Noninvasive Urinary Metabonomic Diagnosis of Human Bladder Cancer

Kishore Kumar Pasikanti,† Kesavan Esuvaranathan,‡ Paul C. Ho,† Ratha Mahendran,‡ Revathi Kamaraj,‖ Qing Hui Wu,‖ Edmund Chiong,‖ and Eric Chun Yong Chan*†

Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, Singapore 117543, and Department of Surgery, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074

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Cystoscopy is considered the gold standard for the clinical diagnosis of human bladder cancer (BC). As cystoscopy is expensive and invasive, it may compromise patients’ compliance and account for the failure in detecting recurrent BC in some patients. In this paper, we investigated the role of urinary metabonomics in the diagnosis of human BC. Gas chromatography/time-of-flight mass spectrometry was applied for the urinary metabolic profiling of 24 BC patients and 51 non-BC controls. The acquired data were analyzed using multivariate principal component analysis followed by orthogonal partial least-squares discriminant analysis (OPLS-DA). Model validity was verified using permutation tests and receiver operating characteristic (ROC) analysis. BC patients were clearly distinguished from non-BC subjects based on their global urinary metabolic profiles (OPLS-DA, 4 latent variables, $R^2_X = 0.420$, $R^2_Y = 0.912$ and $Q^2$ (cumulative) $= 0.245$; ROC AUC of 0.90; 15 marker metabolites). One-hundred percent sensitivity in detecting BC was observed using urinary metabonomics versus 33% sensitivity achieved by urinary cytology. Additionally, urinary metabonomics exhibited potential in the staging and grading of bladder tumors. In summary, urinary metabonomics is amenable for the noninvasive diagnosis of human BC.

Keywords: bladder cancer • gas chromatography mass spectrometry • metabolomics • metabonomics • metabolic profiling • diagnosis

Introduction

The current standard for tumor detection and monitoring of recurrence or progression of bladder cancer (BC) is cystoscopy and urinary cytology. Cystoscopy is invasive and may be associated with a small but definite risk of morbidity. Furthermore, it is known that BC is the most expensive disease to treat from diagnosis to death due to the long-term survival associated with nonmuscle-invasive disease combined with lifelong surveillance. Therefore, a noninvasive test for BC diagnosis and surveillance is desirable. Voided urinary cytology is used frequently as an adjunctive noninvasive detection of BC. However, it is expensive, subjective, and has low sensitivity with considerable variability. While it has a good sensitivity for poorly differentiated tumors, especially for carcinoma in situ, the sensitivity for low grade papillary urothelial carcinoma is low at between 0 and 50%. In recent years, numerous urine-based bladder tumor markers (UBBTMs) have been evaluated to determine whether less invasive follow-up of patients with BC is feasible. The assays evaluated in the past years included the tests for bladder tumor antigen (BTA), urinary nuclear matrix protein 22 (NMP22), fibrin degradation product (FDP), autocrine motility factor receptor, bladder cancer nuclear matrix protein (BCLA-4), cytokeratin 20 (CK20), telomerase, hyaluronic acid, hyaluronidase, Immunocyt, urinary bladder cancer (UBC) test, CYFRA 21–1, chemiluminescent hemoglobin, hemoglobin dipstick, urinary tissue polypeptidesspecific (TPS) antigen, bladder cancer antigen (BCA), beta-human chorionic gonadotropin, tissue polypeptide antigen (TPA), and microsatellite analysis. Sensitivity and specificity of various molecular markers for the diagnosis of BC are reported in recently published review articles. Nevertheless, none of the UBBTMs were as accurate as cystoscopy. As such, the development of an alternative noninvasive and less expensive method to accurately detect BC at high sensitivity and accuracy becomes important.

One recent study demonstrated that well-trained dogs can identify BC patients urine samples from those of non-BC subjects based on urine odor. This study suggested that there exist some urinary metabolites that could be promising markers for BC detection. As urine is bathed in close anatomical proximity to the bladder carcinoma, we hypothesize that urinary metabolic profiling can be applied to the diagnosis of human BC.

Recently, metabonomic analyses are found to have increasing applications in the diagnosis of a number of pathologies, elucidation of clinicopathogenesis of various diseases, and assessment of exposure of biological systems to xenobiotics.
Metabonomics has several major advantages, which include the ready availability and relative ease of analysis of biofluids, such as urine and plasma, as well as the fact that the derived metabolite profiles are sensitive to both environmental and genomic influences affecting the pathogenesis and progression of disease. A recent study reported the feasibility of using liquid chromatography/mass spectrometry (LC/MS) to analyze urinary metabolites to discriminate between controls and BC patients. However, potential marker metabolites responsible for the separation of BC patients from controls were not identified. More importantly, the predictability and robustness of the orthogonal partial least-squares discriminant analysis (OPLS-DA) model were not evaluated and cross-validated. It is also important to note that one key challenge of using LC/MS for the metabolic profiling of complex urine samples is the varied results related to matrix effects.

Gas chromatography/mass spectrometry (GC/MS) has been proven to be a potentially useful metabolic profiling platform based on its high sensitivity, peak resolution and reproducibility. Availability of GC/MS electron impact (EI) spectral library further facilitates the identification of diagnostic biomarkers and aids the subsequent mechanistic elucidation of the pathological phenotypes. Unlike electrospray ionization (ESI) which is used in LC/MS, EI ionization used in GC/MS is less prone to matrix effects. Moreover, we have demonstrated previously that GC/MS-based urinary metabolic profiling shows high inter- and intraday reproducibility. Recently, gas chromatography/time-of-flight mass spectrometer (GC/TOFMS)-based metabonomic analyses were reported for the characterization of diabetes, hepatocellular carcinoma, kidney cancer, colorectal cancer, ovarian cancer, acute coronary syndrome, and Crohn’s disease. These studies reinforced the potential of GC/TOFMS-based metabolomic platform in biomarker discovery and elucidation of pathogenesis of various diseases.

To date, no studies have evaluated the feasibility of GC/TOFMS-based metabolic profiling for the characterization of human BC. This information gap embodies three important research questions: Does human BC generate a signature urinary metabolic phenotype that is discernible using GC/TOFMS? Second, is urinary metabonomics more sensitive compared to urinary cytology? Third, can human BC urinary profiles distinguish the different stages or grades of the carcinoma? The aim of the present study was to determine whether GC/MS-based urinary metabonomics would allow prediction of some aspect of cancer development in the bladder and, thereby, provide a proof-of-principle for its application in the clinical diagnosis of human BC.

Materials and Methods

Clinical Population. The overall workflow of metabolic profiling approach utilized in this study is summarized in Figure 1. The presence of tumors in bladder was initially visualized using cystoscopy. Subsequently, histopathology examination of transurethrally resected specimens was used to diagnose BC and determine stage and grade of these tumors. Patients whose histology did not show any malignancy were placed in the non-BC group. Tumors were classified according to World Health Organization (WHO)/International Society of Urological Pathology (ISUP) classification criteria. The clinicopathological data of BC and non-BC groups are summarized in Table 1. All the volunteers completed confidential health and life style related questionnaires, reported gender and age, and signed informed consent forms to be participants in the study (IRB No. DSRB-B/07/192). All the urine samples were stored in aliquots at -80 °C until further sample processing.

Chemicals. MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) were purchased from Pierce (Rockford, IL), urease of Sigma type III, alkane standard mixture (C10 to C40), and sodium sulfate (anhydrous) were obtained from Sigma-Aldrich (St. Louis, MO), Milli Q water (Millipore, Bedford, MA) was used as blank sample. All other chemicals were of analytical grade.

Urine Preparation for GC/TOFMS Analysis. Prior to sample preparation and GC/TOFMS analysis, all the urine samples were randomized along with 10 quality control (QC) urine samples. QC samples were prepared by mixing equal amounts of urine samples from 5 BC patients and 5 non-BC subjects. The urine samples were thawed at room temperature (23 ± 3 °C). A 100 U of urease was added to 200 µL of urine sample and incubated at 37 °C for 1 h, to decompose and remove excess urea. To precipitate urease and other proteins, 1.7 mL of methanol was added to the mixture. The mixture was vortex-mixed at high speed for 5 min, centrifuged subsequently for 10 min at 10 000× g at 4 °C. A 1.5 mL of supernatant was then carefully separated and evaporated to dryness at 40 °C under a gentle stream of nitrogen gas using TurboVap LV (Caliper...
Life Science, Hopkinton, MA). A 100 µL of toluene (dried over anhydrous sodium sulfate) was added to the dry residue, mixed for 1 min and dried again at 50 °C under nitrogen gas. The dried metabolite extract was derivatized first with 50 µL of methoxamine (20 mg/mL) for 2 h at 60 °C. Subsequently, 100 µL of MSTFA with 1% TCMS was added to the mixture and heated for 1 h at 60 °C to form trimethylsilyl (TMS) derivatives. TMS derivatives were cooled and 100 µL of supernatant was transferred into GC vial and subjected to GC/TOFMS analysis.

**Results**

Representative GC/TOFMS chromatograms acquired using BC and non-BC urine samples analyses are shown in Figure 2A. Our GC/TOFMS-based urinary metabolic profiling method demonstrated retention time (RT) deviation of less than 2 s in overall analysis. The final data table comprised of 398 variables (chromatographic peaks) which were detected consistently in both BC and non-BC samples.

**Validity of Analytical Performance**. PCA performed on all the samples revealed that the QC samples were tightly clustered in PCA scores plot (Supporting Information, Figure 1) confirming that our method was robust. In parallel to using QC samples for qualifying the overall performance of the method, variations in the levels of marker metabolites were further cross-validated using the same metabolites observed in the QC samples. The %RSD of marker metabolites ranged...
from 3.0 to 33.4% with a median RSD of 6.7%. These marker metabolites were selected post-OPLS-DA.

**Urinary Metabonomics of Human BC.** PCA scores and DModX plots revealed that two observations belonging to non-BC cohort were severe outliers and were excluded from further chemometric data analysis. PCA was followed by a supervised analysis technique such as partial least-squares discriminant analysis (PLS-DA) or OPLS-DA that aided the screening of marker metabolites responsible for class separation by removing systematic variation unrelated to pathological status. The PLS-DA model obtained from urine sample analysis contained three latent variables, showing performance statistics of $R^2_X = 0.360$, $R^2_Y = 0.799$ and $Q^2$ (cumulative) = 0.432 (Supporting Information, Figure 2). An OPLS-DA model was calculated based on 24 BC and 49 non-BC urine samples subsequent to PLS-DA analysis. The OPLS-DA model contained four latent variables, showing the performance statistics of $R^2_X = 0.420$, $R^2_Y = 0.912$ and $Q^2$ (cumulative) = 0.245. Clear biochemical distinction between the BC and non-BC cohorts was evident in the OPLS-DA model (Figure 2B). The OPLS-DA was able to correctly classify the urine samples into BC and non-BC groups, and it accurately predicted all BC patients and non-BC subjects showing 100% sensitivity and specificity. While urine cytology offered 100% specificity, its sensitivity was only 33% in detecting BC.

**Selection of Marker Metabolites.** Analysis of OPLS-DA loadings and variable importance plots (VIPs) indicated several discriminatory metabolites that differentiated BC and non-BC urine samples. Totally, 32 metabolites were selected based on the criteria of VIP value greater than one. Unpaired $t$ test and Welch’s $t$ test were employed to select metabolites that dominated the separation between BC and non-BC groups. Fifteen marker metabolites were selected and illustrated in Table 2. Identities of some of the marker metabolites were confirmed by analyzing standard compounds using the same analytical conditions adopted for the urinary metabolic profiling.

**Model Validation.** Goodness of fit ($R^2$ and $Q^2$) of original PLS-DA model and cluster of 100 Y-permutated models were visualized using a validation plot (Figure 2C). The validation plot strongly indicated that the original model was valid as $Q^2$ regression line has a negative intercept and all permuted $R^2$ values to the left were lower than the original point to the right. In addition, area under ROC curve (Figure 2D) of the PLS-DA model calculated using cross validated Y-predicted values resulted in a value of 0.90 confirming validity of the model.

**External Validation.** By incorporation of an orthogonal filter (OPLS), systematic variation unrelated to pathological status could be removed and thereby facilitated the interpretation of BC-related effects. The OPLS-DA model correctly predicted 11 out of 12 BC patient and 4 out of 5 non-BC (Figure 3B) subject urine samples showing the sensitivity and specificity of 92% and 80%, respectively.

**Discussion**

Obtaining an accurate and sensitive noninvasive diagnosis of human BC remains a challenging problem in our healthcare
system. Application of urinary metabonomics for diagnosis of BC is theoretically plausible since pathogenesis of BC may result in dynamic yet discernible changes in the molecular environment of the bladder. Therefore, metabolic profiling of human urine for BC diagnosis is a logical clinical approach. While this idea may appear intuitive, it is somewhat surprising

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Table 2. Marker Metabolites Differentiating Bladder Cancer (BC) and Non-BC Subjects

Figure 3. T-predicted scatter plot of OPLS-DA model obtained from prediction of (A) first pass and evening urine samples collected from 6 BC patients and (B) 5 non-BC spot urine samples collected subsequent to tumor resection.
that the rationale of using urinary metabolic profiling as a mean to diagnose human BC has not been elaborately investigated. In this article, we investigated the use of GC/TOFMS-based urinary metabolic profiling in diagnosing BC patients.

In this study, we adopted the concept of biological QCs reported by Gika et al.\(^47\) wherein PCA was used to check the validity of the data in metabolicomic analysis. The observed close clustering of QC samples (Supporting Information, Figure 1) validated the robustness of our metabolic profiling platform with regards to parameters such as sample derivatization, stability of the detector response, accuracy of sample injection and RT reproducibility. Due to the large and complex data sets, chemometric data analysis was necessary to provide interpretable models for the complex intercorrelation of data.\(^48\) While performance statistics of multivariate model provided information about its validity, there was a possibility that each model was being developed by chance correlation. Results obtained from both permutation tests and ROC analysis of the OPLS-DA model confirmed that our supervised chemometric model was being developed by chance correlation. Furthermore, low variations in the levels of marker metabolites in QC samples confirmed that the perturbations of the biomarkers were due to biological differences and not by chance based on analytical variations. Since the number of females patients in the BC cohort was lower compared to non-BC subjects, it was possible that the observed differences in metabolic phenotypes might be related to gender-based variations. Therefore, an independent OPLS-DA was performed solely based on 20 BC and 28 non-BC male subjects. Performance statistics and marker metabolites identified using the OPLS-DA model (two latent variables R\(^2\)X = 0.379, R\(^2\)Y = 0.862 and Q\(^2\) (cumulative) = 0.257) were similar to the OPLS-DA performed on the complete data set. Moreover, no significance change was observed in separation trends in the OPLS-DA scores plots (Supporting Information, Figure 4). These results further confirmed that observed differences in metabolic profiles were not related to gender-based variations.

The OPLS-DA model revealed several marker metabolites related to BC. As the rate of glycolysis was increased in tumors, the glycerol component was possibly converted to glucose by the liver to provide energy for cellular metabolism. This was consistent with the higher turnover of glycerol as observed in the BC urinary metabolic profiles (Table 2). Similarly, fructose was also found to be reduced in BC urinary profiles possibly due to enhanced glycolysis. Interestingly, serine (or 3-methylcrotonic acid) byproduct during the processing of leucine, was consistently found to be at lower levels in BC urine. This was possibly related to altered amino acid metabolism in the bladder tumors. On the other hand, the urinary levels of uridine, an endogenous metabolite for the synthesis of RNA, was found to be 2-fold higher in BC compared to non-BC urine reflecting the higher energetic state of the tumor. Profiling of these marker metabolites demonstrated the additional capability of metabolomics in the biochemical characterization of BC such as understanding the bioenergetic status of the tumors.

Urinary cytology is conventionally considered as the most important noninvasive method in the detection of BC. However, sensitivity of cytology for low grade tumors is poor.\(^49\) In our current study, we observed that the overall sensitivity of cytology was 33.3%. As cytology was not performed in some patients (3 out of 24 BC patients), these patients were excluded during the calculation of specificity and sensitivity. Moreover, cytology failed to detect any of the low grade tumors in the current study. In contrast, the OPLS-DA model showed 100% specificity and sensitivity in differentiating BC from non-BC, regardless of stage and grade. Good sensitivity (92%) and specificity (80%) was also observed when the OPLS-DA model was applied in discriminating BC from non-BC subjects from an independent set of samples. This external validation study confirmed the clinical utility of the metabolomic platform in diagnosing BC. A clinically relevant diagnostic technique must remain sensitive and specific when applied to an unselected population. The strength of our study design was that it did not overemphasize the diagnostic power of the test by studying highly selected populations. If we had used highly selected and matched patients and controls, it would have emphasized the differences due to disease status by minimizing other sources of variation.

In addition to accurate diagnosis, classification of bladder tumors is critical for patient management. However, reproducibility between pathologists is a major issue.\(^50\) Therefore, the feasibility of using urinary metabolomics for classification of bladder tumors was preliminarily evaluated in the current study. Interestingly, clustering of BC patients according to the stage and grade of the tumors were observed within BC group in the OPLS-DA scores plot (Supporting Information, Figure 3A and B). While further validation needs to be performed using a larger patients cohort, our findings in current study illuminated the potential of urinary metabolomics in the classification of bladder tumors. Focusing on the direct correlation of metabolic components and pathological status, this approach will ultimately allow extraction of biological events at the interface of cancer pathology and metabolic system and provide a mechanistic handle for probing the progression of BC.

In summary, this pilot study provided proof-of-principle that GC/TOFMS-guided urinary metabolomics could accurately distinguish BC patients from non-BC subjects. An elucidation of the association between global metabolic profiles and bladder cancer events in the clinics could open new avenues in the diagnosis and surveillance of BC and other diseases.

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**Supporting Information Available:** Supplemental figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

**References**


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