Present an Educational Webinar
BCR-ABL Testing and IS Standardization
Thursday, March 31, 2011 ● 10:00 am PST

Featuring
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BCR-ABL RQ-PCR for Monitoring Leukemia Treated with Tyrosine Kinase Inhibitors: Clinical Interpretation & Assay Standardization

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Overview of Presentation

• CML: Targeting the causative molecular abnormality (BCR-ABL) with a specific inhibitor (imatinib)

• Monitoring treatment with a targeted diagnostic: BCR-ABL RQ-PCR

• Clinically-relevant RQ-PCR molecular response thresholds

• Loss of response & resistance mutations
  - Both predicted by rising BCR-ABL RNA

• PCR assay standardization
  - How to realize the international scale

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

Chronic myelogenous leukemia
The New Cancer Treatment Paradigm

Imatinib (Gleevec)

- A selective tyrosine kinase inhibitor of
  - KIT
  - Bcr-Abl
  - PDGFR-A/B


\[ C_{29}H_{31}N_7O\cdot CH_3SO_3 \]
Molecular weight 589.7

- Class: Phenylaminopyrimidines
Targeted CML Therapy with Imatinib

Imatinib is the recommended front-line therapy for CML

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

The University of Texas M. D. Anderson Cancer Center database.
Measuring the Imatinib Response

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Threshold</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Blood / bone marrow</td>
<td>Complete Hematologic Response (CHR)</td>
<td>??</td>
</tr>
<tr>
<td>Cytogenetics</td>
<td>Ph chromosome</td>
<td>Complete Cytogenetic Response (CCR)</td>
<td>1: 20</td>
</tr>
<tr>
<td>FISH</td>
<td>Bcr-abl fusion gene</td>
<td>n/a</td>
<td>1: 100 - 1: 500</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Bcr-Abl RNA</td>
<td>Major Molecular Response (MMR; 3 log)</td>
<td>1:10^5 - 1:10^6</td>
</tr>
</tbody>
</table>

The Ideal Cancer Biomarker

- 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
- CML (BCR-ABL) as paradigm
New Targeted Drugs Require More Sensitive Biomarkers to Monitor Treatment

- IM induces a complete cytogenetic response (no detectable Ph) in 87% of pts (5 yrs)
  - Cytogenetic assays not useful for measuring minimal residual disease (MRD) after CCR
- Requires a more sensitive BCR-ABL RT-PCR assay

Bcr-Abl RNA Quantification by Real-Time RT-PCR

- Control Gene: several choices (control for RNA integrity)
- Primers: p210, p190 (single- or multi-plex?)
- Probes: FRET, Taqman, others
- Quantitation calculations: calibration curve vs Delta-Ct
- Wide variability in methods
Reference Genes for BCR-ABL Quantitation
CAP Survey 2010 MRD-A
N = 119 labs

- ABL1: 57%
- G6PD: 20%
- BCR: 8%
- GUSB: 5%
- GAPDH: 4%
- Others: 5%

From Suzanne Kamel-Reid

Reference Genes for BCR-ABL RQ-PCR
CAP 2010 MRD-A-01/03
Expected Log Reduction = 4.0 (1/10,000 dilution)

G6PDH closest to expected value
ABL1 furthest from expected (cross-reacts with BCR-ABL)

From Suzanne Kamel-Reid
BCR-ABL RQ-PCR Analytical OHSU Performance Parameters

- 5 log linear dynamic range
- Limit of detection ~5 logs below “baseline” (diagnosis) levels
- Precision: 95% CI ~3-fold (0.5 log)
- Reported as BCR-ABL/G6PDH ratio (%) & as log-drop from baseline on the international scale (IS)
  - IS units calibrated to 3-log major molecular response level as determined in the pivotal IRIS trial
  - MMR defined as 0.1% IS (baseline = 100%)

“Undetected” BCR-ABL (OHSU)

- A sample is never “negative”, only “undetectable” (to what sensitivity?)
- Sensitivity cutoff of 4.7 logs (IS) for defining “complete molecular response”
  - Defined as the 10th percentile of sensitivity values among all referred samples (ie, 90% of samples had SENS > 4.7)
- Nested PCR (x3) on all samples with RQ-PCR = “undetected” (n=967)
  - 66% (n=634) “negative” by nested PCR (x3)
  - 24% of nested-negative samples with sensitivity < 4.7 (not defined as “CMR”)
  - 44% of nested-positive samples with signals in only 1 of 3 replicates
Bcr-Abl RNA Levels: Bone Marrow vs Peripheral Blood

- Slope = 0.99
- R = 0.95 (P < 0.0001)
- N = 59
- No significant bias between compartments

The Big Picture

- 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 levels that predicts good outcomes?
IRIS Study: PFS by Extent of Molecular Response

Achievement of a 3-log MMR predicts excellent PFS

- No CCR within 12 mo (n=128)
- Reduction of <3 log (n=103)
- Reduction of ≥3 log (n=137) (MMR)

P = 0.007
P = 0.013

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

29 month median follow-up

Achievement of a 3-log MMR Predicts Longer PFS

Best Molecular Response

What does “PCR-undetectable” mean? (How Low Can You Go?)

PCR Negativity (CMR) Predicts Prolonged PFS

- Hazard ratio = 11
- p = 0.0052


“CMR” restricted to samples with sensitivity > 4.7 logs (on IS)
Summary: Prognostic Value of BCR-ABL RQ-PCR

- 3-log drop (MMR) = excellent prognosis (100% progression-free at 5 yrs)
- Failure to achieve MMR @ 18 mo = “suboptimal response”
- PCR-negative (CMR) = excellent prognosis (better than MMR)
- Assay standardization (to IS) remains problematic without reference material

If low levels of MRD are good, then are rising BCR-ABL levels bad?

If so, what amount of rise is worrisome?
Imatinib Resistance

- Significant minority of IM-treated pts eventually become resistant and relapse
- ABL kinase domain mutations are the most common mechanism
  - Abrogate IM binding & re-activate kinase
  - Effective alternative TKI’s available for many mutants (not T315I)
- Can RQ-PCR predict early loss of response & mutations, before frank relapse?

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

4 mutation “hot spots”:

- P-loop
- IM bind
- Cat
- Activation


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

What is the Optimal Rise in BCR-ABL RNA that Should Trigger a Kinase Domain Mutation Screen?

- **2-fold increase?**
  Branford, Blood 104:2926, 2004
  Is a 2-fold change analytically discernable in labs?

- **3.2-fold (half-log) increase?**

- **5-fold increase?**
  NIH consensus group: Hughes, Blood 108:28, 2006

- **10-fold increase?**
  Hughes, Blood 108:28, 2006 ("loss of response")
  NCCN practice guidelines

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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:
  - 2-log drop at time of CCR
  - 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

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**Why Do We Need BCR-ABL Standardization?**

- CAP MRD-A (2010)
  - N=104 Labs
  - 1/10,000 diluted K562 cells

<table>
<thead>
<tr>
<th>Metric</th>
<th>Value</th>
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<tbody>
<tr>
<td>Mean</td>
<td>-2.5</td>
</tr>
<tr>
<td>Median</td>
<td>-2.5</td>
</tr>
<tr>
<td>Range</td>
<td>0.3 to -4.8</td>
</tr>
<tr>
<td></td>
<td>(&gt;5 logs)</td>
</tr>
<tr>
<td>Std dev</td>
<td>0.89 logs</td>
</tr>
<tr>
<td>95% CI</td>
<td>-4.2 to -0.78</td>
</tr>
</tbody>
</table>

Because the inter-lab distribution of results for any given sample is HUGE!
BCR-ABL Standardized Reporting

• IRIS trial defined 3-log drop from median baseline (MMR) as an excellent prognostic threshold
• MMR has become an established 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/calibrators existed with a defined IS value

BCR-ABL IS Reporting

• Without IS-standardized reference materials, IS-validated reporting units can only be defined by laborious sample exchanges with IRIS-participating labs
• IRIS labs (Adelaide, London, Seattle), by definition, are already aligned to the IS
• Sample exchanges allow determination of a “conversion factor” for each lab by:
  ■ Patient samples (~30, broad range of BCR-ABL’s) dually tested by field lab and IS reference lab
  ■ Bias between IS-reference value and field value (log-transformed) determined using Bland-Altman stats
  ■ Anti-log of bias = conversion factor (CF)

IS units = (lab-specific ratio) * CF
OHSU BCR-ABL Ratio (log)

OHSU Conversion Factor Determination
N=29 shared samples

Before Conversion:
13/29 within 2-fold
18/29 within 3-fold
26/29 within 5-fold
Bias = 0.35 logs (P<0.001)

Conversion Factor = 2.22 (anti-log bias)
IS units (OHSU) = (BCR-ABL ratio) * 2.22
MMR level at OHSU (0.1% IS) = 0.045%

OHSU BCR-ABL IS (log)

OHSU Conversion Factor Validation
N=29 shared samples

After Conversion:
15/29 within 2-fold
27/29 within 3-fold
29/29 within 5-fold
Bias = 0.025 logs (1.06-fold) (P=.69)
No significant bias

Validated CF = 2.22
Conversion of BCR-ABL RQ-PCR to Standardized International Scale


Establishment of the first World Health Organization International Genetic Reference Panel for quantitation of BCR-ABL mRNA


blood 2010 116; 111-117
Prepublished online Aug 18, 2010;
doi:10.1182/blood-2010-06-291641
What is the WHO Primary Standard?

- Freeze dried K562 cells (b3a2) diluted at 4 levels into HL-60 cells
- Nominal IS % ratio = average value from 10 different IS labs
- Limited availability (3500 vials; each 1.5M cells)

WHO BCR-ABL reference material:
IS-assigned values

<table>
<thead>
<tr>
<th>Sample</th>
<th>ABL</th>
<th>BCR</th>
<th>GUS</th>
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<tbody>
<tr>
<td>08/198</td>
<td>10.7</td>
<td>16.3</td>
<td>10.1</td>
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<tr>
<td>08/196</td>
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<td>08/194</td>
<td>0.11</td>
<td>0.17</td>
<td>0.07</td>
</tr>
<tr>
<td>08/192</td>
<td>0.012</td>
<td>0.019</td>
<td>0.007</td>
</tr>
</tbody>
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**Commercial IS (WHO)-Traceable Secondary Reference Material**

- Primary WHO reference standard NOT available to individual labs or for inclusion in reagent kits
- Primary standard ONLY available to entities (ie, IVD/kit manufacturers) developing *secondary reference materials* for subsequent commercial distribution
- This secondary reference material is ideally:
  - In a biologically relevant matrix (ie, requires extraction, not just RT-PCR)
  - Produced in large lots with stable storage
  - Assigned a BCR-ABL quantitative value that is directly traceable to the WHO primary reference standard
  - Available at multiple concentrations, one being near the clinically-relevant 0.1% IS threshold

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**Reference Material Traceability**

![Diagram showing the traceability of reference materials from primary to secondary and finally to laboratories.](diagram.png)
Asuragen BCR-ABL Secondary Reference Material (RUO)

- Made from synthetic Armored RNA:
  - Analogous armored RNA controls have been used in FDA-approved kits for quantitating viral load of RNA viruses
  - Manufactured under cGMP
  - Traceable to the WHO BCR-ABL primary reference material
  - Nuclease resistant, stable, and extractable
- 4-level panel for b3a2 & b2a2 (~1 log below MMR to ~2 logs above MMR) and a BCR-ABL1 negative sample
- Tested with an Asuragen assay standardized to the IS by sample exchange
- Field trial (prototype 1) conducted in 2007/2008 by 29 laboratories
- International survey sent to 150 labs in 2009 to gather feedback and finalize panel design (89% of respondents approved the proposed design)
- Reference Panel is currently being evaluated by IS Reference Laboratories worldwide
- Not yet commercially available

Ipsogen Secondary Reference Material (IS-MMR calibrator)

- Composed of extracted RNA from a cell line mixture
- Calibrated and traceable to the WHO primary reference material
- Provided at a single “assigned value” of ~0.1% IS (as determined by Ipsogen’s kit)
- Field-tested in 2010 by 14 labs (abstract in preparation)
- Currently available within the Ipsogen RUO BCR-ABL kit (not packaged separately)
Other Commercial Vendors

• Cepheid: Intending to build an IS conversion factor into their planned FDA-approved assays

• Roche: no immediate plans for IS alignment

• Others?

Stable, well-characterized, locally-available secondary standards will be useful for:

• Easier, more widely available establishment of standardized IS-based reporting for labs that have not participated in sample exchanges with IRIS-validated labs
• Routine monitoring of quantitative drift for assays already aligned with the IS
• Routine monitoring of assay performance, including accuracy, linearity, calibration verification (as required by CAP), & verification of clinically-relevant MMR levels
• Potential standardization of reporting the low-level detection limit for “undetectable” samples
• Long-term goal is universal IS-based reporting in all labs for improved patient care
• Cost?

Current sample-exchange method is laborious and impractical
## Acknowledgements

### Press Lab
- Carole Rempfer
- Rui Yang
- Ashlie Tronnes
- Zac Love
- Chad Galderisi
- Jennifer Laudadio

### Collaborators
- Brian Druker
- Mike Deininger
- Mike Mauro