Clinical Cancer Research

Development and Validation of Urine-based Peptide Biomarker Panels for Detecting Bladder Cancer in a Multi-center Study

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Abstract

Purpose: Urothelial bladder cancer presents high recurrence rates, mandating continuous monitoring via invasive cystoscopy. The development of noninvasive tests for disease diagnosis and surveillance remains an unmet clinical need. In this study, validation of two urine-based biomarker panels for detecting primary and recurrent urothelial bladder cancer was conducted.

Experimental Design: Two studies (total $n = 1,357$) were performed for detecting primary ($n = 721$) and relapsed urothelial bladder cancer ($n = 636$). Cystoscopy was applied for detecting urothelial bladder cancer, while patients negative for recurrence had follow-up for at least one year to exclude presence of an undetected tumor at the time of sampling. Capillary electrophoresis coupled to mass spectrometry (CE-MS) was employed for the identification of urinary peptide biomarkers. The candidate urine–based peptide biomarker panels were derived from nested cross-sectional studies in primary ($n = 451$) and recurrent ($n = 425$) urothelial bladder cancer.

Results: Two biomarker panels were developed on the basis of 116 and 106 peptide biomarkers using support vector machine algorithms. Validation of the urine-based biomarker panels in independent validation sets, resulted in AUC values of 0.87 and 0.75 for detecting primary ($n = 270$) and recurrent urothelial bladder cancer ($n = 211$), respectively. At the optimal threshold, the classifier for detecting primary urothelial bladder cancer exhibited 91% sensitivity and 68% specificity, while the classifier for recurrence demonstrated 87% sensitivity and 51% specificity. Particularly for patients undergoing surveillance, improved performance was achieved when combining the urine-based panel with cytology (AUC = 0.87).

Conclusions: The developed urine-based peptide biomarker panel for detecting primary urothelial bladder cancer exhibits good performance. Combination of the urine-based panel and cytology resulted in improved performance for detecting disease recurrence. *Clin Cancer Res; 22(16); 4077–86. ©2016 AACR.*

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Introduction

Urinary bladder cancer remains the second most frequent cause of mortality among genitourinary cancers, including approximately 430,000 incident cases and 165,000 attributable deaths annually worldwide (1). The striking majority of malignant bladder tumors are of epithelial origin. Depending on the degree of tumor infiltration in the vesical wall, 80% of neoplasms are classified as non-muscle invasive (NMIBC), while the remainder are muscle invasive (MIBC) tumors (2). After initial treatment, up to 70% of NMIBC patients experience disease recurrence (3, 4). Current approaches for detecting both primary and recurrent disease rely on invasive cystoscopy. However, due to high urothelial bladder cancer relapse rates (4) frequent patient monitoring is required (3), leading to diminished patient compliance and augmented associated healthcare costs (5). In an effort to reduce the frequency of cystoscopies conducted, several noninvasive biomarkers have been approved by the FDA, albeit with performance rates remaining insufficient to replace current diagnostic and monitoring practices relying on cystoscopy (6). Therefore, a prevailing need for noninvasive biomarkers which will facilitate the timely diagnosis of primary and recurrent urothelial bladder

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Translational Relevance

Urothelial bladder cancer remains the second most frequent cause of mortality among genitourinary cancers. Because of high relapse rates, frequent patient monitoring is required, leading to augmented associated healthcare costs and diminished patient compliance. A prevailing need for noninvasive biomarkers which will facilitate the timely diagnosis of primary and recurrent urothelial bladder cancer remains unmet to date. This study is focused on the investigation of biomarker panels based on urinary peptides, as screening tools for the diagnosis of urothelial bladder cancer. Two biomarker panels were developed for primary and recurrent urothelial bladder cancer, by employing 1,357 urine samples. Further validation of the peptide panels in patients representing primary and surveillance settings resulted in AUCs of 0.87 and 0.75, respectively. In reference to the peptide biomarker model for detection of recurrence, combination with cytology increased the AUC to 0.87.

cancer (6), including low-grade NMIBC (7, 8), remains unmet to date. Such biomarkers are likely to reduce the frequency of diagnostic cystoscopies conducted, particularly among patients undergoing monitoring for recurrent urothelial bladder cancer.

Capillary electrophoresis coupled to mass spectrometry (CE-MS) has been applied for the investigation of peptides and low molecular weight proteins ($\leq$20 kDa) as urinary biomarkers (9, 10). The biomarkers can be subsequently sequenced and identified by employing CE/MS-MS and liquid chromatography (LC)/MS-MS proteomics platforms (11). Taking advantage of existing ample sized peptide databases available for conducting in-depth biomarker assessment (10), support vector machines (SVM),

shown to be more efficient in the analysis of such complex proteomics datasets in comparison with unsupervised methods (12), are used to generate high-dimensional biomarker classifiers (namely biomarker panels) which exhibit superior accuracy to single protein markers (9, 11, 13–15).

Particularly for urothelial bladder cancer, CE-MS–based biomarker panels have been previously described, exhibiting discriminatory capabilities for primary urothelial bladder cancer (15), nevertheless at advanced stages (15). The present multicenter study aims to expand upon these initial efforts targeting to discover and validate urinary biomarker panels for detecting primary and recurrent urothelial bladder cancer.

Patients and Methods

Patient enrollment and urine collection

Two multicenter cross-sectional studies were conducted to investigate the study objectives according to the REMARK Reporting Recommendations (16) and the recommendations for biomarker identification and reporting in clinical proteomics (17). A schematic representation of the study design is depicted in Fig. 1. The study was performed in accordance with the Declaration of Helsinki and ethical approval was obtained by local Ethics Committees. Proper informed consent procedures under Institutional Review Board–approved protocols were followed. Urine samples were collected in the period 2003–2014 from eligible outpatients visiting the Hospital del Mar in Barcelona, Spain ($n = 526$), Erasmus Medical Center in Rotterdam, the Netherlands ($n = 456$), University Hospital of Virginia, Charlottesville, VA ($n = 304$), Laikon Hospital in Athens, Greece ($n = 47$), and Hannover Medical School, Hannover, Germany ($n = 24$). Voided midstream urine was collected at outpatient visit and prior to any treatment, according to the standard protocol for urine collection defined by the European Kidney and Urine Proteomics (EuroKUP) and Human Kidney and Urine Proteome Project (HKUPP) networks.
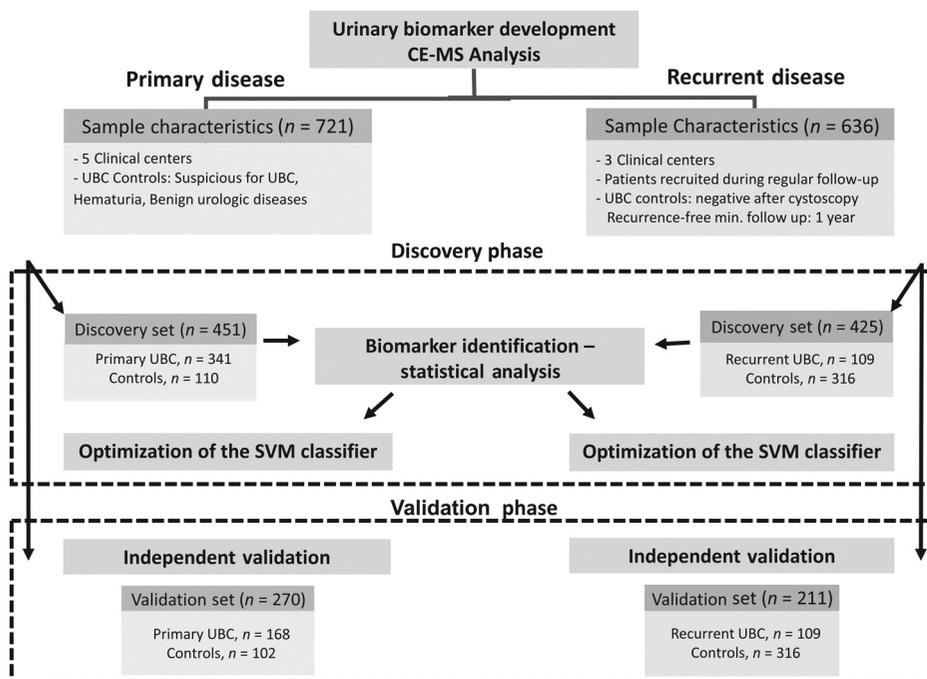

Figure 1.
Schematic representation of the study design and the analytical workflow for the development of urine-based biomarker panels. As shown in the schema, a discovery and validation phase was followed for the urine-based biomarker panels, for detecting primary urothelial bladder cancer, as shown in the left arm of the schema and recurrent urothelial bladder cancer, as displayed in the right arm of the schema. UBC, urothelial bladder cancer.

All urine samples were collected prior to cystoscopy and stored immediately following sample collection at −20°C until CE-MS analysis was performed. Presence of urothelial bladder cancer was confirmed with on-site cystoscopy. Among urothelial bladder cancer patients, tumor stage and grade was defined according to the TNM (tumor nodes metastases) classification (18), following histologic examination of biopsied tumor specimens. To avoid misclassification bias, all patients were reevaluated for at least one year following baseline assessment.

Study cohort for primary urothelial bladder cancer

For assessing primary urothelial bladder cancer, 721 eligible participants, including 509 primary urothelial bladder cancer patients and 212 urologic controls were evaluated. The latter included patients presenting with hematuria and patients suffering from other disorders of the genitourinary tract, such as acute cystitis and nephrolithiasis (Supplementary Table S1). Exclusion criteria were presence of adenocarcinoma and papillary carcinoma ($n = 5$). Forty-seven urine samples (random catch) were collected at Laikon Hospital (Athens, Greece); 304 urine samples were from patients enrolled in the Department of Urology of University of Virginia (Charlottesville, VA). Eighty-five urine samples were from patients undergoing cystoscopy at the Erasmus MC, (Rotterdam, the Netherlands); 267 from Hospital del Mar (Barcelona, Spain), and 18 samples were from patients visiting the Hannover Medical School (Hannover, Germany). All patients were primary referrals, with no prior history of any urinary tract malignancy. The urothelial bladder cancer patients received the following treatment: radical cystectomy (MIBC patients) and TUR-B (NMIBC patients). The primary cohort of 721 patients (mean age of 66 ± 13 years) was randomly separated in discovery and validation sets of 451 (mean age of 66 ± 13 years) and 270 (mean age of 68 ± 12 years) patients, respectively (Table 1). The frequencies of the various stages were similar in the discovery and validation sets.

Study cohort for patients undergoing surveillance

For evaluating recurrent urothelial bladder cancer, 763 patients undergoing urothelial bladder cancer recurrence monitoring, according to the EORTC risk assessment and EAU guidelines (3), were evaluated (Supplementary Table S2). A total of 447 voided urine samples were collected from patients undergoing cystoscopy in at the Erasmus MC, (Rotterdam, the Netherlands). Similarly, 310 urine samples were derived from patients attending the Hospital del Mar and 6 urine samples from Hannover Medical School. Among the 763 patients undergoing urothelial bladder cancer recurrence monitoring, urothelial bladder cancer was confirmed by cystoscopy and histologic diagnosis in 164 cases. Of these 164 relapses, 136 were NMIBC and 28 MIBC cases. Exclusion criteria were presence of adenocarcinoma and papillary carcinoma ($n = 9$). Negative cystoscopy ($n = 599$) was used to exclude recurrence and define controls in this population under surveillance. Controls with follow-up for less than 1 year or relapse within 1 year from sampling were excluded to rule out false negatives at the time of sampling. In total, 127 patients had to be excluded on the basis of the above criteria and the remaining 472 urine samples were included in the analysis. The surveillance cohort (164 confirmed urothelial bladder cancer cases and 472 eligible negative for recurrence controls) presented a mean age of 68 years (SD = ±12) and was separated in a discovery set ($n = 425$; mean age of 69 ± 12) and a validation set ($n = 211$; mean age of 68 ± 13), as shown in Table 1.

The distribution of the different disease stages was similar in the discovery and validation sets. In addition, 55 urine samples (of the 211 validation set samples) originated from urothelial bladder cancer–positive cases, of which 14 or 6.6% were confirmed with MIBC.

Information on supplementary treatment [intravesical Bacillus Calmette-Guerin treatment (BCG), epirubicin, or mitomycin C (MMC)] prior to the urine collection was available for 371 patients undergoing urothelial bladder cancer recurrence monitoring from those attending the Erasmus MC (Supplementary Table S2). The distribution of these treatment modalities was similar in the discovery and validation sets [discovery set included 21.1% treated (out of which 1.6% was BCG, 1.6% MMC, and 17.9% EPI) and 78.9% nontreated; validation set included 21.0% treated (out of which 1.4% was BCG, 0.4 MMC, and 18.1%

Table 1. Patient cohorts and sample sizes involved in the different study phases

	Sample size $n = 1,357$ (%)	Age (SD)[a]
Study arm I: Discovery and validation of biomarker panel for primary UBC	**721**	**66 (±13)**
Overall primary UBC patients	509 (71%)	68 (±12)
Overall urologic controls	212 (29%)	63 (±15)
Discovery phase	451	66 (±13)
Primary UBC patients	341 (76%)	67 (±12)
Urologic controls	110 (24%)	60 (±16)
Validation phase	270	68 (±12)
Primary UBC patients	168 (62%)	69 (±11)
Urologic controls	102 (38%)	65 (±14)
Study arm II: Discovery and validation of biomarker panel for recurrent UBC	**636**	**68 (±12)**
Overall UBC patients with recurrent disease	164 (26%)	70± (11)
Overall UBC patients without recurrent disease ("recurrent controls")	472 (74%)	68± (12)
Discovery phase	425	69 (±12)
UBC patients with recurrent disease	109 (26%)	71± (10)
UBC patients without recurrent disease	316 (74%)	68± (12)
Validation phase	211	68 (±13)
UBC patients with recurrent disease	55 (26%)	69± (13)
UBC patients without recurrent disease	156 (74%)	68± (13)

Abbreviation: UBC, urothelial bladder cancer.
[a]Mean value.

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epirubicin) and 79.0% nontreated, $P = 0.6229$, χ^2 test], as shown in the Supplementary Table S2.

Of the 211 subjects that were included in the validation phase, voided urinary cytology (VUC) results were available for 96 urinary samples.

Sample preparation and CE-MS analysis

Sample preparation was performed according to a standardized protocol (19). Data acquisition was performed by employing a P/ACE MDQ capillary electrophoresis system (Beckman Coulter) coupled on-line to a Micro-time-of-flight mass spectrometry (Micro-TOF-MS; Bruker Daltonic), following the previously described sample injection and acquisition protocol (15). Accuracy, precision, selectivity, sensitivity, reproducibility, and stability have been reported previously (15, 20). Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using the MosaiquesVisu software (21). Normalization of the CE-MS data was performed by using 29 internal peptide standards (10, 13). Detected peptides were deposited, matched, and annotated in a Microsoft-SQL database.

Statistical analysis

Discovery phase. Statistical analysis was performed for identifying discriminatory biomarkers for primary and recurrent urothelial bladder cancer, by analyzing the two discovery sets separately. Peptide intensities were log-transformed, and their difference between cases and controls was evaluated by using the Wilcoxon rank-sum test. To eliminate potential center bias, the biomarkers were further analyzed for their correlation with primary or recurrent urothelial bladder cancer across the various participating clinical centers. All the peptides fulfilling at least one of the two following criteria: (i) significant after multiple testing adjustment using Benjamini–Hochberg and/or (ii) consistent in regulation in at least two clinical centers, were shortlisted. Considering that the significant biomarkers should display differences associated with urothelial bladder cancer disease as a whole, and to increase the SVM statistical power, a pool of significant urothelial bladder cancer associated peptides were shortlisted. Using these later shortlisted peptides, two urine-based biomarker panels were optimized in the two separate training sets, using the SVM-based MosaCluster software (version 1.7.0; refs. 12, 22). The MosaCluster software tool allows for the classification of samples in the high dimensional parameter space by using SVM algorithms, previously shown to be particularly effective in analyzing high dimensional proteomics datasets (22, 23). The software generates a classifier, based on predefined peptides. Each of these peptides allegorizes one dimension in the n-dimensional parameter space (24). Specifically, the following SVM parameters were defined and further applied during validation: for the primary urothelial bladder cancer classifier: C = 5.04494, gamma = 0.008269, epsilon = 0.001; and for the recurrent urothelial bladder cancer classifier: C = 6.40000, gamma = 0.008000, epsilon = 0.001.

Validation phase. The urine-based biomarker panels were subsequently validated for detecting primary or recurrent urothelial bladder cancer in the independent validation sets of 270 and 211 samples, respectively. Sensitivity and specificity of the SVM-based classifier were estimated on the basis of the number of correctly classified samples, as defined by cystoscopy (12). The biomarker score was calculated via SVM-based software, MosaCluster (version 1.7.0). Confidence intervals (95% CI) were based on exact binomial calculations and were calculated in MedCalc Version 12.1.0.0, as were the receiver operating characteristic (ROC) plots. The area under the ROC curve (AUC) was evaluated for estimating the overall accuracy (25, 26). Statistical comparisons of the validation classification scores between the urothelial bladder cancer patients and the control groups, was performed by the Kruskal–Wallis test using MedCalc. For the urothelial bladder cancer patients, the classification scores according to the tumor stages were also investigated. Negative and positive predictive values (NPV/PPV), were calculated accounting for the specific prevalence rates for primary and recurrent disease in this study.

Sequencing of peptides

Urine samples were analyzed on a Dionex Ultimate 3000 RSLS nanoflow system (Dionex) coupled to an Orbitrap Velos instrument (Thermo Scientific; ref. 23). The data files were analyzed using Proteome Discoverer 1.2 (activation type: HCD; min–max precursor mass: 790–6,000 Da; precursor mass tolerance: 10 ppm; fragment mass tolerance: 0.05 Da; S/N threshold: (i) and were searched against the Uniprot human nonredundant database without enzyme specificity. No fixed modifications were selected, oxidation of methionine and proline were selected as variable modifications. The criteria for accepting sequences were high confidence (Xcorr $\geq$ 1.9), absence of unmodified cysteine, and absence of oxidized proline in protein precursors other than collagens or elastin. For further validation of obtained peptide identifications, the strict correlation between peptide charge at the working pH of 2.0 and CE migration time was used to prevent false identifications (27).

Results

Identification of significant urinary biomarkers for urothelial bladder cancer

For assessing primary urothelial bladder cancer, a case–control comparison was conducted in the 451 urine samples of the discovery set, between 341 primary urothelial bladder cancer cases and 110 urologic controls. The statistical comparison enabled the identification of 329 apparently significant peptides ($P < 0.05$, Wilcoxon rank-sum test), shown in Supplementary Table S3. Among those 329, 9 peptides were statistically significant after adjustment for FDR by using Benjamini–Hochberg test (Supplementary Table S3). To further increase the validity of the findings, the 329 biomarkers were assessed for concordant regulation across the different clinical centers. For the primary cohort, 62 potential biomarkers were identified as being concordant in the regulation trend in at least two clinical centers (Supplementary Table S3). Combination of the 9 peptides significant after multiple testing adjustment and the 62 peptides that were identified with concordant regulation in at least two clinical centers resulted in 66 unique peptide biomarkers, apparently significantly associated with the disease. These were included in a peptide panel using the SVM-based software. The biomarker panel exhibited 76% diagnostic accuracy and an estimated AUC value of 0.77 after complete take-one-out cross-validation in the training set of 451 samples. For investigating recurrent urothelial bladder cancer, comparison was performed between the 109 recurrent urothelial bladder cancer cases and 316 negative for recurrence controls. In detail, 327 biomarkers were identified as apparently significantly

altered. Among the latter, 51 were significant after multiple adjustment using the Benjamini–Hochberg test. As described above, the regulation of the 327 potential biomarker peptides was subsequently examined for their regulation in the different clinical cohorts (Supplementary Table S4). In total, 25 peptides were identified as concordantly regulated in the different clinical centers, as shown in Supplementary Table S4. Among the 25 with concordant regulation in the recurrent cohort, 13 had already been shortlisted as remaining statistical significant after multiple adjustment using Benjamini–Hochberg. Therefore, the combination of the 25 consistently regulated peptides with the 51 peptides remaining significant after multiple comparison adjustment resulted in 63 unique peptide biomarkers. These were subsequently combined in a multiple-peptide SVM-based panel. The diagnostic accuracy was initially assessed using cross-validation in the training set of 425 urine samples. The estimated AUC value was 0.70 after complete take-one-out cross-validation. Both the biomarker panels apparently exhibited satisfactory performance when cross-validated in the two separate training sets. Considering that classifiers based on a higher number of biomarkers regularly show increased stability and performance (12), in the next step, all possible available biomarkers (pooling all potential peptide biomarkers defined above, 66 for primary and 63 for recurrent urothelial bladder cancer (including 4 overlapping peptides) were employed aiming to establish SVM-based urothelial bladder cancer–specific classifiers with superior performance. Using the pool of these 125 biomarkers and the SVM-based MosaCluster software, two biomarker panels were generated and optimized using a take-one-out procedure and the two separate training sets of 451 (primary) and 425 (surveillance) urine samples, respectively. In the former (discovery set of 451 urine samples from patients with primary urothelial bladder cancer), 116 peptides were employed to form an optimized SVM-based biomarker classifier (Supplementary Table S5). Similarly, during the optimization of the SVM model for recurrence, out of the 125 peptides, a peptide panel based on 106 peptides (coinciding with 116-peptide model for primary urothelial bladder cancer minus 10 hemoglobin peptide sequences that were proven to carry no value in detecting recurrence) was developed. Both the aforementioned peptide panels (116-peptide model for primary and 106-peptide model for recurrence) exhibited better diagnostic accuracies than the initially developed panels (66-peptide model for primary and 63-peptide model for recurrence). This was indicated by AUC value of 0.88 for detection of primary urothelial bladder cancer after cross-validation in the training set and AUC value of 0.76 after cross-validation in the recurrent training cohort. On the basis of these results, the 116-peptide panel for detecting primary urothelial bladder cancer and the 106-peptide panel for detecting recurrent urothelial bladder cancer were chosen for further validation in the independent validation sets.

Validation of the urothelial bladder cancer biomarker panel for detecting primary urothelial bladder cancer

Subsequent validation of the 116 peptide biomarker panel was conducted in an independent set of 270 samples (including 168 primary urothelial bladder cancer cases and 102 controls) resulting in an AUC of 0.87, (95% CI, 0.83–0.91). At a cut-off level of −0.27, which was selected to allow for high sensitivity in urothelial bladder cancer detection, the biomarker panel's sensitivity was
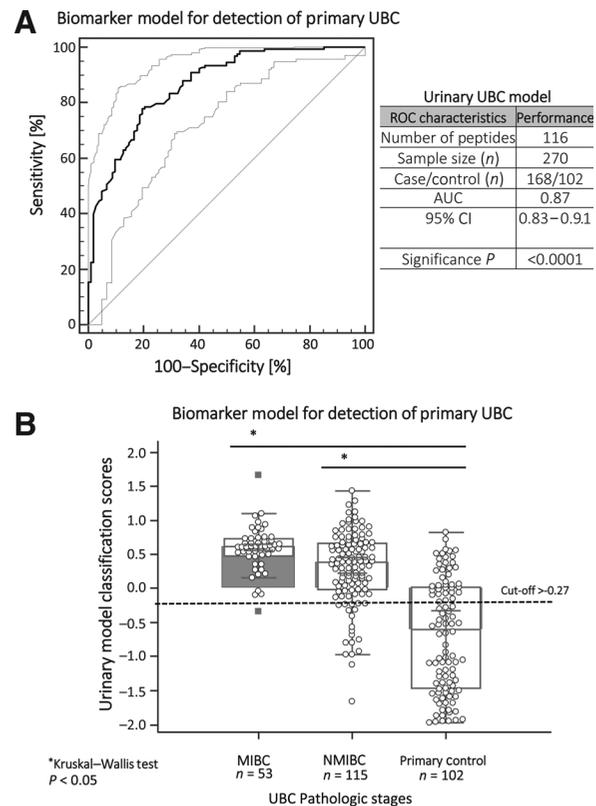

A, Biomarker model for detection of primary UBC

Urinary UBC model	
ROC characteristics	Performance
Number of peptides	116
Sample size (n)	270
Case/control (n)	168/102
AUC	0.87
95% CI	0.83–0.9.1
Significance P	<0.0001

B, Biomarker model for detection of primary UBC

Cut-off >-0.27

*Kruskal–Wallis test
P < 0.05

MIBC n = 53   NMIBC n = 115   Primary control n = 102

UBC Pathologic stages

Figure 2.
A, ROC analysis performed in the independent validation cohort, displaying the performance of the urothelial bladder cancer model for classifying the primary urothelial bladder cancer cases and the respective classification scores, presented in Box-and-Whisker plots (**B**) according to the NMIBC (n = 115) and MIBC (n = 53) stages of the primary urothelial bladder cancer cases. The urothelial bladder cancer model was constructed based on 116 bladder cancer–specific peptides. Other ROC characteristics, such as area under the curve (AUC), 95% confidence intervals (CI), and P value are provided for the classification of urothelial bladder cancer patients. UBC, urothelial bladder cancer.

estimated at 91% with specificity of 68%, respectively (Fig. 2A). Considering a prevalence rate of 63.5%, as estimated on the basis of the participating centers, NPV was estimated at 81.3% (95% CI, 68%–91%) and PPV at 83.2 (95% CI, 75%–89%). Notably, the 116 biomarker panel enabled significant discrimination of urothelial bladder cancer cases from controls regardless urothelial bladder cancer TNM stage (Fig. 2B; $P < 0.001$, Kruskal–Wallis test, Table 2).

Out of the 116 CE-MS–derived peptide biomarkers, sequence could be obtained for 105 peptides (i.e., 90.5%) listed in Supplementary Table S5). Most of the peptide sequences (48/116 or 41%), were collagen fragments, possibly attributed to cancer-related processes (e.g., increased protease activity, extracellular matrix remodeling and increased collagen cleavage). The second most frequent sequences (14/116 or 12%) originated from hemoglobin chains probably due to the presence of hematuria. Additional prominent peptides were derived from apolipoprotein A (5%), CD99 antigen (3%), fibrinogen A (2%), β2-microglobulin (2%), and single peptides from small proline-rich protein 3, insulin, and histidine-rich glycoprotein.

Table 2. Classification scores of the biomarker panels for NMIBC and MIBC, as obtained during the independent validation phase

| (diagnosis[a]) | Urinary biomarker panel for the detection of Primary UBC | | | Urinary biomarker panel for the detection of Recurrent UBC | | |
	Sample Size $n = 270$, (%)[c]	Classification score mean value (SD)	P[b]	Sample size $n = 211$, (%)	Classification score mean value (SD)	P[b]
NMIBC (Ta, T1, CIS[d])	115 (42.6%)	0.29(0.54)	<0.0001	41 (19.4%)	−0.18 (0.48)	<0.0001
MIBC	53 (19.6%)	0.58(0.32)	<0.0001	14 (6.7%)	0.10 (0.50)	<0.0001
Controls[e]	102 (37.8%)	−0.69(0.82)		156 (73.9%)	−0.56 (0.48)	

NOTE: The urinary biomarker panel of 116 peptides was developed for the detection of primary UBC patients and the urinary biomarker panel of 106 was optimized for detecting recurrent UBC patients.

Abbreviation: UBC, urothelial bladder cancer.

[a]According to the 2004 TNM system (18).

[b]Independent samples t test.

[c]Percentage over the number of UBC cases.

[d]According to EAU guidelines (refs. 3, 44).

[e]For the primary UBC, the controls are referring to urologic controls (hematuria, benign urological diseases), while in the recurrent group, the controls included are samples derived from patients negative for recurrence.

Validation of urothelial bladder cancer biomarker panel for detection of recurrent urothelial bladder cancer

The 106 peptide biomarker panel for detection of recurrence was validated in 211 independent samples (including 55 urothelial bladder cancer recurrent cases and 156 recurrent controls), with an AUC of 0.75, (95% CI, 0.68%–0.80%; Fig. 3A). At the ideal cut-off of -0.63, sensitivity and specificity values were 88% and 51%, respectively, while NPV was estimated at 93.6% (95% CI, 85%–98%) and PPV at 32.32% (95% CI, 23%–43%), accounting for a prevalence of 21.2% in the population investigated.

The classification scores of patients with recurrent urothelial bladder cancer significantly differed from the negative for recurrence controls ($P < 0.001$, Kruskal–Wallis test; Fig. 3B). The biomarker panel for detecting urothelial bladder cancer recurrence also presented significant discriminatory ability between patients presenting with NMIBC (Ta, T1, and CIS: $n = 41$, $P < 0.0001$) and MIBC (T2–T4: $n = 14$, $P < 0.0001$), respectively (Table 2).

For a substantial fraction of patients undergoing surveillance, data from the cytologic examination of urine samples to evaluate presence of malignant cells, were available. Out of 211 patients included in the validation phase, cytology had been performed in 96. Sensitivity and specificity of cytology for detecting recurrence was estimated at 31% and 100%, respectively, while sensitivity of the classifier was 92% with a specificity of 53% in this subset of samples. Multivariate analysis, accounting for the available demographical variables (age and gender) showed a superior AUC value of 0.80 for the classifier for detection of recurrence, compared with an AUC value of 0.69 obtained for cytology. Combination of both tests increased the performance, as assessed by an AUC of 0.87, compared with the performances of any single test alone (0.69 for cytology and 0.80 for the classifier; Supplementary Table S6).

The 106-peptide biomarker panel was further evaluated for detecting low risk recurrent urothelial bladder cancer, as this represents a highly relevant biomarker context-of-use during disease surveillance of major anticipated added value to regular clinical practice (potentially decrease in number of cystoscopies; ref. 8). For this assessment, 182 samples were available, including 26 urothelial bladder cancer recurrent cases and 156 recurrent controls. An AUC of 0.72 (95% CI, 0.65–0.78) was observed (Supplementary Table S6). Cytologic examination was available for 88 out of 182 samples. The 106-peptide biomarker panel outperformed cytology, with an AUC of 0.79 compared with an AUC of 0.64 for cytology. Combination of both tests increased the

performance, as assessed by the given AUC of 0.90, compared with the performances of any single test alone, 0.64 for cytology and 0.79 for the classifier.

Data on supplementary treatment (e.g., BCG or chemotherapy) administered prior to the urine collection were available for a total of 123 patients included in the validation set. Logistic regression analysis indicated that the classification score based on the 106 -peptide biomarker was not affected by supplementary treatment (Supplementary Table S7).

Out of 106 peptides included in the biomarker panel, 95 (89.6%) sequences were obtained (Supplementary Table S5). The majority (57%) were collagen fragments, while Apolipoprotein A-I peptides accounted for the second most frequent peptide sequences (6%). Other peptide biomarkers corresponded to fragments of basement membrane-specific heparan sulfate proteoglycan core protein (HSPG2), a disintegrin, and metalloproteinase domain-containing protein 22 (ADAM22), disintegrin, and metalloproteinase with thrombospondin motifs 1 (ADAMTS1).

Discussion

The present multicenter study optimized and validated two unique urinary peptide-based biomarker panels for detecting primary and recurrent urothelial bladder cancer. The findings presented demonstrate the value of a multiple-marker approach for facilitating urothelial bladder cancer diagnosis, particularly considering the increased variability which is likely caused in part by biologic variability and by the high intratumor heterogeneity.

The two classifiers were developed on the basis of a pool of statistically significant different peptides. Even though these initial sets of shortlisted peptides did not largely overlap between the two cohorts, likely due to the applied stringent thresholds in each case (e.g., significant after BH adjustment, being consistent among centers), the final selected peptides forming the two classifiers are identical, with the exception of 10 hemoglobin fragments included solely in the "primary" classifier. Interestingly, out of these 116 peptide biomarkers, 56 were significantly correlated with disease stage and 32 with disease grade (Supplementary Table S8). Moreover, both peptide biomarker panels exhibited superior discriminatory ability in detecting MIBC compared with NMIBC (AUC of 0.94 for MIBC versus 0.84 for NMIBC for the 116 primary panel; AUC of 0.90 for MIBC and 0.70 for NMIBC for the 106-recurrent panel; Supplementary Table S8). Collectively these data suggest that the peptide biomarkers in their vast majority are
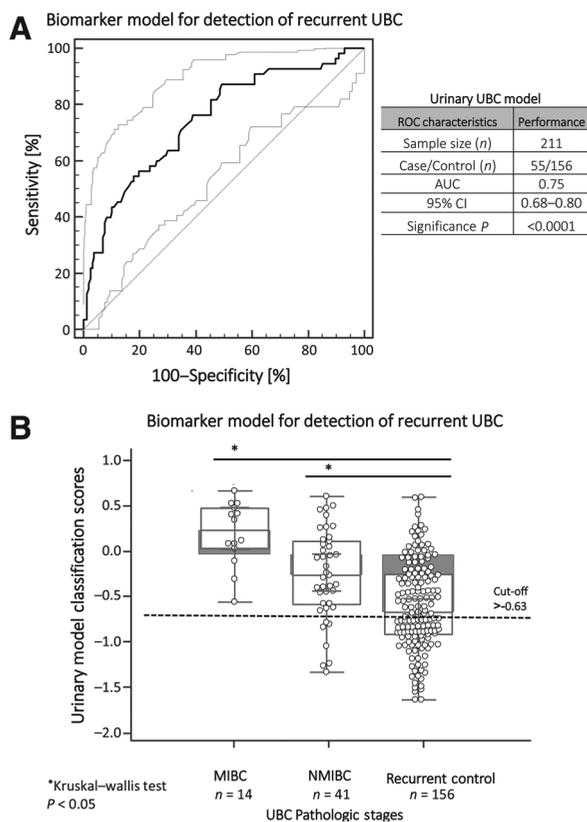
Figure 3.
A, ROC curve for the urinary biomarker panel, consisting of 106 peptides as performed in the independent validation cohort. The AUC, 95% CIs, and *P* value are also provided for the classification of urothelial bladder cancer patients. **B,** classification scores presented in Box-and-Whisker displaying the level of discrimination between the recurrent urothelial bladder cancer cases and negative for urothelial bladder cancer control and the distribution of classification values for NMIBC (*n* = 41) and MIBC cases (*n* = 14). A *post hoc* rank-test was performed using Kruskal–Wallis test. The average rank differences were significantly different (*P* < 0.05) between the controls and the recurrent urothelial bladder cancer case group. UBC, urothelial bladder cancer.

reflective of changes associated with cancer progression, regardless whether that corresponds to a primary or relapsed event.

Several of the biomarkers included in the classifiers were previously reported as biomarkers for the detection of urothelial bladder cancer. Apolipoprotein A-I peptide fragments were found up-regulated in both primary and recurrent disease in line with earlier reported findings on this protein in urothelial bladder cancer following application of two-dimensional electrophoresis (2-DE), or iTRAQ/LC-MS/MS-based proteomics analysis (28–35). In addition, Fibrinogen chains α and β, well known urinary biomarkers for the detection of bladder cancer (30, 31, 33–35), were also found at increased levels in urine from patients with urothelial bladder cancer and included in the classifiers. Beta-2-macroglobulin and basement membrane-specific heparan sulfate proteoglycan core protein (HSPG2) were also confirmed with decreased excretion levels in urine from patients with urothelial bladder cancer, in line with previously reported proteomics data (28, 31, 33). Moreover, PGMRC1 (membrane-associated progesterone receptor component 1) has been previously included as a

peptide biomarker in a CE-MS classifier discriminating between NMIBC and MIBC patients (14). In the presented study, the same PGMRC1 peptide was detected at increased levels in urine of urothelial bladder cancer patients, in comparison to controls.

Of note, in the two biomarker panels for detection of primary and recurrent urothelial bladder cancer, most peptide sequences were derived from collagen fragments. This likely reflects increased extracellular matrix (ECM) turnover, related to the activation of collagen-degrading proteases during tumor invasion (36). Several hemoglobin fragments were significantly associated with primary urothelial bladder cancer, but not found to be significantly altered in the urine from patients presenting recurrence. This observation is in accordance with the hypothesis that hemoglobin fragments most likely indicate hematuria, which is frequently present in the urine of patients with primary tumors, but rarely in recurrence.

Several peptide biomarkers included in the classifiers originate from proteins reported as associated with cancer initiation and/or progression. Small proline-rich protein 3 (SPRR3) was detected at higher levels in the urine of both primary and recurrent urothelial bladder cancer patients, in comparison with controls. SPRR3 protein has not been characterized in the context of bladder cancer yet, however, upregulation of SPRR3 protein levels promotes colorectal tumorigenesis (37) and is associated with tumor cell proliferation and invasion in glioblastoma (38). In addition, 14-3-3 sigma protein is frequently downregulated in a variety of human cancers including invasive urothelial bladder cancer tumors, particularly in lesions undergoing epithelial to mesenchymal transition (39) and this downregulation is attributed to increased methylation of its promoter (40). In the current study, fragments of 14-3-3 sigma were detected at lower urinary levels in patients with either primary or recurrent urothelial bladder cancer in comparison with controls. Similarly, CD99 low protein expression levels, likely due to gene promoter hypermethylation, have been also reported in urothelial bladder cancer (41). In this study, several peptides of CD99 protein were detected at lower levels in urine from patients with urothelial bladder cancer, compared with controls.

A small number (about 10%) of the CE-MS ion peaks included in the classifiers could not be identified by MS/MS. Failure to obtain sequence from these peptides is generally due to either peptides not fragmenting well, or due to unknown post-translational modifications that prevent mapping to the available sequence databases (42). As a higher number of biomarkers confers increased stability of the test (12), the presented biomarker panels include all significant biomarkers identified, irrespective of whether the sequence was obtained, or not. Efforts are ongoing to obtain sequences from all peptides included in the discriminatory panels.

For primary urothelial bladder cancer, CE-MS–derived urinary peptide biomarker panels were previously reported and assessed for detecting urothelial bladder cancer (14, 15). These studies, however, included lower number of samples and mostly MIBC cases (14, 15). When investigating the performance of the previously reported classifier in additional cohorts mainly composed of NMIBC patients (15), the classifier exhibited 71% sensitivity and 40% specificity (data not shown), insufficient for clinical implementation.

In our study, the source population and recruitment procedures very closely represent typical clinical situations. In such settings, high-risk patients (e.g., with hematuria at primary diagnosis and/

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or under surveillance) are most likely to potentially benefit from the adoption of a noninvasive urine test, with a high NPV value, which could accurately guide cystoscopy (3, 8). Considering the prevalence rates for the specific cohorts, the developed biomarker panels present an NPV value of 81.3% (95% CI, 68%–91%) and PPV value of 83.2 (95% CI, 75%–89%) for the primary and NPV of 93.6% (95% CI, 85%–98%) and PPV of 32.32% (95% CI, 23%–43%), for the follow-up cohorts. These performance rates are at least as good as those of other urothelial bladder cancer molecular markers which are FDA-approved and/or are currently under investigation (6). A direct comparison of different markers and their performance, as reported in various studies is difficult, mainly due to differences in the clinical design of the respective studies. A 10-biomarker ELISA-based assay (IL8, MMP9, MMP10, SERPINA1, VEGFA, ANG, CA9, APOE, SDC1, and SERPINE1) provided an overall AUC of 0.85 (95% CI, 0.80–0.91) in discriminating urothelial bladder cancer patients from healthy and benign controls (45), slightly lower than the rates received from the CE-MS classifier for primary urothelial bladder cancer (AUC = 0.87; 95% CI, 0.83–0.91). A three-gene methylation panel (*OTX1*, *ONECUT2*, and *OSR1*) detected low/intermediate risk urothelial bladder cancer with a sensitivity of 74% and specificity of 90% (43). In the current study, when investigating the subpopulation of low/intermediate-risk patients (NMIBC G_1–G_2; $n = 26$), the CE-MS–based classifier provided a sensitivity of 89% in urothelial bladder cancer detection at the preselected cut-off (AUC = 0.72). For those low-intermediate risk patients where information on cytology was available ($n = 7$), an increased sensitivity upon combination of the classifier with cytology could be obtained (AUC of 0.90; 100% sensitivity, 63% specificity). Even though promising, this result is from a small subset of samples, therefore, its further validation is required.

The strengths of the present investigation include that it is the largest multicenter study conducted to date for identifying and validating biomarker panels for primary and recurrent urothelial bladder cancer. The presented urine-based biomarker panels hold promise for facilitating urothelial bladder cancer diagnosis non-invasively in outpatient settings. The proteomics approaches applied for the biomarker panel development including use of an analytically validated platform (19) represent the current state-of-the-art, securing optimal diagnostic performance. The study design employed has diminished the potential effects of both source population and selection biases. In addition, patient classification according to the standard-of-care, cystoscopy, deters misclassification bias while enhancing the external validity and translational potential of study findings.

However, several limitations are present in this study and warrant further consideration. Adjustment for potential confounding effects, including patient characteristics, clinical, and/or treatment variables, upon biomarker panel performance could not be conducted due to data limitations. In detail, clinical variables such as tumor size, multiplicity, presence of hematuria were not available for all samples.

Moreover, multivariable regression analyses to predict urothelial bladder cancer could not be performed, as known risk factors for urothelial bladder cancer, including smoking history, previous upper tract cancer, and history or presence of macroscopic hematuria were also not recorded for all patients.

Collectively, through the current study, we aimed to meet a very clear clinical need in bladder cancer management: the development of biomarker assays to be used for diagnosis of bladder cancer and detection of disease recurrence, particularly among NMIBC patients. NMIBC patients represent the largest bladder cancer subtype and also the group that would benefit most from improvement in recurrence monitoring procedures, as existing approaches are invasive. The specific impact of the noninvasive biomarker classifiers would primarily be to guide cystoscopy and in combination with cytology as suggested by our results, and/or other molecular assays, reduce the number of surveillance cystoscopies. Moreover, in view of a positive test, urologists may be alerted to perform a more thorough investigation of the bladder hence increasing the overall accuracy in disease detection.

Because of the applied cross-sectional study design, the presented findings should be confirmed in a prospective study. Further longitudinal investigations, accounting for potential confounding effects on biomarker performance could confirm the value of the present findings, and possibly allow detecting additional benefits (e.g., value in prognosis of progression).

Conclusions

Two urine-based biomarker panels for detecting primary and recurrent urothelial bladder cancer were developed to support patient screening and monitoring. The urine-based biomarkers for primary urothelial bladder cancer hold promise for facilitating urothelial bladder cancer diagnosis noninvasively in outpatient settings. The urine-based panel for detecting recurrence in combination to cytology resulted in improved non-invasive urothelial bladder cancer detection. Additional prospective investigations accounting for potential confounding effects are planned to evaluate potential clinical implementation.

Disclosure of Potential Conflicts of Interest

E.C. Zwarthoff reports receiving commercial research grants from and is a consultant/advisory board member for MDxHealth. H. Mischak holds ownership interest (including patents) in Mosaiques Diagnostics. No potential conflicts of interest were disclosed by the other authors.

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Development and Validation of Urine-based Peptide Biomarker Panels for Detecting Bladder Cancer in a Multi-center Study

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