Proteomic discovery of biomarkers for diagnosis of acute and chronic outcomes in renal allografts

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Outline
• Causes of graft loss
• Technical work flow of proteomic separation and identification.
• Discuss sample selection and preparation in proteomics workflow.
• Present recent tissue proteomics study from our laboratory.
Biomarkers for causes of graft loss

- Other causes, 46.30%
- Death with function, 41.80%
- Infection, 16.60%
- Renal failure, 11.80%
- 33/39 venous or arterial thrombosis

Incidence of loss as described by El-Zoghby et al. Am J Transplant. 2009

Biomarkers for causes of loss of functioning graft

- Acute rejection, 18/12%
- Infection, 25/10%
- Tumor, 47/31%
- Immune mediated, 7/3%


Proteomic Work Flow: LC/MS

- Proteins → Peptides → HPLC → MS → MS/MS → Database Searching/Protein Identification
How are proteins identified by mass spectrometry and what are the implications?
Identification of a Peptide

Work flow for proteomic comparison

Shotgun Proteomics Identification of Acute Rejection Proteins

Choice of appropriate samples

• “If phenotype specific differences could be identified in urine samples with different etiologies of native and transplant associated renal injury”
• 10 acute rejection
• 10 stable transplant function
• 10 non-specific proteinuria (minimal change)
• 10 healthy children


Shotgun Proteomics Identification of Acute Rejection Proteins

Sample Selection

✅ Samples appropriate to problem
✅ Availability of samples
✅ Appropriate matching of samples
✅ Pooling of samples?


Appropriate matching of samples

• Matched for age, immunosuppression, race and % living donor
• Collection methods matched (second morning, midstream collection)
• Protein/Creatinine ratio AR-0.22, STA-0.14, HC-0.05, NS-0.67
• Difference in eGFR 87 (AR) vs 124 (STA)
• Timing after transplant (protocol or for cause biopsy)
Shotgun Proteomics Identification of Acute Rejection Proteins

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Pooling

- Four pooled samples made for each category using 200 µg from each sample.
  - Not clear if the pools are the same and contain some of each sample or if some pools contain 2 samples and some contain 3.


Pros and Cons of Pooling Samples

**Pros**
- Decrease assay time
- Decrease cost
- Dampen biological variation
- Permits analysis when small amounts of sample available
- May permit identification of low abundance proteins

**Cons**
- Biological variation is an important component of biomarker discovery.
- False positive discoveries can be driven by samples with extreme concentrations
- Pooling may dilute some meaningful proteins below the level of detection
Shotgun Proteomics Identification of Acute Rejection Proteins

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Results

- 1446 identified proteins
- 9 proteins identified only in acute rejection.
- 68 proteins were absent in acute rejection but present in the other three samples.
- 284 proteins ≥2 fold up or down.
- Three proteins confirmed by ELISA.

**Tissue Proteomics to Identify Rejection Associated Proteins**

- Purpose of this study was to look for proteins which are differentially abundant in rejection using tandem mass spectrometry.
- Comparison of renal biopsy specimens from patients with and without acute rejection.

**Methods**

- 5 biopsies with no rejection and 5 biopsies with 1B rejection (Banff 07) were selected retrospectively.
- Tissues preserved in OCT media were prepared for protein extraction.
- Proteins were digested with trypsin and peptides analyzed by tandem mass spectrometry (LC/MS/MS).
- Relative differences in protein abundance were estimated by comparison of normalized spectral counts across samples.
- Statistical significance was determined using a Wilcoxon rank sum test with a p value of < 0.05 considered significant.
### Patient Demographics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1</th>
<th>Group 2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rejection status</td>
<td>Yes (N=5)</td>
<td>No rejection</td>
<td></td>
</tr>
<tr>
<td>Month post transplant</td>
<td>27 ± 24</td>
<td>27 ± 26</td>
<td>1</td>
</tr>
<tr>
<td>(mean ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor age (mean ± SD)</td>
<td>27 ± 7</td>
<td>21 ± 7</td>
<td>0.24</td>
</tr>
<tr>
<td>Recipient age (mean ± SD)</td>
<td>36 ± 7</td>
<td>39 ± 10</td>
<td>0.55</td>
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<tr>
<td>Transplant type</td>
<td>Cadaveric</td>
<td>Cadaveric</td>
<td>1</td>
</tr>
<tr>
<td>Thymoglobulin + IL-2</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td>1</td>
</tr>
<tr>
<td>blockers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (male : female)</td>
<td>4 : 1</td>
<td>4 : 1</td>
<td>1</td>
</tr>
<tr>
<td>Serum creatinine mg/dl (mean ± SD)</td>
<td>3.4 ± 1.0</td>
<td>2.0 ± 0.9</td>
<td>0.05</td>
</tr>
</tbody>
</table>

### Volcano plot of all proteins identified

- 720 total proteins
- False discovery rate 0.3%

### Proteins of Interest

- Out of 720 identified proteins, 21 proteins were lower in relative abundance and 2 proteins were elevated in relative abundance in the rejection group (p value < 0.05)
- **Transglutaminase C**: Calcium-dependent enzyme that cross-links cytosolic and extracellular matrix proteins. Increases extracellular matrix accumulation and collagen deposition.
  - Elevated in the rejection group. This protein has been previously described in chronic allograft nephropathy
- **SERPIN A5**: Is a Glycoprotein and an inhibitor of Activated Protein C, Thrombin, Kallikrein, Urokinase and others. Mediates tissue regeneration.
  - Least abundant protein in the rejection group with p-value (<0.01) and mean fold change of negative 8.92

*1. Johnson T, et al Transplantation 2004*
SERPIN A5 Immunohistochemistry

- Localized in Kidney Tubules.
- Present in Both Rejection and No Rejection.
- No Obvious Differences in Staining Pattern.

Peptide Map of SERPIN A5

- N-terminus
- C-terminus

C-terminal peptides were detected only in non-rejecters

Tissue Proteomic Analysis

- Proteomic analysis of frozen tissue biopsy revealed proteins which could be studied as potential biomarkers of rejection.

- SERPINAS and Tissue transglutaminase C were found to be the most differentially abundant proteins that were consistently found in at least one group.

- Further studies to validate protein data and assess these proteins as biomarkers in tissue and urine are ongoing.
The Cast

The Impressive Clergyman - Joey Alge
Miracle Max - Mike Janech
Vizzini - Ben Neely
Fezzik - Alison Bland
Inigo Montoya – Nithin Karakala
The Grandson – Nihant Bhensdadia
The Grandfather – Milos Budisavljevic
Prince Humperdink – Juan Carlos Velez
Count Rugen – Jessalyne Ierardi
Valerie – Elizabeth Shewfelt
The Albino – Christian Spainhour
ROUS – Peifen Deng
Westley – John Arthur
The Princess Bride – Triple ToF MS
Manish Talwar
Sally Seiff