A Novel Two-Gene Expression Ratio That Predicts Clinical Outcome in Node-negative Breast Cancer Patients Treated With Tamoxifen

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Lecture Outline

- Brief overview of past and present approaches to biomarker discovery.
- Gene expression microarray technologies.
- Application of these technologies to a specific clinical problem.
We are we going?  
Personalized Medicine

The ultimate goal is to identify a biomarker that will predict treatment-specific outcome or treatment-specific response.

Can we identify biomarkers that allow clinicians to match the most effective (appropriate) treatment to the appropriate patient?
Classical Biomarker Discovery: One gene or one protein approach

• Disadvantages
  – Closed system: require the discovery of a new gene or pre-existing reagents- mAbs
  – Time consuming: years to interrogate 100 genes.
  – Costly: reagents expensive and consumption of precious tissue resources
Personalized-Medicine
Classic Breast Cancer Biomarkers

ER

Her-2
Contemporary Biomarker Discovery: Genome-wide approach

• Advantages
  – Time saving: study 30,000 genes in a single experiment
  – Resource conservation: Study 30,000 genes using a single 8 mm tissue section.
  – Open system: does not require pre-existing reagents.
cDNA Microarray Analysis of Gene Expression

mRNA #1 → cDNA #1
RT with Cy3-dUTP

mRNA #2 → cDNA #2
RT with Cy5-dUTP

Hybridize

Genes
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17 18 19 20
21 22 23 24 25

Wash; Read with confocal microscope

Normal

Tumor
The Challenge Facing Pathology

Standard clinicopathological parameters fail to accurately classify breast tumors according to their clinical behavior.
Better Predictor for Outcome to Tamoxifen is an Unmet Clinical Need

- Presence of ER and PR are currently best predictors for response to tamoxifen (and other anti-estrogens)
- However, 30-40% of ER+ cases fail to respond or develop resistance to tamoxifen.

Patient 1
- 1.4 cm
- LN-
- ER+
- PR+
- Adjuvant Tamoxifen
- Metastatic disease 3yrs after initiation of Tam

Patient 2
- 1.2 cm
- LN-
- ER+
- PR+
- Adjuvant Tamoxifen
- Disease free at 12 yrs
Discovery Study Design

60 Patients with Early Stage Invasive Breast Cancer

All patients were hormone receptor positive and received adjuvant tamoxifen monotherapy

Non-recurrences and recurrences were closely matched with respect to tumor size, tumor grade, and nodal status

Comparison of microarray gene expression profiles of non-recurrence to recurrences.

Two Approaches

• Gene expression analysis of whole tumor tissue sections: analysis of tumor cells, stroma, leukocytes and vessels.

• Gene expression analysis of tumor cells only: Microdissection.
Whole Tumor Tissue Section
Approach

Extract RNA
Gene Expression Profile
Microdissection Approach

Extract RNA

Gene Expression Profile
Microarray Data Analysis:

Select genes by t-test (p< 0.001) comparing recurrences vs nonrecurrences

whole tumor tissue sections

19 genes

Recurrences

Non-Recurrences

microdissected tumor cells

9 genes

Recurrences

Non-Recurrences

Receiver Operator Characteristics (ROC) Analysis – Comparison to Known Predictors of Tamoxifen Response

<table>
<thead>
<tr>
<th></th>
<th>Tissue Sections</th>
<th>LCM</th>
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<tbody>
<tr>
<td></td>
<td>AUC</td>
<td>P value</td>
</tr>
<tr>
<td><strong>IL17BR</strong></td>
<td>0.79</td>
<td>1.58E-06</td>
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<td><strong>AI240933</strong></td>
<td>0.81</td>
<td>3.02E-08</td>
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<tr>
<td><strong>HOXB13</strong></td>
<td>0.67</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>ER</strong></td>
<td>0.55</td>
<td>0.277</td>
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<td><strong>PR</strong></td>
<td>0.63</td>
<td>0.036</td>
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<tr>
<td><strong>ERBB2</strong></td>
<td>0.69</td>
<td>0.004</td>
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<tr>
<td><strong>EGFR</strong></td>
<td>0.56</td>
<td>0.2</td>
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HOXB13:IL17BR (H:I) Ratio is a Stronger Predictor of Treatment Outcome

<table>
<thead>
<tr>
<th>Tissue Section</th>
<th>IL17BR</th>
<th>HOXB13</th>
<th>HOXB13:IL17BR</th>
<th>IL17BR</th>
<th>HOXB13</th>
<th>HOXB13:IL17BR</th>
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<td></td>
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<tr>
<td>LCM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IL17BR</td>
<td>4.15</td>
<td>-3.57</td>
<td>-4.91</td>
<td>3.70</td>
<td>-4.39</td>
<td>-5.42</td>
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<tr>
<td>HOXB13</td>
<td>1.15E-04</td>
<td>1.03E-03</td>
<td>1.48E-05</td>
<td>5.44E-04</td>
<td>8.00E-05</td>
<td>2.47E-06</td>
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<tr>
<td>HOXB13:IL17BR</td>
<td>1.58E-06</td>
<td>0.01</td>
<td>1.08E-07</td>
<td>2.73E-05</td>
<td>9.94E-07</td>
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AUC, area under the curve; P values are AUC > 0.5
Univariate and Multivariate Logistic Regression Analysis of HOXB13:IL17BR vs Known Prognostic Factors

### Univariate Model

<table>
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<tr>
<th>Predictor</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>HOXB13:IL17BR</td>
<td>10.17</td>
<td>2.9-35.6</td>
<td>0.0003</td>
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</table>

### Multivariate Model

<table>
<thead>
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<th>Predictors</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td>1.5</td>
<td>0.7-3.5</td>
<td>0.3289</td>
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<tr>
<td>PR</td>
<td>0.8</td>
<td>0.3-1.8</td>
<td>0.5600</td>
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<tr>
<td>ERBB2</td>
<td>1.7</td>
<td>0.8-3.8</td>
<td>0.1620</td>
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<tr>
<td>HOXB13:IL17BR</td>
<td>7.3</td>
<td>2.1-26.3</td>
<td>0.0022</td>
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HOXB13:IL17BR is Highly Predictive of Outcome in Patients Treated with Tamoxifen

Frozen tissue Training Set
Accuracy = 81%

Paraffin Test Set
Accuracy = 80%

Simple Two-Gene PCR Assay using Routine Clinical Breast Cancer Tissues

22,000 Gene Microarray

HOXB13:IL17BR, Validation

t-test $P=0.024$

Disease-free survival: Validation
log-rank $P=0.0018$
Independent Validation of Two-Gene Signature in a Randomized Clinical Trial (Mayo Clinic)

Accuracy = 78.4%

Sgroi et al. ASCO 2004
HOXB13 expression and tumor progression

Relative quantitative HOXB13 gene expression values in normal (N, n=45), DCIS (n=42) and IDC (n=29) cases. Error bars denote 95% confidence intervals.

In situ hybridization of HOXB13 mRNA. DIG11UTP-labeled RNA probes with anti-sense hybridization to human breast epithelium of (i) the normal terminal duct lobular unit (200x magnification), (ii) ductal carcinoma in situ (400x magnification) and (iii) invasive ductal carcinoma (400x magnification), and sense probe hybridization to (iv) invasive ductal carcinoma (400X magnification). Inserts represent select regions of each field at 1000x magnification. L, S, and T denote lobule, stroma and tumor, respectively.
HOXB13 Induces EMT in a Non-Transformed Human Mammary Epithelial Cell Line (MCF10A)
HoxB13 enhances EGF-stimulated migration... and invasion through EHS
HoxB13 Enhances Migration in Cells Expressing ErbB2

Migration Result cells/20X field (50,000 cells/well)

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<thead>
<tr>
<th></th>
<th>AN10-pBabe</th>
<th>AN10-HoxB13</th>
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</thead>
<tbody>
<tr>
<td>AM</td>
<td>4.6</td>
<td>221.6</td>
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<tr>
<td>AP1510</td>
<td>5.8</td>
<td>425.33333333</td>
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<tr>
<td>EGF</td>
<td>52.4</td>
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3D Cell Culture of Epithelial Acini

Introduction of Cells into 3-D Culture System

Mammary Epithelial Cells

Extracellular Matrix Containing Growth Factors, Structural Proteins, Other

Proliferation and Organization

Proliferation

Matrigel

EGF + serum

Growth arrest ~ day 15

Apoptosis ~ day 8

Growth arrest ~ day 15

Matrigel

EGF + serum

Illustrations courtesy of Cassio Lynm, JAMA

Polarization Selective Apoptosis

Acinar Structures

Lumen

Illustrations courtesy of Cassio Lynm, JAMA
3-D Mammary Morphogenesis Assay

ErbB2 +pBabe

Day 8
100X

ErbB2 +HOXB13

ErbB2 +pBabe

Day 22
100X

ErbB2 +HOXB13
Summary

• Microarray-based gene expression profiling is a robust technology for biomarker discovery
• We discovered a novel two-gene expression ratio (HOXB13:IL17BR) that predicts tumor recurrence in node negative breast cancer patients treated with adjuvant tamoxifen monotherapy
• The predictive utility of the signature was demonstrated in two independent cohorts.
• Using a microarray discovery approach we not only identified a novel biomarker, but also a putative functional target in human breast cancer.
Overall Summary

• Microarray-based gene expression profiling is a robust technology for biomarker discovery.

• Real-time quantitative PCR-based biomarkers are readily assessed using standard pathological specimens and can be easily implemented as clinical assays.

• The predictive utility of the different breast cancer signatures should be compared to each other using a common clinical cohort.
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Issues to be addressed before clinical implementation

• Demonstration that these signatures are independent of known clinicopathological parameters.
  – Does the signature improve upon existing predictive biomarkers?
  – Is the signature a mere molecular equivalent of a known biomarker?

• Validation of signature in multiple independent cohorts from different external sources.
  – What is the correct cohort size?
  – What is the minimum follow-up time?
Issues to be addressed before clinical implementation

- Demonstration of reproducibility and standardization.
  - Can clinical labs readily implement this assay?
  - Can one use routine clinical specimens (formalin fixed paraffin embedded) in a reproducible manner?
Other Considerations

• Need for head to head comparison of different signatures in an identical clinical cohort.

• Need to identify treatment predictive signatures.
The Future
Technical Disconnect Between Biopsy Preservation and Gene Expression Microarray Analyses

• Methodologies for gene expression microarrays require RNA from frozen tissue.

• Millions of biopsies are currently stored in hospitals/laboratories but majority are in paraffin blocks and formalin-fixed.
Potential Advantages of Using FFPE Tissues with Microarray Technologies

• The use of archived samples from retrospective clinical trials with well-documented clinical follow-up will accelerate the discovery of potentially useful clinical gene expression signatures.

• Microarray analysis of samples from prospective clinical trials will not require special handling and storage of tissues.
Can one perform microarray gene expression analysis using RNA derived from FFPE tissues?

If so, are the data reproducible and how do the data compare to that generated with RNA derived from fresh tissue?
Reproducibility of Microarray Data Using FFPE Tissue Samples

- Reproducibility on FFPE tissue samples are nearly identical to that of frozen tissue samples.
Comparison of FFPE Microdissected With Frozen Microdissected Tissue

Genes present on both chips

$r = 0.937$
$genes = 16979$
Is it possible to extract an estrogen receptor-associated gene expression signature from FFPE breast cancer tissues?
Signature Discovery with FFPE Breast Cancer Biopsies

Experimental Design:

\[
\begin{align*}
&9 \text{ ER}^+ \text{ Tumors} \\
&8 \text{ ER}^- \text{ Tumors} \\
\end{align*}
\] 1990-2003

- Extract, Isolate and Amplify mRNAs
- From Single 7um Sections
- Hybridize labeled samples to X3P microarray
- Extract Estrogen Receptor Signature
Extracting Signatures from FFPE Tissues

Clustering of 165 ER signature genes on Agilent chips for 17 cases. ER+ cases were labeled red, ER- yellow. Blue arrows are samples with less intact mRNAs.
Overall Summary

• Microarray-based gene expression profiling is a robust technology for biomarker discovery.
• This technology can be readily applied to surgical pathology and cytopathology specimens.
• Several promising prognostic gene expression signatures have been recently identified and these signatures should be further validated in prospective randomized clinical trials.
• Future application of these technologies to the appropriate clinical cohorts should allow for the identification of treatment-predictive biomarkers.
NKI Study Design

78 Sporadic breast tumors:
Untreated patients <55 years
tumor size < 5cm
lymph node negative (LN0)

Distant metastases
<5 years

No distant metastases
<5 years

Gene Expression Profiling: Novel Signature Discovery

The NKI 70-gene Prognosis Signature

Genes associated with poor outcome  Genes associated with good outcome

Patients with good outcome

Patients with poor outcome

83% Accuracy

Subgroup Analysis of NIH High and Low Risk Patients Using 70-Gene Prognosis Signature

The high risk group defined by NIH criteria included many patients who had a good-prognosis signature. Conversely, the low-risk group identified by NIH criteria included patients with a poor-prognosis signature.

Subgroup Analysis: St. Gallen High and Low Risk Patients Using 70-Gene Prognosis Signature

Summary of NKI Study

- The NKI 70-gene signature demonstrated the feasibility and potential usefulness of gene expression in clinical treatment decision-making process in breast cancer.

- The 70-gene signature is a more powerful predictor of outcome in pre-menopausal breast cancer patients than standard systems based on clinicopathological criteria.

- The prognosis signature is superior to the NIH and St Gallen criteria for substratifying patients.