Urinary Lipid Biomarkers for Detecting Canine Transitional Cell Carcinoma Pilot Study

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Urinary Lipid Biomarkers for Detecting Canine Transitional Cell Carcinoma Pilot Study

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Abstract:

Canine Transitional Cell Carcinoma (TCC) makes up 2% of all canine cancers and is the most common cancer of the bladder for dogs. Usually diagnosed in its later stages, TCC is difficult to treat, with low survival times. Current diagnosis involves invasive techniques, and mainstream screening tests are often ineffective. To identify urinary lipids which could be used as biomarkers, this pilot study used 15 dogs in three cohorts (TCC, Urinary Tract Infection, and Health using a lyophilization lipid extraction method. A total of 208 lipids were identified, and the cohorts were distinguishable by Principle Component Analysis. This study provides evidence to investigate urinary lipids as a biomarker profile for cancers.

Introduction:

Bladder Cancer (BC) is believed to be caused by environmental factors (Johansson, 1997), smoking tobacco (Zeegers, 2004), and a high mutation rate of the genome (Zlotta, 2010). In 2016, BC is predicted to affect 76,960 new individuals, and 16,390 of those people are likely to die (Siegel, 2015). In 2010, the cost of treating all cancers was estimated to be $125 billion (Mariotto, 2011). By 2020, this amount is estimated to increase to $155 billion (Mariotto, 2011). Specifically, the cost of treating BC for a lifetime was an average of $109,977 per person in 2005 (Avritscher, 2006). Bladder cancer has a large financial impact on the United States, both because of expensive surgical diagnostics, and its common rate of reoccurrence. It is estimated that 70% of patients diagnosed with BC will develop a reoccurrence within 10 years (Chamie, 2013).

The National Cancer Institute’s cancer information summary Bladder Cancer Treatment (PDQ) describes the symptoms of BC to include: blood in the urine, frequent urination, painful urination, and lower back pain. Current diagnostic tests for BC include imaging techniques such as Intravenous Urography, Computed Tomography (CT) Urography, and abdominal ultrasound. However, the gold standard for diagnosing bladder cancer has been urinary cystoscopy and tissue histology (Sexton, 2010; Babjuck, 2011).
Once bladder cancer has been diagnosed there are a few therapeutic options. These include but are not limited to surgery, radiation therapy, and chemotherapy. Due to the high cost and difficulty of identifying BC, there has been a need for a non-invasive screening test.

In order to reduce costs, increase the capacity of screening, and avoid invasive procedures, urinary screening tests have been developed. These tests include but are not limited to the Bladder Tumor Antigen (BTA) test (Murphy, 1997), ImmunoCyt (Greene, 2006), and the Nuclear matrix protein 22 (NMP22; Fu, 2016). The BTA test looks for the Complement Factor H Related Protein (CFHrp) in urine, and has been noted to have a low specificity due to a high false positive results, specifically in the presence of hematuria (Sözen, 1999). Hence it is not superior to cytology (Murphy, 1997). ImmunoCyt is an expensive test that uses three fluorescent monoclonal antibodies: M334, LDQ10, and 19A211 (Mian, 2006). These antibodies bind onto malignant exfoliated urothelial cells (Greene, 2006). The test has been recommended to be used only to monitor patients rather than screen for bladder cancer (Mian, 2006). In a direct comparison between NMP22 and cytology, cytology had a higher specificity (99%) than the NMP22 test (86%) (Tiki, 2011). It was also reported that NMP22 may be appropriate to screen for BC in humans, but it cannot replace cystoscopy for diagnostic purposes (Fu, 2016). Although these tests have improved the detection, screening, and monitoring of bladder cancer, they are costly and do not improve the current gold standard. Hence there is a critical need for a new screening method for the diagnosis of BC.

In 2005 there were 73 million dogs living in the United States (Rowell, 2011), and with the highest phenotypic diversity of all land mammals next to human beings (Starkey, 2005), they have the highest rate of naturally occurring diseases. Transitional cell carcinoma (TCC) in dogs is estimated to make up 2% of all canine cancers (Mutsaers, 2003) and is caused by environmental exposure, chemical exposure, outdated flea protection products, and obesity (Fulkerson, 2015). Because of similar environmental exposures and the high rate of occurrence, canine TCC has been one of the main animal models for studying human BC. Canine TCC etiology, biological behavior, molecular features, and histopathological characteristics share substantial commonalities with advance stage BC in human (Knapp, 2000).

Detection and diagnosis of canine TCC is often at the T2 Stage (based on the World Health Organization (WHO) criteria; Owen, 1980), which is considered an advanced stage of the disease (Fulkerson, 2015). Figure 1 shows the staging criteria for Canine TCC based on the WHO criteria (Owen, 1980). As in humans, the gold standard for diagnosing canine TCC is urinary cystoscopy and tissue histology. Due to late stage diagnosis, treatments such as chemotherapy and radiation are often ineffective, with response rates of less than 35% and survival times of less than 350 days (Henry, 2003; Boria, 2005; Poirier, 2004).
A specific, non-invasive screening for canine TCC is essential for early detection and monitoring of the disease throughout treatment. Research on both the proteomic profile (Bracha, 2014) and the metabolomics profile (Zhang, 2012) in canine TCC urine have produced encouraging results. The proteomic study predicted canine TCC with 90% confidence and identified 96 proteins unique to TCC. The metabolomics study identified increased amounts of β-hydroxybutyrate, acetone, and citrate, indicating increased activity of the citric acid cycle, which is used to create a high energy phosphate bonds for energy synthesis.

In humans, urinary lipidomics has proven to be a promising avenue of investigation for cancers such as prostate (Roberts, 2011), ovarian (Slupsky, 2010), and colorectal (Chan, 2008). In human bladder cancer, urinary metabolomics has shown to have 92% sensitivity and 80% specificity (Pasikanti, 2010). Specific metabolites such as glycerol, fructose, and senecioic acid had lower concentration in comparison to non-BC patients, which was attributed to the high energy demands of cancer cells.

Although little attention has focused on the lipid profiles of canine TCC, a recent study has shown there are unique differences in the tissues of bladder cancer and normal tissue in canines. Specifically, the cancerous tissues contained upregulated amounts of phospholipids (PL) in comparison to the normal tissue (Dill, 2009).

Urinary Tract Infections (UTI) are common in canines and a study done in 1984 stated that a dog has a 14% chance to have a UTI in its lifetime (Ling, 1984). That number has been recently modified in 2001 to a 4% chance (Ling, 2001) with neutered females
being more susceptible than males (Cohn, 2003). UTI’s can create false positive results in many of the current urinary screening exams, specifically the BTA test (Borjesson, 1999) due to the fact that they present similar to TCC, thus the inclusion of them in the study was imperative in order to identify reliable urinary biomarkers for TCC.

Based on previous research both in humans and in canines, the objective of this study was to analyze the urine lipid profile of canines with TCC in comparison to that of normal dogs and dogs infected with urinary tract infection (UTI).

Materials and Methods

Animal Recruitment:

The study included dogs that were presented to the Veterinary Teaching Hospital at Oregon State University (OSU). Recruitment was done with written consent from the dogs’ owners and in accordance with IACUC guidelines of OSU.

Diagnosing, Collecting, and Categorizing Urine:

Urinary tracts of UTI and TCC dogs were prescreened with ultrasound imaging or CT imaging. Urine was collected in an aseptic manner (trans-abdominal cystocentesis for UTI and healthy dogs, urinary catheter for TCC dogs) and evaluated by urine analysis, cell cytology, bacterial culture and sensitivity test. Diagnosis of TCC was confirmed via cytology or histology. The 15 dogs were assigned to one of the three cohorts: Normal (4), UTI (5), and TCC (9) on their diagnosis.

Extraction of Lipids

1 mL of each urine sample was lyophilized for 12 hours and the resulting powder was dissolved with 0.90 mL of 2:1 (v/v) CHCl₃:CH₃OH by vortexing, and then left at room temperature for 1 hour. Next, 0.18 mL of H₂O was added, followed by centrifugation at 15,700×g for 5 minutes at room temperature. The lower phase, containing lipids, was recovered and dried using a SpeedVac. The extracted lipid powder was dissolved in CH₃OH/CH₃CN (1:1) in a final volume of 500 μL and stored at −20°C (Min, 2011).
**LC-MS Analysis**

A Shimadzu Nexera liquid chromatographic (Shimadzu Corporation, Kyoto, Japan) was applied to perform chromatographic separation on a 2.1 mmX100 mm 1.8 µm C18 ACQUITY HSS T3 column (Waters Corporation, Milford, MA, USA). The flow rate was 0.4 mL/min and the column temperature was held at 55°C. Sample injection volume was 5 mL and lipids were eluted and separated with a 15 minutes gradient (phase A - water: acetonitrile 40:60+0.1% Formic acid+5mM Ammonium formate; phase B - acetonitrile:isopropanol 10:90+ 0.1% Formic acid+5mM Ammonium formate), which started at 40% B and was increased to 100% B at 10.5 mins. B was then held at 100% for 3 minutes and then linearly decreased to 40%B at 14.5 mins. B was then continued to be held at 40% until 15 minutes was reached.

MS and sequential precursor ion fragmentation acquisitions were performed on a TripleTOF™ 5600 (AB SCIEX, Concord, ON) operated in positive and negative ESI mode. The scan range for both modes was from 70 to 1700 m/z. DuoSpray Ion Source parameters included nebulizing gases GS1 at 45, GS2 at 50, curtain gas at 35, positive mode ion spray voltage at 5500, negative mode ion spray voltage at −4500, and at an ESI source operating temperature of 550 °C. Collision energy for each MS/MS step was 45 eV for positive and negative ion mode experiments.

**Addition of Lipid Standard**

In order to interpret the concentration of urinary lipids, 1.5 µg of Internal lipid standards 17:1 Lyso PG (1-(10Z-heptadecenoyl)-sn-glycero-3-phospho-(1’-rac-glycerol)), 17:1 Lyso PI (1-(10Z-heptadecenoyl)-sn-glycero-3-phospho-(1’-myo-inositol)), 17:0 Lyso PC (1-heptadecanoyl-sn-glycero-3-phosphocholine), 17:1 Lyso PS (1-(10Z-heptadecenoyl)-sn-glycero-3-phospho-L-serine), 17:1 Lyso PE (1-(10Z-heptadecenoyl)-sn-glycero-3-phosphoethanolamine), and 17:0 TG (1,2,3-triheptadecanoyl-glycerol) were added to all samples. The lipid standards were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and were dissolved based on the manufacturer’s instructions.

**Data Analysis**

Features of each LC-MS raw data file were extracted by the MarkerViewTM software (Sciex) for retention time alignment, peak detection, and ion intensity integration. The initial lipids list was produced against an in-house lipids database within mass error 10 ppm. To prevent false positive identifications within the dataset, the contribution of isotope peaks was removed. The tandem mass spectrum of each lipid candidate compound was manually checked by combining PeakView (Sciex), MarkerView (Sciex) with LipidView (Sciex) software. The identified lipid list was then converted to a comma separate value (CSV) file format, which was imported into the open source software MetaboAnalyst3.0 for
statistical analysis (Xia, 2015). Distinct features between the three groups were evaluated by univariate statistical analysis and the threshold for the significance was set to fold change <2 with P-value <0.01.

**Results:**

*Lipid Identification*

One hundred and twenty-nine different lipids were identified by Positive Ion Mode LC/MS-MS and 78 lipids were identified by Negative Ion Mode LC/MS-MS. The positive ion mode identified 14 different lipid families (Table 1) in comparison to the negative mode, which identified 8 (Table 1). The lipid families with the most lipids present in the positive ion mode were the triglyceride (TG) and phosphocholine (PC) families, with 41 and 17 lipids respectively (Table 1). In the negative ion mode, the lipid families with the most lipids present were PC, phosphorylethanolamine (PE), and phosphatidylserine (PS) with 19, 19, and 15 lipids present respectively (Table 1).

<table>
<thead>
<tr>
<th>Positive Ion Mode Family’s</th>
<th>Number of Lipids Per Family</th>
<th>Negative Ion Mode Families</th>
<th>Number of Lipids Per Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>41</td>
<td>PC</td>
<td>19</td>
</tr>
<tr>
<td>PC</td>
<td>17</td>
<td>PE</td>
<td>19</td>
</tr>
<tr>
<td>DG</td>
<td>11</td>
<td>PS</td>
<td>15</td>
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<tr>
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<td>8</td>
<td>PI</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total Lipids</strong></td>
<td><strong>129</strong></td>
<td><strong>Total Lipids</strong></td>
<td><strong>79</strong></td>
</tr>
</tbody>
</table>

Table 1 Lipid family distributions of the 208 lipids that were identified through both Positive and Negative Ion Mode LC/MS-MS
**Principle Component Analysis**

Based on the lipid profiles, the Positive and Negative ion mode for each sample was plotted on analyzed using Principle Component Analysis (Figure 2). The TCC, UTI, and Normal samples all clustered into different groups. The Quality Cotnrol (QC) Samples was a mixture of all of the samples together, thus producing a center origin.

![PCA positive data (Supervised, with QC)](image1)

![PCA negative data (Supervised, with QC)](image2)

**Figure 2** The distribution of the cohorts based on their lipid signatures on a principle component analysis graph for both the positive and negative ion mode. The varying distribution of the samples is based on the time of flight and the percent absorption of the lipids.

**Addition of Lipid Standards**

We added lipid standards to the samples in order to determine the concentration of the lipids identified in the urine and provide evidence to if lipids were up or down regulated. However, analysis by positive and negative mode LC/MS-MS demonstrated that the samples were contaminated (Figure 3). The contamination presented as abnormally high peaks between the 7 and 8 seconds (Figure 3). These high peaks masked the results of
the lipids present during those time frames. Due to the contamination, the concentration of the lipids in the samples could not be determined from this experiment.

Figure 3 Contamination present in the standards mix and the quality control samples based on LC-MS/MS analysis. The Y axis represents the relative absorption while the X axis represents time. The standards mix is the combination of each of the 1.5 µg standards with milique water. The QC is the quality control, which is an equal mixture of all the samples together in order to have a sample represent all of the found lipids. The contamination is shown by the abnormally high peaks between the 7 and 8 second mark on the x axis. These high peaks mask the presence of the lipids present in the samples.
Discussion:

Lipidomics and Metabolomics are emerging as tools for cancer detection. Recent research has identified lipid biomarkers for colorectal cancer, gastric cancers, pancreatic cancer, and liver cancer (Patel, 2015). The sub families of the PL family are of particular interest because of their presence in many cancers.

The importance of the TG lipid family has gained increased recognition, specifically due to their increased presence in cardiovascular disease due (Nordestgaard, 2011). High levels of TG in serum have also been associated with prostate (Arthur, 2015) lung, thyroid, and rectal cancer (Ulmer, 2009). The PC lipid family makes up almost 50% of the phospholipid mass in cellular membranes in eukaryotic organisms (Zinser, 1991) and has been hypothesized to assist in cellular membrane production in rapidly proliferating cancerous cells (Wright, 2004). Increased levels of the PC family have been identified as a target for the detection of breast cancer (Eliyahu, 2007). The PE lipid family is important in the late stages of telophase as a recognition site for cytokinesis (Emoto, 1996) and has increased concentrations in the tissues of brain tumors (Kinoshita, 1994). The PS lipid family in particular shows clear down regulation in patients with prostate cancer (Min, 2011), which can be attributed to their role in signaling apoptosis (Fadoc, 1998).

Although the history of phospholipids biomarkers in human cancers is supportive of the idea that there will be unique phospholipids present in TCC, it does not provide concrete evidence that phospholipids can be used for as a marker for TCC. The comparison of TCC tissue to healthy bladder tissue reported that the principle lipids unique to TCC were from the PS, PG, Phosphatidylinositol (PI), and PC families (Dill, 2009). With phospholipids unique history in both human and canine cancers, it can be hypothesized there should be a presence of phospholipids in the urine of canines with TCC.

The TCC, UTI, and Normal samples were examined for their lipid profiles and their distinctive groups were determined through Principle Component Analysis. Of these lipids, there were 4 families with elevated lipid presence in the Positive and Negative Ion Mode of LC/MS-MS. Those were TG, PC, PE, and PS. These lipids are up and down regulated in other cancers due to their crucial roles in the cellular life cycle. However, because we were unable to determine the concentration of the specific lipids in the samples, we could not conclude which lipid families are up regulated and down regulated in the urine of TCC patients in comparison to that of UTI and healthy patients.

The UTI samples presented a unique lipid profile in comparison to that of the normal samples. This was expected, however the exact differences were not predicted ahead of time. The UTI samples also presented differently than that of the TCC samples, thus providing evidence that there are unique differences between the two that could be
identified and exploited. However, because we were unable to determine the concentration of the specific lipids, we could not conclude the specific differences between the UTI and TCC samples.

Due to the similar presence of contamination in the quality control sample and the lipid standards mix, we suspect that the source of the contamination was in the preparation of the lipid standards. This could either be due to poor technique during the mixing process, or because previously contaminated chemicals were used to dissolve the lipid standards.

**Conclusion**

The presence of a variety of lipid families in the urine of canines with Transitional Cell Carcinoma provided the backbone to this research project. The fact that a principle component analysis clearly separated the lipids from the TCC, UTI, and Healthy samples supported the hypothesis that TCC would have a unique urinary lipid profile. Although the concentrations of these lipids were unable to be measured due to contamination, this study has provided the foundation for further research in this field. If future results provide information on unique differences in lipid profiles, a project in conjunction with previous research on TCC urinary proteins from the Bracha laboratory may lead to a lipid biomarker test that screens for a range of components.

**References:**


