Targeted Molecular Diagnostics for Targeted Therapies in Hematological Disorders

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Overview of Presentation

• CML: Targeting the causative molecular abnormality (BCR-ABL) with specific tyrosine kinase inhibitors (imatinib, etc)
• Monitoring TKI treatment with a targeted diagnostic: BCR-ABL RQ-PCR
• Clinically-relevant RQ-PCR thresholds
• Loss of response & resistance mutations
  • Both predicted by rising BCR-ABL RNA
• PCR assay standardization
  • How to realize the international scale
• Paradigm for other cancers: more genes, more drugs, more complexity
Chronic Myeloid Leukemia (CML)

- Clonal myeloproliferative disorder of pluripotent stem cells
  - ↑ proliferation, ↓ apoptosis
  - Cytogenetic hallmark: Ph chromosome
  - Molecular hallmark: Bcr-Abl
  - Bcr-Abl overexpression is causative event
- 7% to 15% of adult leukemias
- Median age @ diagnosis: 55 y
The Cytogenetic Hallmark of CML is the Philadelphia Chromosome (Ph)

22q- = Philadelphia chromosome (Ph)
BCR-ABL: The New Paradigm for the Ideal Molecular Therapeutic Target

- Causative molecular abnormality of CML
- Sole oncogenic event early in the disease
- Leukemic clone dependent on Bcr-Abl for survival
Targeted Leukemia Therapy: Imatinib
Targeted CML Therapy with Imatinib

BCR-ABL

ATP

Substrate

Tyrosine

Phosphate

ADP

Chronic myelogenous leukemia

Imatinib

Substrate

Tyrosine

Chronic myelogenous leukemia
**TKI’s are the recommended front-line therapy for CML**

<table>
<thead>
<tr>
<th>Year</th>
<th>Total</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>1965-1975</td>
<td>123</td>
<td>122</td>
</tr>
<tr>
<td>1975-1981</td>
<td>132</td>
<td>127</td>
</tr>
<tr>
<td>1982-1989</td>
<td>365</td>
<td>266</td>
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<tr>
<td>1990-2000</td>
<td>960</td>
<td>357</td>
</tr>
<tr>
<td>Imatinib</td>
<td>276</td>
<td>14</td>
</tr>
</tbody>
</table>

**Proportion Surviving Years From Referral**

93% progression-free survival (to AP/BC) after 5 years of IM

The University of Texas M. D. Anderson Cancer Center database
The Ideal Cancer Biomarker
CML (BCR-ABL) as paradigm

- Specific (diagnostic) for the cancer cell (or cancer-causing molecule)
- Easily collected sample (blood)
- Fast, ultra-sensitive, precise lab measurement
- Quantitative levels predict:
  - Prognosis
  - Response to therapy
  - Early, impending relapse
Sensitivities Of CML Minimal Residual Disease Monitoring

Diagnosis: $10^{12}$ Leukemia cells

100%
- Blood counts
- Complete hematologic response

10%
- Cytogenetics
- Complete cytogenetic response

1%
- PCR
- Major molecular response

0.1%
- Undetectable range
- Complete molecular response
Bcr-Abl RNA Quantification by Real-Time Quantitative RT-PCR (RQ-PCR)

- 6 log linear dynamic range
- Limit of detection ~5 logs below “baseline” (diagnosis) levels (IS scale)
- Precision: 95% CI ~3-fold (0.5 log)
- G6PDH reference gene
The View from 30,000 Feet (ie, practical clinical utility)

• Do bcr-abl RNA levels predict clinical outcomes?
  • Disease progression
  • Evolving imatinib resistance
  • Survival

• If so, what is the target level for bcr-abl RNA that predicts good outcomes?
Achievement of a 3-log MMR Predicts Longer PFS

Hazard ratio = 7.3; 95% CI, 2.8 - 19  
$p < 0.0001$

## ELN Consensus Guidelines: Therapeutic Milestones (as defined by the lab)

<table>
<thead>
<tr>
<th>Month</th>
<th>Optimal</th>
<th>Suboptimal</th>
<th>Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>CHR</td>
<td>N/A</td>
<td>&lt;CHR</td>
</tr>
<tr>
<td>6</td>
<td>&lt;35% Ph+</td>
<td>Ph+ &gt;35%</td>
<td>&gt;95% Ph+</td>
</tr>
<tr>
<td>12</td>
<td>CCR (0% Ph)</td>
<td>Less than CCR</td>
<td>&gt;35% Ph+</td>
</tr>
<tr>
<td>18</td>
<td>MMR (&lt;0.1% IS)</td>
<td>Less than MMR</td>
<td>Less than CCR</td>
</tr>
</tbody>
</table>

Baccarani et al on behalf of the ELN. J Clin Oncol. 2009;27(35):6041-51
## Therapeutic Milestones

<table>
<thead>
<tr>
<th>Month</th>
<th>Optimal</th>
<th>Suboptimal</th>
<th>Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Favorable outcome likely: keep going!</td>
<td>Favorable outcome uncertain: consider alternative!</td>
<td>Favorable outcome unlikely: change strategy!</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
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Baccarani et al on behalf of the ELN. J Clin Oncol. 2009;27(35):6041-51
What does “PCR-undetectable” mean?

How Low Can You Go?

Translation:

My assay is better than yours…
PCR Negativity (CMR) Predicts Prolonged PFS


49 month median IM follow-up

“CMR” restricted to samples with sensitivity > 4.7 logs (on IS)

Hazard ratio = 11  
\(p = 0.0052\)
If low levels of MRD are good, then are rising BCR-ABL levels bad?

If so, what amount of rise is worrisome?
A Half-Log (3.2–fold) RQ-PCR Rise is a Risk Factor For Future Relapse

49 month median follow-up

No RQ-PCR rise (N=48)

≥0.5 log RQ-PCR rise (N=42)

Hazard ratio = 4.9

p = 0.0017

TKI Resistance: BCR-ABL Mutations

4 mutation “hot spots”:

- P-loop
- IM bind
- Cat
- Activation

KD mutations predict a poor prognosis (now often overcome with other TKI’s)

Progression-Free Survival (%)

Months After Mutation Screen

No mutation (n=67)
IM-treated pts
KD mutation (n=34)

P < 0.0001
Hazard ratio = 3.2

A 2.6-fold BCR-ABL Rise Optimally Predicts a Concomitant KD Mutation (ROC analysis)

$J_{\text{max}}$ (2.6-fold)

2-fold

3-fold

5-fold

10-fold

N=150 pts with mutation screening
2.6-fold BCR-ABL rise was optimal by ROC
97% NPV (3% with mutation but no transcript rise)

Summary

• BCR-ABL RNA levels are a sensitive predictive biomarker of the response of CML to targeted therapy

• Clinically-proven prognostic thresholds include:
  • 3-log drop (MMR; 0.1% on the IS)
  • PCR-negativity (CMR)
  • Half-log rise during CCR
  • Presence of kinase domain mutations
  • Optimized threshold for mutation screening = >2.6-fold rise

• But can labs reliably measure this RNA?
Why Do We Need BCR-ABL Standardization?

CAP MRD-A (2010)
N=102 labs tested blinded samples
1/10,000 diluted K562 cells (close to 0.1% IS=MMR)
Labs report: Log-drop relative to undiluted K562 cells

Because the inter-lab distribution of results for any given sample is HUGE!

Here are the statistics:

<table>
<thead>
<tr>
<th>BCR-ABL Log Drop</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>2.8</td>
</tr>
<tr>
<td>Median</td>
<td>2.6</td>
</tr>
<tr>
<td>Range</td>
<td>-1.4 to 5.3 (&gt;6 logs)</td>
</tr>
<tr>
<td>Std dev</td>
<td>0.99 logs (9.7-fold)</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.83 to 4.7 (3.9 logs)</td>
</tr>
</tbody>
</table>
BCR-ABL Standardized Reporting

• IRIS trial defined 3-log drop from median baseline (MMR) as a good prognostic threshold

• MMR is an accepted therapeutic goal, now defined (by consensus) as 0.1% “international scale” (IS)
  • Baseline pretreatment level (CP-CML) = 100% IS (median)

• Until recently, no reference materials existed with a defined IS value (WHO standard recently available)
OHSU Conversion Factor Determination
N=29 shared samples

Conversion Factor = 2.22 (anti-log bias)

IS units (OHSU) = (BCR-ABL ratio) * 2.22

MMR level at OHSU (0.1% IS) = 0.045%

Before Conversion:
- 13/29 within 2-fold
- 18/29 within 3-fold
- 26/29 within 5-fold

Bias = 0.35 logs (P<0.001)

After Conversion:
- 15/29
- 27/29
- 29/29

No Bias
Conversion of BCR-ABL RQ-PCR to Standardized International Scale by inter-lab sample exchanges

Freeze dried K562 cells (b3a2) diluted at 4 levels into HL-60 cells

Nominal IS % ratio = average value from 10 different IS-standardized labs

Limited availability (3500 vials; each 1.5M cells)
Stable, well-characterized, locally-available secondary standards will be useful for:

• Easier, more widely available establishment of standardized IS-based reporting
• IS-based reporting is required for assessment of MMR, a consensus and actionable treatment goal
• Standardize the definition of the low-level detection limit for “undetectable” samples
  □ Current sample-exchange method for conversion to IS is impractical
  □ Only 10 of 136 (7%) US labs currently use IS-scale reporting [CAP survey MRD-A(2011)]
  □ So how do most labs assess treatment response goals (ie, MMR)?
Patient MK

BCR-ABL RQ-PCR (IS)(%) vs. Months on Imatinib

- CCR
- MMR

Data points:
- 0 months: 110
- 3 months: 9
- 6 months: 0.8
- 9 months: 0.26

Graph shows a downward trend in BCR-ABL RQ-PCR (IS)(%) over time.
Patient MK

BCR-ABL RQ-PCR (IS)(%)

Months on Imatinib

CCR

4-fold↑

MMR

WT

G250E (minor)
(not ordered)
Patient MK

Dasatinib or Nilotinib?

G250E (no WT)

CCR & CHR lost
RELAPSE
Low-Hanging Fruit: CML & BCR-ABL
Other Cancers will be MUCH harder
Cancer Genotyping for Personalized Cancer Therapy

Receptor tyrosine kinases

Finding drug targets

- SHC
- GRB2
- SOS
- NRAS
- KRAS
- BRAF
- MEK
- ERK
- STAT
- PI3K
- PDK
- AKT
- mTOR
- PTEN
- S6K
- mTOR
- p53

Drugs:
- Erlotinib
- Lapatinib
- PF299804
- Afatinib
- Imatinib
- RAF265
- Vemurafenib
- AZD6244
- PD0325901
- ARRY162
- BRAF
- MEK
- ERK
- STAT
- PI3K
- PDK
- AKT
- mTOR
- PTEN
- S6K
- p53

Genes:
- ALK
- p53

Other molecules:
- BEZ235
- BKM120
- BGT226
- BYL719
- MK2206
- SR13668
Mass Spectrometry-Based Detection of Genomic Mutations

PCR targets of interest
~ 100 bp amplicons

Clean-up steps

Primer extension reaction

De-salting step

MALDI-TOF Mass spectrometry
Mass Spec Leukemia Panel: 370 mutations / 31 genes

<table>
<thead>
<tr>
<th>ABL</th>
<th>FLT3</th>
<th>KRAS</th>
</tr>
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<tbody>
<tr>
<td>AKT1</td>
<td>FMS</td>
<td>MET</td>
</tr>
<tr>
<td>AKT2</td>
<td>GATA1</td>
<td>MPL</td>
</tr>
<tr>
<td>AKT3</td>
<td>HRAS</td>
<td>NOTCH1</td>
</tr>
<tr>
<td>BRAF</td>
<td>IDH1</td>
<td>NPM1</td>
</tr>
<tr>
<td>CBL</td>
<td>IDH2</td>
<td>NRAS</td>
</tr>
<tr>
<td>CBLB</td>
<td>JAK1</td>
<td>NTRK1</td>
</tr>
<tr>
<td>FBXW7</td>
<td>JAK2</td>
<td>PAX5</td>
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<td>FES</td>
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<td>PDGFRB</td>
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<td>FGFR4</td>
<td>KIT</td>
<td>PTPN11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SOS1</td>
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Mutation Spectrum in AML (108 OHSU cases)

Normal cytogenetics: 78% mutation frequency
Abnormal cytogenetics: 43% mutation frequency

Mutation discovery with 31-gene mass spec panel plus single gene in-del assays (FLT3, CEBPA, KIT)

J Dunlap, in press
Next Generation (Massively Parallel) Sequencing?

- Broader coverage, including tumor suppressors
- Better sensitivity, through deeper reads
- Lower costs, through multiplexing
- More comprehensive cancer genome characterization for targeted therapeutic, prognostic, (diagnostic) discovery

Single gene assays | Multiplexed hotspots | Multigene panels | Whole exome | Whole genome
739 hotspots covered by 190 amplicons
Single tube amplification
Average amplicon length: 119 bp (100-169 bp)
Input DNA: 10 ng (Fresh or FFPE)
Turn-around time: 48 hours
46 genes:

ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNAS, HNF1A, HRAS, IDH1, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL
### The Future: NGS Disease-Specific Gene Panels

<table>
<thead>
<tr>
<th>Cancer Site</th>
<th>Target genes</th>
<th># Exons</th>
<th>Kilobases</th>
<th>Ampli-cons</th>
<th>New Genes (not in Ampliseq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>23</td>
<td>224</td>
<td>34.3</td>
<td>502</td>
<td>9</td>
</tr>
<tr>
<td>Colon</td>
<td>16</td>
<td>157</td>
<td>31.8</td>
<td>405</td>
<td>6</td>
</tr>
<tr>
<td>Melanoma</td>
<td>21</td>
<td>113</td>
<td>13.9</td>
<td>231</td>
<td>9</td>
</tr>
<tr>
<td>AML / ALL / MDS</td>
<td>42</td>
<td>342</td>
<td>62.6</td>
<td>863</td>
<td>28</td>
</tr>
</tbody>
</table>

- Ion Torrent custom primer design software used to design primers for library prep
- Proprietary primer modifications allow massive multiplexing: 2-4 PCR reactions per library prep
Challenges for Targeted Molecular Diagnostics

• Assay Standardization
• Gene Patents (restricted licensing)
• Costs & Reimbursements
• Regulations
  • LDT’s vs IVD’s
• Quality Control (proficiency testing)
Acknowledgements

**Press Lab**
Carole Rempfer
Rui Yang
Ashlie Tronnes
Zac Love
Chad Galderisi
Jennifer Laudadio
Fei Yang

**Collaborators**
Brian Druker
Mike Deininger
Mike Mauro
Chris Corless
Carol Beadling
Oregon Health & Science University