Protein Biomarker Discovery in Organ Transplantation

A Proteomics Approach

Tara Sigdel, PhD

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Organ Transplantation

• Optimal Choice for end stage organ failure
• Significant number of transplant occur every year
  Total transplantation in the US January - June 2011 = 13,969
  (source: ttp://optn.transplant.hrsa.gov)
• Causes of organ dysfunction after transplantation:
  ▪ Donor related
  ▪ Ischemia reperfusion injury
  ▪ Recipient’s Immune response
  ▪ Drug toxicity
• Improvements in organ procurement & Immunosuppressive drugs have contributed to short term outcome
• Long-term outcome is still not very satisfactory and needs to be improved

http://www.mode.org.in/organ_transplantation.html
**Biomarkers in Transplantation**

**Biomarker:** Substance (molecules such as gene, proteins, metabolites etc) that can be measured to determine the biological, pathological, pharmacological state

**Current monitoring relies on dated technologies**
- Serum creatinine level
- Histopathology of biopsy samples

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**DISCOVERY (microarrays)**

**Data Analysis**

**Validation (qPCR)**


**Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling.**

Gene Expression & Protein Biomarkers

Differentially Expressed RNA from Public Microarray Data Identifies Serum Protein Biomarkers for Cross-Organ Transplant Rejection and Other Conditions

Rong Chen1,2, Tara K. Sigdel1,2, Li Li1,2, Neeraja Kambham3, Joel T. Dudley1,2, Szu-chuan Hsieh1,2, R. Bryan Klassen1,2, Amery Chen1,2, Tuyen Caohuu4, Alexander A. Morgan1,2, Hannah A. Valantine4, Kiran K. Khush4, Minnie M. Sarwal1,2*, Atul J. Butte1,2*†

1 Department of Pediatrics, Stanford University School of Medicine, Stanford, California, United States of America, 2 Lucie Packard Children’s Hospital, Palo Alto, California, United States of America, 3 Department of Pathology, Stanford University School of Medicine, Stanford, California, United States of America, 4 Division of Cardiovascular Medicine, Department of Medicine, Stanford University School of Medicine, Stanford, California, United States of America

45 up-regulated AR genes across solid-organ transplant:
- Commercial ELISA kits
- ELISA in the serum samples of 19 acute rejection vs. 20 stable after renal transplantation
- ELISA in the plasma samples of 32 acute rejection vs. 31 stable after heart transplantation
- Immunohistochemistry on multi-organ AR vs. stable biopsies

3 AR protein biomarkers for renal transplant

Cross-organ AR pathway
- 3 success of 5 in pathway
- 0 success of 5 outside pathway

Biofluid proteome databases
- 2 success of 6 in database
- 1 success of 4 outside database
Hypothesis

There is a signature of proteins present in the blood and urine which could serve as non-invasive biomarkers for organ transplant injury.
The Goal

Acute Rejection

Chronic Injury

Stable Graft

ELISA

Blood

Urine

SRM

BK Virus Infection
Urinary Protein Biomarkers

Urine Proteomics

Shotgun Proteomics Identifies Proteins Specific for Acute Renal Transplant Rejection

Tara K. Sigdel1, Amit Kaushal2, Marina Gritsenko2, Angela D. Norbeck2, Wei-Jun Qian2, Wenzhong Xiao2, David G. Camp3, Richard D. Smith4, and Minnie M. Sarwal1

1 Department of Pediatrics, 360 Pasteur Dr, S376 Grant Bldg, Stanford University School of Medicine, Stanford, CA 94305
2 Department of Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, CA 94143
3 Satellite, Pacific Northwest National Laboratory, Richland, WA 99352

BASIC RESEARCH

www.jasen.org

Integrative Urinary Peptidomics in Renal Transplantation Identifies Biomarkers for Acute Rejection

Xuefeng B. Ling,† Tara K. Sigdel,† Kenneth Lau,† Litlua Ying,‡ Irwin Lau,*, James Schilling,* and Minnie M. Sarwal†

Divisions of †Biotechnology Core and ‡Nephrology and Department of Pediatrics, Stanford University School of Medicine, Stanford University, Stanford, California

*Stanford Genomics and Proteomics in Organ Transplantation

NIH Public Access

Author Manuscript

Proteomics Clin Appl. Author manuscript; available in PMC 2010 June 10.

Optimization of Proteomics Protocol


- Subjects: Renal Transplant Patients
- Urine Samples
- Centrifugal Filtration (10 kDa cutoff)
- Peptidomics (<10 kDa peptides)
- Urinary Proteins (>10 kDa)
- Fractionation
- Mass spectrometric and Bioinformatics analysis
- Biomarker Candidates

Proteomics (>10 kDa proteins)
Patient demographic information

Patient information on 60 renal transplant patients (30 AR, 30 STA).

<table>
<thead>
<tr>
<th></th>
<th>Acute Rejection (AR) (n=30)</th>
<th>Stable Graft Function (STA) (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age</td>
<td>12±5</td>
<td>14±5</td>
<td>0.21</td>
</tr>
<tr>
<td>Age Range</td>
<td>3–19</td>
<td>6–21</td>
<td></td>
</tr>
<tr>
<td>Immunosuppression, %SF#</td>
<td>66%</td>
<td>50%</td>
<td>0.19</td>
</tr>
<tr>
<td>Race *</td>
<td>63%,13%,0%,17%,7%</td>
<td>59%,7%,10%,17%,7%</td>
<td>0.45</td>
</tr>
<tr>
<td>Donor, % living donor</td>
<td>40%</td>
<td>53%</td>
<td>0.44</td>
</tr>
<tr>
<td>Mean GFR (mL/min/1.73m²)</td>
<td>87.45±38.46</td>
<td>124±29.86</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

# SF: Steroid-free immunosuppression treatment, consisting of daclizumab induction + mycophenolate mofetil + tacrolimus

* Race: 1=Caucasian; 2= Hispanic; 3=Asian; 4=African American; 5=Other
High throughput proteomics using shotgun proteomics identified a total of 1446 urinary proteins which included a number of AR specific proteins.

We have identified alterations in a number of specific urinary proteins in AR, primarily relating to MHC antigens, the complement cascade and extra-cellular matrix proteins.

Selected candidates were verified by ELISA in an independent urine sample set.
Identification of More Proteins in Urine


This work (1340)

Adachi et al (1543)

Gonzalez et al (1160)

560
414
583
239
127
307
487
### Table 4A: List of proteins identified only in AR urine

<table>
<thead>
<tr>
<th>S. No.</th>
<th>IPI ID</th>
<th>Gene Symbol</th>
<th>Protein Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IPI00103082.7</td>
<td><em>HLA-DBP</em></td>
<td>HLA class II histocompatibility antigen, DP(W4) beta chain</td>
</tr>
<tr>
<td>2</td>
<td>IPI00005180.2</td>
<td><em>IgHM</em></td>
<td>HLA class II histocompatibility antigen, DRB1-8 beta chain</td>
</tr>
<tr>
<td>3</td>
<td>IPI0021727.1</td>
<td><em>C4BPA</em></td>
<td>C4b-binding protein alpha chain</td>
</tr>
<tr>
<td>4</td>
<td>IPI00641889.1</td>
<td><em>K1AA1522</em></td>
<td>25 kDa protein</td>
</tr>
<tr>
<td>5</td>
<td>IPI00746396.1</td>
<td></td>
<td>302 kDa protein</td>
</tr>
<tr>
<td>6</td>
<td>IPI00760688.2</td>
<td><em>HLA-DR</em></td>
<td>MHC class II antigen (Fragment)</td>
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<tr>
<td>7</td>
<td>IPI00027255.1</td>
<td><em>MYL6B</em></td>
<td>Myosin light chain 1, slow-twitch muscle A isoform</td>
</tr>
<tr>
<td>8</td>
<td>IPI00783351.1</td>
<td><em>SUMF2</em></td>
<td>sulfatase modifying factor 2 isoform d</td>
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<tr>
<td>9</td>
<td>IPI00743218.1</td>
<td><em>HLA-DQBI</em></td>
<td>HLA class II histocompatibility antigen, DQ(3) beta chain</td>
</tr>
</tbody>
</table>
Transplantation and AR Specific Proteins


Venn diagrams showing the proteomic changes in healthy control, renal transplantation, stable graft, and acute rejection.

- Healthy Control (1340)
  - Renal Transplantation (1350)
    - Overlapping Proteins: 87
- Stable Graft (1325)
  - Acute Rejection (1001)
    - Overlapping Proteins: 25
    - Additional Proteins: 976
Verification of Urine Protein Markers
UMOD, SERPINF1 and CD44 (ELISA Assay)

We validated observation made in discovery step by ELISA assay performed on independent set of samples.

Shotgun proteomics approach for analyzing urinary proteome in normal and disease states is a robust and sensitive method for detection of urinary proteins for serial, non-invasive clinical monitoring for graft rejection after kidney transplantation.
Integrative Urinary Peptidomics in Renal Transplantation Identifies Novel Biomarkers for Acute Rejection

Ling and Sigdel et al JASN 2010 Apr;21(4):646-53

Peptidomics approach for biomarker discovery (70 urine samples)
(50 renal transplant and 20 controls)

We identified a panel of 40 peptides that discriminates AR from non-AR, ROC AUC>0.96
Verification of Urine Peptide Markers

Ling and Sigdel et al JASN 2010 Apr;21(4):646-53

SRM verification of two UMOD peptide biomarkers
## Discovery of Disease Specific Proteolytic Activity

### Collegen Proteases

<table>
<thead>
<tr>
<th>COL1A1</th>
<th>COL1A2</th>
<th>COL1A3</th>
<th>COL1A5</th>
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<tr>
<td>1. 1235.56</td>
<td>2. 1251.55</td>
<td>3. 1322.57</td>
<td>4. 1316.59</td>
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<tr>
<td>COL1A1</td>
<td>COL1A2</td>
<td>COL1A3</td>
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<tr>
<td>5. 1409.66</td>
<td>6. 2048.92</td>
<td>7. 2064.91</td>
<td>8. 2192.97</td>
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<td>COL1A1</td>
<td>COL1A2</td>
<td>COL1A3</td>
<td>COL1A5</td>
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<tr>
<td>9. 2362.12</td>
<td>10. 2378.10</td>
<td>11. 2645.24</td>
<td>12. 1709.79</td>
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<tr>
<td>COL1A1</td>
<td>COL1A2</td>
<td>COL1A3</td>
<td>COL1A5</td>
</tr>
<tr>
<td>13. 2031.95</td>
<td>14. 2221.97</td>
<td>15. 2205.99</td>
<td>16. 2277.91</td>
</tr>
<tr>
<td>COL1A1</td>
<td>COL1A2</td>
<td>COL1A3</td>
<td>COL1A5</td>
</tr>
<tr>
<td>17. 2293.01</td>
<td>18. 2617.15</td>
<td>19. 2086.93</td>
<td>20. 2157.96</td>
</tr>
<tr>
<td>COL1A1</td>
<td>COL1A2</td>
<td>COL1A3</td>
<td>COL1A5</td>
</tr>
<tr>
<td>COL1A1</td>
<td>COL1A2</td>
<td>COL1A3</td>
<td>COL1A5</td>
</tr>
<tr>
<td>25. 2086.93</td>
<td>26. 2157.96</td>
<td>27. 3014.41</td>
<td>28. 1266.58</td>
</tr>
<tr>
<td>COL1A1</td>
<td>COL1A2</td>
<td>COL1A3</td>
<td>COL1A5</td>
</tr>
<tr>
<td>29. 2179.99</td>
<td>30. 2017.93</td>
<td>31. 2086.93</td>
<td>32. 1266.58</td>
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</table>

### Uromodulin Proteases

<table>
<thead>
<tr>
<th>UMOD</th>
<th>VIMNLGPRV</th>
<th>IDQHSVNLGPI</th>
<th>GDQHSVNLGPI</th>
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<tbody>
<tr>
<td>1. 962.59</td>
<td>2. 1047.48</td>
<td>3. 1211.66</td>
<td>4. 1225.69</td>
</tr>
<tr>
<td>5. 1324.76</td>
<td>6. 1423.83</td>
<td>7. 1468.82</td>
<td>8. 1510.87</td>
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<tr>
<td>9. 1567.91</td>
<td>10. 1581.91</td>
<td>11. 1684.91</td>
<td>12. 1688.98</td>
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<tr>
<td>13. 1755.96</td>
<td>14. 1768.01</td>
<td>15. 1912.07</td>
<td>16. 2040.16</td>
</tr>
</tbody>
</table>
Decreased Fragments of COL and UMOD in AR

Ling and Sigdel et al J Am Soc Nephrol. 2010

SAMPLE: URINE PEPTIDES

AR | STA | BK | NS | HC

1. THP 982.59  VLNLPITR
2. THP 1047.48 SGVIDQSRV
3. THP 1211.66 DQSRVLNLGI
4. THP 1225.69 SRVNLGIITR
5. THP 1324.76 IDQSRVLNLGI
6. THP 1423.83 VIDQSRVLNLGI
7. THP 1468.82 DQSRVLNLGIITR
8. THP 1510.87 VIDQSRVLNLGI
9. THP 1567.91 GSVIDQSRVLNLGI
10. THP 1581.91 IDQSRVLNLGIITR
11. THP 1654.91 SGVIDQSRVLNLGI
12. THP 1680.98 VIDQSRVLNLGIITR
13. THP 1755.96 SGVIDQSRVLNLGIITR
14. THP 1768.01 SVIDQSRVLNLGIITR
15. THP 1912.07 SGVIDQSRVLNLGIITR
16. THP 2040.16 SGVIDQSRVLNLGIITRK

Sarwallab
Transcription-Translation-Proteolysis
Is there an altered gene expression for the precursor gene of biomarker peptides in AR?

Microarray data (Affymetrix HU133 plus 2) on 82 biopsies

Biopsy arrays chosen for study on overlapping urine peptidomic samples for 20 AR, 20 STA and 10 HC.

Ling and Sigdel et al J Am Soc Nephrol. 2010
A Panel of genes as biomarkers specific for AR
(Q-PCR validation)

Kidney gene expression analysis:
1. Upregulation of COL1A2, COL3A1, MMP7, SERPING1, and TIMP1
2. Downregulation of UMOD

Q-PCR validation
A New Understanding

Biopsy Gene Expression
GSE 14328

Integrated Analysis

Decreased Collagen Activity In AR tissue

Decreased Collagen Breakdown in AR

Increased Collagen Deposition in AR

More Graft Fibrosis After an AR episode?

Urine Peptidomics

Urine

Decreased Collagen Peptides In AR

Urine Peptide Analysis by MS
Protein Biomarkers in the Blood

Serum Proteomics
Serum Protein Biomarkers for Renal Transplant Injury Samples (n=78)

Transplant Injury Phenotype
- AR
- CAN
- BKV

Transplant Non-injury Phenotype
- STA

Shotgun Proteomics

Data Analysis

Discovery → Verification → Validation
### Patient Demography

#### Discovery (n=60) (50 transplantation 10 healthy normal)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>AR</th>
<th>STA</th>
<th>CAN</th>
<th>CNIT</th>
<th>BKV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Patients</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Steroid-free/Steroid-based</td>
<td>5/5</td>
<td>4/6</td>
<td>3/7</td>
<td>8/2</td>
<td>6/4</td>
</tr>
<tr>
<td>Recipient Gender (M/F)</td>
<td>6/4</td>
<td>7/3</td>
<td>6/4</td>
<td>8/2</td>
<td>7/3</td>
</tr>
<tr>
<td>Recipient Age*</td>
<td>12 ± 5 (14; 10-19)</td>
<td>16 ± 3 (16; 10-19)</td>
<td>12 ± 6 (9; 8-18)</td>
<td>11 ± 6 (11; 3-17)</td>
<td>13.5 ± 7 (18; 1-20)</td>
</tr>
<tr>
<td>Living/Deceased</td>
<td>3/7</td>
<td>5/5</td>
<td>1/2</td>
<td>3/7</td>
<td>6/4</td>
</tr>
<tr>
<td>Donor Gender (M/F)</td>
<td>5/5</td>
<td>6/4</td>
<td>4/6</td>
<td>6/4</td>
<td>7/3</td>
</tr>
<tr>
<td>Donor Age*</td>
<td>28 ± 8 (29; 17-37)</td>
<td>28 ± 10 (27; 14-47)</td>
<td>24 ± 8 (25; 16-31)</td>
<td>28 ± 10 (28; 17-37)</td>
<td>29 ± 9 (30; 16-47)</td>
</tr>
</tbody>
</table>

#### Verification (n=71) (71 transplantation sera)

<table>
<thead>
<tr>
<th>Number of Patients</th>
<th>16</th>
<th>15</th>
<th>17</th>
<th>10</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroid-free/Steroid-based</td>
<td>9/7</td>
<td>9/6</td>
<td>8/9</td>
<td>5/5</td>
<td>9/4</td>
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<tr>
<td>Recipient Gender</td>
<td>12/4</td>
<td>10/5</td>
<td>6/11</td>
<td>5/5</td>
<td>8/5</td>
</tr>
<tr>
<td>Recipient Age*</td>
<td>12 ± 5 (14; 3-19)</td>
<td>13 ± 7 (16; 1-20)</td>
<td>8 ± 6 (7; 1-17)</td>
<td>13 ± 5 (15; 4-20)</td>
<td>11 ± 8 (15; 1-19)</td>
</tr>
<tr>
<td>Living/Deceased</td>
<td>13/3</td>
<td>2/13</td>
<td>10/7</td>
<td>6/4</td>
<td>11/2</td>
</tr>
<tr>
<td>Donor Gender</td>
<td>8/8</td>
<td>8/7</td>
<td>11/6</td>
<td>7/3</td>
<td>10/3</td>
</tr>
<tr>
<td>Donor Age*</td>
<td>24 ± 5 (14; 5-38)</td>
<td>23 ± 6 (23; 14-36)</td>
<td>30 ± 10 (28; 14-44)</td>
<td>35 ± 5 (37; 29-44)</td>
<td>27 ± 16 (29; 1-49)</td>
</tr>
</tbody>
</table>

Heart transplantation sera (n=29)
Lung transplantation sera (n=27)
Work Flow

Sera Retrieval

\[ \downarrow \]

Immunodepletion

To deplete 20 highly abundant proteins from serum

\[ \downarrow \]

Protein Assay

\[ \downarrow \]

Trypsin Digestion

\[ \downarrow \]

MS MS/MS Analysis

\[ \downarrow \]

Data Analysis
There is slightly increased protein presence in AR Overall
Antibody Biomarkers in the Blood

High-density Protein Arrays

“Antibiomics”

Identifying compartment-specific non-HLA targets after renal transplantation by integrating transcriptome and “antibodyome” measures

Li Li¹, Persis Wadia², Rong Chen³, Neeraja Kambham⁴, Maarten Naesens⁵, Tara K. Sigdel⁶, David B. Miklos⁷, Minnie M. Sarwal⁸,¹, and Atul J. Butte²,³,⁴

¹Department of Pediatrics, Blood and Marrow Transplantation Division, Departments of ²Medicine and ³Pathology, and ⁴Center for Biomedical Informatics Research, Stanford University, 300 Pasteur Drive, Stanford, CA 94304
Immune Response Markers for Chronic Allograft Injury

172 unique sera + 172 matched renal txp bx

60 Protoarrays

Discovery Set; 60 serial samples
10 pts with CAI and 10 pts with nCAI
Samples at 0, 6, 24 mo

98 unique transplant pts
37 pts with serial bx at 0, 6, 24 mo
61 pts with cross sectional bx at 12 mo

Blinded CADI scores for CAI
66 pts with CAI; 30 pts with nCAI (STA)

Data filtration, normalization
M-statistic; p<0.05

Localization of antibodies to anatomical kidney compartments by enrichment analysis

Selection of 4 CAI-specific antibodies

Reverse ELISAs on 4 Antibody Markers

Cross-Sectional Validation Set; 61 samples
31 pts with CAI; 30 pts with nCAI (STA)
Samples at 12 mo

Longitudinal Validation Set; 51 serial samples
17 pts with CAI
Samples at 0, 6, 24 mo
Conclusion

- There is a dire need of more sensitive and specific biomarkers

- Carefully designed proteomics studies on appropriate samples could provide potential biomarkers for diagnosis and monitoring of transplant dysfunction

- Our effort in this field has yielded a number of potential biomarker proteins and peptides that could provide more specific and sensitive biomarkers that can be used in clinical setting
Acknowledgements

- Minnie Sarwal, Dept of Pediatrics
- David Camp and Wei-Jun Qian (Pacific Northwest National Lab, Richland, WA)
- Bruce Ling, Ken Lou, and Jim Schlling(Dept of Pediatrics, Stanford Univ)
- Van Dinh , Tim Tran, Bryan Klassen, Many Mohindra, and other Sarwal Lab members
- SUMS , Stanford Univ
- NIH for funding