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HR-MAS NMR Tissue Metabolomic Signatures Cross-Validated by Mass Spectrometry Distinguish Bladder Cancer from Benign Disease

Pratima Tripathi^{1,#}, Bagganahalli S Somashekar^{1,#}, M. Ponnusamy⁴, Amy Gursky², Stephen Dailey², Priya Kunju³, Cheryl T. Lee², Arul M. Chinnaiyan^{2,4,5,6}, Thekkelnaycke M. Rajendiran^{3,4,†}, and Ayyalusamy Ramamoorthy^{1,†,*}

¹Department of Chemistry and Biophysics, University of Michigan, Ann Arbor, Michigan

²Urology, University of Michigan, Ann Arbor, Michigan

³Pathology, University of Michigan, Ann Arbor, Michigan

⁴Michigan Center for Translational Pathology, University of Michigan, Ann Arbor, Michigan

⁵Comprehensive Cancer Center, University of Michigan, Ann Arbor, Michigan

⁶Howard Hughes Medical Institute, University of Michigan, Ann Arbor, Michigan

Abstract

Effective diagnosis and surveillance of Bladder Cancer (BCa) is currently challenged by detection methods that are of poor sensitivity, particularly for low-grade tumors, resulting in unnecessary invasive procedures and economic burden. We performed HR-MAS NMR-based global metabolomic profiling and applied unsupervised principal component analysis (PCA) and hierarchical clustering performed on NMR dataset of bladder derived tissues and identified metabolic signatures that differentiate BCa from benign disease. A partial least-square discriminant analysis (PLS-DA) model (leave-one-out cross-validation) was used as diagnostic model to distinguish benign and BCa tissues. Receiver operating characteristic curve generated either from PC1 loadings of PCA or from predicted Y-values resulted in an area under curve of 0.97. Relative quantification of more than fifteen tissue metabolites derived from HR-MAS NMR showed significant differences ($P < 0.001$) between benign and BCa samples. Noticeably, striking metabolic signatures were observed even for early stage BCa tissues (Ta-T1) demonstrating the sensitivity in detecting BCa. With the goal of cross-validating metabolic signatures derived from HR-MAS NMR, we utilized the same tissue samples to analyze eight metabolites through gas chromatography-mass spectrometry (GC-MS)-targeted analysis, which undoubtedly complements HR-MAS NMR derived metabolomic information. Cross-validation through GC-MS clearly demonstrates the utility of straightforward, non-destructive and rapid HR-MAS NMR technique for clinical diagnosis of BCa with even greater sensitivity. In addition to its utility as a diagnostic tool, these studies will lead to a better understanding of aberrant metabolic pathways in cancer as well as the design and implementation of personalized cancer therapy through metabolic modulation.

*Corresponding Author Prof. Ayyalusamy Ramamoorthy Biophysics and Department of Chemistry University of Michigan 930 North University Avenue Ann Arbor, Michigan 48109-1055, USA Phone: (734) 647-6572 Fax: (734) 764-3323 ramamoor@umich.edu.

#Authors contributed equally

†Authors share senior authorship

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Keywords

HR-MAS NMR; GC-MS Bladder Cancer; metabolites; metabolomics; energy metabolism

Introduction

Bladder Cancer (BCa) is the second most frequent genito-urinary malignancy and fifth leading new cancer diagnosed in the United States ¹. Nearly 75% of newly diagnosed patients have non-muscle invasive disease (i.e. restricted to urothelium or lamina propria). Within this population, a subgroup of high-risk patients will progress to invasive and possibly metastatic disease resulting in significant morbidity. Currently the initial diagnosis and surveillance of BCa rely on cystoscopy, urinary markers and histopathology of tissue specimens obtained from tumor biopsies. Urinary cytology has historically being used as the primary adjunct to cystoscopy. Its specificity is high but its sensitivity is modest particularly for low-grade tumors. Given the major economic burden of BCa ², novel strategies to reduce the cost of bladder cancer surveillance are greatly needed. The development of a cost-effective bladder cancer marker that could reduce cystoscopy utilization in low risk patients, identifies early tumor development or recurrence, and predicts progression would have major impact in clinical practice. Multiple potential biomarkers for BCa have been detected in urine and excised tissue specimens by various investigators originating at the genomic and epigenetic levels, as well as on the RNA, miRNAs, and protein levels ³. Although our knowledge about the biology of bladder cancer has been increased in recent years, most of the biomarkers identified have been investigated only in small cohorts and therefore it is unclear how clinically relevant these biomarkers, particularly single molecule based biomarkers, will be in larger populations for effective monitoring and disease management. Therefore, the development of more accurate biomarker-based tools for early detection, surveillance and treatment monitoring is critical.

Similar to various “Omics” technologies such as genomic and proteomics approaches to discover global, unbiased genetic and protein aberrations respectively, the expressed metabolome in a cell can be investigated using metabolomics approach to understand the global changes in small-molecule biochemicals (metabolites) that occur in response to various environmental and cellular signals. Information obtained from metabolomics is complementary to those from genomics and proteomics in the context of systems biology for the global assessment of metabolic network within a living system ⁴. Metabolic profiling also provides opportunities for developing diagnostic/ prognostic biomarkers for specific diseases ⁵. Ever since its inception, metabolomics approaches have been applied to a wide variety of biological problems and to delineate metabolic biomarkers for cancer detection ⁶. Among various analytical platforms, Nuclear Magnetic Resonance (NMR) and Mass spectrometry (MS)-based metabolomics offer innovative, non-invasive platforms for the development of metabolic biomarker panels that are characteristic of disease phenotypes or cellular processes that are readily measured in biofluids/tissues ⁷. Both NMR and MS spectroscopic techniques provide complementary and supplementary metabolic information that can be used to develop disease-specific multiplex biomarker panel ⁷. Although NMR technique has substantially lower sensitivity than MS, the greatest and unparalleled advantage of NMR has been high reproducibility and the characterization of intact tissue specimens by high-resolution magic angle spinning (HR-MAS) NMR. The HR-MAS NMR requires only minimal sample preparation and leaves the tissue sample intact so it can be used subsequently for histopathological and/or MS analysis. Several significant advantages combined with well-defined protocols make HR-MAS NMR technique ^{8,9} amenable for translational research, specifically for cancer detection. HR-MAS NMR technique can reveal a more detailed landscape of tumor metabolome and has been used to investigate

various cancer types such as head and neck¹⁰, breast^{11, 12}, prostate^{13, 14}, lung^{15, 16}, colorectal¹⁷, cervical^{18, 19}, hepatic²⁰, gastric²¹ and renal carcinomas^{22, 23}.

Given the importance and necessity of effective biomarkers in BCa, metabolomics is an excellent platform for identifying biomarkers from aberrant metabolic pathways during BCa development and progression. Since metabolic markers are less complex than proteomic/genomic markers, a metabolite-based detection assay for BCa could be easily transitioned to a clinical setting. A limited number of metabolomics studies have been explored in bladder cancer research using urine^{24, 25} and serum²⁶ samples. Recently, Putluri et al.,²⁷ performed LC-MS (liquid chromatography-mass spectrometry)-based global metabolic profiling of BCa tissues/urine and identified several potential biomarkers, however the sample preparation involved cumbersome tissues metabolite extraction procedure. In general, most of the metabolomics studies published to date utilized either NMR or MS for the development of diagnostic/prognostic biomarkers. None of the studies hitherto have explored the utility of the well-established HR-MAS NMR to analyze intact tissues requiring minimal sample preparation for BCa detection. Moreover, the clinical utility is improved when a panel of metabolic biomarkers analyzed by NMR is independently validated by MS or vice versa. Towards the goal of developing of tissue-based biomarkers for BCa detection, here we performed global metabolic profiling of BCa and benign adjacent tissues by non-destructive HR-MAS NMR and identified differentially expressed metabolites that distinguish BCa from benign. Using the same tissues that were analyzed by HR-MAS NMR, we further cross-validated a panel of potential BCa biomarkers by targeted GC-MS analysis. A panel of multiple BCa biomarkers addressed in the present study more comprehensively captures multiple metabolic pathways involved in BCa development and progression than individual biomarkers alone. Therefore we anticipate that a multiplex panel of biomarkers will predict BCa status with greater accuracy and sensitivity.

Materials and Methods

Tissue Sample Collection and Pathologic Evaluation

Frozen bladder tissues were collected during transurethral resection of the bladder tumor (TURBT) from patients enrolled in the bladder cancer tissue bank in the Department of Urology at the University of Michigan. All samples were collected according to prior institutional review board approval and informed consent from patients. All tissue samples were embedded in Optimal Cutting Temperature (OCT) solution (composed of 10.24% w/w polyvinyl alcohol, 4.26% w/w polyethylene glycol and 85.50% w/w nonreactive ingredients), stored at -80°C and later evaluated by a genitourinary pathologist who confirmed the presence or absence of urothelial carcinoma with or without mixed or variant histology. For specimens with malignant disease, tumor stage and grade was established according to world health organization and International Society of Urologic Pathology standards²⁸. In total 59 OCT-embedded tissue samples were collected and were characterized based on histology and clinical outcomes: Tissues were characterized into benign (no malignancy, normal urothelium), non-muscle invasive (Ta and T1) cancer without subsequent stage progression, and any stage cancer that was T2 at TURBT or that demonstrated stage progression to T2 disease, local or distant metastasis, or cancer-specific death during follow-up²⁹. The detailed information of the patients and pathologic stage are included in Table S1.

High-Resolution Magic Angle Spinning (HR-MAS) ^1H NMR

Each specimen (15-25 mg wet weight) was rinsed twice with 100 μL deuterium oxide (D_2O) (Sigma Aldrich) to wash out water soluble OCT. Tissue sample was inserted into a 4 mm Zirconia MAS rotor (40 μL capacity) followed by the addition of ~ 10 μL D_2O and excess

fluid was removed when assembling the Kel-F plastic cap. The total preparation time for each sample was ~6 minutes. For all the tissue samples, NMR experiments were performed on Varian Inova 600 MHz NMR spectrometer equipped with gHX nano-probehead (Varian Inc.) at 298 K temperature. The spinning rate of the rotor was regulated by MAS controller (Varian Inc.) and also verified by measuring frequency separation between spinning side bands from ^1H spectra with an accuracy of ± 1.0 Hz. A standard one-dimensional (1D) spectrum and a 1D Carr-Purcell-Meiboom-Gill (CPMG) spectrum with spin-echo pulse sequence $[\pi/2-(\tau-\pi-\tau)_n\text{-acquisition-recycle delay}]$ were acquired for all the samples with a spinning rate of 2.6 KHz. The spectral width used for both the experiments was 8.5 KHz and water signal was presaturated using a weak continuous radio frequency irradiation on water resonance during the recycle delay. The free induction decay (FID) of CPMG spin-echo spectra were acquired into 32K data points during 1.89 s acquisition delay and 3s recycle delay; 256 transients were coadded. T_2 filtering was obtained with an echo time of 100 μs (τ) repeated 320 times, resulting in an effective echo time of 64 ms ($2n\tau$). The total acquisition time for each sample was <30 min. The acquired data were processed using Topspin 2.1 software (Bruker Biospin, Karlsruhe, Germany). The FIDs were multiplied by an exponential window function with a line broadening of 0.3 Hz prior to Fourier transformation and the frequency domain spectra were manually phase and baseline corrected. The proton chemical shifts were calibrated to CH_3 signal of lactate at 1.33 ppm.

A two-dimensional (2D) ^1H - ^1H total correlation spectroscopy (TOCSY) spectrum was acquired for one of the tumor tissue samples for chemical shift assignment of the tissue metabolites. The spectra were acquired in the phase-sensitive mode using time proportional phase incrementation (TPPI) with MLEV17 spin lock corresponding to mixing time of 80 ms. 2048 data points with 16 transients per increment and 256 increments were acquired with a spectral width of 8.5 KHz in both the dimensions and a recycle delay of 1.5 s. The resulting data were zero filled to a 2048×1028 matrix and Fourier transformed along both dimensions after multiplying the data by a squared sine-bell window function shifted by $\pi/2$.

Multivariate Statistical Analysis

Tissues CPMG NMR spectra were subjected to multivariate statistical analysis. The chemical shift region of 0.75 - 8.85 ppm was converted to ASCII files and the spectral regions of residual water 4.68 - 5.05 ppm and regions where spinning side bands appear (5.56 - 5.75 ppm) were excluded from the analysis. The regions containing OCT signals (3.62 - 3.76 ppm and 1.52 - 1.87 ppm) were also removed from the spectra. The spectral regions were normalized by the sum of all intensities over the entire spectrum and principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were performed with mean-centered scaling (The Unscrambler V10.1, CAMO, Oslo, Norway). The results were presented as 3D principal component scores plot (each point represents an individual sample) and loadings plot in which metabolite peaks are shown as positive and negative signals to indicate differential changes of metabolites. The quality of the model was described by the cross-validation parameters R^2 and Q^2 , representing the explained variance and predictive capability of the model respectively.

Relative Quantification and Statistical Analysis

For relative quantification, the ^1H peak intensities of metabolites were calculated separately for benign and urinary bladder cancer patients using total area normalized spectra. A selective non-overlapping resonance from each metabolite was used to calculate relative intensities. ^1H peak corresponding to branched chain amino acids (BCAA) (0.93-1.07 ppm), alanine (Ala) (1.45-1.51 ppm), acetate (Ace) (1.91-1.93 ppm), glutamate (Glu) (2.31-2.40 ppm), glutamine (Gln) (2.42-2.49 ppm), glutathione (GSH) (2.51-2.61 ppm), aspartate (Asp) (2.62-2.72 ppm), creatine (Cre) (3.02-3.07 ppm), taurine (Tau) (3.39-3.47 ppm), glycine

(Gly) (3.55-3.57 ppm), phenylalanine (Phy) (7.30-7.46 ppm), and tyrosine (Tyr) (6.85-6.92 ppm) were used. Since the triplet CH₂ signal of taurine (at 3.26 ppm) is overlapping with that of choline containing compounds (ChoCC), it is not possible to directly measure the intensity of peaks originating from ChoCC. Therefore, to obtain the intensity of peaks from ChoCC, the intensity of another CH₂ triplet signal of taurine (3.39-3.47 ppm) was subtracted from the total intensity of all peaks in the chemical shift region of 3.16-3.31 ppm. Similarly, the CH₂ (4.12 ppm) signal of lactate (Lac) was also overlapping with CH₂ (4.08 ppm) of triglycerides (TG); therefore, Lac concentration was measured by subtracting the spectral region (4.044.15 ppm) with CH₂ (4.23-4.37 ppm) of TG. Unsupervised hierarchical clustering (Pearson correlation) and heat map was drawn using R statistical package “gplot”. Statistical comparisons were made using Mann-Whitney U-test followed by a correction for multiple hypothesis testing (Benjamini-Hochberg correction) at 5% level.

GC-MS Analysis

Immediately after HR-MAS NMR measurement, tissue samples were taken out of zirconium rotors transferred into clean vials, flash frozen in liquid nitrogen and stored at -80°C until used for GC-MS targeted analysis. The procedure described previously⁵ was adapted for quantifying tissue metabolites. For every metabolite being assessed, a labeled (deuterium or ¹³C) isotope (Sigma, St Louis, MO) was also used as an internal standard for validation. Tissue samples were homogenized in methanol after spiking with labeled internal standards and mixed on a shaker overnight at 4°C. The extraction was carried out using 1:1 molar ratio of water/chloroform. The aqueous methanolic layer was collected and dried completely under nitrogen. The methanolic dried extract containing metabolites (amino acids) were further analyzed by GC-MS after derivatization using N-methyl-N-(tert-butylmethylsilyl)trifluoroacetamide (MtBSTFA) and dimethylformamide (DMF). The dried methanolic residue underwent azeotrope twice by adding 100 µL DMF, vortexing, and then drying in a Speedvac for 30 minutes. 100 µL of DMF and MtBSTFA + 1% t-butyl-dimethylchlorosilane was added to the dried sample, capped, and incubated at 80°C for 1 hr and then re-suspended with ethyl acetate and 1 L injected into a GC-MS.

Selective Ion Monitoring (SIM) was used for quantification. The amount of metabolite in the sample was calculated by comparing the peak area of the native metabolite to the area of the peak for the isotope-labeled internal standard. The levels of metabolites in tissues were normalized to tissue weight.

Results

Clinical characteristics of patients and tissue samples used for metabolomics

Frozen OCT-embedded tissues from 59 patients were selected from the Bladder Cancer Program tissue bank at University of Michigan. Twenty-six patients had benign disease and thirty-three patients had bladder cancer of which 17 patients were of Ta-T1 grade and 16 patients had evidence of muscle invasion at diagnosis or during surveillance. Patient clinical characteristics are shown in Table 1.

Metabolic Profiling of intact tissues by HR-MAS ¹H NMR

Metabolic perturbations derived from tissue degradation are possible during HR-MAS NMR spectral measurement and may significantly interfere with native metabolic profile of the samples. Although previous reports revealed that tissue specimens are metabolically stable during HR-MAS measurements¹⁰, metabolic stability of bladder-derived tissues had not been demonstrated. To test the metabolic stability of bladder-derived tissues over the period of HR-MAS ¹H NMR spectral measurement, two consecutive ¹H CPMG spectra were measured independently on benign and BCa tissues over 25 min. of data collection. No

significant metabolic variations were observed between two ^1H CPMG spectrum measured within a 1 hr. time period confirming that metabolic landscape is intact during HR-MAS NMR measurements performed at 298 K (data not shown).

HR-MAS ^1H NMR-based metabolic profiling was performed on bladder-derived tissues (33 BCa and 26 Benign) obtained from fifty-nine patients (Table 1) and visual inspection of all the ^1H CPMG spectra revealed striking differences between benign and BCa tissues. Representative ^1H CPMG spectra of a benign and various pathological stage BCa tissues (Ta/T1 and T2) are shown in Figure 1 along with chemical shift assignments of various metabolites. Hematoxylin and eosin (H&E)-stained photomicrographs obtained of dissected tissues used for HR-MAS NMR is also shown alongside each ^1H CPMG spectra. Although it is common practice to perform histopathological evaluation for the same tissue after HR-MAS NMR measurements^{10, 30} here they were utilized for MS analysis to validate the NMR results. The ^1H chemical shift assignments of tissue metabolites were carried out using the combination of previous reports^{10, 21, 30}, human metabolome database (HMDB)³¹, Biological Magnetic Resonance Data Bank (BMRB)³² and 2D ^1H - ^1H TOCSY spectrum. Triglycerides (TG) were detected in most of the bladder-derived tissues in addition to other small metabolites such as leucine (Leu), isoleucine (Ile) and valine (collectively termed as branched chain amino acids (BCAA)), lactate (Lac), alanine (Ala), acetate (Ace), lysine (Lys), glutamate (Glu), glutathione (GSH), glutamine (Gln), aspartate (Asp), creatine (Cre), choline-containing compounds (ChoCC) (choline, phosphocholine and glycerophosphocholine), taurine (Tau), glycine (Gly), *myo*-Inositol (*myo*-Ins), uridine diphosphate sugars (UDP-sug), phenylalanine (Phe) and, tyrosine (Tyr).

All the ^1H CPMG spectra of bladder-derived tissues showed differential metabolic profile between BCa and benign, with lower TG levels and higher aqueous metabolites in BCa compared to benign tissues. Though the BCa tissues were of various pathological stages, the overall NMR spectral features appeared similar. To better understand the metabolic variation between benign and BCa of various pathological stages and to develop a statistical model, we performed multivariate statistical analysis on the entire NMR data set.

Metabolic profiling differentiates benign and BCa: Statistical Model

PCA and PLS-DA statistical analysis were performed on the entire NMR datasets after removing unwanted spectral regions as described in methods section. Figure 2A depicts two-dimensional PCA score plot derived from ^1H CPMG spectra of the entire fifty-nine bladder derived tissues. Clear group discrimination was observed along principal component 1 (PC1) between benign and BCa tissues, in which most of the benign tissue samples segregated more compactly compared to BCa tissues, suggesting that BCa tissues exhibit more metabolic deviations whereas benign tissues exhibit stable metabolic patterns. In PCA score plot (Figure 2A), PC1 displays highest variation of 87% followed by PC2 and PC3 with 7% and 3% of variations respectively. Out of 26 benign samples, only a few segregated as BCa samples (indicated by arrows in Fig. 2A and 2B) that were partially contaminated with BCa tissues during resection. These samples were further evaluated by pathologist to confirm the presence of few cells of urothelial carcinoma with predominantly benign urothelium. Although the BCa group was well separated from the benign group in PCA score plot, samples from various pathological stages (Ta-T1, T2) were indistinguishable, suggesting that metabolic patterns at different BCa stages are not easily separable. Further assessment of PC1 loadings plot (Figure 2C) suggested decreased TG levels (negative loadings) and elevated levels of other metabolites (positive loadings) such as BCAA, Lac, Ala, Ace, Lys, Glu, GSH, Gln, Asp, Cre, ChoCC, Tau, Gly, *myo*-Ins, UDP-sug, Phe, Tyr and His in BCa tissues. To address the overall variations in metabolic patterns, comparisons were made between mean spectra of all ^1H CPMG spectra of benign (Figure 2D) and BCa (Figure 2E), where the visually observed spectral difference between these two mean spectra

were clearly in agreement with the PC1 loadings plot (Figure 2C). PCA was also performed separately on ^1H CPMG spectra of Ta/T1 and T2 samples (Figure S2) but no clear segregation was observed between these BCa tissue samples.

Following PCA Analysis, PLS-Discriminant Analysis (PLS-DA) was performed for the same dataset where PLS-DA score plot also showed a clear group separation between benign and BCa groups (Figure 2B) and was characterized by a strong predictive model supported by $R^2=0.93$ and $Q^2=0.86$.

Quantitative assessment of tissue metabolites detected in HR-MAS NMR

Although ^1H CPMG NMR has been the preferred method for NMR-based metabolomics analysis of tissues to obtain the metabolic profile of small biochemicals by filtering the signals from macromolecules (proteins, lipoproteins), it cannot be used to obtain absolute quantitative information due to attenuation of metabolite intensities. Nevertheless, the relative signal intensities (after total area normalization) of metabolites can be compared between the spectra of different tissue samples⁹. The peak intensities of metabolites were calculated for benign and cancer (Ta/T1 and T2) tissues from normalized total area of CPMG NMR spectra. Due to signal overlapping of Val, Leu and Ile (Figure 1), these signal intensities were summed and represented collectively as BCAA. Similarly the peak intensities of choline, phosphocholine and glycerophosphocholine were also combined and depicted as ChoCC. The representative non-overlapping ^1H signal from each metabolite (as described in methods section) was used to ascertain the intensities and total intensities of fifteen metabolite entities were established. Further, hierarchical clustering was performed on the intensity values (represented by z-score) of these fifteen metabolites. The heat map showing the variation of metabolites from benign and BCa tissues arranged by unsupervised hierarchical clustering (Figure 3) revealed that most of the benign and BCa tissues clustered separately. Few benign tissues were clustered among BCa tissues (Figure 3) which were partially contaminated with BCa tissues during resection. Results from both unsupervised hierarchical clustering and PCA models performed on the intensities and whole NMR dataset are both consistent in differentiating benign and BCa tissues. The peak intensities of fifteen differential metabolites between benign and Ta/T1, benign and T2, and Ta/T1 and T2 samples were further assessed using Mann-Whitney U test followed by Benjamini–Hochberg correction, and all the fifteen metabolites were found to be statistically significant ($P < 0.001$) where TG levels were depleted and rest of the metabolites were elevated in Ta/T1 and T2 compared to benign tissues (Figure S1). However, among all the metabolites, only a significant decrease in acetate and an increase in lactate levels were observed in Ta/T1 compared to T2 tissues (Figure S1).

Targeted GC-MS Validation of Tissue Metabolite Markers derived from HR-MAS NMR

HR-MAS NMR-based metabolic profiling revealed several metabolites, including amino acids that were elevated in BCa tissues. To validate our NMR findings and to explore the potential of the putative biomarkers of BCa for clinical utility, we performed targeted GC-MS analysis on the tissues samples that were recovered after HR-MAS NMR analysis. GC-MS analysis was performed on select glycolytic/ TCA cycle intermediates namely alanine, glutamate, glutamine, valine, isoleucine, phenylalanine and tyrosine. Tissues were homogenized and metabolites were extracted and GC-MS analysis was performed on 30 bladder-derived tissues (10 benign, 10 bladder cancer Ta-T1 and 10 bladder cancer T2 stage patients) using isotopic dilution method as described in the Methods section. For selected ion monitoring (SIM) analysis, the m/z for native and isotopically-labeled molecular peaks for various target metabolites quantified were: 232/235 (alanine), 432/437 (glutamate), 43/436 (glutamine), 200/203 (leucine and isoleucine), 466/470 (tyrosine) and 186/187 (valine). The levels of these amino acids were normalized to tissue weight and

benign, Ta/T1 and T2 tissue groups were analyzed using Mann-Whitney U test followed by Benjamini–Hochberg correction (Figure 4). All the amino acids analyzed were found to be significantly elevated in Ta/T1 and T2 compared to benign tissues, however, no significant changes were observed between Ta/T1 and T2 tissues, consistent with the HR-MAS NMR findings from intact tissues.

Diagnostic Performance of HR-MAS NMR metabolic signature

Two distinct ROC curves were generated (Fig. 5) using PC1 scores and predicted Y-values of PLS-DA model which yielded AUC values of 0.978 and 0.979 respectively.

Discussions

In the present study, we performed metabolic profiling of bladder-derived tissue specimens to develop tissue-specific metabolomic signatures for early and specific detection of bladder cancer. We initially performed HR-MAS NMR of intact tissues followed by multivariate statistics to identify a panel of metabolites that are specifically altered in bladder cancer followed by GC-MS-based targeted validation of differential metabolites identified by NMR. Although NMR is highly reproducible and straight forward technique for analyzing intact tissues, our study focused on validating NMR with MS analysis in order to strengthen the predictive value of a multiplex metabolic biomarker panel for use as a clinical diagnostic/prognostic tool. Here, we showed that HR-MAS NMR-based metabolic profiling cross-validated with GC-MS analysis can distinguish benign patients from BCa patients with greater sensitivity (AUC > 0.97) than conventional techniques³³. BCa tissues were characterized with decreased TG levels and increased levels of BCAA, Lac, Ala, Ace, Glu, GSH, Gln, Asp, Cre, ChoCC, Tau, *myo*-Ins, Phe, and Tyr ($P < 0.001$ for all). Multivariate statistical models (PCA and PLS-DA) of NMR dataset clearly distinguished BCa and benign tissues with good predictive capabilities ($R^2 = 0.93$ and $Q^2 = 0.86$). Altered metabolite levels identified through NMR also showed a trend similar to GC-MS analysis for eight amino acids ($P < 0.001$ for Ala, and Glu and $P < 0.02$ for Phe, Gln, Tyr, Val, Leu and Ile), suggesting that HR-MAS NMR can be reliably used to detect BCa. This method involves no tissue exhaustion, minimal sample preparation (< 5 min) and rapid data collection (5–15 min). Additionally, it has been demonstrated in other cancers that only biopsy size tissue specimens are required for HR-MAS NMR^{14, 34} which makes this technique ideally suited in clinical settings.

Early biochemical studies showed that tumors have altered metabolic activity and display high rates of glucose metabolism and catabolism³⁵. This phenomenon is likely due to the heterogeneous nature of tumors that are often comprised of micro-ecological niches that vary significantly in gradients of metabolites like glucose, oxygen, growth factors, etc. Most tumor tissues generate cellular energy through break down of glucose by glycolysis at a vastly higher rate than in normal tissues, even in the presence of high oxygen (that promotes aerobic glycolysis), a phenomenon termed the Warburg effect³⁵. Through aerobic glycolysis, tumor cells convert large portion of pyruvate to lactate which accumulates in tumor tissues. In the present study, we observed significantly elevated levels of lactate in BCa tissues, the end product of aerobic glycolysis attributed to the Warburg effect. Elevated lactate levels were also observed in other tumor tissues^{10, 14}; notably, increase in lactate levels were not observed in recent metabolomics study on BCa tissues²⁷. However, glucose, the precursor of glycolysis, was not detected in any bladder-derived tissues (benign/BCa) even as BCa tissues facilitate glucose entry into the cells through glucose transporters^{36, 37}. Our study also showed increased level of alanine, another important end product of glycolysis/glutaminolysis and found be major source of energy required for tumor proliferation and growth, consistent with other LC-MS-based metabolomic studies of BCa tissues²⁷ as well as in other tumors^{10, 14}. BCa tissues showed elevated creatine in present

study as well as in previous report²⁷, further evidence of altered energy metabolism in tumor tissues¹⁰. High acetate levels detected in BCa tissues is likely associated with enhanced lipid synthesis for the formation of cell membranes³⁸. BCa tissues also exhibit elevated UDP-sugars (UDP-GlcNAc and UDP-GalNAc), these metabolites are associated with biosynthesis of complex extracellular N-glycans and O-GlcNAcylation that confer growth advantage to cancer cells³⁹. Overall the rate of glycolysis is highly elevated in bladder cancer, comparable to other tumors.

Cancer cells can also shift to alternate energy sources for their growth, proliferation and survival, such as glutaminolysis to allow cancer cells to maintain sufficient anaplerotic flux into TCA cycle and has been well documented in various cancer types^{39, 40}, however, its biological role in bladder cancer was not known. Our results showed elevated glutamine and glutamate levels in BCa tissues. Amino acids such as leucine, isoleucine, valine, phenylalanine and tyrosine were also elevated BCa tissues by both HR-MAS NMR and GC-MS analysis confirming that TCA cycle anaplerosis contribute significantly towards tumor cell proliferation and growth. We also observed increase in anaplerotic amino acids in our recent HR-MAS NMR study of head and neck cancer¹⁰.

One of the hallmarks of cancer cells is aberrant membrane choline phospholipid metabolism (MCPM) leading to altered levels of choline-containing metabolites such as choline, phosphocholine, glycerophosphocholine and phosphatidylcholine⁴¹. The production of these cell membrane metabolites is facilitated by the activation of both biosynthetic (*choline kinase*) and catabolic enzymes (*phospholipases A, C and D*)³⁹. One of the most significant pathways in bladder cancer is *phospholipase C (PLC)* among others and de-regulation of these pathways leads to abnormal gene regulation⁴². Increase in choline-containing metabolites was observed in varieties of tumor cells and tissues mostly using NMR technique^{10, 39}. Multiple studies confirmed that *phospholipases* are associated with malignant transformations of many human cancers, which makes these enzymes potential molecular targets for anti-cancer chemotherapies⁴³. Consistent with these findings, elevated levels of choline-containing metabolites observed in BCa tissues reflect altered MCPM.

In addition to altered metabolic pathways, cancer cells often undergo oxidative stress associated with generation of reactive oxygen species including oxidation-induced mutations, malignant transformation and alterations in metabolic activity⁴⁴. Oxidative stress results in elevated expressions of antioxidant enzymes⁴⁵ such as *glutathione peroxidase*, *glutathione reductase* and *glutathione-S transferase* which produce glutathione (GSH), a tripeptide that plays a key role against pro-inflammatory processes and acts as intra- and extracellular protective antioxidant. Elevated GSH levels were observed in BCa tissues and correlates with previously reported GSH levels in bladder cancer cell lines and tissues⁴⁶. BCa tissues also showed elevated taurine and *myo*-inositol, both of these metabolites act as osmoprotectants and substrates for volume-activated organic efflux pathway⁴⁷. Taurine also display antineoplastic and antioxidant properties⁴⁸. *Myo*-inositol is also associated with phosphatidylcholine turnover and modulation of phospholipids (phosphatidylinositol) in mammalian cells⁴⁹. Recent studies have suggested taurine as biomarker of bladder cancer⁵⁰ and hepatocellular carcinoma⁴⁸.

In contrast to increased aqueous metabolites, BCa tissues clearly showed decreased triglyceride levels similar to other cancers like colorectal, oral and head and neck cancer¹⁰. Cancer cells also exhibit increased fatty acid oxidation to generate bioenergy for rapid proliferation and growth that may lead to cancer-associated cachexia⁵¹. We hypothesize that lower TG levels in BCa tissues could be associated with enhanced β -oxidation of lipids to satisfy energy needs for rapid growth.

Overall, HR-MAS NMR-based metabolic profiling of bladder derived tissues showed improved performance in distinguishing BCa from benign and even at early pathologic stage BCa (Ta, T1). Therefore, we believe that HR-MAS NMR-based metabolic profiling of intact tissues is quite promising that warrants exploration of its eventual application towards clinical diagnosis. In addition to various advantages of sampling and rapid data collection, HR-MAS NMR can be cost effective. With recent advancement in NMR hardware technology (higher magnetic field, cryogenic probe technology), metabolic information even for low abundant metabolites can be obtained within few minutes of data collection using only a needle biopsy tissue sample. GC-MS analysis further confirmed the metabolomic findings from HR-MAS NMR. Most of our findings on BCa tissues agree with previously reported LC-MS-based BCa tissue profiling. In addition to its potential as a diagnostic tool, metabolic information procured from HR-MAS NMR can uncover aberrant metabolic pathways associated with BCa development and progression and reveal novel therapeutic targets through metabolic modulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative 600 MHz ¹H HR-MAS CPMG NMR spectra (area normalized) of benign (A), and various bladder cancer pathologic stages Ta/T1 (B and C) and T2 (D). The vertical scale of all the spectra were kept constant and spectral regions containing spinning side bands (5.6 – 6.0 ppm) and OCT (3.6 – 3.8 ppm) were removed from the figure. The intensity of peaks in the chemical shift region 6.0 – 8.5 ppm was enhanced equally in all the spectra to show the low abundant metabolites. The triglycerides signals were indicated as 'TG'. The corresponding H & E photomicrographs obtained from a section dissected from the same tissues are also shown alongside of each ¹H CPMG spectrum.

Figure 2.

(A) Unsupervised 2D PCA score plot (B) supervised 2D PLS-DA score plot and (C) PC1 loadings of a total of 59 tissues generated from ^1H CPMG spectra of 26 benign, 17 bladder cancer Ta-T1 and 16 bladder cancer T2 stage. Both PCA and PLS-DA score plots showed clear segregation of benign and BCa groups, predominantly due to PC1 loading. From PC1 loadings, TG levels were clearly decreased (negative loadings) while all other aqueous metabolites were elevated (positive loadings) in BCa compared to benign tissues. Few benign samples (indicated by black arrows) categorized under BCa samples were predominantly benign urothelium partially contaminated with urothelial carcinoma. Figure 2D and 2E show the average ^1H CPMG spectrum of all 26 benign spectra and 33 bladder cancer spectra, respectively. The spectral difference between the two average spectra is consistent with PC1 loadings.

Figure 3.

Heat map showing z-score of 15 quantified metabolite entities in 26 benign and 33 bladder cancer tissues. An unsupervised hierarchical classification showing separate cluster for benign and bladder cancer tissues is also included in the heat map. Only few benign samples fall under BCa group those were partially contaminated with BCa tissues during resection. Heat map shows significant differences in the concentration of all metabolites between benign and cancer tissues. The difference in concentration of metabolites was further assessed using Mann-Whitney U test followed by Benjamini–Hochberg correction, and all the fifteen metabolites were found to be statistically significant ($P < 0.001$).

Figure 4.

Box plot showing the levels of select metabolites that were measured in benign, Ta/T1 and T2 tissues (normalized to the tissue weight) by targeted GC-MS analysis. Analysis was performed on the same tissues that were recovered from HR-MAS NMR analysis. The levels of metabolites were compared using Mann-Whitney U test followed by Benjamini–Hochberg correction and all the metabolites were found to be significantly elevated in Ta/T1 and T2 cancer tissues, however, no significant changes were observed between Ta/Ta and T2 tissues, consistent with HR-MAS NMR findings. Benign Vs Ta/T1: * P 0.05, ** P 0.01 and *** P 0.001 ; Benign Vs T2: ■ P 0.05, ■■ P 0.01 and ■■■ P 0.001.

Figure 5. ROC curves generated from PC1 scores (A) and predicted Y-values of PLS-DA (B). AUC values of 0.978 and 0.979 respectively, represent a good predictability of PCA and PLS-DA model in differentiating benign and bladder cancer tissues.

Table 1

Clinical characteristics of the patients

Total number of patients = 59			
	No. of patients	Sex (Male/Female)	Age in years [Mean (range) $\pm$ S.D.]
Benign	26	22 / 4	69.4 (51-87) $\pm$ 10.6
BCa: Ta-T1	17	15 / 2	60.1 (33-78) $\pm$ 11.5
BCa: T2	16	14 / 2	68.8 (43-87) $\pm$ 11.7