Urinary Lipid Biomarkers for Detecting Canine Transitional Cell Carcinoma Pilot Study

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Goal of Today’s Presentation

- Human Bladder Cancer (BC)

- Canine Transitional Cell Carcinoma (TCC) as a Model for BC

- What did we find and How did we find it

- What is on the Research Horizon
Cancer Statistics and Costs

- **Cost of Treating Cancer in 2010**
  - $125 Billion

- **Cost of Treating Cancer in 2020**
  - $155 Billion

### Estimated New Cases

<table>
<thead>
<tr>
<th>Type</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>160,890</td>
<td>21%</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>117,920</td>
<td>14%</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>70,820</td>
<td>8%</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>58,950</td>
<td>7%</td>
</tr>
<tr>
<td>Melanoma of the skin</td>
<td>46,870</td>
<td>6%</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>40,170</td>
<td>5%</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>39,650</td>
<td>5%</td>
</tr>
<tr>
<td>Oral cavity &amp; pharynx</td>
<td>34,780</td>
<td>4%</td>
</tr>
<tr>
<td>Leukemia</td>
<td>34,090</td>
<td>4%</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>28,410</td>
<td>3%</td>
</tr>
<tr>
<td>All Sites</td>
<td>841,390</td>
<td>100%</td>
</tr>
</tbody>
</table>

### Estimated Deaths

<table>
<thead>
<tr>
<th>Type</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung &amp; bronchus</td>
<td>85,920</td>
<td>27%</td>
</tr>
<tr>
<td>Prostate</td>
<td>26,120</td>
<td>8%</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>26,020</td>
<td>8%</td>
</tr>
<tr>
<td>Pancreas</td>
<td>21,450</td>
<td>7%</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>16,280</td>
<td>6%</td>
</tr>
<tr>
<td>Leukemia</td>
<td>14,130</td>
<td>4%</td>
</tr>
<tr>
<td>Esophagus</td>
<td>12,720</td>
<td>4%</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>11,820</td>
<td>4%</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>11,520</td>
<td>4%</td>
</tr>
<tr>
<td>Brain &amp; other nervous system</td>
<td>9,440</td>
<td>3%</td>
</tr>
<tr>
<td>All Sites</td>
<td>314,290</td>
<td>100%</td>
</tr>
</tbody>
</table>

Siegel, 2016

Mariotto, 2011
Impact of Bladder Cancer

Rate of Recurrence:
- 60% after 5 Years
- 90% after 15 Years

Life Time Cost:
- $109,977

76,960 New Bladder Cancer
18,010
58,950
16,390

Siegel, 2016
Van Rhin, 2009
# What is Bladder Cancer

<table>
<thead>
<tr>
<th>T</th>
<th>Primary Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tis</td>
<td>Carcinoma in Situ</td>
</tr>
<tr>
<td>T0</td>
<td>No Evidence of Primary Tumor</td>
</tr>
<tr>
<td>T1</td>
<td>Superficial Primary Tumor</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor invading bladder wall</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor invading neighboring organs</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>Regional Lymph Node</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>No Regional Lymph Node Involved</td>
</tr>
<tr>
<td>N1</td>
<td>Regional Lymph Node Involved</td>
</tr>
<tr>
<td>N2</td>
<td>Regional and Juxtaregional Lymph Node Involved</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M</th>
<th>Distant Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No evidence of Metastases</td>
</tr>
<tr>
<td>M1</td>
<td>Distant Metastases Present</td>
</tr>
</tbody>
</table>

Owen, 1980
Diagnosing

Intravenous Urography

- Cross section of right kidney
- The dye in the urinary tract is shown up on x-ray pictures
- Urinary Tract
- Left kidney
- Ureter
- Bladder
- Prostate (men only)

Computed Tomography (CT) Urography

- CT Scan

Cystoscopy

A cystoscope is a flexible tube used to view the structure of the bladder. This procedure is often associated with biopsies. To minimize pain, the procedure is done under local anesthesia.

Invasive and Expensive

http://patient.info/health/intravenous-urography-pyelography


https://www.healthtap.com/doctor_advice/cystoscopy/10004
464-dr-simon-kimm

“Gold Standard”
Current Urinary Screening Tests

Bladder Tumor Antigen (BTA)

ImmunoCyt

Nuclear Matrix Protein 22 (NMP22)

Antibodies:
- M334
- LDQ10
- 19A211


Greene, 2006

http://numonthly.com/898.fulltext

Fu, 2016

Sözen, 1999

Not Reliable
False Positive
Not up to Standards
# Perspective for Developing a BC Screening Test

**Bladder Cancer Issue**
- High Lifetime Cost
- High Rate of Recurrence

**Current Detection and Diagnosing**
- “Gold Standard”
  - Invasive
  - $350 or More
- Unspecific Urinary Screening

**We Can Do Better**
“Preventative care and early detection is key for present and future cancer care”
- Beant Gill MD
Canine Transitional Cell Carcinoma TCC

A Model For Human BC

<table>
<thead>
<tr>
<th>Similar Clinical Signs</th>
<th>Similar Molecular Features</th>
<th>Current Model for BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Hematuria</td>
<td>• Concentrated bFGF</td>
<td>• Androgen Receptors</td>
</tr>
<tr>
<td>• Urinary Tract Infection</td>
<td>• Cox-2 Overexpression</td>
<td>• Estrogen Receptors</td>
</tr>
<tr>
<td></td>
<td>• Survivin Expression</td>
<td></td>
</tr>
</tbody>
</table>

Knapp, 2000

Knapp, 2014
Canine Transitional Cell Carcinoma

2% of Canine Cancers

Caused By:
- Environmental
- Chemical
  - Pesticides
  - Insecticides
- Obesity

Cost:
Treatment usually costs more than $4,000
Euthanasia:
$400

Mutsaers, 2003
Fulkerson, 2015

https://www.pinterest.com/bluestaffy/canines-william-wegman/

http://pets.costhelper.com/
Diagnosing, Staging, and Treating

### Diagnosing

- **Cystoscopy**
  - A cystoscope is a flexible tube used to view the structure of the bladder. This procedure is often associated with biopsies. To minimize pain, the procedure is done under local anesthesia.

### Staging

<table>
<thead>
<tr>
<th>T</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>No Evidence of Primary Tumor</td>
</tr>
<tr>
<td>T1s</td>
<td>Carcinoma in Situ</td>
</tr>
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</tr>
<tr>
<td>T3</td>
<td>Tumor invading neighboring organs</td>
</tr>
</tbody>
</table>

- Fukerson, 2015

### Treating

- <35% Response
- <350 Days Survival Time

- Henry, 2003
- Boria, 2005
- Poirier, 2004

Latest Research in TCC

**Urinary Proteins**
- CON
- TCC
- UTI
- 8
- 38
- 39
- 5
- 131
- 62
- 96

**Urine Metabolites**
- Citrate
- β-hydroxybutyrate
- Acetone

**Tissue Comparison:**
- Phosphatidylserine (PS)
- Phosphatidylglycerol (PG)
- Phosphatidylinositol (PI)
- Phosphocholine (PC)

Bracha, 2014
Zhang, 2012
Dill, 2009
Urinary Proteins

- 379 Proteins Identified
  - 96 Unique to TCC

- 3 Special Proteins
  - Macrophage Capping Protein
    - Tumor Invasion
  - PRX5
    - High levels present in Mammary Cancer
  - Heterogeneous Nuclear Ribonucleoprotein A2/B1
    - Linked to Lung and Colon Cancers
    - mRNA Posttranscriptional Regulation
Urine Metabolites


https://en.wikipedia.org/wiki/Ketogenesis
Tissue Comparison

Desorption Electron Spray Ionization

Dill, 2009
**Lipids Review**

<table>
<thead>
<tr>
<th>Synthesis</th>
<th>What are they used for</th>
<th>Common Types</th>
</tr>
</thead>
</table>
| - Produced by the Smooth Endoplasmic Reticulum | - Plasma Membrane  
- Cell Signaling | - Triglycerides 
- Phospholipids |
Triglycerides

- Glycerol + Three Fatty Acids
- Saturated Versus Unsaturated
- Association with Heart Disease

http://friedbiochem.weebly.com/metabolism.html
Phospholipids

- Major Component of Cell Membrane
- Amphiphilic
- Some can be cleaved into signaling molecules

http://www.abpischools.org.uk/page/modules/chemistryoflife/chemistryoflife8.cfm?coSiteNavigation_allTopic=1
Types of Phospholipids

- Phosphocholine (PC)
- Phosphatidylserine (PS)
- Phosphatidylglycerol (PG)
- Phosphatidylinositol (PI)
- Phosphorylethanolamine (PE)

http://medcell.med.yale.edu/lectures/introduction_cell_membrane.php
Review of Canine TCC

- There are components in Human Urine

- Research has shown Canine TCC tissue has unique lipids
  - PG, PI, PS, PC

- Research has shown Canine TCC urine has unique proteins

- Research has shown Canine TCC urine has a unique lipid profile
Objective of this study

- Analyze the urine lipid profile of canines with TCC in comparison to those with Urinary Tract Infections (UTI) and Healthy Bladders

http://www.preventivevet.com/dogs-going-to-the-vet-how-to-collect-your-dogs-urine-sample
Materials and Methods

- Animal Recruitment
- Diagnosing and Collecting
- Extraction of Lipids
- LC-MS/MS Analysis
- Data Analysis
Animal Recruitment

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

- Urinary tracts of UTI and TCC dogs were prescreened with ultrasound imaging or CT imaging.

- Urine was collected in an aseptic manner and evaluated by urine analysis, cell cytology, and bacterial culture.
  - Trans-Abdominal Cystocentesis → UTI and Healthy
  - Urinary Catheter → TCC

- Diagnosis of TCC was confirmed via cytology and tissue histology.
Extraction of Lipids

TCC: 9
UTI: 2
Healthy: 4
Total: 15

1 mL

0.90 mL of 2:1 (v/v) CHCl₃:CH₃OH

0.18 mL of H₂O

Centrifugation at 15,700×g for 5 minutes RT

CH₃OH:CH₃CN (1:1)

FV of 500 μL

Stored at −20 °C
Extraction of Lipids

Shotgun lipidomics for candidate biomarkers of urinary phospholipids in prostate cancer
LC-MS/MS Analysis

- A Shimadzu Nexera liquid chromatographic (Shimadzu Corporation, Kyoto, Japan) was applied to perform chromatographic separation on a 2.1 mm × 100 mm μ 1.8 m 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 is 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 increased to 100% B at 10.5 minutes and hold B 100% for 3 mins; then linearly decreased to 40% B at 14.5 mins and hold B 40% until 15 mins.

- MS and sequential precursor ion fragmentation acquisitions was performed on a TripleTOF™ 5600 (AB SCIEX, Concord, ON) and 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.
LC-MS/MS Simplified

1. Separation by Liquid Chromatography
   a) 2.1 mm X 100 mm μ 1.8 m C18 ACQUITY HSS T3 column
   b) Flow Rate of 0.4 mL/minute

https://en.wikipedia.org/wiki/Tandem_mass_spectrometry
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. Extracted features relating to identified the lipid list were 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.
Data Analysis Simplified

1. Raw Data was Extracted via MarkerView
2. Raw Data was compared to House Lipid List
3. Manually double checked against:
   1. PeakView
   2. MarkerView
   3. LipidView
4. Uploaded to MetaboAnalyst 3.0 for Statistical Analysis
## Results: Extraction of Lipids

<table>
<thead>
<tr>
<th>Scan Type</th>
<th>Number of Lipids Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Ion</td>
<td>129</td>
</tr>
<tr>
<td>Negative Ion</td>
<td>79</td>
</tr>
<tr>
<td>Total</td>
<td>208</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipid Family</th>
<th>Number of Lipids Per Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>41</td>
</tr>
<tr>
<td>PC</td>
<td>17</td>
</tr>
<tr>
<td>DG</td>
<td>11</td>
</tr>
<tr>
<td>SM</td>
<td>8</td>
</tr>
<tr>
<td>PI</td>
<td>8</td>
</tr>
<tr>
<td>PE</td>
<td>8</td>
</tr>
<tr>
<td>PS</td>
<td>7</td>
</tr>
<tr>
<td>FFNE</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
</tr>
<tr>
<td>LPC</td>
<td>5</td>
</tr>
<tr>
<td>MAG</td>
<td>5</td>
</tr>
<tr>
<td>FFA</td>
<td>1</td>
</tr>
<tr>
<td>FA</td>
<td>1</td>
</tr>
<tr>
<td>LPG</td>
<td>1</td>
</tr>
</tbody>
</table>

| Total Lipids | 129                         | Total Lipids | 79  |

*Notes: Positive Ion Mode Families highlighted in green, Negative Ion Mode Families highlighted in red.*
# Lipid Families in other Cancers

<table>
<thead>
<tr>
<th>TG</th>
<th>PC</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>- High Serum Levels</td>
<td>- Makes up 50% of Cellular Membrane</td>
<td>- Important in Cytokinesis</td>
</tr>
<tr>
<td>Associated with:</td>
<td>- Elevated levels in Breast Cancer</td>
<td>- Elevated levels in Brain Tumors</td>
</tr>
<tr>
<td>- Prostate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Thyroid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Unique Lipids to TCC?

http://cliparts.co/smiley-face-sad-face
Principal Component Analysis (PCA)
PCA negative data (Supervised, with QC)

Scores for D1 (47.0%) versus D2 (45.5%), Log | Paretto (DA)

Norm:

TCC:

UTI:

QC:
What is PCA?

- A comparison of the largest variation (Extend in Average) in a study compared to the next largest variation while keeping that one the same.

- Teapot Example: https://www.youtube.com/watch?v=BfTMmoDFXYyE
1.5 µg of internal lipid standards were added to all samples.

“Many long chain, saturated acidic lipids are difficult to solubilize in chloroform. We suggest adding a small amount of methanol (2%) and deionized or distilled water (0.5-1%).”
## Additional Samples

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCC</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>UTI</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Normal</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>16</td>
</tr>
</tbody>
</table>
Extraction of Lipids: Trial 2

1 mL of Sample

*9ug of Lipid Standard *

0.90 mL of 2:1 (v/v) CHCl₃:CH₃OH

0.18 mL of H₂O

Centrifugation at 15,700×g for 5 minutes RT

CH₃OH:CH₃CN (1:1)

FV of 500 μL

Stored at −20 °C
PCA Negative
PCA Positive
Contamination Strikes

- Standards mix
- Contamination

QC
What Have We Covered So Far

✓ A General understanding of where Bladder Cancer is Today

✓ A General understanding of Canine Transitional Cell Carcinoma

✓ What did we find and how did we find it

□ What is on the research horizon
Avoiding Contamination

- Purchase New Lipid Standards
- Remake using predetermined clean chemicals
- Use proper aseptic technique
Putting it all Together

96 Unique Proteins

Unique Lipid Profile

http://www.huffingtonpost.co.uk/2013/11/29/urine-test-help-cancer-diagnosis_n_4358965.html

Other Cancers?
Adding New Cancers


Osteosarcoma
## What I Didn’t Tell You Before

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCC</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>UTI</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Norm</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>
What Did We Observe

Negative mode

Principal Components Analysis

202.473.2821 mz

202.304.1404 mz

202.304.1404 mz

1.31.304.1404 mz
What Did We Observe

Positive mode
Does it Make Sense?

Canine Skeletal System

http://imes.32-b.it/skeletal-system-of-a-dog/


Prognostic and predictive biomarkers of canine osteosarcoma

“Many proteins that could reflect disease are not released or do not leak from diseased tissue into the circulation, and biomarkers are typically those substances that can be detected in samples such as serum, plasma or urine.”
What I have learned

1. It won’t always work the first time

2. Just because it worked the first time doesn’t mean it will work the second

3. Sometimes you have to be a child
   1. Question the Why

4. Teamwork
I would like to thank Dr. Shay Bracha and all of his associates who dedicated their time and funding in order to help me throughout this process.

The OSU mass spectrometry facility and core lab is supported in part by a grant from the National Institute of Environmental Health Sciences.

I would like to thank Kate Field and Wanda Crannell for all of their help in teaching me how to write and present science.


Questions?
What Is Lyophilization

https://malouffschemblog.wordpress.com/2013/10/18/phase-changes-and-pressure-a-miscellaneous-compilation/