ULTRA-DEEP SEQUENCING OF THE BCR-ABL KINASE DOMAIN FOR BETTER THERAPEUTIC TAILORING OF PHILADELPHIA CHROMOSOME-POSITIVE LEUKEMIA PATIENTS

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BCR-ABL KD mutations

![Diagram of BCR-ABL kinase domain]

**1st generation**
- Imatinib
  - M237V
  - M244V
  - L248R
  - G250E/R
  - Q252R/H
  - Y253F/H
  - E255K/V
  - E258D

**2nd generation**
- Nilotinib
  - Y253F/H
  - T315I
- Dasatinib
  - V299L
  - T315I
- Bosutinib
  - E255K
- Ponatinib
  - ?

**3rd generation**
- V299L
- T315I
- F317L/V/I/C
- ?

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<table>
<thead>
<tr>
<th>BCR</th>
<th>ABL</th>
</tr>
</thead>
<tbody>
<tr>
<td>kinase domain</td>
<td></td>
</tr>
<tr>
<td>Ex4</td>
<td>Ex4-5</td>
</tr>
<tr>
<td>P-loop</td>
<td>C-helix</td>
</tr>
</tbody>
</table>

**Keys to the mutations:**
- Ex4
- Ex4-5
- Ex5-6
- Ex 6
- Ex 6-7
- Ex 7
- Ex 8-9
- P-loop
- C-helix
- SH3 contact
- IM binding site
- SH2 contact
- C-loop
- A-loop
- C-terminal lobe

**Molecular sites:**
- BCR
- ABL
- 1st generation
- 2nd generation
- 3rd generation

**Mutations:**
- M237V
- L248R
- G250E/R
- Q252R/H
- Y253F/H
- E255K/V
- E258D
- M273L
- E355D/G
- F317L/V/I/C
- D363Y
- L364I
- A365V
- F359V/I/C
- L370P
- V371A
- Y393C
- H396R/P
- F486S

**Other notes:**
- Y253F/H
- T315I
- F359V/I/C
- V299L
- T315I
- F317L/V/I/C
- E255K
- V299L
- ?

**T315I = ?**
Advantages of next-generation vs. conventional sequencing for BCR-ABL KD mutation screening

- Higher sensitivity* conjugated with the possibility to fully characterize the spectrum of minor mutated variants

- Characterization of the clonal architecture of the mutated populations when multiple mutations fall within the same sequence read (compound vs polyclonal mutations)

- Quantitative analysis of the dynamics of resistant clones over time

* Detection limit may be as low as 1%
The IRON (Interlaboratory RObstustness of Next-Generation Sequencing) study

International consortium of 10 laboratories from 8 countries engaged in the standardization and validation of a common UDS protocol for BCR-ABL KD mutation screening based on the Roche Titanium chemistry

1. Bologna
2. Munich
3. Jena
4. London
5. Madrid
6. Salamanca
7. Brno
8. Prague
9. Vienna
10. Istanbul
The IRON (Interlaboratory RO bustness of Next-Generation Sequencing) study

1. Sensitivity

<table>
<thead>
<tr>
<th>T315I dilution</th>
<th>Detected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>YES (54.47%)</td>
</tr>
<tr>
<td>25%</td>
<td>YES (22.11%)</td>
</tr>
<tr>
<td>10%</td>
<td>YES (8.28%)</td>
</tr>
<tr>
<td>5%</td>
<td>YES (5.04%)</td>
</tr>
<tr>
<td>2%</td>
<td>YES (1.75%)</td>
</tr>
<tr>
<td>1%</td>
<td>YES (0.95%)</td>
</tr>
</tbody>
</table>

R² = 0.99

Dilution of T315I into unmutated BaF3 cells

Variant % detected
The IRON (Interlaboratory RObustness of Next-Generation Sequencing) study

2. Repeatability

<table>
<thead>
<tr>
<th>RT</th>
<th>1st amplification step</th>
<th>2nd amplification step</th>
<th>Equimolar pooling of</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td>BCR-ABL KD</td>
<td>Amp</td>
<td></td>
</tr>
<tr>
<td>ALL-27-04</td>
<td>cDNA</td>
<td>BCR-ABL KD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amp</td>
<td>cDNA</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>BCR-ABL KD</td>
<td>Amp</td>
<td></td>
</tr>
<tr>
<td>LBC-17-01</td>
<td>cDNA</td>
<td>BCR-ABL KD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amp</td>
<td>cDNA</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>BCR-ABL KD</td>
<td>Amp</td>
<td></td>
</tr>
<tr>
<td>MBC-12-01</td>
<td>cDNA</td>
<td>BCR-ABL KD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amp</td>
<td>cDNA</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>BCR-ABL KD</td>
<td>Amp</td>
<td></td>
</tr>
<tr>
<td>CP-05-02</td>
<td>cDNA</td>
<td>BCR-ABL KD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amp</td>
<td>cDNA</td>
<td></td>
</tr>
</tbody>
</table>
3. Accuracy

✓ 554 CML samples analyzed, including:
   - 517 clinical samples analyzed in parallel by UDS and SS;
   - 30 clinical samples analyzed in parallel by UDS, SS and conventional pyrosequencing

✓ 394/398 (99%) variants detected by SS were also detected by UDS

✓ Very good concordance in the estimation of variant abundance between UDS, SS and conventional pyrosequencing
4. Inter-laboratory reproducibility

- identical aliquots of 22 plasmids containing wild-type or mutated BCR-ABL distributed and analyzed in parallel by the 10 laboratories

<table>
<thead>
<tr>
<th></th>
<th>Lab 1 (Prague)</th>
<th>Lab 2 (Bologna)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No mutations</td>
<td>No mutations</td>
</tr>
<tr>
<td>2</td>
<td>E255V (36.9%)</td>
<td>F311L (60%)</td>
</tr>
<tr>
<td>3</td>
<td>M244V (33.1%)</td>
<td>E282K (14.7%)</td>
</tr>
<tr>
<td></td>
<td>No mutations</td>
<td>T315I (18.3%)</td>
</tr>
<tr>
<td>4</td>
<td>M244V (99.8%)</td>
<td>E282K (41.4%)</td>
</tr>
<tr>
<td>5</td>
<td>H396P (11.5%)</td>
<td>H396P (12.7%)</td>
</tr>
<tr>
<td>6</td>
<td>No mutations</td>
<td>No mutations</td>
</tr>
<tr>
<td>7</td>
<td>No mutations</td>
<td>No mutations</td>
</tr>
<tr>
<td>8</td>
<td>No mutations</td>
<td>No mutations</td>
</tr>
<tr>
<td>9</td>
<td>F311L (2.1%)</td>
<td>F311L (1.8%)</td>
</tr>
<tr>
<td>10</td>
<td>L387M (4.3%)</td>
<td>L387M (4.9%)</td>
</tr>
<tr>
<td>11</td>
<td>No mutations</td>
<td>No mutations</td>
</tr>
<tr>
<td>12</td>
<td>E255V (3.3%)</td>
<td>E255V (2.7%)</td>
</tr>
<tr>
<td>13</td>
<td>M351T (18.2%)</td>
<td>M351T (18.5%)</td>
</tr>
<tr>
<td>14</td>
<td>No mutations</td>
<td>No mutations</td>
</tr>
<tr>
<td>15</td>
<td>E255K (7.2%)</td>
<td>E255K (8.6%)</td>
</tr>
<tr>
<td>16</td>
<td>M351T (45.1%)</td>
<td>M351T (45.5%)</td>
</tr>
<tr>
<td>17</td>
<td>E255K (68.5%)</td>
<td>E255K (67.8%)</td>
</tr>
<tr>
<td></td>
<td>K357E (16.5%)</td>
<td>K357E (12.4%)</td>
</tr>
<tr>
<td>18</td>
<td>T315I (99.7%)</td>
<td>T315I (100%)</td>
</tr>
<tr>
<td>19</td>
<td>M244V (64.4%)</td>
<td>M244V (61.8%)</td>
</tr>
<tr>
<td>20</td>
<td>E282K (35.4%)</td>
<td>E282K (32.9%)</td>
</tr>
<tr>
<td>21</td>
<td>Y253F (17.9%)</td>
<td>Y253F (19.8%)</td>
</tr>
<tr>
<td>22</td>
<td>M351T (81.1%)</td>
<td>M351T (81.8%)</td>
</tr>
</tbody>
</table>
Mutation(s) detectable by conventional sequencing may be the tip of the iceberg.
UDS at the time of switchover allows to detect DAS/NIL-resistant mutations in more cases

How many of these mutations could have been detected at switchover using a more sensitive UDS approach?

Pts harboring BCR-ABL mutations:

<table>
<thead>
<tr>
<th></th>
<th>By SS</th>
<th>By UDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pts</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td>harboring</td>
<td>28</td>
<td>48</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutations:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number of BCR-ABL mutations:

<table>
<thead>
<tr>
<th></th>
<th>By SS</th>
<th>By UDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20% (low level)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>&gt;20%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Soverini et al, Blood, submitted
Resistance profile of the 60 low level mutations detected at switchover by UDS

- IM-resistant (22%)
- Unknown or silent (28%)
- IM- and 2GTKI-resistant (50%)

**T315I**
- Outgrew in all cases
- Outgrew in all cases who happened to receive NIL
- Res to DAS only*
  - Res to DAS only**
  - Outgrew in all cases who happened to receive DAS

* F317L/V/I/C, V299L
** Y253H, E255K/V, F359V/I/C
The landscape of mutated populations is highly dynamic

> Did the original Y253H-positive cells persist at very low levels (undetectable even by UDS) until they happened to gain a selective advantage again?
> Y253H and T315I were already detectable by UDS two months before

male, Ph+ ALL 66 yrs DAS after IM failure

Y253H
Y253H+T315I
Y253H+F317L(ttc>ctc)
Y253H+F317L(ttc>ttc)

Sanger Sequencing

100%
20%
0.1%
15.36%
4.04%
1.30%
1.19%
99.79%
78.77%

exitus
Two biologically different scenarios
Compound mutants cannot easily be inferred by Sanger sequencing

- G>A → G250E, 100%
- C>T → T315I, 100%
- C>G → F317L, 100%
- A>G → H396R, 60%
- T>C → Y253H, 40%
- A>T → E255V, 40%

must be a single clone G250E+T315I

must be two clones, one with F317L+H396R, 60% and one with F317L alone, 40%
The two hits are most frequently the result of sequential TKI therapy

Relapse to 1st TKI → Relapse to 2nd TKI

1. Two mutations are sequentially acquired within a short timeframe

Very rare

2. The baseline mutant is eradicated and later replaced by another one conferring resistance to the 2nd TKI

3. The baseline mutant persists and later acquires an additional mutation further increasing its fitness
Multiple mutations often hide complex mixtures of single and compound mutants

Frequency of single and compound mutants in 33 patients (CML or Ph+ ALL) who had experienced sequential relapses with selection of one or more TKI-resistant mutations:

- Single mutants: 49.6%
- Double mutants: 38.3%
- Triple mutants: 10.6%
- Quadruple mutants: 1.5%

Some compound mutants might be particularly tough to defeat

The Italian ‘NEXT-IN-CML’ study

STUDY TITLE:
“NEXT-GENERATION SEQUENCING FOR BCR-ABL KD MUTATION SCREENING IN PHILADELPHIA CHROMOSOME-POSITIVE LEUKEMIAS”

STUDY ACRONYM: “NEXT-IN-CML”

Prospective Investigational Multi-Center Tissue Study

✓ The primary objective of phase A (technical validation phase) is to assess the inter-laboratory reproducibility of NGS for BCR-ABL KD mutation screening and to identify the optimal coverage and sensitivity thresholds below which NGS results should not be considered reliable.

✓ The primary objective of phase B (clinical validation phase) is to prospectively assess the added value of NGS technology over conventional SS by estimating the frequency and clinical relevance of minor mutated clones and compound mutants that NGS technology allows to identify in patients with failure or warning to TKI therapies. […]

NGS results will be correlated with:
1) baseline disease features, therapy and level of response at the time of sampling
2) response to subsequent therapy(ies) and twelve-month outcome
Thank you!

**Dept of Hematology/Oncology**
*University of Bologna:*
- Caterina De Benedittis
- Fausto Castagnetti
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- Gianantonio Rosti
- Giovanni Martinelli
- Michele Baccarani

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- Domenico Russo
- Federica Cattina

**IRON STUDY II LABORATORIES:**
- Naples, Munich, Mannheim, Jena, London, Vienna, Prague, Istanbul

**GIMEMA**
**CML WP**

all GIMEMA friends & colleagues!