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NMR-based metabolomics study of canine bladder cancer

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ABSTRACT

Bladder cancer is one of the leading lethal cancers worldwide. With the high risk of recurrence for bladder cancer following the initial diagnoses, lifelong monitoring of patients is necessary. The lack of adequate sensitivity and specificity of current noninvasive monitoring approaches including urine cytology, other urine tests, and imaging, underlines the importance of studies that focus on the detection of more reliable biomarkers for this cancer. The emerging area of metabolomics, which deals with the analysis of a large number of small molecules in a single step, promises immense potential for discovering metabolite markers for screening and monitoring treatment response and recurrence in patients with bladder cancer. Since naturally-occurring canine transitional cell carcinoma of the urinary bladder is very similar to human invasive bladder cancer, spontaneous canine transitional cell carcinoma has been applied as a relevant animal model of human invasive transitional cell carcinoma. In this study, we have focused on profiling the metabolites in urine from dogs with transitional cell carcinoma and healthy control dogs combining nuclear magnetic resonance spectroscopy and statistical analysis methods. ^{1}H NMR-based metabolite profiling analysis was shown to be an effective approach for differentiating samples from dogs with transitional cell carcinoma and healthy controls based on a partial least square-discriminant analysis of the NMR spectra. In addition, there were significant differences in the levels of six individual metabolites between samples from dogs with transitional cell carcinoma and the control group based on the Student's *t*-test. These metabolites were selected to build a separate partial least square-discriminant analysis model that was then used to test the classification accuracy. The result showed good classification between transitional cell carcinoma and control groups with the area under the receiver operating characteristic curve of 0.85. The sensitivity and specificity of the model were 86% and 78%, respectively. These results suggest that urine metabolic profiling may have potential for early detection of bladder cancer and of bladder cancer recurrence following treatment, and may enhance our understanding of the mechanisms involved.

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1. Introduction

Bladder cancer (BC), and specifically transitional cell carcinoma (TCC) was one of the leading lethal cancers worldwide. It was estimated that 69,250 new cases of BC would be diagnosed and 14,990 deaths would result from this disease in the United States alone in 2011 [1]. The current standard approach to diagnose BC is cystoscopy and histopathologic examination of the tissues collected at cystoscopy. Following diagnosis and treatment, there is a high risk of BC recurrence. Patients with BC are monitored lifelong for recurrence through urine cytology, other analyses performed on urine, imaging and periodic cystoscopy [2–4]. However, none of the monitoring techniques provide a non-invasive test with high sensitivity and specificity for clinical use [5–7]. Recently, a few studies have focused on BC biomarker discovery, with the goal of finding reliable metabolites or proteins that could make a significant impact in the management of the malignancy [4,8–11]. Urine contains tumor cells and metabolites shed from the TCC mass in the bladder wall. BC biomarker discovery in urine may prove useful in detection of the cancer and in providing a better understanding of how the cancer develops and progresses.

Metabolomics, also commonly known as metabolic profiling and metabonomics, is a growing field in systems biology [12,13], and offers a powerful and promising approach to identify biomarkers associated with numerous cancers [14–17] and other diseases [18–24].

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The information-rich analytical techniques of nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) combined with multivariate statistical analyses are the premier methods for metabolomics-based studies [13,25]. While utilization of both NMR and MS methods leads to the analysis of over 1000 small molecules (molecular weight <1000 Da), various unsupervised and supervised statistical methods create robust mathematical models to detect significant differences between cancer and healthy subjects arising from perturbations caused by disease processes, toxins, therapy or even diet [26]. Key metabolites can be identified from the statistical results and then validated as biomarker candidates [13]. However, there are few BC metabolomic studies that have been reported to date [5,27]. Issaq and co-workers analyzed human urine from BC patients and healthy controls using liquid chromatography-mass spectrometry (LC-MS) and stated the approach could be used as a potential noninvasive early BC detection test [28]. Srivastava et al. observed a significant elevation in the concentration of taurine in urine of BC patients using NMR, and proposed it as a possible biomarker for the non-muscle invasive form of BC [29]. Two studies using gas chromatography–mass spectrometry (GC–MS), one based on global metabolomics and another based on an amino acid targeted approach, have explored urinary metabolites as diagnostic markers for human BC [30,31]. More recently, the Huang group used two complementary LC separation techniques to profile human urinary metabolites and found carnitine C9:1 and an unknown metabolite provided effective indication of BC using MS detection [32]. However, to decrease the mortality rate of BC, the development of methods to detect the cancer earlier and to detect its recurrence in a timely fashion, especially the exploitation of biomarkers that offer high sensitivity and specificity, is still in great demand. In addition, information provided by metabolomics in the area of cancer biology may be useful in further understanding the disease and how to prevent and control it more effectively.

Naturally-occurring canine TCC of the urinary bladder has been found to be very similar to human invasive BC not only in its histopathologic characteristics and molecular features, but also in its biological behavior including sites and frequency of metastasis, response to medical therapy, and prognosis [33,34]. Spontaneous canine TCC has been considered a highly relevant animal model of human invasive BC [35], and it has been applied in studies of antitumor drug activity [36,37]. Several canine TCC cell lines have been established for mechanism studies [38].

In this study, NMR combined with statistical analyses was used to profile and compare the urine metabolites from dogs with TCC and healthy control dogs with no bladder disease, and the approach was proven to be a powerful method for TCC biomarker discovery. Six highly sensitive biomarker candidates, including urea, choline, methylguanidine, citrate, acetone, and β-hydroxybutyrate, were identified. The power of these metabolites to distinguish the TCC and control groups, in terms of their sensitivity, specificity, and level comparisons were made. Factors such as different ages, genders and breeds were also evaluated. Based on the identified biomarker candidates, intrinsic disease-related mechanisms were discussed and suggested for further studies. In particular, emerging methodologies in TCC metabolomics in this study were shown to be effective, and open a number of potential avenues for further development and clinical applications.

2. Materials and methods

2.1. Chemicals

Deuterium oxide (D_2O, 99.9% D) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Trimethylsilylpropionic acid-d_4 sodium salt (TSP), sodium azide (NaN_3), disodium hydrogen phosphate (Na_2HPO_4), and monosodium phosphate (NaH_2PO_4) were purchased from Sigma-Aldrich (analytical grade, St. Louis, MO). Deionized water was obtained from an EASYpure II UV water purification system (Barnstead International, Dubuque, IA).

2.2. Animal handling and urine collection

This work was approved by the Purdue Animal Care and Use Committee. Dogs participating in the study were evaluated at the Purdue University Veterinary Teaching Hospital. Participating dogs included privately owned dogs with naturally-occurring invasive TCC of the urinary bladder and/or urethra diagnosed by histopathology, and privately owned healthy control dogs of similar age, breed, and gender. In the dogs with TCC, thoracic radiography and abdominal ultrasonography were used to help determine tumor stage. The TNM stage was defined following criteria established by the World Health Organization (WHO) [39].

The control dogs had no evidence of TCC other cancer, any bladder disease, major organ dysfunction, or illness. To help confirm that the control dogs did not have occult urinary tract disease, urine samples were analyzed by dip stick exam (Multistix 10SG, Siemens, Washington, DC) and urine specific gravity. If any abnormalities were noted in the urine of the control dogs, such as urinary tract infection or blood in the urine, the dogs were excluded from the study.

Midstream free catch urine samples (10 mL) were collected from the dogs. Sodium azide (0.1%) was added to the samples. The urine samples were centrifuged, and the supernatants stored at −80 °C until metabolomic analyses were performed.

2.3. NMR spectroscopy

The experimental methods used for NMR analysis have been published elsewhere [18]. Briefly, frozen urine samples were thawed, and 300 μL was mixed with 300 μL phosphate buffer (pH 7.4, 0.5 M). Each resulting solution was centrifuged at 13,200 rpm for 10 min to remove particulate matter, if any was present. 550 μL of the supernatants were transferred to 5-mm NMR tubes. A 50 μL of TSP (0.06 mg/mL in D_2O) was also transferred into each tube, and utilized as the chemical shift reference ($\delta = 0.00$). All ^1H NMR experiments were carried out at 25 °C on a Bruker DRX-500 spectrometer equipped with a cryogenic probe and triple axis magnetic field gradients. ^1H NMR spectra were acquired using the standard one-dimensional NOESY pulse sequence with water presaturation. Each dataset was averaged over 64 transients using 16 K time domain points and 7 kHz spectral width. The data were Fourier transformed after multiplying by an exponential window function corresponding to a line broadening of 1 Hz. The spectra were then phase and baseline corrected using Bruker TopSpin software (version 3.0).

2.4. Data analysis

NMR spectral were binned to 4 K buckets of equal width (0.0034 ppm) to minimize errors due to any fluctuations of chemical shifts arising from pH or ion concentration variations. Each spectrum was aligned to the methyl peak of alanine at 1.48 ppm, and was normalized using the integrated creatinine signal at 4.05 ppm. Spectral regions within the range of 0.3 to 10.0 ppm were used for the analysis after deleting the region containing water residual signals (4.5 to 5.0 ppm). Univariate analysis was performed by applying the unpaired Student's *t*-test to identify significantly different spectral bins among samples from dogs with TCC and healthy controls. The characteristic spectral regions for each metabolite were then integrated, and *p*-values and fold changes between different groups were calculated.

To classify cancer and control groups, statistical analysis was performed on the whole (binned) spectral data using partial least square discriminant analysis after orthogonal signal correction (OSC-PLS-DA). For this analysis, the binned NMR data were imported into Matlab (R2008a, Mathworks, MA) installed with a PLS toolbox

Table 1
Summary of clinicopathological characteristics of dogs with invasive transitional cell carcinoma (TCC) and controls.

Characteristics	TCC dogs	Control dogs
Number	40	42
Chronological age (yrs, mean ± SD)	9.8 ± 2.1	6.7 ± 2.8
Standardized age in human equivalents[a] (yrs, mean ± SD)	55.5 ± 7.6	42.7 ± 11.8
≤45 yrs	3	21
>45 yrs	37	21
Gender (male/female)	30/10	17/25
Breed		
Scottish Terrier	9	8
Shetland Sheepdog	8	6
West Highland White Terrier	1	1
Others	22	27
Cancer stage and grade[b]		
T0	2	
T2	29	
T3	9	
N0	37	
N1	3	
M0	31	
M1	9	
Nodal or distant metastasis developed before death		
Yes	26	
No	10	
Unknown	4	

[a] Converted from chronological age according to Reference [41].
[b] Stage defined by World Health Organization criteria for canine bladder tumors [39].

(version 4.1, Eigenvector Research, Inc). The X matrix consisted of OSC corrected NMR spectral variables; the Y matrix consisted of "0" and "1" for TCC and control samples, respectively. We then performed PLS-DA using peak integrals of the selected metabolites as X matrix inputs. Subsequently, the performance of the PLS-DA models was assessed by Monte Carlo Cross Validation (MCCV) analysis [40] using 100 iterations as well as another 100 iterations for permutation analysis. For MCCV analysis, all samples were randomly divided into two sets, 60% of the samples were used as the training set and 40% as the test set with and without (for permutation analysis) true class assignment. For each iteration, the training set was 5-fold cross-validated and used to predict the class membership of the test set samples. Selected metabolites with $p<0.05$ were also analyzed using the same approach. Predictions were made visually using a Y-predicted scatter plot with a cut-off value chosen for potential class membership. The R statistical package (version 2.8.0) was used to generate box-and-whisker plots and receiver operating characteristics (ROC) curves, calculate and compare the sensitivity, specificity and the area under the ROC curve (AUROC).

3. Results and discussion

3.1. Biomarker discovery and evaluation

Clinical data were obtained from 40 dogs with TCC and 42 controls. The clinicopathological characteristics are summarized in Table 1. To minimize the effect of breed and body size on dog age, each dog's age was converted to human year equivalents as previously described [41].

Representative ^{1}H NMR spectra of samples from the TCC group and the control group are shown in Fig. 1, along with the metabolite assignments. As listed in Table S1, a total of 21 metabolites were assigned to the corresponding resonances by comparing chemical shifts and multiplicities of peaks to the previously reported data [42,43]. In order to remove the influences attributed to muscle mass and urine concentration [43,44], the NMR data were normalized to the integrated creatinine resonance at 4.05 ppm. The normalized NMR whole spectral data were further analyzed by orthogonal signal correction pretreated PLS-DA (OSC-PLS-DA) to differentiate the urine of dogs with TCC from healthy controls. The OSC-PLS-DA score plot shows a clear separation, indicating a metabolic difference between the two dog groups (Fig. 2A). In the figure, TCC dogs are represented by an open square and healthy controls are represented by a solid circle. Monte Carlo Cross Validation (MCCV) was then applied to further evaluate the classification ability of the whole NMR spectra. The predictions for the true class assigned models and permuted models were plotted in an ROC space as shown in Fig. 2B. The permuted model results, which clustered at the center of the ROC, indicate no discrimination. And the true class assigned models all showed high sensitivity and specificity, which gave evidence for the good classification ability of the PLS-DA model. Analysis of the loading plots indicates that a number of metabolites including all of those shown in supplementary Table S1 (except taurine), along with bile acids, threonine, dimethylamine and betaine contribute to the discrimination.

We decided to also pursue a more targeted metabolomics approach in this study to focus on individual metabolites with individual statistical significance. A major drawback of multivariate model building such as OSC-PLS-DA using the full data as shown in Fig. 2 is that such models typically rely on the contributions from numerous small peaks, many of which are currently unknown metabolites, or even chemical noise. In

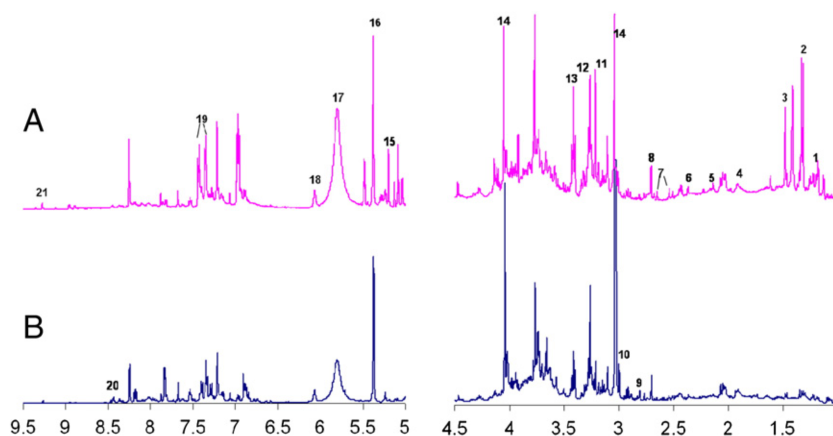

Fig. 1. Typical 500 MHz ^{1}H NMR spectra of urine obtained from (A) a dog with transitional cell carcinoma (TCC) and (B) a healthy control dog. Key: 1, β-hydroxybutyrate; 2, lactate; 3, alanine; 4, acetate; 5, acetone; 6, pyruvate; 7, citrate; 8, dimethylamine; 9, methylguanidine; 10, creatine; 11, choline; 12, trimethylamine N-oxide; 13, taurine; 14, creatinine; 15, glucose; 16, allantoate; 17, urea; 18, cis-aconitate; 19, (phenylacetyl)glycine; 20, formate; 21, N-methylnicotinamide.

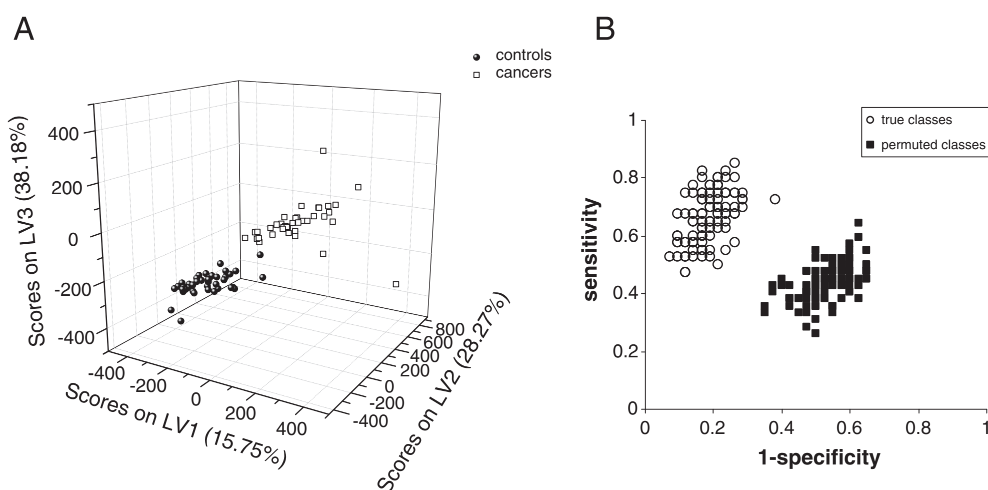

Fig. 2. (A) OSC-PLS-DA score plot, and (B) Monte Carlo Cross Validation (MCCV) prediction results of the PLS-DA model plotted as sensitivity *vs* 1-specificity based on the ^{1}H NMR spectra of samples from 42 control dogs and 40 dogs with TCC.

addition, such an approach can be susceptible to errors arising from imperfect spectral baselines, peak misalignments, and strong uneven solvent backgrounds. The positions of NMR signals specifically in urine are often sensitive to subtle differences in parameters such as pH, ionic strength, temperature or concentration of macromolecules. Peak shifts combined with baseline distortions can translate into spectral bins that do not represent true peak intensity and pose a significant challenge to the accuracy of the outcome. The problem becomes more severe when the metabolite peaks involved are of low intensity. The quantitative metabolomics approach that we have pursued here alleviates these issues by correctly identifying peak regions, followed by application of local base line corrections wherever needed, and then peak integration.

As a result of this process, individual peaks from 21 known metabolites (Supplementary Table S1) were integrated, and their *p*-values between the TCC and control groups were calculated. Six metabolites, urea, choline, methylguanidine, citrate, acetone and β-hydroxybutyrate, were significantly different between dogs with TCC and control dogs ($p<0.05$). These 6 metabolites were considered as biomarker candidates in the following analysis and discussion, and their relative concentrations in both the TCC and control groups, *p*-values and fold changes are shown in Table 2. Also, the ranges of each metabolite concentration for the TCC and control samples are shown as box-and-whisker plots in Fig. 3. Both the results from Table 2 and Fig. 3 indicate that all the median levels of the 6 marker candidates increased from controls to dogs with TCC. PLS-DA was then utilized to build a multivariate model to evaluate the potential biomarker candidates. The 6 metabolites were selected as the variables to build this second PLS-DA model. Leave-one-out cross-validation was performed to obtain the best model and avoid over-fitting. Six LVs were used based on the RMSECV (Root-Mean-Square Error of Cross-Validation) procedure using Matlab, and the cross validation error was estimated as 20.9%. The need for 6 LVs is likely due to the fact that most of the metabolite correlations are modest and mostly decrease in TCC compared to normal (Fig. S2). The prediction plot of the group membership from the PLS-DA model is shown as in Fig. 4A, in which most samples were well classified using a cut-off of 0.0034. In Fig. 4B, ROC curve analysis using the cross-validated predicted Y (predicted class) values was utilized to judge the sensitivity and specificity of the PLS-DA model. The AUROC was 0.85, and the sensitivity and specificity for TCC detection were 86% and 78%, respectively. In a similar validation process as described above for the whole spectral data, MCCV was applied to the group of 6 marker candidates, and the results shown in Fig. 4C validate the good sensitivity and specificity of the true class assigned models and the non-discriminating permuted models.

Table 2
Metabolites that are significantly different between dogs with transitional cell carcinoma (TCC) and controls.

Metabolite	Relative concentration[a] (mean ± SD)		*p*-Value	FC[b] (TCC/control)
	Control	TCC		
Urea	1452.8 ± 615.4	2000.0 ± 970.0	3.04*E*−03	1.37
Choline	65.9 ± 31.2	109.7 ± 99.2	9.86*E*−03	1.63
Methylguanidine	31.4 ± 7.9	37.3 ± 16.1	3.44*E*−02	1.19
Citrate	325.6 ± 281.9	857.8 ± 801.8	7.71*E*−04	2.56
Acetone	44.1 ± 15.3	55.2 ± 26.8	2.92*E*−02	1.24
β-hydroxybutyrate	72.6 ± 25.5	119.5 ± 50.1	1.51*E*−06	1.62

[a] μM/mM of creatinine.
[b] Fold change.

3.2. Effect of gender, age, breed and TNM stage

Previous reports have shown that a series of factors, including gender, age, breed, and TNM stage, may result in distinctive canine metabolic differences [33,43,45]. Therefore, to further evaluate the marker candidates, *p*-values for the 6 metabolites comparing age, gender and breed between dogs with TCC and controls were calculated and shown in Table 3A, B and C, respectively. The *p*-values of the marker candidates between ages, genders and breeds within the control and TCC groups were also calculated as shown in Table S2. In terms of different ages, genders and breeds, the 6 metabolites in TCC group rarely showed much difference.

Typically, canine TCC is a disease found in older dogs. In this study, dogs with a standardized age of 45 and higher were considered as the old group and the rest as the young group. Within the control dogs, the number of old and young subjects was both 21, and the *p*-values of all 6 marker candidates between old and young dogs indicated no significant difference as listed in Table S2. However, as shown in Table 3A, urea, choline, citrate and β–hydroxybutyrate showed significant differences ($p<0.05$) between TCC and control dogs within the old group, while no metabolite had a low *p*-value in the young group (although this may partly be due to the small TCC sample number). These data indicate that the markers are not age-dependent, and urea, choline, critate and β-hydroxybutyrate could be used to distinguish dogs with TCC from controls, at least for the older dogs.

Unlike humans, female dogs are confirmed to have an increased risk of developing BC [34]. All marker candidates showed $p>0.05$

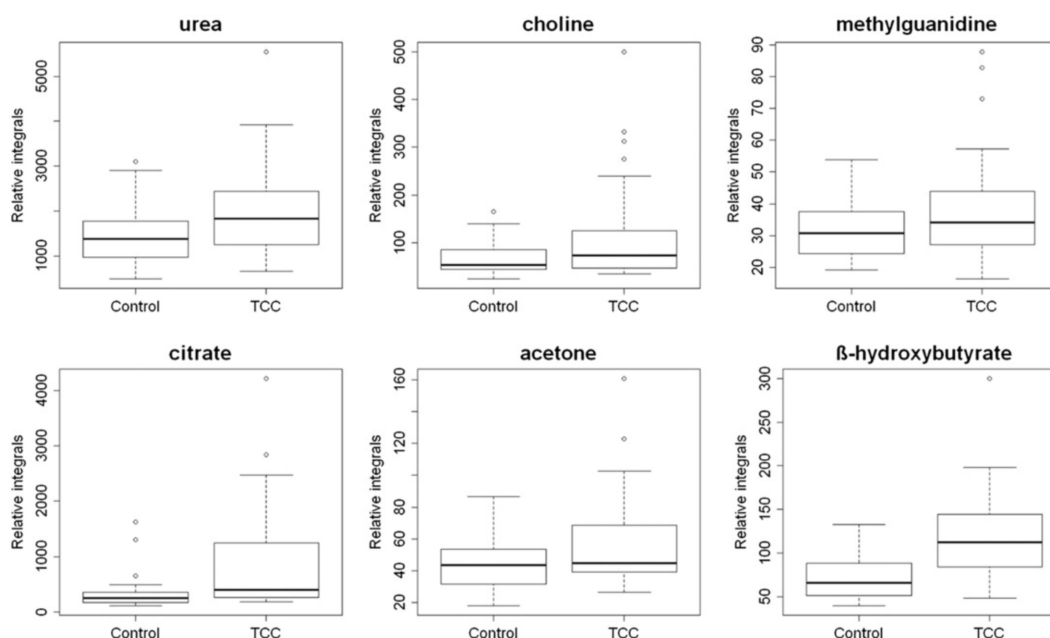

Fig. 3. Box-and-whisker plots of the candidate metabolite markers. The *y*-axis represents the relative concentration level of each metabolite normalized by the concentration of creatinine (μM/mM). Horizontal line in the middle portion of the box, median; bottom and top boundaries of boxes, lower and upper quartile; wiskers, 5th and 95th percentiles; open circles, outliers.

between female and male dogs, which indicates they are not gender-dependent (Table S2). However, as shown in Table 3B, all 6 marker candidates are more sensitive to the male dogs, and only β-hydroxybutyrate is useful in distinguishing TCC from control in female dogs.

The risk for TCC is much higher in Scottish Terriers (20× increased risk), and West Highland White Terriers and Shetland Sheepdogs (3–5× increased risk) vs other breeds implying a strong genetic risk for the cancer [34]. Based on genetic risks for BC, interest was also focused on metabolite level differences between Scottish

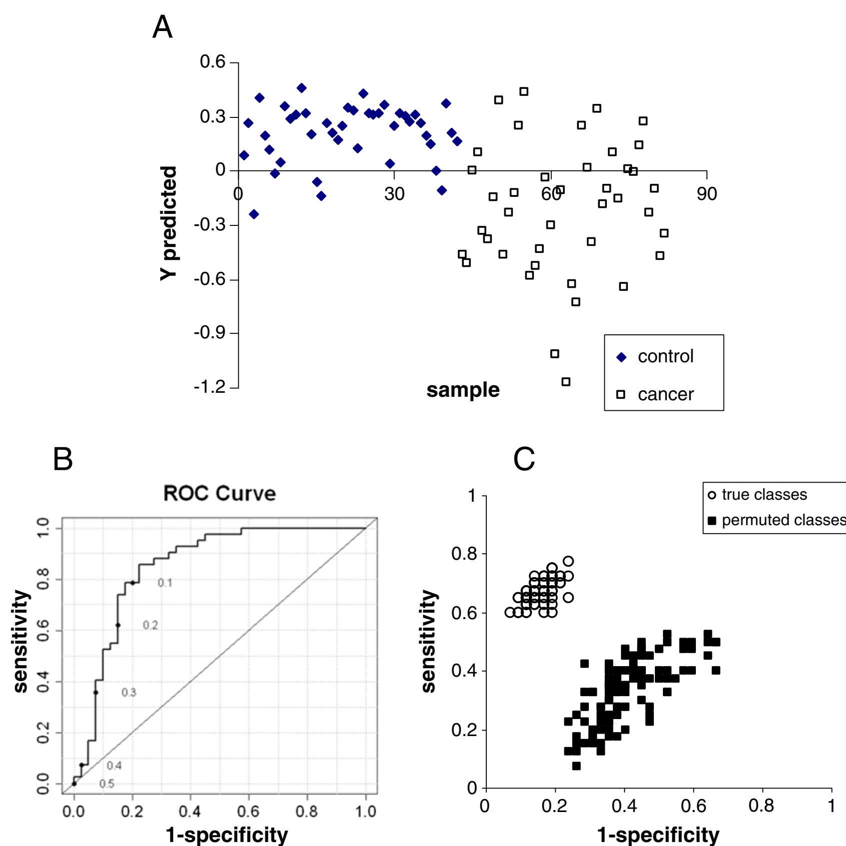

Fig. 4. (A) Prediction result of the group membership from the PLS-DA model of 6 metabolite markers, and (B) receiver operating characteristics (ROC) curve using the cross-validated predicted class value. The area under the ROC curve (AUROC) was 0.85; the sensitivity and specificity were 86% and 78%, respectively. (C) Monte Carlo Cross Validation (MCCV) prediction results of the PLS-DA model.

Table 3
Student's *t*-test results for comparisons among various groups.

Metabolite	*p*-Value (TCC vs control)					
	(A) Age		(B) Gender		(C) Breed	
	≤45 yrs[a]	>45 yrs[a]	Female	Male	At-risk breeds[b]	Other breeds
Urea	0.22	6.5*E*−04	0.12	0.013	0.18	0.0048
Choline	0.84	0.021	0.14	0.024	0.30	0.024
Methylguanidine	0.35	0.19	0.34	0.021	0.13	0.22
Citrate	0.47	0.0013	0.23	0.00069	0.26	0.0010
Acetone	0.26	0.14	0.16	0.020	0.25	0.056
β-hydroxybutyrate	0.26	2.4*E*−05	0.0054	2.5*E*−05	0.011	0.00017

[a] Standardized age.
[b] At-risk breeds including Scottish Terrier, Shetland Sheepdog and West Highland White Terrier.

Terriers, West Highland White Terriers, Shetland Sheepdogs *vs* other breeds. Herein, these three breeds were grouped as at-risk breeds, in which β-hydroxybutyrate was associated with a small *p*-value in comparing the TCC dogs and controls. For the other breeds, the *p*-values of urea, choline, citrate and β-hydroxybutyrate were all below 0.05 (Table 3C and Fig. S3). Interestingly, even within the control dogs, the levels of methylguanidine, citrate, acetone and β-hydroxybutyrate were significantly different (Table S2). The results showed that the marker levels in the at-risk breed group (either controls or TCC dogs) are larger than their counterparts in non-risk controls and comparable to their counterparts in non-risk TCC dogs. Therefore, the *p*-values of at-risk control *vs* at-risk TCC are large; however, in the other breeds, the *p*-values are small. This result may suggest why Scottish Terriers, West Highland White Terriers and Shetland Sheepdogs are at-risk breeds for TCC at the molecular level: the at-risk dogs may have different metabolism, which contributes to their susceptibility to developing TCC.

In addition, lymphatic spread and distant spread effects were also investigated among the samples from dogs with TCC. Large *p*-values were observed between T0/T2 *vs* T3; N1 or M1 *vs* others; cases that never developed metastases *vs* those that did develop metastases along the course of their disease; and distant metastases *vs* no distant metastases for each of the six markers, which indicates that none of these factors is significant in the classification (data not shown).

3.3. Metabolic pathway analysis

Altered pathways in canine TCC were identified based on the metabolites that showed significant level changes in samples from dogs with TCC versus control samples. A simplified pathway map is shown in Fig. 5 based on the KEGG online database (http://www.genome.jp/kegg/pathway.html).

Increased amounts of β-hydroxybutyrate, acetone and citrate indicate upregulation of the synthesis of ketone bodies and activity of the TCA cycle in the tumor cells. These elevated levels were also found in human serum of esophageal cancer patients and plasma of mice with prostate tumors [17,46]. The Cori cycle might fail to convert lactate back to glucose in the liver when the lactate is abundant, which results in the accumulation of Acetyl-CoA and citrate up-regulation in the TCA cycle sequentially [47]. If the Acetyl-CoA is not well accommodated by the TCA cycle, ketogenesis will take place and ketone bodies, including acetone and β-hydroxybutyrate, will be converted [46], thus, levels of acetone and β-hydroxybutyrate would increase in dogs with TCC compared with healthy controls.

As an important precursor of cell membrane constitution, choline was also found to be present at higher levels in the TCC specimens. Similar observations have been reported in a number of other NMR-based disease studies such as studies of human colorectal cancer [48,49], cerebral gliomas [50], cervical cancer [51], and bipolar disorder [52]. One possible reason for the choline upregulation could be membrane turnover [50]; while another key factor could be the increased glycerophospholipid metabolism due to the lactate accumulation discussed above.

Moreover, elevations of urea and methylguanidine levels were observed in the urine of dogs with TCC. Methylguanidine is synthesized from creatinine concomitant with the synthesis of hydrogen peroxide from endogenous substrates in peroxisomes, and is considered as a uremic toxin accumulated in uremic patients [53]. As the principal end product of protein catabolism, urea is formed in the urea cycle, where amino groups donated by ammonia and L-aspartate are converted to urea.

4. Conclusions

A metabolomics approach based on ^{1}H NMR coupled with the statistical methods of OSC-PLS-DA and univariate Student's *t*-test based feature (metabolite) selection provides a powerful methodology for metabolic profiling of urine to differentiate dogs with TCC from healthy controls. Six candidate metabolite markers, including urea, choline, methylguanidine, citrate, acetone and β-hydroxybutyrate were found to have good discriminatory ability and low *p*-values. Good sensitivity and selectivity was shown using these markers to predict the healthy control and disease samples, which indicates the promise of this approach towards the development of a metabolic profile for TCC and potentially its earlier detection. Further analysis involving a larger

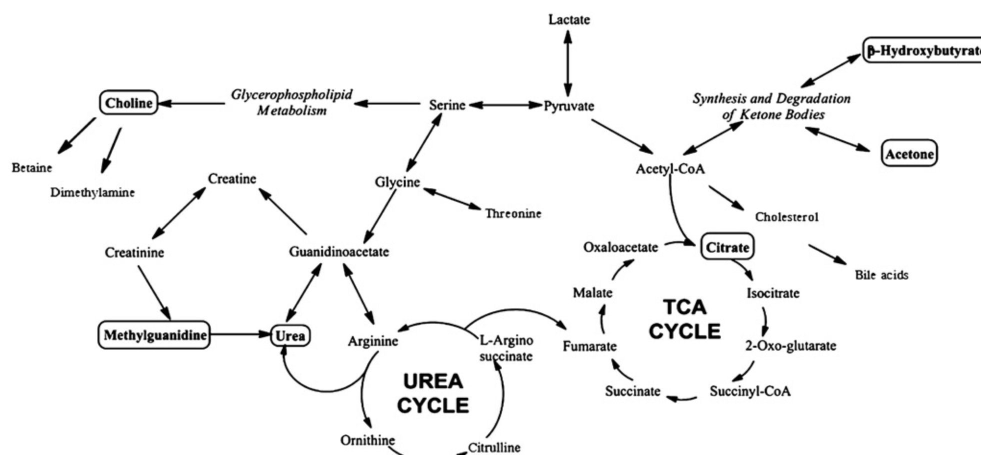

Fig. 5. Simplified altered metabolism pathways for the most relevant metabolic differences between dogs with TCC and controls. The metabolites highlighted with rounded boxes were biomarkers observed as being upregulated in dogs with TCC.

number of subjects is required to determine the accuracy and widespread applicability of this and similar approaches in guiding treatment of TCC.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2012.08.001.

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