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

John Arthur, MD, PhD
Medical University of South Carolina
Charleston, SC
Disclosures

• Advisory Board- AbbVie
• Research grant- Bristol Meyers Squibb
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

Incidence of loss as described by El-Zoghby et al. Am J. Transplant. 2009
Biomarkers for causes of loss of functioning graft

Proteomic Work Flow: LC/MS

Proteins → Peptides → HPLC → MS → MS/MS → Database Searching/Protein Identification

NEIPEEALYK
Example: Identification of Candidate Markers in AKI

<table>
<thead>
<tr>
<th>Protein</th>
<th>Study Species</th>
<th>RRT Human</th>
<th>Early Human</th>
<th>MICU Human</th>
<th>Glycerol Rat</th>
<th>Ischemia/Reperfusion Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensinogen</td>
<td>9.7</td>
<td>0.002 ↑</td>
<td>Div/0</td>
<td>5.2</td>
<td>0.06 ↑</td>
<td>10.5</td>
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<tr>
<td>Apolipoprotein A-IV</td>
<td>9.1</td>
<td>0.007 ↑</td>
<td>Div/0</td>
<td>7.7</td>
<td>0.03 ↑</td>
<td>5.3</td>
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<tr>
<td>Vitamin D-binding</td>
<td>12.1</td>
<td>0.009 ↑</td>
<td>4.9</td>
<td>3.2</td>
<td>0.11 ↑</td>
<td>6.4</td>
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<tr>
<td>Complement C4-B</td>
<td>8.6</td>
<td>0.009 ↑</td>
<td>Not observed</td>
<td>2.9</td>
<td>0.63 ↓</td>
<td>4.4</td>
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<tr>
<td>Superoxide dismutase [Cu-Zn]</td>
<td>2.4</td>
<td>0.09 ↑</td>
<td>Div/0</td>
<td>1.1</td>
<td>0.34 ↓</td>
<td>1.6</td>
</tr>
<tr>
<td>Complement C3</td>
<td>5.7</td>
<td>0.009 ↑</td>
<td>2.0</td>
<td>1.1</td>
<td>1.0 ↓</td>
<td>3.2</td>
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<td>Proline-1</td>
<td>41.1</td>
<td>0.02 ↑</td>
<td>Div/0</td>
<td>1.0</td>
<td>0.97 ↓</td>
<td>Div/0</td>
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<tr>
<td>Increased Pigment epithelium-derived factor</td>
<td>9.1</td>
<td>0.03 ↑</td>
<td>Div/0</td>
<td>5.5</td>
<td>1.0 ↑</td>
<td>Not observed</td>
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<tr>
<td>Thrombin Beta 4</td>
<td>7.6</td>
<td>0.05 ↑</td>
<td>Div/0</td>
<td>1.7</td>
<td>0.34 ↑</td>
<td>Not observed</td>
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<tr>
<td>Insulin-like growth factor-binding protein 1</td>
<td>28.6</td>
<td>0.06 ↑</td>
<td>Div/0</td>
<td>2.3</td>
<td>0.49 ↓</td>
<td>Div/0</td>
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<td>Myoglobin</td>
<td>9.0</td>
<td>0.10 ↑</td>
<td>Div/0</td>
<td>1.2</td>
<td>0.49 ↓</td>
<td>Div/0</td>
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<td>Glutathione peroxidase 3</td>
<td>18.6</td>
<td>0.20 ↑</td>
<td>Div/0</td>
<td>4.9</td>
<td>0.66 ↑</td>
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<td>Neutrophil defensin</td>
<td>12.6</td>
<td>0.22 ↑</td>
<td>Div/0</td>
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<td>0.17 ↑</td>
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<td>Antithrombin-III</td>
<td>2.4</td>
<td>0.37 ↑</td>
<td>Div/0</td>
<td>1.2</td>
<td>0.69 ↑</td>
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<tr>
<td>Decreased Secreted Ly-6/uPAR-related protein 1</td>
<td>1.4</td>
<td>0.03 ↓</td>
<td>3.0</td>
<td>1.0 ↓</td>
<td>Not observed</td>
<td>Not observed</td>
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<td>Non-secretory ribonuclease</td>
<td>2.2</td>
<td>0.04 ↓</td>
<td>2.0</td>
<td>1.2</td>
<td>0.69 ↓</td>
<td>Not observed</td>
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<td>Uromodulin</td>
<td>1.7</td>
<td>0.30 ↓</td>
<td>3.2</td>
<td>12.6</td>
<td>0.03 ↓</td>
<td>10.7</td>
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<td>CD59 glycoprotein</td>
<td>2.0</td>
<td>0.38 ↓</td>
<td>Div/0</td>
<td>1.0 ↓</td>
<td>Div/0</td>
<td>30.5</td>
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<td>Pro-Epidermal Growth Factor</td>
<td>10.4</td>
<td>0.11 ↑</td>
<td>Not observed</td>
<td>3.0</td>
<td>0.11 ↑</td>
<td>Not observed</td>
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<td>NGAL</td>
<td>2.6</td>
<td>0.09 ↑</td>
<td>2.1</td>
<td>1.9</td>
<td>0.89 ↓</td>
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<td>Clusterin</td>
<td>10.4</td>
<td>0.11 ↑</td>
<td>Not observed</td>
<td>3.0</td>
<td>0.11 ↑</td>
<td>Not observed</td>
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<tr>
<td>L-FABP</td>
<td>10.4</td>
<td>0.11 ↑</td>
<td>Not observed</td>
<td>3.0</td>
<td>0.11 ↑</td>
<td>Not observed</td>
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</table>
How are proteins identified by mass spectrometry and what are the implications?
Angiotensinogen (P01019) 53.1548 kDa

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<th></th>
<th>A</th>
<th>L</th>
<th>Q</th>
<th>D</th>
<th>Q</th>
<th>L</th>
<th>V</th>
<th>L</th>
<th>V</th>
<th>A</th>
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<td>MRKRAPQSEM</td>
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<td>ILCLAWAGL</td>
<td>AAGDRVYIH</td>
<td>FHL*VH</td>
<td>NEST</td>
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<td>60</td>
<td>CEQLAKANAG</td>
<td>KPKDPTFIPA</td>
<td>PIQAKTSPVD</td>
<td>EKALRQDQLVL</td>
<td>VAAKLDTEDK</td>
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<td>110</td>
<td>LRAAMVGMLA</td>
<td>NFLGFRIYGM</td>
<td>HSELWGVVHG</td>
<td>ATVLSPTAVF</td>
<td>GTLASLYLGA</td>
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<td>160</td>
<td>LDHTADRLQA</td>
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<td>CTRSLDAHKV</td>
<td>LSALAQCQVGL</td>
<td>LVAQGRADSQ</td>
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<td>210</td>
<td>AQLLLSTVVG</td>
<td>VFTAPGLHLK</td>
<td>QPFVQGLALY</td>
<td>TPVVLPRSLD</td>
<td>FTELDVAAEK</td>
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<td>IDRFMQAVTG</td>
<td>WKTGCSLMGA</td>
<td>SVDSTLAFNT</td>
<td>YVHFQGKMKG</td>
<td>FSSLAEPQEF</td>
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<td>310</td>
<td>WVDNSTSVSV</td>
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<td>HWSDIQDNFS</td>
<td>VTQVPFTEA</td>
<td>CLLLIQPHYA</td>
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<td>360</td>
<td>SDLKVEGLT</td>
<td>FQQNSLNMWK</td>
<td>KLSPRTIHLT</td>
<td>MPQLVGLYGSY</td>
<td>DLQDLLAQAEG</td>
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<td>410</td>
<td>LPAILHTELN</td>
<td>LQKLNSNDRIR</td>
<td>VGEVLNSIFF</td>
<td>ELEADEREPT</td>
<td>ESTQQNLKPE</td>
<td></td>
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<tr>
<td>460</td>
<td>LLEVTLNRPF</td>
<td>LFAVYDQSAT</td>
<td>ALHFLGRVAN</td>
<td>PLSTA</td>
<td></td>
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<td></td>
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</tbody>
</table>
Total Ion Current
Identification of a Peptide
Work flow for proteomic comparison

Sample Selection
- Samples appropriate to problem
- Availability of samples
- Appropriate matching of samples
- Pooling of samples?

Separation of Peptides
- Prefractionation
- Chromatography
- Tandem mass spectrometry

Interpretation of Data
- Confident identification of proteins
- Comparison of abundance
- Rational selection of candidates
Shotgun Proteomics Identification of Acute Rejection Proteins

Sample Selection

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

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

- 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

Sample Selection

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

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
AUC=0.79

Not Pooled

AUC=0.88

Pooled into 4 groups
Shotgun Proteomics Identification of Acute Rejection Proteins

Sample Selection

✔ • Samples appropriate to problem
✔ • Availability of samples
+/− • Appropriate matching of samples
✔ • Pooling of samples?

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 $\geq 2$ fold up or down.
• Three proteins confirmed by ELISA.

Tamm-Horsfall Protein

Pigment Epithelium Derived Factor (SERPINF1)

CD44

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>Characteristics</th>
<th>Group 1</th>
<th>Group 2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rejection status</td>
<td>1B (N=5)</td>
<td>No rejection (N=5)</td>
<td></td>
</tr>
<tr>
<td>Month post transplant (mean ± SD)</td>
<td>27 ± 24</td>
<td>27 ± 26</td>
<td>1</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>
</tr>
<tr>
<td>Transplant type</td>
<td>Cadaveric</td>
<td>Cadaveric</td>
<td>1</td>
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<tr>
<td>Thymoglobulin : IL-2 blockers</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td>1</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%

log₂ (mean fold change)

-log₁₀(p-value)

SERPINA 5

Transglutaminase C

P<0.05
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\(^1\)

- **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

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

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.

- SERPINA5 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 – Nishant Bhensdadia
The Grandfather – Milos Budisavljevic
Prince Humperdink – Juan Carlos Velez
Count Rugen – Jessalyn Ierardi
Valerie – Elizabeth Shewfelt
The Albino – Christian Spainhour
ROUS – Peifeng Deng
Westley – John Arthur
The Princess Bride – Triple ToF MS

Manish Talwar
Sally Self