Molecular transplant pathology

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Gene expression, i.e. transcription:
The techniques
Method platforms of transcriptomics

– transcriptomics mainly relays on the high density DNA and oligonucleotide **microarrays** ("chips")
  – genome-spanning tiling arrays (detection of novel coding and non-coding RNAs),
  – 3’ expression arrays (i.e. analysis of the global mRNA expression),
  – whole transcript coverage expression arrays, (i.e. exon arrays, detection of splicing events – mRNA maturation and a step towards proteomics),
  – miRNA arrays (i.e. detection of the regulatory RNAs, next step in the post-translational regulation of mature mRNAs)
  – ChiP on chip arrays, insight into the transcription factors - promoter interaction: the genomics/proteomics interface.
  – The **high throughput quantitative RT-PCR**, and various proteomics approaches are used to confirm the findings obtained from these platforms.
3’ expression arrays

- Expression microarrays are now the standard technology to interrogate the transcriptome (global mRNA expression).

- The advantages are the great number of simultaneously analyzed transcripts (up to 55,000) and the versatility (bacteria, plants, mammals, and human specific chips).

- Data obtained from so called one color microarrays is very reproducible, both in terms of technical and biological replicates, and the unprocessed raw data files can be compared across different laboratories and used for the data mining at remote sites.
Base-pairing allows DNA:DNA or DNA:RNA

http://www.accessexcellence.org/AB/GG/nucleic.html

Nucleic Acid Hybridization
Red-Green

Overview of DNA Microarrays

- Experimental RNA
  - Reverse Transcribe
  - Red Probe

- Reference RNA
  - Reverse Transcribe
  - Green Probe

DNA microarray

- Hybridize
- Wash
- Scan

- Green = Decreased Expression
- Red = Increased Expression
- Yellow = Equal Expression
- Dark = No Expression

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Affymetrix Arrays
Figure 1. Workflow for NimbleGen Gene Expression Microarray Analysis – Steps in the process and estimated time for each step, based on the processing of one array, are shown in the boxes. Incubation times are indicated beneath the appropriate process times.
Analysis of the gene expression data
(and all other large scale (‘OMICS’) data)

• Class (phenotype) **discovery**:
  – unbiased approach that serves to identify a collection of clinical samples that represent a distinct disease type or stages of disease progression.

• Class (phenotype) **comparison**:
  – identification of interesting features (genomics events, transcripts, proteins, metabolites), that significantly differ between the discovered or *a priori* established classes (phenotypes).

• Class (phenotype) **prediction**: the final proof of clinical/pathological indications, *building of the classifier*.
  – The classifier is trained on the gold standard that is reflecting the class discovery, class comparison, pathology and/or clinical data.
  – It returns a set of genes called a metagene, or other metamarker or a single gene or protein marker that predicts the phenotypes with a high specificity, sensitivity, and accuracy.
Supervised vs. Unsupervised analysis

**Supervised** = learning from examples, classification
- Known structure in the data needs to be generalized to new data.
- Groups with distinct phenotypes (healthy vs. sick) are known: Now let’s diagnose the next person walking into the hospital.

**Unsupervised** = Exploratory analysis in data where no structure is known, e.g. by clustering
- Are there groups of genes that behave similarly in all conditions?
Gene Expression:
The Molecules and their (tissue-based) Application to Transplantation
Gene expression analysis in renal allograft rejection


Flechner et al. AJT 2004; 4: 1475-1489

TX: transplanted kidneys with stable function
C: normal living kidney donors
AR: acute rejection
NR: acute renal dysfunction without rejection
Microarray analysis of human biopsies: The concept of Pathogenesis Based Transcript sets

• Concept: altered expression of any gene in a disease state is part of a pathogenesis-based transcript sets (PBTs) reflecting a certain biological event

• PBT is not just a collection of genes but the fingerprint of a biological process
  – e.g. a cytotoxic T lymphocyte has GzmB but MUST have a TCR

reviewed in Halloran et al. AJT 2010, in press
Generation of human Cytotoxic T-cell (CD8)-Associated Transcript set (hCAT)

For available PBTs including their algorithms go to:
http://transplants.med.ualberta.ca/Nephlab/data/gene_lists.html

Algorithm:

**hCD8** – expression 5-fold higher in isolated hCD8 ETCs vs Neph and p-value < 0.05 by ANOVA

**hB cell** – expression 5-fold higher in isolated B cells from WB vs Neph and p-value < 0.05 by ANOVA

**THP-1** – expression 5-fold higher in isolated THP-1 vs Neph and p-value < 0.05 by ANOVA

**hCAT** – 509 unique transcripts based on highest expression in CD8$^+$ CTL and avoiding cross-hybridizing probe sets when possible
## Pathogenesis-based transcript sets (PBTs) in allograft rejection

<table>
<thead>
<tr>
<th>PBT</th>
<th>PBT name</th>
<th>Biological description</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT</td>
<td>human cardiac, heart transcripts</td>
<td>Cardiac selective transcripts showing high expression in normal control mouse heart but low expression in inflammatory cells</td>
<td>Mengel et al. Am J Transplant. 2010 In press.</td>
</tr>
<tr>
<td>ENDAT</td>
<td>Endothelium associated transcripts</td>
<td>Literature based transcript set, in which genes were identified based on their selective expression in cultured human endothelial cells when compared with non-endothelial cells</td>
<td>Sis B et al. Am J Transplant 2009; 9(10):2312-2323.</td>
</tr>
<tr>
<td>NKAT</td>
<td>NK cell associated transcripts</td>
<td>Set of transcripts with increased expression in NK cells</td>
<td>Hidalgo et al. Am J Transplant. 2010 In press.</td>
</tr>
</tbody>
</table>

The probe sets in each PBT as well as the related algorithms showing how the PBTs were derived are available at (http://transplants.med.ualberta.ca/Nephlab/data/gene_lists.html).
"The ICON"

PBTs reveal a stereotyped structure of molecular ‘Disturbance’ in rejection

‘The jaws of death’

Biopsies for cause ordered by CTL associated transcripts

T Mueller and G Einecke et al. AJT 7:2712, 2007
The molecular phenotype of transplant biopsies
Biopsies for cause ordered by 7 gene average

Individual CAT or GRIT transcripts provide similar information to PBTs
All biopsies aligned by T cell burden (QCATs): Standardized with 8 control kidneys, PBTs from IQR filtered set

Comparing extreme phenotypes ‘sick versus well’

limited challenge bias
Discovery of new and refinement of existing diagnostic categories using molecules
The Banff concept of diagnosing rejection

by Lorraine Racusen & Kim Solez
Diagnostic labels are unstable: they are only estimates of the probability of a disease.

- Symptoms
- Signs
- Laboratory tests
- Imaging e.g. MRI
- Function
- Outcomes

**The Truth**
true disease process
(Mechanisms of injury
response to injury
consequences)

**Tissue biopsy**
read by histopathology

- Assess features ("lesions")
  (arbitrary rules for grading)
- Assign "diagnosis" (label)
  (arbitrary rules based on context)
Predictive Classifiers

• Machine learning methods for combining multiple input variables (features) in order to predict a categorical or binary phenotype
• Must provide a relatively unbiased estimate of how well they will perform on future datasets from a similar population
• Independent external test sets are desirable but seldom available – some form of internal cross validation must be used to assess accuracy, sensitivity, specificity, etc.
• (Optionally) Should provide an indication of which input variables are most important in making the predictions
Building a rejection classifier:

PAM (predictive analysis of microarrays) with Cross-validation through multiple resampling

**Training set.** Build classifier based on gene expression values and histopathology diagnoses.

**Test set.** Apply classifier equation from training set to test set expression values. Predict diagnoses.

Repeat 1000x.
ABMR  Borderline  TCMR  BK virus  Other

Banff Rejection  Banff Non-rejection

Mixed

Banff - PAM+

Banff+ PAM-

 clinical episode of rejection
 Treatment before biopsy

Biopsies grouped by pathology diagnosis

## Top 20 genes used by PAM

<table>
<thead>
<tr>
<th>GENE</th>
<th>Pathogenesis Based Transcript (PBT) membership</th>
<th>Percentage of the 1000 training sets that used the gene in the PAM classifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL11</td>
<td>interferon-γ induced</td>
<td>100</td>
</tr>
<tr>
<td>GBP1</td>
<td>T-cell associated</td>
<td>99</td>
</tr>
<tr>
<td>CXCL9</td>
<td>interferon-γ induced</td>
<td>99</td>
</tr>
<tr>
<td>INDO</td>
<td>interferon-γ induced</td>
<td>97</td>
</tr>
<tr>
<td>CXCL10</td>
<td>interferon-γ induced</td>
<td>97</td>
</tr>
<tr>
<td>FAM26F</td>
<td>interferon-γ induced</td>
<td>95</td>
</tr>
<tr>
<td>GBP5</td>
<td>T-cell associated</td>
<td>95</td>
</tr>
<tr>
<td>{235229_at}</td>
<td>Epithelial cell associated</td>
<td>93</td>
</tr>
<tr>
<td>GBP2</td>
<td>interferon-γ induced</td>
<td>80</td>
</tr>
<tr>
<td>UBD</td>
<td>interferon-γ induced</td>
<td>76</td>
</tr>
<tr>
<td>NLRC5</td>
<td>Epithelial cell associated</td>
<td>66</td>
</tr>
<tr>
<td>GBP4</td>
<td>interferon-γ induced</td>
<td>62</td>
</tr>
<tr>
<td>LCP2</td>
<td>T-cell associated</td>
<td>58</td>
</tr>
<tr>
<td>{238725_at}</td>
<td>Epithelial cell associated</td>
<td>44</td>
</tr>
<tr>
<td>WARS</td>
<td>interferon-γ induced</td>
<td>40</td>
</tr>
<tr>
<td>PSMB9</td>
<td>interferon-γ induced</td>
<td>38</td>
</tr>
<tr>
<td>LILRB1</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>GZMA</td>
<td>T-cell associated</td>
<td>37</td>
</tr>
<tr>
<td>CCL5</td>
<td>interferon-γ induced</td>
<td>36</td>
</tr>
<tr>
<td>TAP1</td>
<td>interferon-γ induced</td>
<td>34</td>
</tr>
</tbody>
</table>
Marginal variability within the top 50 transcripts
Influence of extreme phenotype comparisons

Extreme phenotype classifier

Full spectrum classifier

Probability of TCMR

Influence of extreme phenotype comparisons
Interdisciplinary and iterative approaches are necessary to develop and validate new integrated diagnostic and predictive standards.
‘Canonical’ TCMR characterized by molecular features in mice with typical histology of TCMR

- High expression of AMA associated transcripts
- Strong interferon gamma effects
Typical (canonical) TCMR

High GRIT1-AMAT1 (cTCMR)

Typical (canonical) TCMR is treatable

Is TCMR the same in all organ transplants?
Kidney rejection classifier applied to EMB from heart allografts
Using the molecular phenotype for assigning risk for allograft failure to individual patients
Building a prognostic classifier:

Supervised PCA (principle component analysis) with Cross-validation through multiple re-sampling
Build a predictive model based on 9/10ths of the samples.

Each sample then has 1000 different risk score assignments, generated from 1000 different sets of “training” data. Use the average of these as the final predicted risk score.
Relationship of gene expression risk score with graft loss

No effect of varying numbers of probe sets used by the classifier
PBT annotation of transcript sets used by the molecular classifier to predict risk of graft loss

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>Transcript Set</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reversible tissue injury</td>
<td>IRIT</td>
<td>&lt; 9.99E-15</td>
</tr>
<tr>
<td>Reversible tissue injury (intermediate phase)</td>
<td>IRIT3</td>
<td>&lt; 9.99E-15</td>
</tr>
<tr>
<td>Severe tissue injury</td>
<td>GST</td>
<td>9.99E-15</td>
</tr>
<tr>
<td>Endothelial cell activation</td>
<td>ENDAT_GE</td>
<td>5.67E-14</td>
</tr>
<tr>
<td>TGF-beta signalling</td>
<td>TGFB1</td>
<td>6.25E-11</td>
</tr>
<tr>
<td>TGF-beta signalling</td>
<td>TGFB2</td>
<td>2.49E-10</td>
</tr>
<tr>
<td>Fibroblast activation</td>
<td>FIBET</td>
<td>4.47E-10</td>
</tr>
<tr>
<td>Ifng effects</td>
<td>GRIT2</td>
<td>1.77E-07</td>
</tr>
<tr>
<td>Endothelial cell activation</td>
<td>ENDAT</td>
<td>3.17E-07</td>
</tr>
<tr>
<td>Severe tissue injury</td>
<td>NAT</td>
<td>2.00E-06</td>
</tr>
<tr>
<td>Severe tissue injury</td>
<td>CIST</td>
<td>9.49E-06</td>
</tr>
<tr>
<td>Reversible tissue injury (late phase)</td>
<td>IRIT5</td>
<td>1.47E-05</td>
</tr>
<tr>
<td>Alternative macrophage activation</td>
<td>AMAT</td>
<td>1.52E-05</td>
</tr>
<tr>
<td>Epithelial cell dedifferentiation</td>
<td>CECAT</td>
<td>1.58E-05</td>
</tr>
<tr>
<td>Classical macrophage activation</td>
<td>IMAT</td>
<td>0.00011</td>
</tr>
<tr>
<td>Alternative macrophage activation</td>
<td>E-AMA</td>
<td>0.002</td>
</tr>
<tr>
<td>Fibroblast activation</td>
<td>FIBTG</td>
<td>0.002</td>
</tr>
<tr>
<td>Severe tissue injury</td>
<td>NIRIT</td>
<td>0.007</td>
</tr>
<tr>
<td>Classical macrophage activation</td>
<td>QIMAT</td>
<td>0.009</td>
</tr>
<tr>
<td>Ifng effects</td>
<td>GRIT1</td>
<td>0.042</td>
</tr>
</tbody>
</table>
No overlap of probesets significant in rejection/non-rejection vs. graft failure/non-failure
Which will be the relevant molecules?
The AUCs of probesets positively associated with survival (higher expression = better survival) are shown as 1-AUC i.e. those on the bottom half of the graph.
Will we have a molecular Banff classification? Yes!

- Why? – there are unmeet needs and limitations with histopathology
- How can molecules improve diagnostics? – they represent a more robust and objective measurement and they can provide mechanistic insights
- Will molecules replace histology, be adjunctive or even only a transient part of diagnostics? - adjunctive
- What methods/platforms could be used in daily routine? – depending on the number of molecules, cost will come down significantly, new platforms will emerge
- Which will be the relevant molecules? – will have to be identified in a consensus process
- **Interdisciplinary and iterative approaches are necessary to develop and validate new diagnostic and predictive standards on a data-driven basis**
Establishment of Banff Working Groups

Data-driven & Validated Refinement of the Banff Guidelines

- Isolated v-lesion Working Group
- IHC Quality Assurance Working Group
- Fibrosis Scoring Working Group
- Glomerular Lesion Working Group
- Polyoma Virus Nephropathy Working Group
- Molecular Pathology Working Group