Inc.). Univariate logistic regression was used to study the predictive ability of the 11 markers on the cancer status with the odds ratios (ORs) with 95% confidence intervals (CIs), area under the receiver operation characteristic (ROC) curve (AUC), and p-value. Multiple logistic regression with backward stepwise elimination using stopping rule of entering p-value = 0.25 and leaving p-value = 0.05 was performed to reduce the panel of markers. A reference marker was included in the model as a sample adequacy control and to normalize cell numbers.  $C_t$  values of 3-marker signature (*ROBO1*, *WNT5A*, and *CDC42BPB*) were used for calculating the probability of bladder cancer score ( $P_{BC}$ ) of each sample:  $P_{BC} = \exp [A]/(1+\exp [A])$  with  $A = 19.82 - 0.43 \times ROBO1 C_t - 0.56 \times WNT5A C_t + 0.33 \times CDC42BPB C_t$ . Receiver operating characteristic (ROC) curve and AUC for the 3-marker panel were generated and calculated with the JMP Pro 12 software. Empirical ROC curve for the cytology report was estimated from ordinal empirical data with 4 categories (negative, atypical, suspicious, and malignant) (16). Sensitivity and specificity for each category was determined and the ROC curve was generated with 4 sets of data point connected by straight line. AUC of the ROC curve was calculated using R software. The statistical significance of the difference between two AUCs was evaluated as described (17).



## Results

### Study participants

Between 2013 and 2016, 186 human subjects were recruited and 226 urine samples were collected and processed. Subject demographic and clinicopathologic characteristics are shown in Table 1. Urine samples were collected from 1) patients undergoing bladder cancer evaluation (BC-evaluation) who presented with hematuria (n=78), suspicious urine cytology (n=2) or suspicious mass in computer tomography (n=3); 2) patients with known history of bladder cancer undergoing surveillance cystoscopy (BC-surveillance, n=118); 3) patients with non-neoplastic urological diseases including benign prostatic hyperplasia (n=2), urolithiasis (n=2), urinary tract infections (n=1) and indwelling ureteral stents (n=3) (other non-neoplastic urological diseases); and 4) healthy male volunteers age > 35 years with no prior history of cancer or active urological issues (healthy controls, n=17).

### Urinary biomarker discovery

To identify candidate urinary biomarkers, RNA-seq was applied to 10 urine samples from patients with HG bladder cancer, 3 samples from patients with LG bladder cancer, and 10 control samples (Table 2). To reduce non-urothelial cell sequences related to the blood-cell-associated transcriptome, RBCs and WBCs were depleted prior to total RNA isolation for sequencing. Notably, more RNA was extracted from cancer samples (mean concentration 0.98 ng/ml) than from controls (0.08 ng/ml). This difference is likely due to a higher concentration of urothelial cells in urine of cancer patients. The RNA integrity number (RIN) ranged from 2.5 to 9.5 independent of sample type. As shown in Table 2, 41-313 million paired-reads were generated per sample and 13-72.5% of the reads could be mapped to human genome. Two control samples had a low percentage of mapped reads, sample 4 with 13% and sample 9 with 27%, suggestive of sample contamination and were excluded from further analysis. Standard differential analysis based on FPKM values was performed for pairwise comparison of the gene expression profiles of control, HG, LG and combined HG and LG bladder cancer. Comparison of control and combined bladder cancer RNA-seq data identified 418 differentially expressed genes, 281 over-expressed and 137 under-expressed in bladder cancer. Comparison of control and HG samples yielded 105 differentially expressed genes, 74 over-expressed and 31 under-expressed in HG. Comparison of control and LG samples identified, 17 differentially expressed genes, 8 over-expressed and 9 under-expressed in LG. When comparing LG to HG samples, 3

genes were over-expressed in HG. The full panel of differentially expressed genes, prioritized by fold change of FPKM value is listed in Supplementary Table S1-4.

### **Biomarker selection based on RNA-seq**

To minimize false positive signals due to hematuria and inflammation, genes known to be highly expressed in blood cells were excluded as candidate biomarkers (15). Sixteen candidate cancer-specific genes were chosen from the control vs. HG and control vs. combined HG + LG comparisons. Fifteen of the candidate genes selected (*CP*, *PLEKHS1*, *MYBPC1*, *ROBO1*, *RARRES1*, *WNT5A*, *AKR1C2*, *AR*, *IGFBP5*, *ENTPD5*, *SLC14A1*, *FBLN1*, *SYBU*, *STEAP2*, and *GPD1L*) were overexpressed in HG samples with fold-change above control ranging from 3.10 to 7.39. One bladder cancer specific gene, *BPIFB1*, identified in control vs. combined HG + LG comparison had a 6.65-fold increase in cancer. All of the candidate cancer specific genes were among the top 30 genes in the control vs. combined HG + LG comparison. The Cuffdiff output for the 16 bladder cancer-specific genes selected for the validation in the training study cohort is shown in Supplementary Table S5. In order to find a suitable reference gene to control urinary RNA quantity, 5 genes (*QRICH1*, *CDC42BPB*, *USP39*, *ITSN1*, and *DNMBP*) with uniform expression level, mean FPKM value  $\sim 4$ , and standard deviation (SD)  $\leq 0.25$  among all RNA-seq samples were selected for investigation (Supplementary Table S6).

### **Biomarker validation in the training cohort**

Candidate biomarkers were validated in a training cohort of cancer and control urine samples to confirm expression level and select a panel with best diagnostic performance for bladder cancer. In contrast to the biomarker discovery phase, sample preparation during the biomarker validation phases were performed with RNA isolation from total urine sediment without blood cell depletion, thereby facilitating future clinical translation. Gene expression of an initial panel of 16 cancer-specific and 5 reference genes was determined by qPCR in 29 urine samples (16 cancer and 15 controls). Uniform expression of the candidate reference genes was evaluated and the qPCR  $C_t$  values from control and cancer samples were collected and compiled (Supplementary Figure S1). Among the candidates reference genes, *QRICH1*, *CDC42BPB* and *DNMBP* had the most similar  $C_t$  values ( $\sim 28$ ) and least variability (SD range 2.0 to 2.6), indicating they are stably expressed and suitable for data normalization in qPCR experiments. Based on the relative expression of the cancer genes normalized to the reference genes ( $\Delta C_t$ ), 8 of the cancer genes (*WNT5A*, *RARRES1*, *ROBO1*, *CP*, *IGFBP5*, *PLEKHS1*, *BPIFB1*, and

*MYBPC1*) were selected for additional testing. These 8 cancer and 3 reference genes were evaluated in an additional 73 urine samples (34 cancer and 39 controls).

To confirm that qPCR validation results were not biased by the reverse transcriptase method used to generate cDNA from urinary RNA, qPCR experiments with the 11 candidate genes were run on 4 samples (2 cancer, and 2 controls) with cDNAs produced using two different kits (NuGEN Technologies and Applied Biosystems). After the qPCR data were normalized using the geometric average of the 3 reference genes, the relative expression levels of the 8 cancer genes were consistent between methods (data not shown) suggesting reverse transcriptase kit did not introduce bias in the gene expression analysis.

### **Construction of the diagnostic model**

Univariate logistic analysis of  $C_t$  values of the 11 candidate genes in the training cohort was performed to evaluate predictive accuracy for bladder cancer for each candidate. The 8 cancer markers were all significant predictors ( $p < 0.0001$ ) with *WNT5A*, *RARRES1*, *ROBO1* and *CP* the strongest predictors of bladder cancer with odds ratios ranging from 1.65 to 2.12 and AUCs  $\geq 0.9$  (Supplementary Table S7). Although the reference markers were chosen as sample adequacy and reference levels for the number of cells in the sample, two of the reference markers, *CDC42BPB* ( $p = 0.0476$ ) and *DNMBP* ( $p < 0.0001$ ), were significant predictors of bladder cancer, likely due to higher concentration of urothelial cells in bladder cancer samples.

Multiple logistic regression analysis of  $C_t$  values of the 11 candidate genes in the training cohort was used to construct a diagnostic model equation. *ROBO1*, *WNT5A* and *CDC42BPB* were identified as having relevant, non-redundant diagnostic values for constructing an equation to calculate a score for probability of bladder cancer ( $P_{BC}$ ):

$$P_{BC} = \exp [A] / 1 + \exp [A]$$

$$A = 19.82 - 0.43 \times ROBO1 C_t - 0.56 \times WNT5A C_t + 0.33 \times CDC42BPB C_t$$

Using this equation, the  $P_{BC}$  for each sample in the training cohort was calculated (Figure 2A). A  $P_{BC} \geq 0.45$ -cutoff was designated a positive test as it gave the best overall combination of sensitivity and specificity at 88% and 92% respectively (Table 3). In 81 samples, the diagnostic accuracy of the 3-marker panel using  $P_{BC} \geq 0.45$  cutoff was compared to cytology. While the overall specificity of the 3-marker panel was modestly lower than cytology, the overall sensitivity was much better, 88% for the 3-marker panel compared to 21% for cytology.

### **Validation of the diagnostic model**

The 3-marker panel of *ROBO1*, *WNT5A*, and *CDC42BPB* was evaluated by qPCR in an independent validation set of 101 urine samples (47 cancer and 54 controls) from 86 patients (Table 1, Figure 2B). Similar to sample preparation in the training cohort, total urine sediment was used for RNA isolation and analysis. Using  $P_{BC} \geq 0.45$  as the threshold for a positive test, the overall sensitivity and specificity for the 3-marker panel was 83% and 89%, respectively (Table 3). Notable the sensitivity for the 3-marker panel was the same for HG and LG disease (83% sensitivity). The diagnostic performance of 3-marker panel was also compared with cytology on a subset of samples ( $n = 89$ ) with an AUC of 0.87, which was significantly more accurate than the diagnosis by cytology with an AUC of 0.68 ( $p < 0.01$ ) (Figure 2C). Cytology had high specificity (100%) but lower overall sensitivity (25%). While the 3-marker panel performed equally well for HG and LG, the sensitivity of cytology for HG disease (50%) was considerably better than for LG (12%).

### **Using the 3-marker panel for bladder cancer surveillance**

To explore the potential of using the 3-marker panel urine test for bladder cancer surveillance, we evaluated its test performance in serially collected urine samples from six patients. For each patient, 2 to 4 urine samples were collected over 7 to 18 months. The results from the 3-marker panel were compared with cystoscopic and/or pathologic findings. In all patients, the 3-marker panel was concordant with cystoscopic and/or pathologic results, both in cancer positive and negative scenarios (Figure 3).

In a patient with LG Ta bladder cancer (Figure 3A), the 3-marker panel was positive at the initial diagnosis and two subsequent cancer recurrences, whereas cytology remained negative throughout, indicating that the 3-marker panel is a better adjunct to cystoscopy for this patient. In another patient with prior history of LG with focal HG bladder cancer, the patient had 3 negative cystoscopy and 3 matched negative 3-marker urine tests (Figure 3B). At the time of tissue-confirmed recurrence 16 months later, the 3-marker panel also turned positive. The concordance of the 3-panel marker with cystoscopy suggest that the use of the panel may reduce the frequency of cystoscopic surveillance in selected patients. Similar findings are seen in two other patients with Ta LG cancer (Figure 3C-D), in which the 3-marker panel paralleled negative cystoscopies and biopsy-proven recurrences.

In patients with HG T1 (Figure 3E) and TIS (Figure 3F) at the time of study entry, both cytology and the 3-marker panel were positive at cancer diagnosis and negative during surveillance.

Notably, the patient in Figure 3F underwent induction BCG following the diagnosis of TIS. The surveillance cystoscopy following BCG identified an erythematous patch on the anterior bladder wall. The appropriately negative 3-marker panel (Figure 3F, test 2) suggests that in this case the test remained reliable after BCG and did not falsely identify inflammation as bladder cancer.

## Discussion

While most bladder cancers are non-muscle invasive at initial diagnosis, the high recurrence rate and potential to progress to invasive disease necessitates frequent surveillance cystoscopy, contributing to bladder cancer as one of the most expensive cancers to treat (18). To date, a non-invasive test with sufficient accuracy to reduce the frequency of cystoscopy in low-risk patients, while providing timely treatment in high-risk patients, has remained elusive. For development of a urine-base bladder cancer test, we reasoned that direct analysis of exfoliated urothelial cells, rather than tissue biopsies, would yield higher translational potential for biomarker discovery. We applied RNA-seq for unbiased gene expression analysis of urinary cells and demonstrated the success of extracting high quality RNA and generating high quality sequencing for identifying a new 3-marker panel (*ROBO1*, *WNT5A* and *CDC42BPB*) for molecular diagnosis of bladder cancer.

Identification of differentially expressed genes between cancer and benign tissues is a common starting point for biomarker discovery. Development of next generation sequencing technologies that allow for high sensitivity, resolution, throughput and speed have advanced research on biomarker discovery for cancer diagnosis, assessing prognosis, and directing treatment monitoring (19-22). RNA-seq has emerged as a powerful tool for unbiased interrogation of gene expression as well as identification of splice variants and non-coding RNAs (14). An innovative aspect of our study lies in the discovery approach of using urine as the starting material for RNA-seq. Direct application of RNA-seq to urine has been limited, given the relatively low cellularity and heterogeneity of urine samples that may impact RNA integrity. To address these issues, we processed the entire volume urine sample within 2 hours of collection to maximize the number of cells and the yield of total RNA. We observed RIN values that spanned almost the entire range of 1 to 10 (Table 2), suggesting variable levels of RNA degradation. By not excluding samples with low RINs, we aimed to improve the translational potential of the assay as the transcripts identified are likely to remain stable even in partially

degraded RNA samples. Furthermore, the variable degrees of RNA degradation did not appear to compromise the number of sequencing reads or percentage of mapped reads.

Through the blood cell depletion steps, our sample preparation protocol for RNA-seq analysis was designed to enrich urothelial cells and genes specific to bladder cancer while reducing potentially confounding markers of inflammation commonly found in urine (e.g. urinary tract infection, post-intravesical BCG administration). Additionally, candidate genes identified by RNA-seq that are also known to be highly expressed in blood cells were excluded from marker validation. Targeting transcripts likely to be specific to urothelial cells specific during the discovery phase allowed us to use total urine sediment RNA without blood cell depletion at the validation phases, thereby simplifying the sample preparation and increasing the translational potential.

Our discovery strategy allowed us to concentrate on a small panel of genes with the highest diagnostic yield that are stable in urine from the myriad of differentially expressed genes in bladder tumors. Although this study aims to identify robust urinary diagnostic markers rather than causative markers for cancer biology, several of the bladder cancer specific genes identified in our approach have been linked to bladder and other cancers. *CP*, which has the highest fold increase in cancer compared to control in our screen (Supplementary table S1), encodes a ferredoxin oxidase enzyme and was previously identified in a proteomic screen as a urinary biomarker of bladder cancer (23) and as a serum biomarker in other cancers (24). *IGFBP5*, another top candidate gene, was previously found to be upregulated in bladder cancer by tissue microarray analysis and is part of the Cxbladder 5-marker panel for bladder cancer diagnosis described below (8,9). The two cancer specific genes in our 3-marker panel were also previously implicated in tumor formation and progression. *ROBO1* is a promoter of tumor angiogenesis and overexpressed in both human bladder cancer tissue and cultured cell lines (25,26). *WNT5A* is a secreted glycoprotein that plays an important regulatory role in embryogenesis, including regulation of cell polarity and migration. *WNT5A* expression decreases after development and upregulation in adult tissue has been implicated in oncogenesis (27). In bladder cancer, *WNT5A* protein expression correlated positively with the histological grade and pathological stage (28,29).

Several urine tests have been approved for clinical use in bladder cancer. However, due to inadequate sensitivity (particularly for LG cancer) and specificity in inflammatory conditions,

current guidelines on NMIBC do not recommend their routine use for surveillance or initial work-up (6,30). Fluorescence *in situ* hybridization (UroVysion) and immunocytochemistry (ImmunoCyt) incorporate molecular markers with microscopic evaluation of urine cells with overall better sensitivity but lower specificity than conventional cytology (11,31). However, these tests, like cytology, are subject to interobserver differences in interpretation (32). Protein biomarker assays nuclear matrix protein 22 (NMP22) and bladder tumor antigen (BTA) offer the potential for simple, more objective tests (33). Both tests have higher sensitivity but lower specificity than cytology, especially in patients with inflammation and infection in the urinary tract (34,35).

Recent efforts to improve urine-based diagnostics for bladder cancer have focused on multiplex detection of mRNAs that are differentially expressed between cancer and non-cancerous tissues. A general strategy uses microarray analysis of bladder cancer tissue samples for target selection, followed by validation in urine samples. One panel, Cxbladder (Pacific Edge, Dunedin, New Zealand), assays urinary expression of bladder cancer markers *CDC2*, *HOXA13*, *MDK* and *IGFBP5*, as well as inflammation biomarker *CXCR2* to reduce false positive tests (9). In a multicenter prospective study of 485 patients presenting with gross hematuria, the Cxbladder assay had an overall sensitivity of 81% (97% for HG, 69% for LG) and specificity of 85% (8). Another assay under development by BiofinaDX (Madrid, Spain) uses a 2, 5, 10 or 12 gene signature for urinary detection of bladder cancer (12). The 12-gene signature was first identified by microarray analysis of bladder cancer tumor tissue then validated in urine samples (10). In a multicenter prospective study of 525 samples, the 12-marker panel was narrowed to two (*IGF2* and *MAGEA*) with an overall sensitivity of 81% (89% for HG, 68% for LG) and specificity of 91% (7,12).

Improving the diagnostic sensitivity of LG is one of the central goals of urine-based diagnostics, as the majority of bladder cancer patients present with LG disease. While LG tumors are typically not life threatening, the diagnosis and treatment of these lesions is crucial to prevent morbidity and reduce the risk of progression. Our diagnostic model consisting of *ROBO1*, *WNT5A*, and *CDC42BPB*, had an overall sensitivity of 83% and specificity of 89%. Compared to Cxbladder and BiofinaDX, our overall sensitivity was similar and subset analysis showed improved sensitivity 83% for LG cancer compared to 69% for Cxbladder and 68% for BiofinaDX (8,12). The improved sensitivity may be due in part to our urine-based biomarker discovery strategy to target mRNA that are not only differentially expressed in bladder cancer but also

maintain stability in urine. Additionally, concentrating the cellular fraction from the entire urine sample may account for superior detection of LG tumors that shed fewer cells.

One strength of our study is the proof of concept demonstration of serial testing for a cohort of patients over their course of bladder cancer surveillance (Figure 3). The consistent results between cystoscopy and the 3-marker panel suggest that the test may be a dependable adjunct for cancer surveillance. This may be especially true in the setting of an initial positive 3-marker urine test indicating that the markers are upregulated in the tumor.

Based on our dataset, we set the threshold for a positive test at  $P_{BC} \geq 0.45$  in both bladder cancer evaluation and surveillance populations. In the clinical scenario of using the urine test to prescreen patients before cystoscopy, sensitivity may be considered more important than specificity as the clinical outcome of missing cancer is worse than negative cystoscopy. To maximize the sensitivity, the threshold for a positive test may be set lower for surveillance than in evaluation populations as recurrent bladder tumors tend to be smaller than primary tumors (6), which may result in a lower cancer  $P_{BC}$  value. For example, using a lower cutoff for bladder cancer surveillance than evaluation was found to improve sensitivity of the NMP22 test (36). Other efforts that may improve the accuracy of bladder cancer diagnostics include integration of the urine tests with the clinical characteristic (37-39). For example, Kavalieris *et al.* developed an integrated model consisting of both Cxbladder gene expression urine test and patient characteristic variables such as gender, age, smoking history and frequency of gross hematuria for use to triage patients for hematuria workup but with a low probability of bladder cancer (40).

To further evaluate the 3-marker panel in the future, a prospective, multicenter study is required. A broader study will further allow us to assess assay performance under a range of urologic conditions. It will be especially valuable to evaluate the 3-marker panel in patients undergoing BCG where the performance of urine cytology is poor due to an increase of inflammatory cells in urine (6,41). Our approach of selecting against markers of inflammation suggests our 3-marker may be useful for assessing patient response to BCG treatment. Further, as subjects were selected retrospectively for the current study, valid bladder cancer prevalence estimates could not be obtained. A prospective study is necessary to allow us to calculate negative and positive predictive values of the test and set a  $P_{BC}$  cutoff to maximize the negative predictive value, which may be useful for reducing the need for cystoscopy. With a larger sample size, we can also assess whether supplementing our gene expression model with a phenotypic model of risk



stratification provides an improved resource for clinical decisions, particularly for patients with scores near the  $P_{BC}$  threshold (42). Further interrogation of our RNA-seq dataset may yield insights into bladder cancer biology, identify rare splice variants and other RNA targets (e.g. lncRNA) that were enriched through our sample preparation strategy. Lastly, RNA-seq of urinary RNA could be employed to discover urinary biomarkers for differentiation of HG and LG bladder cancer, detection other urinary tract diseases, and evaluating response to treatments.

## Conclusions

Using RNA-seq as a discovery tool, we have demonstrated the feasibility of obtaining high quality sequencing data from urine sediments for RNA expression profiling. Through qPCR evaluation and multiple logistic analysis, we generated an equation to predict bladder cancer probability based on the urinary expression of *ROBO1*, *WNT5A* and *CDC42BPB*. The overall sensitivity for both the HG and LG samples was superior to urine cytology. A prospective multicenter clinical study should be conducted to further validate the 3 marker signature for detection, surveillance, and post-BCG populations.

## Acknowledgements

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**Table 1. Demographic and clinicopathologic features of the study cohorts.**

		Biomarker Discovery		Diagnostic Model		Validation	
Demographic features		Benign (n = 10)	Cancer (n = 13)	Benign (n = 52)	Cancer (n = 50)	Benign (n = 54)	Cancer (n = 47)
Average age (range) <sup>a</sup>		> 35	72.8 (58-90)	67.3 (30-89)	71.8 (53-93)	70.8 (29-100)	71.4 (55-91)
Gender: male/female, n		10 / 0	13/0	52/0	50/0	53/1	47/0
BC-evaluation		-	8	15	23	22	15
BC-surveillance		-	5	23	27	31	32
Healthy/ other controls		10	-	14	-	1	-
Clinicopathologic features <sup>b</sup>		Cancer (n = 13)		Cancer (n = 50)		Cancer (n = 47)	
Grade	Low <sup>c</sup>	3		19		29	
	High	10		31		18	
Clinical Stage	Papillary						
	Ta	8		28		36	
	T1	1		10		4	
	≥ T2	2		3		5	
	Papillary + CIS						
	Ta	-		1		2	
	T1	1		-		-	
	T2	1		2		-	
	CIS	-		6		-	

Abbreviation: CIS, carcinoma in situ.

<sup>a</sup>Average age and range does not include healthy controls as specific ages were not collected for this group.

<sup>b</sup>Clinicopathologic features are available only for bladder cancer patients.

<sup>c</sup>All LG cancer samples were with stage pTa.

**Table 2. Summary of urine samples used for RNA-seq transcriptome profiling.**

Sample Number	Clinicopathologic features	Urine volume (ml)	Total RNA concentration (ng)	RIN (1-10) <sup>a</sup>	Number of reads	% of mapped reads
1	Control	200	7.1	9.4	88,922,624	35.3
2	Control	75	13.6	5.3	84,926,624	37.8
3	Control	190	6.1	9.2	82,152,466	58.3
4	Control	150	11.6	2.5	202,122,232	13.0
5	Control	200	11.1	6.6	211,454,432	47.6
6	Control	435	51.0	5.6	261,575,308	48.4
7	Control	327	27.5	2.7	313,362,582	44.3
8	Control	750	17.4	3.7	59,641,782	54.8
9	Control	460	13.3	4.9	54,411,982	27.0
10	Control	500	81.7	6.6	73,908,808	52.9
11	Ta HG	50	9.7	9.5	77,299,864	35.2
12	Ta HG	176	57.4	7.6	100,212,450	72.5
13	Ta HG	140	8.8	3.1	76,097,546	54.1
14	Ta HG	125	28.0	2.7	90,664,046	59.5
15	Ta HG	82	72.8	3.8	57,041,308	59.8
16	T1 HG	110	128.8	7.0	41,764,318	64.6
17	T2 HG	60	63.7	6.7	95,286,642	70.1
18	T2 HG	115	110.2	8.6	67,061,502	68.1
19	T1 HG +CIS	125	101.4	6.9	91,298,356	39.0
20	T2 HG + CIS	133	603.8	6.2	70,401,502	65.9
21	Ta LG	80	79.4	7.7	58,109,042	65.3
22	Ta LG	215	110.0	6.3	55,461,696	46.8
23	Ta LG	68	67.9	6.2	48,072,074	61.8

Abbreviations: CIS, carcinoma in situ.

<sup>a</sup>The RNA integrity number (RIN) is an algorithm for evaluating the integrity of RNA with a value of 1 to 10, with 10 being the least degraded.

**Table 3. Summary of diagnostic performance for bladder cancer prediction on urine based on the 3-marker panel using *ROBO1*, *WNT5A*, and *CDC42BPB* and cytology in both training and validation cohorts with the cutoff of  $P_{BC} \geq 0.45$  giving a positive test.**

		Training Cohort		Validation Cohort	
		3-Marker Panel	Cytology <sup>a</sup>	3-Marker Panel	Cytology <sup>a</sup>
Sensitivity	All Cancer	88% (44/50)	21% (9/42)	83% (39/47)	25% (10/40)
	HG	94% (29/31)	35% (8/23)	83% (15/18)	50% (7/14)
	LG	79% (15/19)	5% (1/19)	83% (24/29)	12% (3/26)
Specificity	All Non-Cancer	92% (48/52)	97% (38/39)	89% (48/54)	100% (49/49)
	Negative BCevaluation	93% (14/15)	100% (15/15)	86% (19/22)	100% (19/19)
	Negative BCsurveillance	87% (20/23)	96% (22/23)	90% (28/31)	100% (30/30)
	Healthy/ Other Controls	100% (14/14)	100% (1/1)	100% (1/1)	N/A

<sup>a</sup>Cytology was only available for a subset of samples.

## Figure legends

**Figure 1. Study design.** For the biomarker discovery in part 1, urine samples from 13 bladder cancer patients and 10 control subjects were collected for RNA-seq analysis. For model construction in part 2, a subset of genes that were differentially expressed in bladder cancer compared to controls was selected for qPCR validation in 102 urine samples. A model for computing a probability of bladder cancer score ( $P_{BC}$ ) based on the gene expression of the 3-marker panel in urine was constructed using multivariate logistic regression. For model validation in part 3, the diagnostic performance of the 3-marker panel was evaluated in an independent study cohort of 101 urine samples.

**Figure 2. Diagnostic performance of the 3-marker panel for bladder cancer prediction.**

The probability of bladder cancer score ( $P_{BC}$ ) based on the diagnostic equation using the 3-marker (*ROBO1*, *WNT5A*, *CDC42BPB*) urine assay was measured in **A**, the training cohort (n=102) and **B**, the validation cohort (n=101).  $P_{BC} \geq 0.45$  (the black line in **A** and **B**) as the threshold for a positive test gave the best concordance with clinical findings for patients without evidence of bladder cancer (Neg cysto, BC-evaluation; Neg cysto, BC-surveillance; Neg cysto, others (other non-neoplastic urological diseases); and Healthy controls) and patient with bladder cancer (HG and LG). **C**, comparison of the diagnostic performance of the 3-marker in the validation cohort (n= 101) with cytology on a subset of samples (n=89) using ROC curves resulting in AUCs of 0.87 for the 3-marker panel and 0.68 for cytology. Neg cysto, Negative cystoscopy.

**Figure 3. Bladder cancer surveillance using the 3-marker urine test.** Serial urine samples were collected from 6 patients and the probability of bladder cancer score ( $P_{BC}$ ) based on the 3-marker (*ROBO1*, *WNT5A*, *CDC42BPB*) diagnostic equation was determined.  $P_{BC} \geq 0.45$  (black line) was considered positive for bladder cancer. Corresponding bladder cancer pathology (stage, grade) or cystoscopy (if no bladder cancer detected) was indicated above urine test result. **A**, urine test can accurately detect persistent bladder cancer. Test 1 for bladder cancer evaluation accurately detected bladder cancer as did follow up surveillance tests after 5 months (test 2) and another 6 months (test 3). **B**, urine test can accurately detect bladder cancer recurrence in patient disease free for >16 months. Test 1 for bladder cancer surveillance was negative consistent with negative cystoscopy, as were tests 2 and 3 at 3 month intervals, test 4 accurately detected bladder cancer recurrence 10 months later. **C**, urine test was reliable for prediction of alternating pattern of positive and negative tests. Test 1 for bladder cancer

evaluation accurately detected bladder cancer, follow up surveillance at 3 months was negative by both urine test and cystoscopy, bladder cancer recurrence was accurately detected after another 9 months, followed by negative results from both urine test and cystoscopy after another 5 months. **D, E and F**, after initial positive bladder cancer test, the subsequent urine tests accurately predict disease free survival. Test 1 for bladder cancer surveillance (**D**) or bladder cancer evaluation (**E and F**) accurately detected bladder cancer, subsequent surveillance tests were negative by both urine test and cystoscopy. LG, low grade; HG, high grade.

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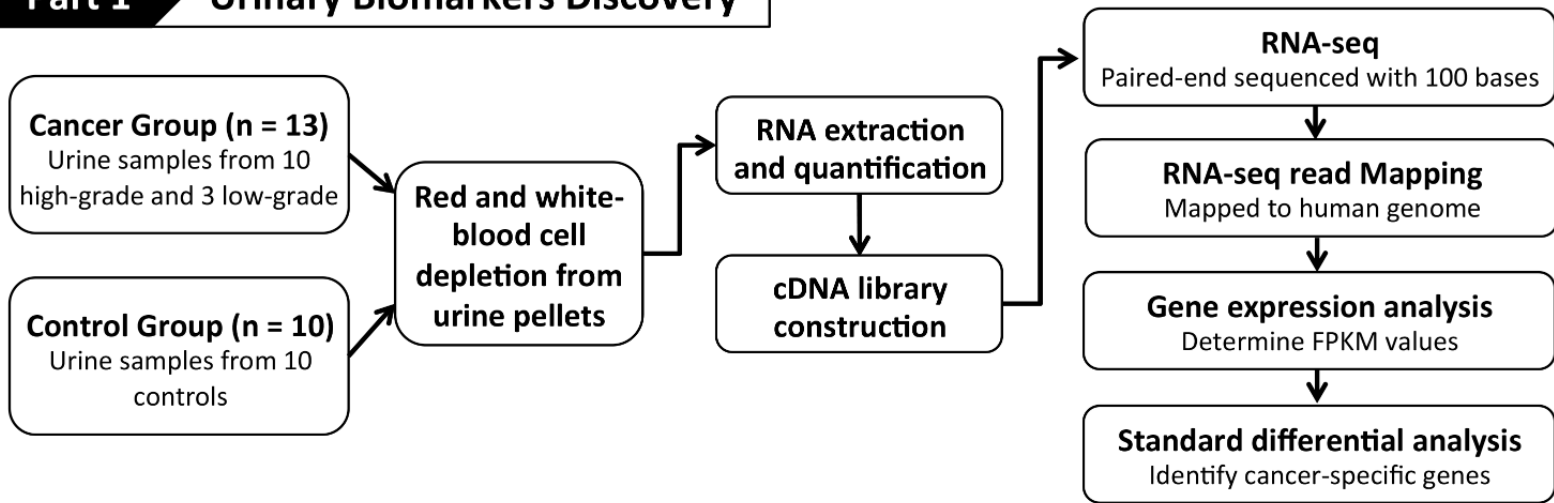
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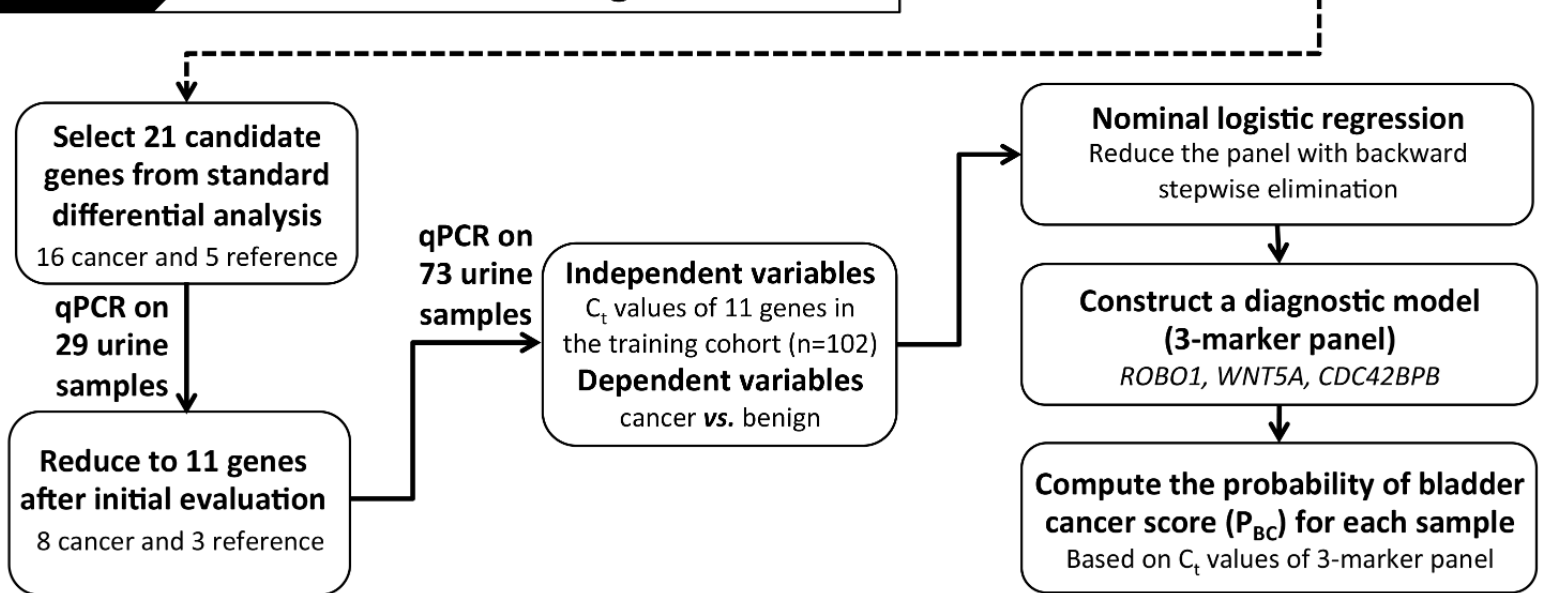
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Figure 1

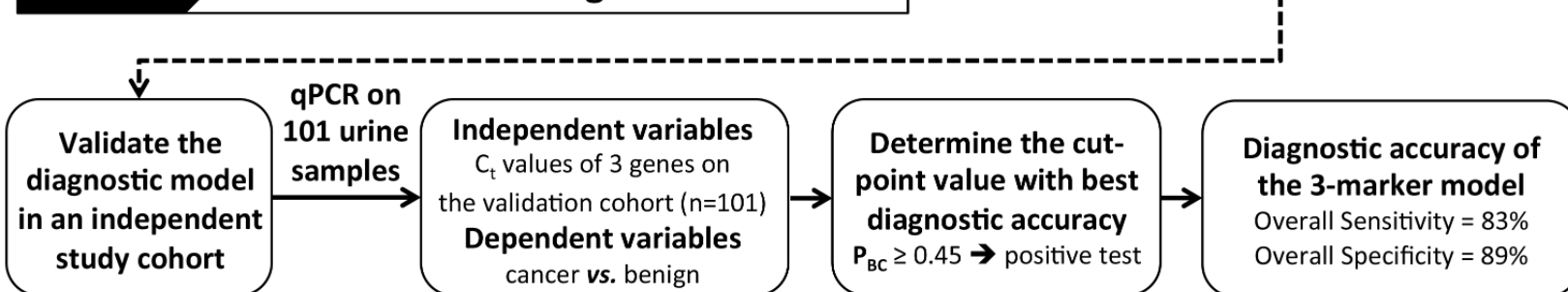
**Part 1 Urinary Biomarkers Discovery**



**Part 2 Construction of the Diagnostic Model**



**Part 3 Validation of the Diagnostic Model**



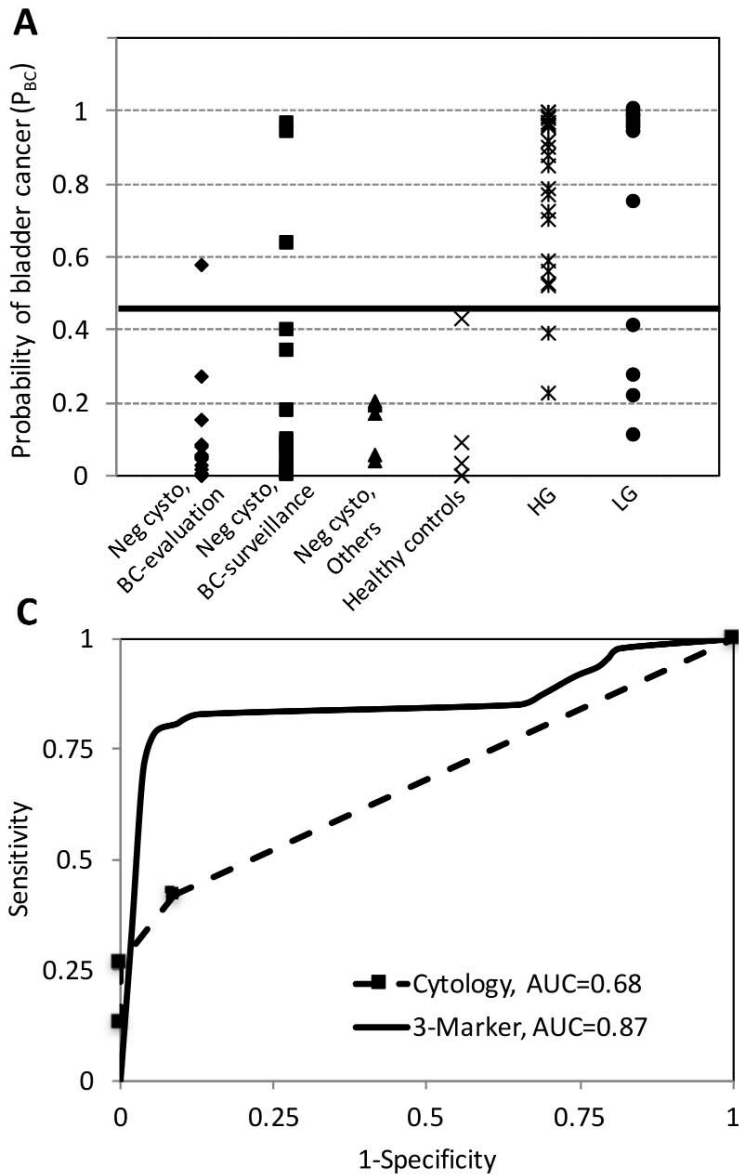
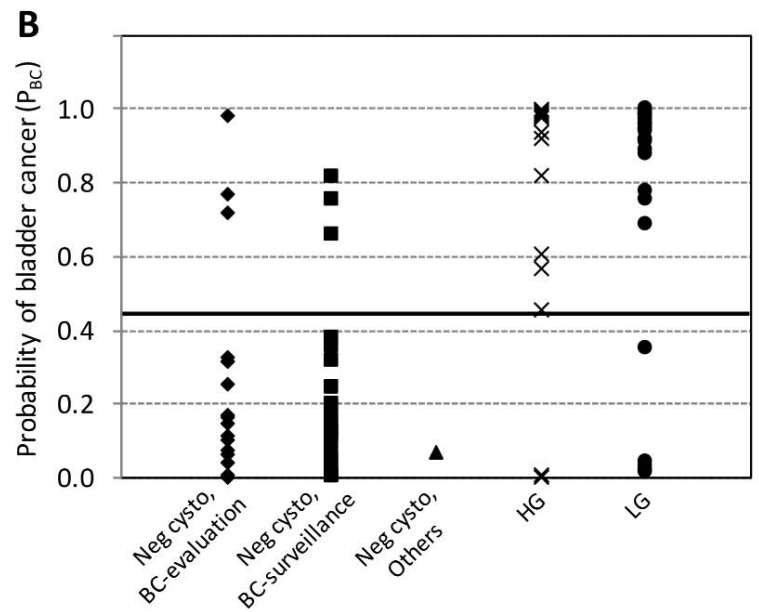
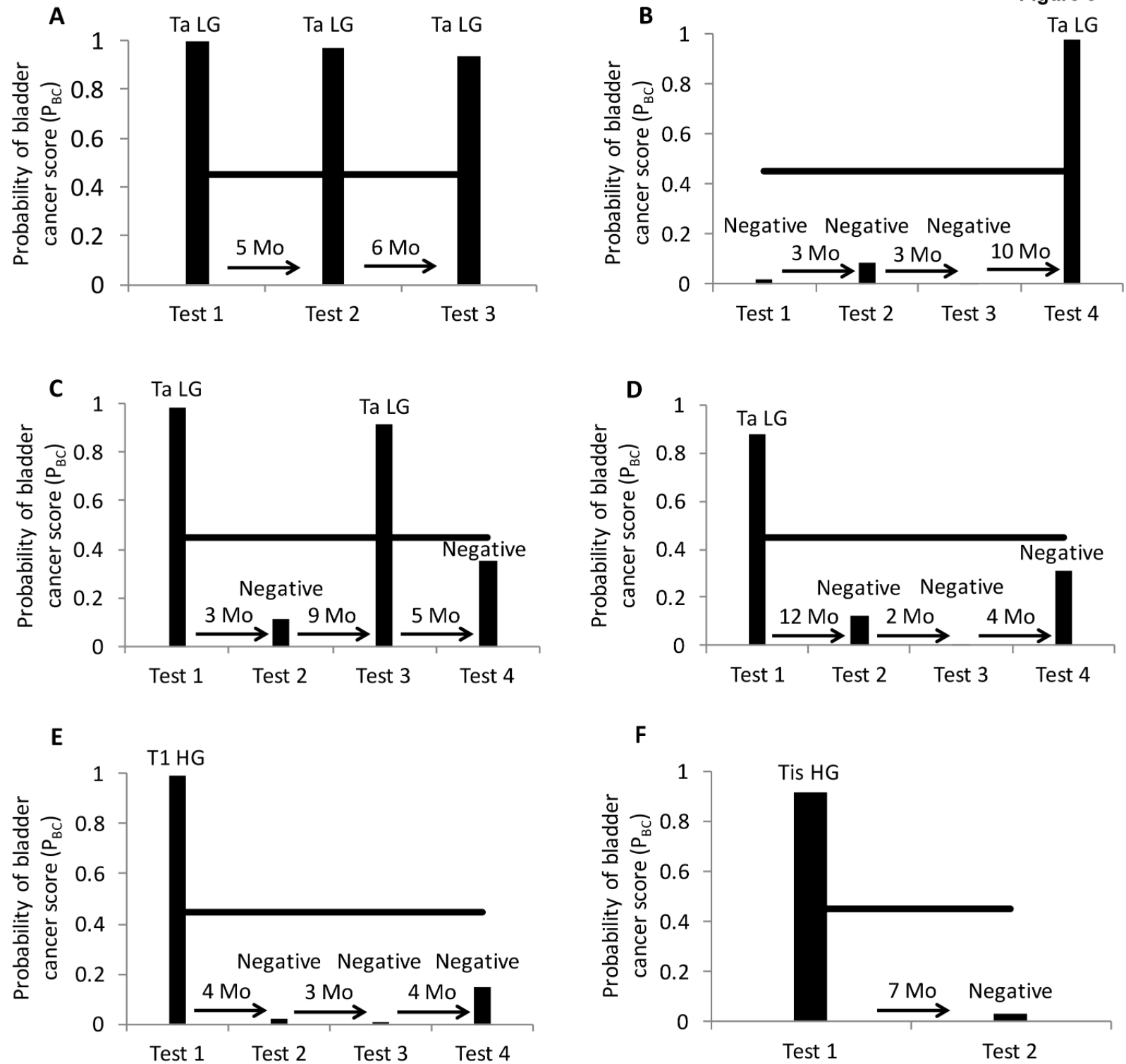


Figure 2



**Figure 3**



# Clinical Cancer Research

## Deep Sequencing of Urinary RNAs for Bladder Cancer Molecular Diagnostics

Mandy L Y Sin, Kathleen E Mach, Rahul Sinha, et al.

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