Prediction of Muscle-invasive Bladder Cancer Using Urinary Proteomics

Eric Schiffer,1 Antonia Vlahou,2 Andreas Petrolekas,3 Konstantinos Stravodimos,3 Robert Tauber,4 Jürgen E. Geschwend,4 Jochen Neuhaus,5 Jens-Uwe Stolzenburg,5 Mark R. Conaway,6 Harald Mischak,1 and Dan Theodorescu7,8

Abstract

Purpose: Minimally invasive methods of predicting the risk of muscle-invasive urothelial bladder carcinoma may expedite appropriate therapy and reduce morbidity and cost.

Experimental Design: Here, capillary electrophoresis coupled mass spectrometry was used to identify urinary polypeptide bladder cancer biomarkers in 127 patients. These markers were used to construct a panel discriminating muscle-invasive from noninvasive disease, which was refined in 297 additional samples from healthy volunteers, patients with malignant and nonmalignant genitourinary conditions. Sequencing of panel polypeptides was then done. Finally, the ability of the panel to predict muscle-invasive disease was evaluated prospectively in 130 bladder carcinoma patients. Four sequenced polypeptides formed a panel predictive of muscle-invasive disease.

Results: Prospective evaluation of this panel revealed a sensitivity of 81% [95% confidence interval (CI), 69-90] and specificity of 57% (95% CI, 45-69) for muscle-invasive disease. Multivariate analysis revealed the panel (P < 0.0001) and tumor grade (P = 0.0001), but not urine cytology, predict muscle invasion. A model including grade and panel polypeptide levels improved sensitivity [92% (95% CI, 82-97)] and specificity [68% (95% CI, 55-79)] for muscle-invasive disease. A model score of >0.88 provided a negative predictive value of 77% and positive predictive value of 90% for muscle invasion.

Conclusions: Use of urinary peptides seems promising in estimating the probability a patient harbors muscle-invasive urothelial bladder cancer. These peptides may also shed novel insights into the biology of bladder tumor progression not obtainable by other methods. Clinical trials seem warranted to evaluate the effect of this approach on practice.

Bladder cancer (BCa) is a common malignancy worldwide (1). The urothelial carcinoma subtype constitutes 95% of all cases in Western countries (2). At initial presentation, ~70% of tumors do not invade the bladder muscle wall, whereas the remainder present with muscle-invasive disease. In addition, up to 30% of the former patients recur with muscle-invasive disease during follow up (3). The standard method used to determine the local stage of the neoplasm is transurethral resection (TUR) of the bladder tumor (4). This procedure provides treatment for patients with non–muscle-invasive disease and staging information for those with muscle-invasive disease. In addition, if patients in the latter group were to select organ-sparing approaches (5) for the treatment of their disease, the TUR would also provide a therapeutic contribution.

Prior efforts have identified urinary protein biomarkers of bladder cancer (6–9). However, markers that can predict tumor stage have not been identified. Such markers would have several clinical uses. They would provide pre-TUR information that could be used for patient counseling and determination of the extent of the TUR required in cases where muscle-invasive disease is predicted. In such cases, if the patient wishes to have...
radiochemotherapy, then a complete TUR would be attempted. In contrast, if the patient would prefer to have radical cystectomy, given the benefit from neoadjuvant chemotherapy (10), immediate initiation of such treatment would take place thus minimizing delays.

Here, we designed a study to test the hypothesis that stage-specific markers exist and can be helpful in predicting the presence of muscle-invasive disease with the hope that such knowledge can eventually guide therapeutic choice.

Materials and Methods

**Patient and tumor characteristics.** The urothelial cancer “training” set used for the determination of the muscle-invasive bladder cancer (defined as stages T2-T4; ref. 11) polypeptide panel (MI-BCA) included 127 patients [81 male, 46 female; median age, 68 ± 17 y; interquartile range (IQR), 38-86 y] with urothelial bladder cancer. All patients had a negative metastatic workup (NOM0) by imaging criteria (computed tomography or magnetic resonance imaging). These patients underwent radical cystectomy and/or TUR and were prospectively enrolled in the current study in the Departments of Urology of University of Virginia, Charlottesville, Virginia, USA; Laikon Hospital, Athens, Greece; University of Leipzig, Leipzig, Germany; or Technische Universität München, Munich, Germany. These patients did not have genitourinary stones by imaging criteria. The local ethics committees approved the study, and all subjects gave informed consent. The study was done in accordance with the Helsinki Declaration.

**Capillary electrophoresis coupled to mass spectrometry analysis.** Detailed Materials and Methods section and data of analytic platform validation are available in Supplementary Data. Samples consisted of spontaneously voided urine stored at -20°C. Preparation was done as described (6, 13) using ultrafiltration followed by desalting and lyophilization. Shortly before capillary electrophoresis coupled to mass spectrometry (CE-MS) analysis, lyophilisates were resuspended in high performance liquid chromatography-grade water to a final protein concentration of 0.8 μg/μl checked by BCA assay (Interchim). CE-MS analysis was done as described (6, 13). The analytic precision was assessed by performing repeatability, intermediate precision, temperature stability, postpreparation stability, frost/defrost stability, and time course experiments. The reproducibility of the findings as it relates to intraindividual variability was also evaluated (see Supplementary Data).

**Data processing.** Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaiquesVisu software (14). For normalization of analytic and urine dilution variances, MS signal intensities are normalized relative to 29 “housekeeping” peptides generally present in at least 90% of all urine samples with small relative standard (15). The obtained peak lists characterize each polypeptide by its molecular mass (Da), normalized CE migration time (min), and normalized signal intensity. All detected peptides were deposed, matched, and annotated in a Microsoft SQL database allowing further statistical analysis. For clustering, peptides in different samples were considered identical if mass deviation was <50 ppm for small or 75 ppm for larger peptides. Due to analytic diffusion effects, CE peak widths increase with CE migration time. In the data clustering process, this effect was considered by linearly increasing cluster widths over the entire electropherogram (19-45 min) from 2% to 5%. These clustering parameters showed minimal error rates and resulted in the tentative definition of 116,869 different peptides and proteins. Each peptide was assigned a unique identification number (Protein ID). As described previously (16), several of these peptides appeared sporadically, being observed in only one or a few samples. To eliminate such peptides of apparently low significance, only those peptides detected in >20% of the urine samples in at least one group (samples from patients with same disease) were included for the tentative definition of the muscle-invasive bladder cancer panel. The obtained peak lists were analyzed and clustered using MedCalc software (17). The ROC curve plot was obtained by plotting all sensitivity values (true positive fraction) on the Y axis against their equivalent (1-specificity) values (false-positive fraction) for all available thresholds on the X axis. The area under the ROC curve (AUC) was evaluated as it provides the single best measure of overall accuracy independent of any threshold. P-values were calculated using the base 10 logarithm-transformed intensities and the Gaussian approximation to the t-distribution. For multiple testing corrections, P values were corrected using the Westfall and Young maxT-procedure (18) for proteins that were detected in at least 9 patients.

**Statistical analysis.** Sensitivity, specificity, and 95% confidence intervals (95% CI) for the identification of muscle-invasive disease were calculated using receiver operating characteristic (ROC) plots (17). The ROC curve plot was obtained by plotting all sensitivity values (true positive fraction) on the Y axis against their equivalent (1-specificity) values (false-positive fraction) for all available thresholds on the X axis. The area under the ROC curve (AUC) was evaluated as it provides the single best measure of overall accuracy independent of any threshold. P-values were calculated using the base 10 logarithm-transformed intensities and the Gaussian approximation to the t-distribution. For multiple testing corrections, P values were corrected using the Westfall and Young maxT-procedure (18) for proteins that were detected in at least 9 patients.

**Translational Relevance.** Minimally invasive and accurate methods of predicting the risk of muscle-invasive urothelial bladder carcinoma at presentation are not available. We used mass spectrometry to identify urinary polypeptide biomarkers in 127 patients and construct a panel discriminating muscle-invasive from noninvasive disease. This panel was refined using 297 additional samples from healthy volunteers and patients with malignant and nonmalignant conditions. The ability of the panel and tumor grade to predict muscle-invasive disease was then evaluated prospectively in 130 bladder cancer patients, revealing a sensitivity of 92% and specificity to 68% for muscle-invasive disease. Urinary peptide evaluation is a novel and promising strategy for estimating the probability a patient harbors muscle-invasive urothelial bladder cancer.
70% of samples. The maxT function computes permutation-based step-down adjusted $P$ values. In addition to the maxT procedure, we verified that the $P$ values by the minP procedure of Westfall and Young were of similar magnitude (18). Both independently done tests were implemented as macros in SAS\textsuperscript{10} and are part of the multitest R-package\textsuperscript{11} (19).

**Classification.** MosaCluster (version 1.6.5;\textsuperscript{12} ref. 20) allows the classification of samples in the high dimensional parameter space by using support vector machines (SVM). MosaCluster generates polypeptide models that rely on polypeptides displaying statistically significant differences when comparing data from patients with a specific disease to controls or other diseases. Each of these polypeptides allegorises one dimension in the n-dimensional parameter space (21–24). Classification of a urine sample as derived from a muscle-invasive lesion is done by determining the Euclidian distance defined as the SVM score of the polypeptides in the MI-BCa panel.

**Nomogram development.** For the nomogram, the association between predictors (polypeptide biomarker panel SVM score, tumor grade (high/low according to the contemporary WHO system; ref. 25), urine cytology (positive/negative), and tumor stage was tested by multivariate logistic regression. Backward stepwise regression seems to be the preferred method of exploratory analyses, where the analysis begins with a full or saturated model and variables are eliminated from the model in an iterative process. However, this kind of regression may be associated with some risk of overfitting. Therefore, we have chosen a forward regression approach, where significant predictors are entered sequentially and after entering a variable, those that become nonsignificant are removed. Nevertheless, we evaluated both strategies and obtained identical models (data not shown). To assess the nomogram for overfitting, nomogram establishment was repeated 10 times by randomly excluding 33% of all available samples per diagnostic group in the training set. The obtained permutation nomograms were subsequently applied to the test set to assess classification performance, which was expressed by the obtained AUC values in ROC statistics. The probability of invasive disease was calculated according to

$$P = \frac{1}{1 + e^{-\left(\sum \beta_i x_i\right)}}$$

with $\beta_0$ as constant and $\beta_i$ as the classification coefficient of the predictor $x_i$. To enable mapping of the probability to detect muscle-invasive tumor for a given SVM score and tumor grade, a graphical nomogram was developed. Predictive accuracy of the resulting nomogram was quantified with ROC statistics. Statistical tests were done

<table>
<thead>
<tr>
<th>Table 1. Patient and tumor characteristics and uses in study</th>
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<tr>
<td><strong>A. Patient cohort</strong></td>
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<td>Clinical condition</td>
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<td>-------------------</td>
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<tr>
<td>Training set</td>
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<tr>
<td>Tis-T1*</td>
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<td>T2-T4*</td>
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<tr>
<td>Control set 1</td>
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<tr>
<td>Healthy volunteers</td>
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<tr>
<td>Genitourinary disorders\textsuperscript{†}</td>
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<tr>
<td>Control set 2</td>
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<tr>
<td>Healthy volunteers</td>
</tr>
<tr>
<td>Nephrolithiasis</td>
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<tr>
<td><strong>Test (validation) set</strong></td>
</tr>
<tr>
<td>Tis-T1*</td>
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<tr>
<td>T2-4*</td>
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<tr>
<td><strong>Total</strong></td>
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\textsuperscript{*}Bladder tumor stage (Sobin et al., 1997).
\textsuperscript{†}Disorders of the genitourinary tract such as cystitis, prostate cancer, or prostatism.
\textsuperscript{‡}Bladder tumor stage (Sobin et al., 1997).
\textsuperscript{§}Bladder tumor grade (Sauter et al., 2004).

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\textsuperscript{10}http://www.sas.com
\textsuperscript{11}http://www.bioconductor.org
\textsuperscript{12}http://www.proteomiques.com
using MedCalc version 8.1.1.0. Two-sided tests with significance at 0.05 were used.

**Sequencing of peptides.** To identify the defined staging biomarkers, we applied tandem mass spectrometry (MS/MS) peptide sequencing using either CE- or liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis (26, 27) or electron transfer dissociation (28). Obtained MS/MS data were submitted either to MASCOT for a search against human entries in the MDSB Protein Database. Accepted parent ion mass deviation was 50 ppm; accepted fragment ion mass deviation was 25 ppm. Hits with MASCOT peptide score of ≥20 were accepted, which also met ion coverage stipulations as related to the main spectral features. The number of basic and neutral polar amino acids of the peptide sequences was used to correlate peptide sequencing data to CE-MS/MS features. Then the number of basic and neutral polar amino acids of the peptide sequences was used to correlate peptide sequencing data to CE-MS/MS features. The number of basic and neutral polar amino acids of the peptide sequences was used to correlate peptide sequencing data to CE-MS/MS features.

**Results**

**Development of biomarker panel identifying muscle-invasive tumors.** To define polypeptides associated with muscle-invasive cancer, we used a training set of 71 samples from patients with noninvasive (pTis-1) tumors and 56 from those with invasive (pT2-4) bladder cancer (BCa; Table 1A). In a first step, pTis-1 BCa (n = 71) and pT2-4 BCa (n = 56) were independently compared with control set 1 (n = 121) to define Bca-specific polypeptides. These were then required to show BCA specificity in a second control group (n = 130; Table 1). In a second step, BCA-specific markers that were able to discriminate Tis-1 (n = 71) from T2-4 (n = 56) tumors (Table 1B) were defined as muscle-invasive markers and formed the MI-BCa panel. This panel was composed of four urinary polypeptides (Table 2; Fig. 1A-B).

To identify the four MI-BCa polypeptides, we applied MS/MS peptide sequencing using either CE-, LC-MS/MS analysis (26, 27), or electron transfer dissociation (28). The polypeptides were successfully identified as fragments of Uromodulin, Collagen α-1 (I), Collagen α-1 (III), and membrane-associated progesterone receptor component 1 (Table 2). All identified fragments were found to be down-regulated in muscle-invasive tumors compared with non-muscle-invasive tumors. One of these fragments, membrane-associated progesterone receptor component 1, was also found to be down-regulated as a function of tumor stage at the gene expression level in human BCa (Fig. 1C-D).

The ability of MI-BCa to discriminate non-muscle-invasive from invasive tumors in the training set (71 noninvasive and 56 invasive tumors) was evaluated by ROC analysis and found to have an AUC of 84% (95% CI, 77-90; Fig. 2A). By plotting sensitivity and specificity for detecting muscle-invasive disease against the SVM score provided by MI-BCa (MI-BCa Score; Fig. 2B), a classification threshold was developed for use in the subsequent blinded validation. A threshold of 0.87 was selected to ensure good predictive ability for muscle-invasive tumors.

**Validation of the MI-BCa panel in identifying muscle-invasive tumors.** The MI-BCa was examined in blinded fashion in a cohort of 149 samples from patients with urothelial BCa prospectively collected in four different clinical centers. Using the classification threshold score of >0.87 derived above, 90 samples scored as noninvasive and 40 samples scored as invasive tumors.
tumors. After unblinding 59 of 68 noninvasive tumors [specificity, 90% (95% CI, 80-96)] and 32 of 62 invasive tumors [sensitivity, 52% (95% CI, 39-65)] were correctly identified resulting in an AUC value of 74% (95% CI, 66-81; \( P < 0.0001 \); Fig. 2C). Analysis of these classification results revealed that accuracy for invasive cancers trended upwards the higher tumor stage (Table 3). Although 49% of T2 are correctly identified, 80% of T4 tumors were classified as invasive, whereas non-muscle-invasive tumors were identified with good accuracy (pTis, 83%; Ta, 90%; T1, 68%; Table 3). Low-grade noninvasive tumors had lower MI-BCa scores (median, -0.86) compared with high-grade noninvasive tumors (median, -0.66), but this did not reach significance (\( P = 0.777 \)). Although low-grade tumors are rarely muscle-invasive, high-grade lesions can be either invasive or non–muscle-invasive. We thus evaluated MI-BCa in classifying high-grade tumors and ROC analysis revealed an AUC of 72% (95% CI, 61-81; \( P = 0.0001 \)), which suggested that MI-BCa offers additional classification benefit in addition to tumor grade.

Given the above result and because tumor grade can be obtained with a minimally invasive office biopsy (29), we wondered, if combining grade with MI-BCa proteomic classification improves tumor stage prediction. For this purpose, a nomogram was established using logistic regression procedures to evaluate this idea (Fig. 3A). Both MI-BCa and tumor grade were significant independent predictors of tumor stage in the training set (Fig. 3C). However, urine cytology information did not provide additional predictive value (data not shown). The
resulting nomogram based on the MI-BCa score and tumor grade identified invasive tumor in the training set with sensitivity of 96% (95% CI, 87-99) and specificity of 72% (95% CI, 60-83) using a \( P \) value of 0.40 as the nomogram classification threshold or a sensitivity of 31% and specificity of 99% using a \( P \) value of 0.88 as the threshold.

To validate the nomogram, we evaluated test set patients. This resulted in a sensitivity of 92% (95% CI, 82-97) and specificity of 68% (95% CI, 55-79; \( P = 0.40 \) cutoff). The predictive accuracy of the nomogram [AUC, 87%; 95% CI, 79-92; \( P < 0.0001 \)] was found to be superior to that obtained proteomic classification alone (\( P = 0.0001 \)) or grading alone (\( P = 0.015 \); Fig. 3B). To evaluate positive predictive value (PPV) and negative predictive value (NPV) rates for invasive tumor, we assumed an estimate that 30% of tumors at initial presentation were muscle invasive (30-32). Using the \( P = 0.40 \) cutoff for the nomogram yielded, an NPV of 93% and a PPV of 62% for muscle-invasive disease while using a \( P \) value of 0.88 provided PPV and NPV for invasive tumor of 90% and 77%, respectively. To assess the nomogram for overfitting, nomogram establishment was repeated 10 times by randomly excluding blocks and were found to be associated with tumor grade and invasiveness (34). In addition, different keratins and psoriasin were associated with the degree of differentiation of bladder squamous cell carcinomas (35, 36). The same technology was applied to define biomarkers indicative for tumor heterogeneity among low-grade Ta noninvasive tumors (37). Although these results revealed interesting insights into tumor pathology, their effect in predicting tumor stage using body fluids remains unclear.

Here, we established and validated in a blinded setting a panel of four urinary polypeptides that seem promising for the noninvasive detection of muscle-invasive disease. Sequencing of the polypeptides in the panel provides insights into the

**Table 3. MI-BCa tumor staging classification performance in patients in test set (\( n = 130; \) Table 1A)**

<table>
<thead>
<tr>
<th>Tumor stage*</th>
<th>Specificity (95% CI)</th>
<th>Sensitivity (95% CI)</th>
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<tbody>
<tr>
<td>Tis (( n = 6 ))</td>
<td>83% (36-97)</td>
<td>—</td>
</tr>
<tr>
<td>pTa (( n = 40 ))</td>
<td>90% (76-97)</td>
<td>—</td>
</tr>
<tr>
<td>T1 (( n = 22 ))</td>
<td>86% (65-97)</td>
<td>—</td>
</tr>
<tr>
<td>T2 (( n = 39 ))</td>
<td>—</td>
<td>49% (32-65)</td>
</tr>
<tr>
<td>T3 (( n = 18 ))</td>
<td>—</td>
<td>50% (26-74)</td>
</tr>
<tr>
<td>T4 (( n = 5 ))</td>
<td>—</td>
<td>80% (29-97)</td>
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*Bladder tumor stage (Sobin et al., 1997).
pathology of urothelial malignancy as well as the future potential to establish high throughput multiplexed and inexpensive assays to evaluate these peptides in the routine laboratory setting. We successfully identified fragments of Uromodulin, Collagen α-1 (I), Collagen α-1 (III), and membrane-associated progesterone receptor component 1 as biomarkers. Progesterone receptor membrane component 1 is a member of the so-called membrane-associated progesterone receptor and is involved in different cellular processes at various subcellular locations (38). However, it has yet to be shown that progesterone receptor membrane component 1 exhibits specific progesterone binding. As an adaptor protein, progesterone receptor membrane component 1 might be involved in regulating protein interactions, intracellular signal transduction, and/or membrane trafficking (38). Finally, consistent with our data, the RNA expression of this protein is also reduced as a function of tumor stage in two independent microarray studies (39, 40). We found Collagen α-1 (I) and α-1 (III) fragments down-regulated in invasive compared with noninvasive tumors. Both Collagen α-1 (III) and Collagen α-1 (I) are substrates of collagenases, members of the matrix metalloproteinases family, a group of zinc finger endopeptidases with partially overlapping substrate specificity (41). Deregulation of Collagen or of matrix metalloproteinase activity have been found for different cancers (42–44). Our data support the hypothesis that with increasing stage, the tumor displays increasing protease activity, resulting in an increased degradation of collagen fragments that are usually present in body fluids under benign and/or non-muscle-invasive tumors. Uromodulin or Tamm-Horsfall protein is the most abundant protein in normal urine. Upon excretion in urine, proteolytic cleavage occurs of the ectodomain of its glycosyl phosphatidylinositol-anchor component that is located on the luminal cell surface of the loop of Henle. Physiology of Tamm-Horsfall protein is not well-understood. It may act as a constitutive inhibitor of calcium crystallization in renal fluids (45). A role in nephrolithiasis remains under debate (46). Excretion of uromodulin in urine may provide defense against urinary tract infections caused by uropathogenic bacteria (47). Its biological role in BCa remains unclear.

The ability to determine the risk of muscle-invasive disease noninvasively has several practical applications in urologic practice. On occasion, the pathologist will have difficulty determining whether the tumor has invaded the muscularis propria. As this is important in determining therapy, these patients need
to undergo a resection of the base of the tumor (48). For patients initially diagnosed with superficial bladder tumors upstaging from superficial to invasive BCA occurs in approximately one third of patients (30–32) with often fundamental implications for management of patients. Although several urine-based markers for the detection of BCA have been described (6, 49), there have been until now, no single or set of biomarkers that can predict BCA stage.

In this report, we show for the first time that urinary proteomics offer predictive value in distinguishing muscle-invasive and non–muscle-invasive tumors of the bladder. Therefore, this work should be viewed as an important first step and not the definitive answer. Nevertheless, although the ability to correctly predict patients based on a single urine test as to whether they have muscle-invasive disease is the ultimate goal, the currently described tool may be presently useful in the risk stratification of patients with aggressive non–muscle-invasive tumors. For example, if patients with a Ta or T1 lesion have a high probability of muscle-invasive disease according to the MI-BCa panel, the physician would be more likely to lean toward more aggressive treatment options. The converse would be true for patients with predictions in the opposite direction, where intravesical and/or surveillance options would be more prominently recommended.

Our study has limitations. The majority of patients with non–muscle-invasive tumors did not have radical cystectomy following the proteomic evaluation. Because as many as 40% of patients undergoing TUR have been shown to be understaged (30, 50), it is possible that our training may have been vulnerable to this variable. In future work, we hope to reevaluate our biomarkers in patients who have exclusively undergone cystectomy following the proteomic assessment to determine, whether the characteristics of peptide composition in the panel are altered. Such an updated panel may help to further improve the detection of muscle-invasive disease. Another limitation is the lack of uniform review of the cytology and pathology across all the participating institutions. This is in process but because of the international nature of this work, the large number of samples and the multitude of hospitals involved, the legal and logistic issues are very significant and not all resolved. Although for this study, we used the tumor grade obtained from the TUR specimen, the eventual grade used with the urine test would conceivably be obtained by office cold cup biopsy. However, given the heterogeneity of some tumors, it remains to be seen if this type of sampling is as effective as the grade from the TUR in improving the urinary biomarker predictions. Finally, despite the multiple centers accruing patients, the assay has been carried out at one single site, and thus, determination of the interlaboratory variation of the assay is the objective of ongoing work.

In summary, the use of a specific polypeptide panel seems promising for the noninvasive detection of muscle-invasive urothelial BCA. Longitudinal prospective multicenter studies are desirable to validate this tool against current staging algorithms. Furthermore, these peptides may also shed novel insights into the biology of bladder tumor progression not obtainable by other methods.

Disclosure of Potential Conflicts of Interest


Acknowledgments

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References

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