Evaluation of an Epigenetic Profile for the Detection of Bladder Cancer in Patients with Hematuria

Kim E. M. van Kessel,*,† Leander Van Neste,†,‡ Irene Lurkin,* Ellen C. Zwarthoff* and Wim Van Criekinge*,§

From the Departments of Pathology, Erasmus Medical Center (KEMvK, IL, ECZ), Rotterdam and GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center (LVN), Maastricht, The Netherlands, MDxHealth, Inc. (LVN, WVC), Irvine, California, and Laboratory of Bioinformatics and Computational Genomics, Ghent University (WVC), Ghent, Belgium

Purpose: Many patients enter the care cycle with gross or microscopic hematuria and undergo cystoscopy to rule out bladder cancer. Sensitivity of this invasive examination is limited, leaving many patients at risk for undetected cancer. To improve current clinical practice more sensitive and noninvasive screening methods should be applied.

Materials and Methods: A total of 154 urine samples were collected from patients with hematuria, including 80 without and 74 with bladder cancer. DNA from cells in the urine was epigenetically profiled using 2 independent assays. Methylation specific polymerase chain reaction was performed on *TWIST1*. SNaPshot™ methylation analysis was done for different loci of *OTX1* and *ONECUT2*. Additionally all samples were analyzed for mutation status of *TERT* (telomerase reverse transcriptase), *PIK3CA*, *FGFR3* (fibroblast growth factor receptor 3), *HRAS*, *KRAS* and *NRAS*.

Results: The combination of *TWIST1*, *ONECUT2* (2 loci) and *OTX1* resulted in the best overall performing panel. Logistic regression analysis on these methylation markers, mutation status of *FGFR3*, *TERT* and *HRAS*, and patient age resulted in an accurate model with 97% sensitivity, 83% specificity and an AUC of 0.93 (95% CI 0.88–0.98). Internal validation led to an optimism corrected AUC of 0.92. With an estimated bladder cancer prevalence of 5% to 10% in a hematuria cohort the assay resulted in a 99.6% to 99.9% negative predictive value.

Conclusions: Epigenetic profiling using *TWIST1*, *ONECUT2* and *OTX1* results in a high sensitivity and specificity. Accurate risk prediction might result in less extensive and invasive examination of patients at low risk, thereby reducing unnecessary patient burden and health care costs.

Key Words: urinary bladder neoplasms, hematuria, epigenomics, diagnosis, risk

Abbreviations and Acronyms

BCa = bladder cancer
MSP = methylation specific PCR
NPV = negative predictive value
PCR = polymerase chain reaction

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§ Correspondence: 15279 Alton Pky., Suite 100, Irvine, California 92618 (telephone: 949-812-6979; FAX: 949-788-0014; e-mail: wim.vancriekinge@mdxhealth.com).

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Hematuria, which accounts for up to 20% of all urological visits, is the presence of red blood cells in urine.[1] Urological cancer is found to be the cause of hematuria in 5% of patients with microscopic hematuria and in up to 20% with macroscopic hematuria.[2,3] Therefore, it is an important clinical question to discriminate between a malignant and nonmalignant cause of hematuria since early detection of bladder cancer correlates with

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an increased likelihood of bladder preservation and improved overall survival.[4]

The typical diagnostic analysis of hematuria includes cystoscopy, which has 68% to 83% sensitivity to detect BCa recurrence. For the primary diagnosis of BCa sensitivity was described to be 87% in patients with gross hematuria.[5] Cystoscopy is invasive and causes pain and discomfort.[6] Cytology is considered specific but with low sensitivity and it is typically used in combination with cystoscopy.[7] Patients diagnosed with early stage BCa undergo frequent monitoring currently based on cystoscopy and cytology, resulting in BCa becoming one of the most costly of all cancers to manage.[8]

To improve current clinical practice noninvasive and more sensitive screening methods should be applied to improve patient treatment and reduce the cost, morbidity and mortality of the sixth most common cancer.[9] Much effort has focused on trying to identify urine based markers for the diagnosis of BCa. While urine cytology is specific but lacks sensitivity, especially for low grade BCa,[10,11] and with cystoscopy being only 68% to 83% sensitive and invasive as well as costly and often associated with discomfort[12] an accurate, noninvasive assay could complement current clinical practice.

Cancer results from interactions between the environment, and genetic and epigenetic factors. Although genetic mutations are often the subject of investigation,[13] such genetic alterations account for only a small percent of most cancers.[14] The epigenetic component of cancer represents the most frequent DNA alteration that can lead to the development and progression of cancer.[15] More specifically DNA hypermethylation occurs when DNA becomes methylated at CpG-rich regions located in the gene promoter regions, leading to gene inactivation. DNA methylation of critical genes such as tumor suppressors is a frequent and early event in neoplastic development.[16]

MSP is widely used to detect hypermethylated genes originating from cancer cells in tissues and body fluids such as serum, urine, stool and saliva.[17] A large number of studies have identified methylated genes linked to BCa.[18–24]

The aim of the current study was to combine 2 previously published methylation marker panels to assess their complementarity and validate the minimal panel with the best clinical performance. Detection of the methylated *TWIST1* gene in urine sediments using MSP provides a greater than 90% sensitive and specific noninvasive approach to detect primary BCa.[20,21,25,26] Methylation of *OSR1*, *SIM2*, *OTX1*, *MEIS1* and *ONECUT2* was shown to be a significant predictor of the presence of urothelial cell carcinoma.[27]

MATERIALS AND METHODS

Patients and Urine Samples

Voided urine samples were collected from 154 patients undergoing cystoscopy for the diagnostic evaluation of hematuria. Urine samples were collected at Erasmus Medical Center, The Netherlands, between 2006 and 2013. These samples were used according to The Code for Proper Secondary Use of Human Tissues in the Netherlands (http://www.federa.org/). All patients were primary referrals with no history of any urinary tract malignancy. Of these 154 patients 74 were diagnosed with BCa and 80 had a benign cause of hematuria (table 1).

Urine samples were collected prior to cystoscopy and stored at 4C. The samples were centrifuged at 3,000 rpm. The cell pellets were washed with 10 ml phosphate buffered saline twice and spun down for 10 minutes at 3,000 rpm. The cells were then resuspended in 1 ml phosphate buffered saline, transferred to an Eppendorf vial and centrifuged for another 5 minutes. Supernatant was discarded and the cell pellet was stored at −80C until DNA isolation.

Analyses

Methylation. DNA was isolated from urine sediments using the QIAamp® DNA Micro Kit and quantified with the Quant-iT™ PicoGreen® dsDNA Quantification Kit according to manufacturer instructions. DNA (100 ng) was used for bisulfite modification with the EZ DNA Methylation-Lightning™ Kit. Modified DNA was eluted into 20 μl tris-HCl (1 mM, pH 8.0) and stored at −80C for further processing.

Blinded MSP analyses were performed using a multiplex reaction for *TWIST1* on the antisense chain (Integrated DNA Technologies, Coralville, Iowa). Supplementary table 1 (http://jurology.com/) lists the sequences. Quantitative PCR mix (Quantitect® Multiplex Mastermix) was used and reactions were run on a Rotor-Gene® Q. Copy numbers were calculated based on linear regression of a standard curve obtained from plasmid DNA containing the bisulfite modified sequences of interest (GeneArt™ Gene Synthesis).

SNaPshot assays consisted of multiple probes covering 4 CpG sites in *OTX1* and 5 sites in *ONECUT2*. After bisulfite specific PCR for the sites of interest for

Table 1. *Characteristics of patients and tumors included in analysis*

	Urothelial Cell Ca		Benign	
No. pts	74		80	
Mean age (range)	68 (38–91)		58 (21–86)	
No. gender (%):				
Male	59	(80)	50	(63)
Female	15	(20)	30	(37)
No. stage (%):			–	
Ta	36	(49)		
T1	14	(19)		
T2–4	24	(32)		
No. grade (%):			–	
G1	11	(15)		
G2	29	(39)		
G3	34	(46)		