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# Detection of Bladder Cancer in Human Urine by Metabolomic Profiling Using High Performance Liquid Chromatography/Mass Spectrometry

Haleem J. Issaq,\* Ofer Nativ, Timothy Waybright, Brian Luke, Timothy D. Veenstra, Elias J. Issaq, Alexander Kravstov and Michael Mullerad

From the Laboratory of Proteomics and Analytical Technologies (HJI, TW, TDV) and Advanced Biomedical Computing Center (BL), SAIC Frederick, Inc., NCI-Frederick, Frederick, Maryland, and Department of Urology, Bnai-Zion Medical Center (ON, EJI, AK, MM), Haifa, Israel

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**Purpose:** The current use of cystoscopy for screening and detecting bladder cancer is invasive and expensive. Various urine based biomarkers have been used for this purpose with limited success. Metabolomics, ie metabonomics, is the quantitative measurement of the metabolic response to pathophysiological stimuli. This analysis provides a metabolite pattern that can be characteristic of various benign and malignant conditions. We evaluated high performance liquid chromatography coupled online with a mass spectrometer metabolomic approach to differentiate urine samples from healthy individuals and patients with bladder cancer.

**Materials and Methods:** Urine specimens were collected from 48 healthy individuals and 41 patients with transitional cell carcinoma, and stored at  $-80^{\circ}\text{C}$ . Samples were analyzed using an Agilent 1100 Series high performance liquid chromatography system (Agilent Technologies, Santa Clara, California) coupled online with a hybrid triple-quad time-of-flight QSTAR<sup>®</sup> XL mass spectrometer. At the time of analysis samples were thawed and centrifuged. The resulting total ion chromatograms of each sample were submitted for statistical analysis. For data interpretation in this study 2 statistical methods were used, that is principal component analysis and orthogonal partial least square-discriminate analysis.

**Results:** Using positive ionization mass spectrometry orthogonal partial least square-discriminate analysis correctly predicted 48 of 48 healthy and 41 of 41 bladder cancer urine samples, while principal component analysis, which is an unsupervised profiling statistical method, confirmed these results and correctly predicted 46 of 48 healthy and 40 of 41 bladder cancer urine samples.

**Conclusions:** The results of this proof of concept study in a relatively small number of subjects indicate that metabolomics using high performance liquid chromatography-mass spectrometry has the potential to become a noninvasive early detection test for bladder cancer.

*Key Words: bladder; carcinoma, transitional cell; metabolism; chromatography, high pressure liquid; mass spectrometry*

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Bladder cancer is the second most common genitourinary malignancy. The American Cancer Society estimated that 61,420 new cases of bladder cancer would be found in the United States during 2006 and about 13,060 individuals would die of the disease.<sup>1</sup>

Many patients with bladder cancer experience recurrence but do not die of the disease. While bladder cancer is only the fourth most common cancer in men after lung, colorectal and prostate cancers, in terms of incidence it is the second most

prevalent malignancy in middle-aged and elderly men after prostate cancer.<sup>2,3</sup>

The current standard of care for detecting and monitoring bladder tumors is cystoscopy, voided urine cytology and imaging.<sup>4</sup> However, cystoscopy is invasive, painful and costly. Therefore, it is not suitable as a screening test. Although urine cytology is a noninvasive test, it is limited by its low sensitivity of 20% to 40% for low grade tumors. Several methods have been reported for the early detection of bladder cancer using various potential markers.<sup>5-8</sup> The specificity and sensitivity of these tests vary between 50% and 100%. Therefore, they are not adequate for screening patients.

Ideally a urine based bladder tumor marker would be noninvasive, inexpensive and nonuser dependent, and have high accuracy. Optimal markers would serve for screening, initial diagnosis, and monitoring recurrence and progression as well as predicting prognosis.

Metabolomics, also known as metabonomics, simply stated, is the study of the metabolic response of the body to drug toxicity or disease. Global metabolomics relies on nonbiased, quantitative analysis of all or a large number of metabolites

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\* Correspondence: Laboratory of Proteomics and Analytical Technologies, SAIC Frederick, Inc., NCI-Frederick, P. O. Box B, Frederick, Maryland 21702 (telephone: 301-846-7186; FAX: 301-846-6037; e-mail: [issaqh@mail.ncifcrf.gov](mailto:issaqh@mail.ncifcrf.gov)).

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in biological samples from healthy (control) and affected individuals.<sup>9–11</sup> The aim is to identify disease related differences in 1 or more of the numerous endogenous metabolites found in biological fluids. Metabolomic analysis of accessible biofluids provides information on all tissues that deliver to and obtain metabolites from those fluids.<sup>12–14</sup> Target or metabolite specific studies depend on a priori knowledge of the biological system or pathway used for use as a successful diagnostics tool.

In this metabolomics study of HPLC coupled on line with MS we identified metabolite variations that may be used to discriminate between urine specimens from healthy individuals and patients with bladder cancer.

## MATERIALS AND METHODS

### Patients and Study design

Urine specimen were collected from 48 healthy volunteers and 41 patients with bladder TCC, and stored at  $-80^{\circ}\text{C}$ . The TCC group included 36 males who were 56 to 93 years old (average age 77.1) and 5 females who were 51 to 90 years old (average age 74.6). The control group included 21 males who were 31 to 86 years old (average age 58.0) and 27 females who were 20 to 80 years old (average age 59.8). All patients with TCC had a confirmed tissue diagnosis. Most cases were of low grade and low stage disease, including Ta in 28, Cis in 1, T1 in 9, T2 in 3, low grade in 28 and high grade in 13. All patients with TCC in this study had a history of TCC.

Patients were detected with TCC at routine followup cystoscopy and urine samples were taken before hospital admission for transurethral resection. Midstream voided urine samples were collected from patients with TCC 3 to 4 weeks after cystoscopy at the Department of Urology, Bnai-Zion Medical Center, Haifa, Israel and approved by the institutional review board. Samples were frozen as is and shipped on dry ice via air to NCI-Frederick, Frederick, Maryland, where they were stored at  $-80^{\circ}\text{C}$  and analyzed at the Laboratory of Proteomics and Analytical Technologies.

## MATERIALS

Acetonitrile (HPLC grade) was obtained from Fisher Scientific, Somerville, New Jersey. Formic acid was obtained from Sigma-Aldrich<sup>TM</sup>. Distilled water was obtained from an in-house NANOpure<sup>®</sup> water filtration system.

### Instrumentation

An Agilent 1100 Series HPLC system equipped with an autosampler and a BetaBasic-18 reversed phase column (Thermo Electron Corp., Waltham, Massachusetts) 100 mm long with a 1 mm inner diameter and packed with 5  $\mu\text{m}$  silica based particles was coupled online with a hybrid triple-quad time-of-flight QSTAR XL mass spectrometer for the HPLC-MS analysis of urine samples. All samples were scanned over a mass range of 100 to 2,000  $m/z$  with an ion spray voltage of 5,500, declustering potential of 160, declustering potential 2 of 20, focusing potential of 200, ion source gas of 20, curtain gas of 25 and collision gas of 3. The HPLC instrument was placed in line from the liquid chromatography system and attached directly to the MS electrospray ionization source. A flow rate of 50  $\mu\text{l}$  per minute was maintained for all sample analyses.

### Sample Preparation and Analysis

We decided to use neat urine without dilution for HPLC/MS analysis because in a previous study we found that 1:4 dilutions may not allow the detection of low abundant metabolites.<sup>15</sup> However, the injection of neat urine on the HPLC column required the introduction of a column wash step after each analysis.

At the time of analysis urine samples were thawed on ice and centrifuged at 3,000 rpm for 20 minutes at  $5^{\circ}\text{C}$ . Before HPLC analysis the chromatographic system was optimized for optimum separation by selecting the right column, mobile phase and gradient conditions.<sup>15</sup> The column was equilibrated with 98% mobile phase A (0.1% formic acid in  $\text{H}_2\text{O}$ ) in mobile phase B (0.1% formic acid in acetonitrile). Neat urine (10  $\mu\text{l}$ ) was injected onto the column. A step gradient of solvents A in B, including 0 to 10 minutes with 95% A, 65 minutes with 60% A, 70 minutes with 2% A and 80 minutes with 98% A, was used to elute the metabolites from the column at room temperature. MS using the positive ionization mode was used to analyze the eluate. The resulting total ion chromatograms of each urine sample were submitted for statistical analysis. All samples were analyzed randomly under the same experimental conditions.

### Reproducibility

The HPLC/MS procedure used in this study was evaluated for interday and intraday reproducibility by injecting the same urine sample on 3 consecutive days using 3 injections per day. Results indicated that the method is robust and provides highly reproducible data (data not shown).

### Data Interpretation and Analysis

Due to the large number of peak intensities and the relatively small number of samples we selected 2 popular dimensionality-reduction statistical methods, that is OPLS-DA using SIMCA-P (Umetrics, Kinnelon, New Jersey) and PCA. OPLS-DA is a supervised procedure that constructs a linear combination of all peak intensities, which maximizes the separation between healthy and diseased samples. Other variances in the data set, such as intragroup variability, are explained by the second and higher orthogonal linear combinations.

In contrast, PCA is an unsupervised procedure in which linear combinations of all peak intensities are constructed to produce orthogonal components that maximize the total variance in the samples independent of their group labels. The linear combination that yields the greatest variance is projected on the first axis, called the first principal component, while the second greatest variance is projected on the second axis and so on. PCA decreases dimensionality while it keeps the characteristics that contribute most to the variance. It does this by giving more importance to lower order principal components and ignoring higher order ones using the assumption that low order components often contain the most important aspects of the data.

Data collected from the HPLC-MS chromatograms were subjected to a logarithmic weighting step and scaled using a beta version of MarkerView<sup>TM</sup> software before performing PCA. The weighting step determines the mean peak intensity for each peak and then calculates the natural logarithm of peak intensities divided by the mean. This has the effect of converting a random distribution of peak intensities into

a natural or Gaussian distribution. To account for differences in the ranges of weighted intensities the autoscale function takes the weighted response for each peak from all samples, calculates the mean and SD, and then subtracts each value by the mean and divides by the SD. This conversion of weighted peak intensities into z-scores means that the coefficient of a peak in the principal component is a direct measure of its contribution to the overall variance and it is not affected by the magnitude of the peak.

For the OPLS-DA method 3 healthy and 3 diseased samples were removed from the data set to construct a blind validation set. The orthogonal components were constructed using the remaining 45 healthy samples and 38 samples of bladder cancer. A plot of the first 2 components was used to obtain a discriminating vertical line using the first component that distinguished the 2 groups. The 6 testing samples were then examined with respect to this discriminating line. All samples were used to construct the principal components for PCA analysis. The first 2 principal components were then used to plot all samples to determine whether this unsuper-

vised procedure could distinguish healthy from cancerous samples, again using a linear separator.

If the healthy sample with the smallest projection on the first component was placed in the testing set, the position of the discriminating vertical line would be less certain. It would be possible that this sample could be misclassified upon testing. This situation is true for any study that performs a priori division of samples into a training set and a testing set since it must be assumed that the training set correctly represents the full range of intensities for each group in each peak. Therefore, such studies not only measure the accuracy of the proposed classifier, but also the extent to which the full range is properly represented.

## RESULTS

Examination of the total ion chromatograms of each urine sample indicated the difficulty of differentiating between cancer and control urine specimens by the naked eye. Figure 1 shows the total ion chromatograms of 3 normal and 3 blad-

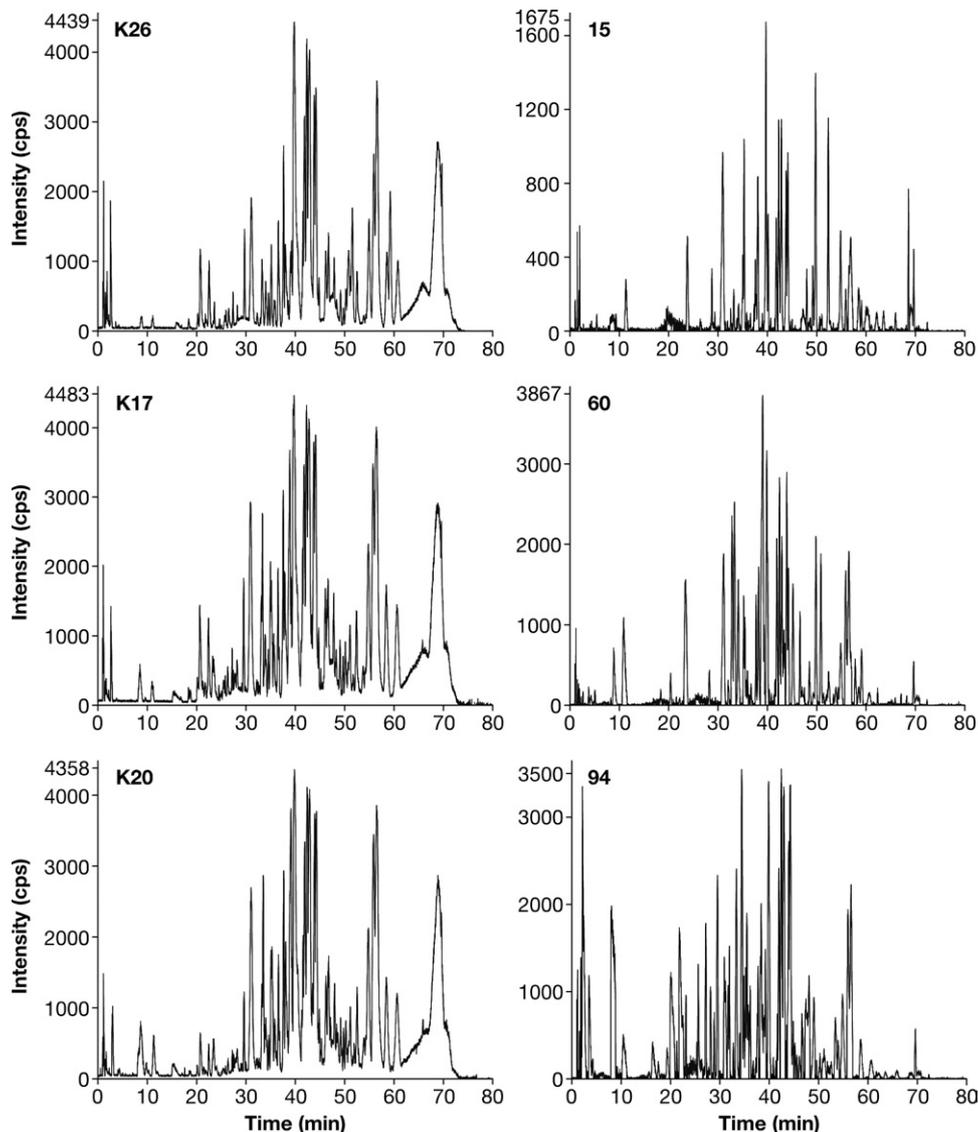


FIG. 1. Chromatographic comparison of MS spectrum of urine from healthy patients (left) vs patients diagnosed with bladder cancer (right).

der cancer urine samples. Although each had similar profiles, there were differences that could not be detected by the naked eye but could be detected by statistical methods.

Figure 2 is a plot of OPLS-DA statistical analysis of all urine samples from control patients and those with bladder cancer, as analyzed by HPLC-ESI/MS in the positive mode. The algorithm was able to correctly separate the urine samples into 2 groups corresponding to normal and cancer, and it correctly predicted 41 of 41 bladder cancer specimens (100% sensitivity) and 48 of 48 healthy specimens (100% specificity). These results included 3 samples per group that were analyzed as unknown (blind) to verify the robustness of this method.

Since such a degree of accuracy in bladder cancer detection based on urine testing is uncommon, we were interested in confirming the classifications by using 1 or more different statistical procedures. PCA correctly predicted 40 of 41 urine samples from patients with bladder cancer and 46 of 48 urine samples from healthy controls (fig. 3). Although the results are excellent, they are not as accurate as those produced using OPLS-DA. Figures 2 and 3 show that the division between the control and TCC groups was less pronounced using the PCA procedure.

## DISCUSSION

Metabolomics is the detection and quantitation of metabolites in biological samples that are the result of the metabolic response of living systems to drug toxicity or disease. Technological advances in nuclear magnetic resonance spectroscopy, MS, chromatography and electrophoresis enables the measurement of alterations in the concentrations of metabolites found in biological samples even at low levels.<sup>16</sup> In global metabolomics the entire metabolite component of a biological sample, such as urine, serum, cerebrospinal fluid or tissue, is separated, detected, quantified and identified by various analytical methods. Currently 2 of the most commonly used platforms for metabolite analysis are nuclear magnetic resonance spectroscopy and HPLC coupled on line with MS.<sup>17</sup>

As with other “-omics” techniques (proteomics, genomics and transcriptomics), metabolomic analysis of biological systems typically results in a plethora of data that can be overwhelming in its abundance. For any meaningful data

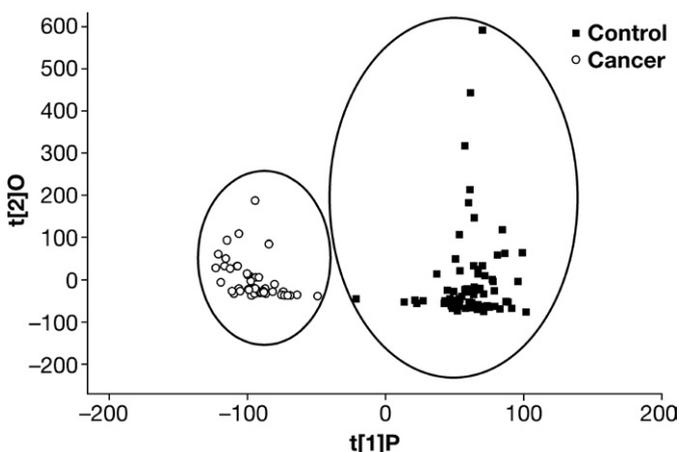


FIG. 2. OPLS-DA results of HPLC-MS data on 48 control and 41 TCC urine samples.

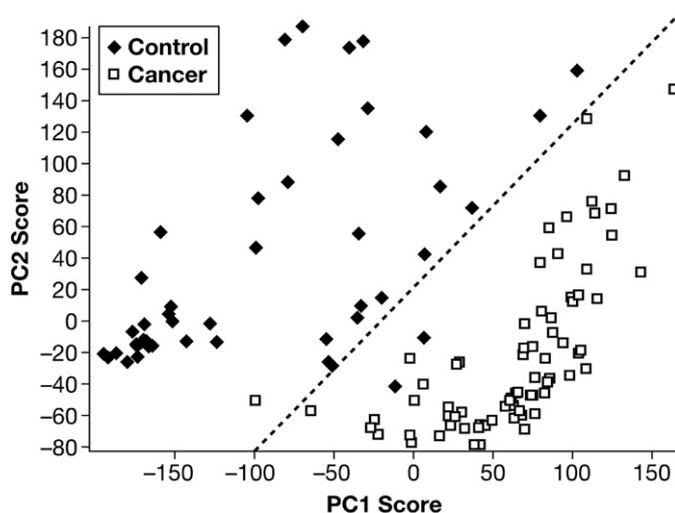


FIG. 3. PCA results of HPLC-MS data on 48 control and 41 TCC urine samples using log mean center method.

interpretation the appropriate statistical tools must be used to process the large raw data sets to provide a useful, understandable and workable format. Different multidimensional and multivariate statistical analyses and pattern recognition programs have been developed to distill the large amounts of data in an effort to interpret the complex metabolic pathway information from the measurements.<sup>18–20</sup>

This study included 41 urine samples from patients with TCC and 48 control urine samples. Using the OPLS-DA and PCA programs resulted in 2 groups, corresponding to normal and cancer urine samples, and correctly predicted 41 of 41 bladder cancer samples (100% sensitivity) and 48 of 48 healthy samples (100% specificity) by OPLS-DA, while PCA analysis resulted in predicting 40 of 41 cancer samples (98% sensitivity) and 46 of 48 control samples (96% specificity).

The ultimate diagnostic procedure for any disease is one that gives no false-positive and no false-negative prediction, that is 100% sensitivity and specificity. It seems that this is more an ideal than an attainable goal for disease biomarkers, especially for heterogeneous neoplasms such as bladder cancer, for which sensitivity and specificity are much less than 100%.

We developed a noninvasive procedure that can discriminate bladder cancer from normal (control) urine at a level of sensitivity and specificity that would challenge cystoscopy and urine cytology as the primary diagnostic tool. The limitations of this study include the relatively small sample size, the difference in sex and age distribution, and comorbidities between normal controls and patients with TCC, and the lack of nonTCC control patients with benign disease of the urinary collecting system, such as infection, urolithiasis or an indwelling catheter, or after the incorporation of a bowel segment.

In our study the PCA algorithm confirmed the results of the OPLS-DA approach. Results indicate that no single classification method is significantly superior, although OPLS provided better sensitivity and specificity than PCA. As mentioned, urine specimens were collected from patients with TCC 3 to 4 weeks after cystoscopy. Therefore, we assumed that the trauma to tissue at cystoscopy was probably not the cause of the differences found in our research.

Although the results of this study are preliminary, they have the potential to become a clinical test pending further evaluation. To achieve such recognition a test requires a prospective cohort group as well as application of the markers found in the study in a new patient group and control group. Another ongoing task is the identification of the individual metabolite as a potential biomarker. It may improve our understanding about the pathophysiology of the disease, identify correlations with disease aggressiveness and potentially serve as a future target for therapy.

## CONCLUSIONS

Our study demonstrates that urine metabolomics can differentiate patients with TCC from normal controls. Thus, it is a proof of principle for the use of metabolomics for the early detection of TCC.

### Abbreviations and Acronyms

HPLC	=	high performance liquid chromatography
MS	=	mass spectrometry
OPLS-DA	=	orthogonal partial least square-discriminate analysis
PCA	=	principal component analysis
TCC	=	transitional cell carcinoma

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