Molecular Monitoring of CML: International Standardization of BCR-ABL Quantification

Y. Lynn Wang, MD, PhD, FCAP
Pathology and Laboratory Medicine
Weill Cornell Medical College
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Levels of BCR-ABL Transcript Decrease Over Treatment Course


Terminology-NCCN (Version 1.2013)

- Major molecular response (MMR): BCR-ABL/Control gene ≤ 0.1% on the International Scale (≥3 log reduction)
- Complete molecular response (CMR): Undetectable BCR-ABL mRNA by quantitative PCR (assay sensitivity 4.5 logs below the standardized baseline)

Prognostic Significance of Molecular Responses-IRIS EFS Data

- Better event-free survival (EFS) is associated with a BCR-ABL level of:
  - ≤10% at 6 mo
  - ≤1% at 12 mo
  - ≤0.1% (MMR) at 18 mo

Prognostic Significance of Molecular Responses-IRIS PFS Data

- Lower probability of progression to AP/BP is associated with a BCR-ABL level of:
  - ≤10% at 6 mo
  - ≤1% at 12 mo
  - ≤0.1% (MMR) at 18 mo


Prognostic Significance of Molecular Responses-Summary

- Achieving MMR is a clinically significant event
  - IRIS trial: Compared with patients without MMR, patients who had MMR at the 18-month landmark exhibited a significantly superior EFS rate and lack of progression to AP/BP at 7 years (Blood. 2010, 116:3758-65).
  - ENESTnd trial (nilotinib): At 12 month, the rate of MMR for nilotinib was twice that for imatinib (NEJM 2010, 362, 2251-9; Leukemia. 2012 May 18. Epub).
  - DASISION (dasatinib): At 18 month, the rate of MMR for dasatinib was 1.84 fold of that for imatinib (NEJM 2010, 362, 2260-70).

Prognostic Significance of Molecular Responses-Summary

- The prognostic significance of CMR is not yet known.
  - If patients achieve CMR, are they cured? Can therapy be stopped?
  - Two clinical trials showed that 60% of CMR patients had molecular relapse in 4 months after imatinib cessation (Lancet Oncol 2010, 11, 1029-35).
  - To date, there is insufficient evidence that TKIs can be discontinued in clinical practice.

Indications for cytogenetics and RQ-PCR in patients receiving tyrosine kinase inhibitors

NCCN Version 1.2013 Practice Guidelines

<table>
<thead>
<tr>
<th>Indication</th>
<th>Testing</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>At diagnosis prior to therapy</td>
<td>RQ-PCR and BM cytogenetics</td>
<td>Every 3 months</td>
</tr>
<tr>
<td>If BM is not feasible, FISH on PB is acceptable</td>
<td>BM FISH, if BM is not available, FISH on PB is acceptable</td>
<td>Every 3 months</td>
</tr>
<tr>
<td>During therapy</td>
<td>BM cytogenetics</td>
<td>At 3, 12 and 18 months if CCyR is achieved</td>
</tr>
<tr>
<td>ABL kinase domain mutation analysis</td>
<td>BM cytogenetics</td>
<td>At 3, 12 and 18 months if CCyR is achieved</td>
</tr>
<tr>
<td>After complete cytogenetic response</td>
<td>BM cytogenetics</td>
<td>At 3, 12 and 18 months if CCyR is achieved</td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>BM cytogenetics to detect clonal evolution</td>
<td>As clinically indicated</td>
</tr>
<tr>
<td>BM FISH</td>
<td>BM FISH</td>
<td>BM FISH not recommended</td>
</tr>
<tr>
<td>Rising level of BCR-ABL transcripts</td>
<td>RQ-PCR in those with MMR BM cytogenetics in those without MMR ABL kinase domain mutation analysis</td>
<td></td>
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<tr>
<td>(1 log)</td>
<td>RQ-PCR in those with MMR BM cytogenetics in those without MMR</td>
<td>As clinically indicated</td>
</tr>
</tbody>
</table>

Imatinib Resistance and ABL Mutation Detection


Imatinib-Resistant ABL Kinase Domain Mutations

Lancet Oncol, 8, 1018, 2007
**FDA-approved second-line Therapies**

- Second Generation of TKI
- Allogeneic transplantation

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**RQ-PCR**

**Patient PB specimens**
- Cell preparation
- RNA preparation
- Reverse transcription into cDNA
- **RQ-PCR** (BCR-ABL and Internal Controls)
- (Standard Curves)
- (Reference Materials)
- Data Analysis and Reporting

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**RQ-PCR**

- RQ-PCR is technically challenging due to its multi-step nature. Different materials and methods may be used for each step with different instruments.
- Different units for reporting are used.

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**Oct 2006 CAP Survey**

- 34 labs reported data on two samples created with RNA from BCR-ABL containing K562 cell line and a normal individual
- **1st sample-mimic of untreated**

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**Laboratory Guidelines**

- NIH Consensus Meeting
  - October 25-26, 2005
  - Organized by Dr. John Goldman
  - ~30 participants from academic institutions and hospitals
- Guidelines are summarized in
  - Leukemia, 20, 1925-30, 2006

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**Oct 2006 CAP Survey**

- **2nd sample-mimic of MRD**

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RQ-PCR

Patient PB specimens
- Cell preparation
- RNA preparation
- Reverse transcription into cDNA
- RQ-PCR
  (BCR-ABL and Internal Controls)
  (Standard Curves)
  (Reference Materials)
- Data Analysis and Reporting

Laboratory Guidelines

• Sample requirement
  - 5-10 mL PB specimen or 10-20 million cells in EDTA-anticoagulated tubes
  - Specimens sent to labs in 24-36 hours
    - >50% RNA is degraded by 48 hours
  - Switching between PB and BM specimens are not recommended
    - In some patients, BCR-ABL levels in the bone marrow and peripheral blood are different.

Imatinib response

Branford et al, Leukemia, 20, 1925-30, 2006

Role of Internal Control

• Control genes in quantitative PCR
  - Internal caliber to control for RNA loss in both quantity and quality from the step of cell preparation to the step of PCR.
  - BCR-ABL/Control gene x100%

Criteria Used For Control Gene Evaluation

• EAC major criteria for inclusion
  - Absence of pseudogenes.
  - High or medium expression, excluding very high or low expression.
  - No significantly different control gene expression between normal PB samples and leukemic sample.
  - No significantly different control gene expression between PB and BM.
  - In a follow-up paper by EAC, stabilities of ABL, GUS, and B2M were studied.

ABL and GUSB are recommended

Leukemia. 2003,17(12):2474-86
### Criteria Used For Control Gene Evaluation

- Criteria in our studies
  - Express at similar levels to BCR-ABL
  - Exclude those with pseudogenes
  - Perform well on serial sample testing.
  - Perform as expected on an MRD model.
  - Degrade with similar kinetics as BCR-ABL.
  - Does not change significantly with cellular conditions.

### Control Gene Selection

- Compared 14 potential controls genes with 6 most relevant criteria
- Concluded that *GUSB* gene is the most appropriate control for CML monitoring

### Recommended Control Genes

- Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting *BCR-ABL* transcripts and kinase domain mutations and for expressing results
  - ABL, BCR and *GUSB* are recommended

### GUSB is the Most Suitable Control Gene

- *GUSB* is the most suitable control for *BCR-ABL* quantification

### CAP Surveys Demonstrated That *GUSB* And *BCR* Are The Most Suitable Control Gene
What is a CAP survey

What is Proficiency Testing

Updated June 25, 2007

The CAP Surveys and Anatomic Pathology Education/EXCLl Program are the largest laboratory peer comparison program in the world. The programs allow laboratories to regularly evaluate their performance and improve the accuracy of the patient results they provide. Through this program, the CAP provides individual laboratories with unknown specimens for testing. The participants analyze the specimens and return the results to the CAP for evaluation. In turn, each participating laboratory receives a report of their performance as well as a report summarizing the results of all participating laboratories.

How are surveys conducted?

• Sample preparation
  – RNA is isolated from cell lines using Trizol reagents
  – BCR-ABL-containing K562 RNA is diluted in HL-60 RNA at various defined ratios
  – RNA pellets in 100% EtOH are shipped to participating labs

2009 CAP Survey of 80 Labs that performs QBCR-ABL

Recommended Control Genes

• GUSB and BCR
  • ABL may be used when leukemia burden measured by cytogenetic is ≤10%

Reference Materials

• “Reference materials” are external controls with defined quantities.
  • These materials are key in standardization. They serve as a universal ruler that unifies measurements of BCR-ABL regardless of specific processes.
    – They allow different labs to calibrate their lab-specific measurements to common standards no matter what materials and what instruments they use to generate their values.
International Standardization

- Led by Nick Cross/John Goldman from UK and Susan Branford/Tim Hughes from Australia
- ~30 labs around the world including 3 from the US participated in the initial meeting at Bethesda in October of 2005
  - Define International Scale as new unit of BCR-ABL measurement
  - Two-arm study to convert local lab values to International Scale (IS)
    - Generation of laboratory-specific conversion factors
    - Creation of reference materials

Definition of International Scale

- Baseline: The mean BCR-ABL level of 30 diagnostic specimens used in IRIS Trial is defined as 100%.
- MMR: 3 log reduction from the baseline
- CMR: 4.5 log reduction from the baseline

<table>
<thead>
<tr>
<th>BCR-ABL/Ctl ratio (%)</th>
<th>100 (Baseline)</th>
<th>10</th>
<th>1</th>
<th>0.1 (MMR)</th>
<th>0.01 (CMR)</th>
<th>0.001</th>
</tr>
</thead>
</table>

International Standardization

- Two-arm study
  - Generation of laboratory-specific conversion factors
  - Creation of reference materials

International Standardization - Conversion Factor Method

- Led by Branford & Hughes
- Methodology
  - Establishment of a few reference labs that maintain materials with IS values
  - Sample exchanges between reference labs and individual testing labs
  - Results comparison and calculation of lab-specific conversion factors by the reference laboratories
  - Conversion of lab-specific BCR-ABL values to IS using the conversion factor

Before

After

Blood. 2008;112:3330-8

International Standardization

- Two-arm study
  - Generation of laboratory-specific conversion factors
  - Creation of reference materials
International Standardization-Reference Materials Method

- Led by Cross & Goldman
- Large-scale production of well-calibrated reference materials with defined BCR-ABL IS values
  - Primary reference materials—tested intensively, certified by WHO, and made in a limited quantity
  - Secondary reference materials—manufactured by commercial sources, calibrated to the primary references, and made in large quantity for purchase by individual testing labs.

International Standardization-Generation of Primary Reference Materials

- Four levels of primary reference materials would be generated
- Mixtures of wild-type and mutant-containing at defined ratios
  - Cells
  - RNA (unstable)
  - Chemically stabilized RNA—armored RNA
  - cDNA (not going through RT step)
  - plasmid DNA (not going through RT step)

International Standardization-Generation of Primary Reference Materials

- Cells and armored RNA were selected
  - They may control all steps of RQ-PCR including RNA extraction
  - Stable forms can be made either in the form of lyophilized cells or chemically protected RNA
  - They are compatible with widely used methods for BCR-ABL determination.
- Gabert and Cornell labs validated cell-based reference materials
- A commercial company has manufactured armored RNA

International Standardization-Generation of Primary Reference Materials

- Cell-based reference materials
  - Mixtures of K562 with 30 non-BCR-ABL cell lines were tested.
  - HL60 was selected as the cell line to be mixed with K562 since it resembles normal leukocytes.
  - Mixtures were generated with 10%, 1%, 0.1% and 0.01% BCR-ABL on the IS.
  - Multiple labs participated in the field trial.

Stability of the Reference Materials

Blood, 116, e111-7, 2010

Table 3: Percent BCR-ABL/control genes assigned to each reference material

<table>
<thead>
<tr>
<th>Reference Material</th>
<th>% BCR-ABL</th>
<th>% BCR-ABL</th>
<th>% BCR-ABL</th>
<th>% BCR-ABL</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCR-ABL, 1/6/100</td>
<td>0.0139</td>
<td>0.0195</td>
<td>0.0171</td>
<td>0.0171</td>
</tr>
<tr>
<td>BCR-ABL, 2/6/100</td>
<td>0.1112</td>
<td>0.1753</td>
<td>0.0754</td>
<td>0.0754</td>
</tr>
<tr>
<td>BCR-ABL, 3/6/100</td>
<td>1.1672</td>
<td>1.6527</td>
<td>0.8205</td>
<td>0.8205</td>
</tr>
<tr>
<td>BCR-ABL, 4/6/100</td>
<td>10.1468</td>
<td>16.0159</td>
<td>13.1235</td>
<td>13.1235</td>
</tr>
</tbody>
</table>

Blood, 116, e111-7, 2010
~3,500 vials of cell-based primary reference materials were made.

### International Standardization-Generation of Primary Reference Materials

- Primary reference materials have become available since 2010 through NIBSC (www.nibsc.ac.uk)
- Provided only to commercial companies with intention to manufacture secondary reference materials in large quantities.

### Summary

- Molecular response is prognostically significant. The information is used to determine whether patients obtain milestone responses at the appropriate time.
- Various techniques are used to monitor the levels of BCR-ABL.
- Guidelines have been developed to direct laboratory practice.
- International Scale has become the standardized reporting unit.
- Secondary reference materials will be available soon to aid the implementation of IS reporting.

### Acknowledgements

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