NGS-based Assessment of Clonality & MRD Determination in Acute Lymphoblastic Leukemia

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COI disclosure

Name of author: In-Suk Kim

I have no personal or financial interests to declare:
I have no financial support from an industry source at the current presentation.
Overview

- Minimal residual disease (MRD)
  - A Proven Powerful Prognostic Indicator

- Immunoglobulin (IG) and TCR gene in ALL

- NGS based clonality and MRD determination in ALL

- Experience of NGS based TCR clonality & MRD Determination in ALL
MRD: A Proven Powerful Prognostic Indicator in Leukemias

When do we know a “negative marrow” is really negative?
Response Decision After Therapy

• Standard morphology - aspirate smears
• Cytogenetics
  – Routine karyotyping
  – FISH
• Flow cytometry
• Molecular techniques
  – Quantitative PCR or RT-PCR
  – NGS based test

MRD is Important
Long-term follow-up in childhood ALL patients, classified according to MRD measurements.

A. MRD-Low Risk (1/55)
   MRD-Medium Risk (13/55)
   MRD-High Risk (16/19)

B. MRD-Low Risk (21/24)
   MRD-Medium Risk (10/16)
   MRD-High Risk (0/14)

C. Event-free survival
   BCP-ALL
   N. pts N. events 5y EFS 7y EFS
   SR 1348 81 92.3% (0.9) 91.1% (1.2)
   MR 1647 288 77.6% (1.3) 76.0% (1.4)
   HR 189 86 50.1% (4.1) 46.6% (5.1)
   p-value<0.001

D. Event-free survival
   T-ALL
   N. pts N. events 5y EFS 7y EFS
   SR 75 4 94.7% (2.6) 94.7% (2.6)
   MR 290 50 82.5% (2.3) 82.5% (2.3)
   HR 96 48 47.4% (5.4) 47.4% (5.4)
   p-value<0.001

International BFM study TP1 33 days, TP2 78 days
Issues with MRD Detection

• Different methods/targets used by different centers
  – Difficult to compare across studies
• Comparison of different methods across patients
• Standardization & reproducibility
• Reference lab vs individual centers
• Viable vs dead/dying leukemia cells
• What do we do with the results?
Characteristics of Ideal Assay for MRD

• Patient (or leukemia) specific
• High sensitivity: $10^{-5} \sim 10^{-6}$
• Broad applicability to majority of patients
• Feasibility
• Intra- & inter-laboratory reproducibility
• Precise quantification of MRD levels

Szczepanski T. Leukemia 2007 21, 622-626
## Detection of minimal residual disease in acute leukemia

<table>
<thead>
<tr>
<th>Technique</th>
<th>Applicability</th>
<th>Detection limit</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometry (4 to 6 colors)</td>
<td>BCP-ALL: 85%</td>
<td>$(10^{-3}) - 10^{-4}$</td>
<td>Fast, but variable sensitivity because of similarities between normal (regenerating) cells and malignant cells</td>
</tr>
<tr>
<td></td>
<td>T-ALL: 90%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AML: 60-70%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR of Ig/TCR genes</td>
<td>BCP-ALL: 95%</td>
<td>$10^{-4} - 10^{-5}$</td>
<td>Time consuming and relatively expensive (junctial region sequencing), but applicable in $\geq 95%$ of lymphoid malignancies</td>
</tr>
<tr>
<td></td>
<td>T-ALL: 95%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AML: 10-15%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR of fusion transcripts and mutations</td>
<td>BCP-ALL: 40%</td>
<td>$10^{-4} - 10^{-6}$</td>
<td>Limited applicability in ALL, but potentially useful in specific subgroups, e.g. BCR-ABL cases in specific protocols</td>
</tr>
<tr>
<td></td>
<td>T-ALL: 25%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AML: 25-40%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Overview

- Minimal residual disease (MRD)
  - A Proven Powerful Prognostic Indicator

- Immunoglobulin (IG) and TCR gene in ALL

- NGS based clonality and MRD determination in ALL

- Experience of NGS based TCR clonality & MRD Determination in ALL
Immunoglobulin and T Cell Receptor Gene Rearrangements

Early B cell precursor → Pre-B → B cell → Mature PC

IgH + IgL GR

TCR δ and γ GR → TCR β and α GR

Early thymocytes → Common thymocytes → Cytotoxic T → Helper T

Lymphoid stem cell
**IgH Gene Rearrangement**

Stepwise rearrangement of V, D, and J gene segments

This intron is removed by splicing.

IG light chain and TCR genes rearrange in a similar manner.

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**Table 1** Genes encoding the T cell receptor (TCR) chains

<table>
<thead>
<tr>
<th>TCR gene</th>
<th>Chromosome</th>
<th>V segments</th>
<th>D segments</th>
<th>J segments</th>
<th>C segments</th>
</tr>
</thead>
<tbody>
<tr>
<td>α (A)</td>
<td>14q11-12</td>
<td>70</td>
<td>0</td>
<td>61</td>
<td>1</td>
</tr>
<tr>
<td>β (B)</td>
<td>7q32-35</td>
<td>67</td>
<td>2</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>γ (G)</td>
<td>7p15</td>
<td>14</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>δ (D)</td>
<td>14q11-12</td>
<td>8</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

C, constant; D, diversity; J, joining; V, variable.
## Frequencies and stability of MRD-PCR targets in childhood precursor-B-ALL and T-ALL

<table>
<thead>
<tr>
<th>Gene</th>
<th>Rearrangement type</th>
<th>Precursor-B-ALL</th>
<th>T-ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>frequency</td>
<td>oligoclonality</td>
</tr>
<tr>
<td><strong>IGH</strong></td>
<td>VH-JH</td>
<td>93%</td>
<td>30-40%</td>
</tr>
<tr>
<td></td>
<td>DH-JH</td>
<td>20%</td>
<td>50-60%</td>
</tr>
<tr>
<td></td>
<td>total <em>IGH</em></td>
<td>98%</td>
<td>~40%</td>
</tr>
<tr>
<td><strong>IGK</strong></td>
<td>V&lt;sub&gt;k&lt;/sub&gt;-Kde</td>
<td>45%</td>
<td>5-10%</td>
</tr>
<tr>
<td></td>
<td>intron RSS-Kde</td>
<td>25%</td>
<td>5-10%</td>
</tr>
<tr>
<td></td>
<td>total <em>Kde</em></td>
<td>50%</td>
<td>5-10%</td>
</tr>
<tr>
<td><strong>TCRB</strong></td>
<td>Vβ-Jβ</td>
<td>21%</td>
<td>10-15%</td>
</tr>
<tr>
<td></td>
<td>Dβ-Jβ</td>
<td>14%</td>
<td>10-15%</td>
</tr>
<tr>
<td></td>
<td>total <em>TCRB</em></td>
<td>33%</td>
<td>10-15%</td>
</tr>
<tr>
<td><strong>TCRG</strong></td>
<td>Vγ-Jγ</td>
<td>55%</td>
<td>~15%</td>
</tr>
<tr>
<td><strong>TCRD</strong></td>
<td>Vδ-Jδ or Dδ-Jδ1</td>
<td>&lt;1%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Vδ2-Dδ3 or Dδ2-Dδ3</td>
<td>40%</td>
<td>20-25%</td>
</tr>
<tr>
<td></td>
<td>total <em>TCRD</em></td>
<td>40%</td>
<td>20-25%</td>
</tr>
<tr>
<td>TCRD/A</td>
<td>Vδ2-Jα</td>
<td>46%</td>
<td>~45%</td>
</tr>
</tbody>
</table>
Cross-lineage rearrangement

• **Prevailing dogma**
  - B cell $\rightarrow$ *IG* genes rearrangement
  - T cell $\rightarrow$ *TCR* genes rearrangement

• **Cross-lineage rearrangement = lineage infidelity = lineage promiscuity**

  - much more prevalent in *immature or precursor neoplasms*, as compared with more differentiated or peripheral neoplasm
  - Occurs in same neoplastic cells
  - $>70\%$ of B cell ALL : monoclonal *TCR* gene rearrangements
  - 5 to 10% of mature B-cell lymphoma : monoclonal *TCR* gene rearrangements
  - 20-30% of T cell ALL : monoclonal *IgH* gene rearrangements
  - provide a most convenient phenomenon for the study of minimal residual disease, particularly ALL
Pattern of clonal progression

Massive evolution of the *IGH* chain locus in children with B ALL
[Blood. 2012;120(22):4407-4417]
Clonality & MRD Determination techniques

A
- polyclonality
- oligoclonality
- clonality in poly/oligo-clonal background
- clonality

B

C

Fragment analysis
Diversity of IG/TR repertoire
EuroClonality (BIOMED-2) consortium

RQ-PCR based methods: Low-throughput methodology-based detection of repertoire, MRD, and clonality testing

NGS based methods: How high-throughput methodology discloses the full IG/TR sequence information of the entire cell population -> repertoire analysis, MRD monitoring, and clonality assessment

Clonotype
RQ-PCR for *TCR* & *IG*

- *TCRγ, TCRδ, TCRβ, IgH* etc..
- Applicability: most ALL’s
- DNA
- Sensitivity: $10^{-4}$ to $10^{-5}$
- Patient’s clone is sequenced & primers made to detect patient’s particular sequence
- Look for clonal signal against polyclonal background
- The technique used in Europe
RQ-PCR for TCR & IG

• Advantages
  – Applicable for virtually all patients
  – High sensitivity
  – Patient specific
  – Relatively rapid turn around time in follow up (2-3 days)

• Disadvantages
  – Time consuming at diagnosis - identifying the junctional regions
  – Relatively expensive
  – Need for preferably 2 PCR targets per patient - beware clonal evolution & mutations of junctional regions
  – At very low levels, may get nonspecific amplification of normal cells there - false positives
  – Difficult to increase sensitivity against normal polyclonal background -false negative
NGS-based MRD assessment

- Correct MRD quantification.
  - number of index sequences / total number of sequenced amplicons

- Correct MRD quantification is dependent on the background level of polyclonal normal lymphocytes
  - adequate internal controls have to be included

- Presence of evolved clonotypes during treatment or at relapse.
Validation, quality control, and standardized interpretation of NGS-based MRD results.

- **Minimal technical requirements**
  - Higher sensitivity of NGS compared to other methods
    - DNA amount or the number of cells analyzed in a single sample.
- **Clear definition of MRD positivity/negativity**
  - technical assay performance
  - the number of good quality reads
  - the total number of cells analyzed
- **Participation in external quality control**
  - EuroMRD Consortium twice yearly
EuroClonality-NGS Consortium

• **Primary purpose**
  – setting standards in IG/TR NGS methodology
  – its applications in hemato-oncology, hematology and immunology.

• **EuroClonality-NGS**
  – part of the EuroClonality Consortium.

• **EuroClonality** ([www.euroclonality.org](http://www.euroclonality.org))
  – ESLHO (European Scientific foundation for Laboratory HematoOncology)
    • EuroFlow ([www.euroflow.org](http://www.euroflow.org))
    • EuroMRD ([www.euromrd.org](http://www.euromrd.org)).
NGS-based clonality assessment

- Identification of the correct **index clone** (Clonotype)

Figure 2. Clone frequencies in B-ALL (green and red) and normal bone marrow controls (blue), with each dot representing a single clone. The dashed line indicates the 5% threshold for index clone selection; green represents B-ALL clones; and red, other clones.
NGS-based \textit{IgH} & \textit{TCRγ} rearrangements

- Next generation sequencing-based \textit{IgH} & \textit{TCRγ} rearrangements

- Reagents: LymphoTrack\textsuperscript{TM} \textit{IgH} and \textit{TCRγ} DeepSeq Assay (InVitroScribe Technologies, San Diego, CA, USA)

- Scanning: Illumina MiSeq platform, which targeted all potential V-J rearrangement combinations (Illumina\textsuperscript{®}, San Diego, CA, USA)

- Data Analysis: LymphoTrack\textsuperscript{TM} Bioinformatics software package (Invivoscribe)
Clonotypes detected by NGS
PCR based vs. NGS based MRD detection
Relationship Between DNA Input, Read Frequency, & Level of Confidence

FIGURE 2B. CONFIDENCE LEVELS FOR CLONOTYPE DETECTION AT $10^{-5}$ TESTING VARIOUS DNA QUANTITIES AS A FUNCTION OF READ DEPTH
Relationship Between DNA Input, Read Frequency, & Level of Confidence
- Reagents: LymphoTrack™ IgH and TCRγ DeepSeq Assay (InVitroScribe Technologics, San Diego, CA, USA)
- Scanning: Illumina MiSeq platform, which targeted all potential V-J rearrangement combinations (Illumina®, San Diego, CA, USA)
- **Flow cell:** V3 600 cycles (25 million) : for 24 samples
  V2 500 cycles (15 million)

**Table 1. General Guidance**

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>DNA per Replicate</th>
<th># Replicates</th>
<th>Read Depth per Replicate</th>
<th># of Different Samples for Clonotype Tracking per Run</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x10^-4</td>
<td>0.250 µg, 0.500 µg</td>
<td>1 sample of 0.250 µg, 1 sample of 0.500 µg</td>
<td>&gt;310,000, &gt;190,000</td>
<td>21 samples per run plus 3 controls</td>
</tr>
<tr>
<td>1x10^-5</td>
<td>1 µg</td>
<td>3 replicates of 1 µg each</td>
<td>&gt;1,080,000</td>
<td>7 samples per run* plus 3 controls or 5 samples per run plus 3 controls</td>
</tr>
<tr>
<td>1x10^-6</td>
<td>2.5 µg</td>
<td>9 replicates of 2.5 µg each</td>
<td>&gt;4,000,000</td>
<td>1 sample over 2 runs**</td>
</tr>
</tbody>
</table>

Note: A replicate is an independent PCR reaction with input DNA from the same subject.

*25,000,000 total reads (for the v3 600 cycle flow cell) divided by ~1,000,000 required reads per sample ~24 samples; utilizing the 24 indices provided in the LymphoTrack Assay Panels for the MiSeq® and including 3 controls = 21 indices remaining for samples; requiring 3-4 replicates to obtain 95% probability of detecting 5 target reads allows 5-7 independent clonotype tracking samples to be run on the same flow cell.

**To obtain a 95% probability of detecting 5 reads of the target sequence (if the total number of reads is at least 24,000,000) requires 9 replicates of 2.5 µg each of input DNA and a minimum of 2 flow cells to assess a sample for the presence of a clonal sequence in a subsequent sample. Adding the 3 controls to the flow cell may reduce the number of sample reads below the required read depth thus reducing the 95% confidence.”
Detection of minimal residual disease in B ALL by high-throughput sequencing of \textit{IGH}.

Clin Cancer Res. 2014; 20(17): 4540-4548

Pre-Tx (92/98 = 94%)  
Post 29 days (N=91)  
N=40/91 (44%)  N=28 (31%)  N=23 (25%)

Pre- and day 29 post-treatment B lymphoblast frequencies by high-throughput sequencing (HTS) versus multi-parametric flow cytometry (mpFC)
Clinical utility of next-generation sequencing-based minimal residual disease in pediatric B-cell acute lymphoblastic leukaemia

(British Journal of Hematology, 2017, 176, 248-257)

MRD-Standard risk (SR): MRD was negative at both day 33 and day 80
MRD-High risk (HR): Patients with a MRD >10^{-3} at day 80
MRD-Intermediate risk (IR): Other patients

(A) Sensitivity 10^{-4}

(B) Sensitivity 10^{-6}
Overview

- Minimal residual disease (MRD)
  - A Proven Powerful Prognostic Indicator
- PCR techniques for Immunoglobulin (IG) and TCR gene in ALL
- NGS based MRD determination in ALL
- Experience of NGS based TCR clonality & MRD Determination in ALL
**TCRG** gene rearrangement in T-ALL with Korean patients

- A total of 31 T-ALL initial specimens was tested.

<table>
<thead>
<tr>
<th>Discrepancy between Multiplex PCR and NGS for <strong>TCRG</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial specimen (n =31)</td>
</tr>
<tr>
<td>Clonality</td>
</tr>
</tbody>
</table>

*This two specimen shows **TCRB** or **D** gene clonality in PCR assay

**TCRG** gene rearrangement in B-ALL with Korean patients

N =13, Detected TCR clonality (5/13 =38.5%)

Low frequency in Korean T-ALL and B-ALL

*European 95% (T-ALL), 55% (B-ALL)
**Sensitivity: Read depth per replicate**

### TABLE 1. GENERAL GUIDANCE

<table>
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<tr>
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<th>DNA per Replicate</th>
<th># Replicates</th>
<th>Read Depth per Replicate</th>
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<tr>
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<td>21 samples per run plus 3 controls</td>
</tr>
<tr>
<td>$1 \times 10^{-5}$</td>
<td>1 µg</td>
<td>3 replicates of 1 µg each, 4 replicates of 1 µg each</td>
<td>&gt;1,080,000, &gt;820,000</td>
<td>7 samples per run plus 3 controls or 5 samples per run plus 3 controls</td>
</tr>
<tr>
<td>$1 \times 10^{-6}$</td>
<td>2.5 µg</td>
<td>9 replicates of 2.5 µg each</td>
<td>&gt;4,000,000</td>
<td>1 sample over 2 runs</td>
</tr>
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</table>

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**To obtain a 95% probability of detecting 5 reads of the target sequence (if the total number of reads is at least 24,000,000) requires 9 replicates of 2.5 µg each of input DNA and a minimum of 2 flow cells to assess a sample for the presence of a clonal sequence in a subsequent sample. Adding the 3 controls to the flow cell may reduce the number of sample reads below the required read depth if reducing the 95% confidence."
T-ALL 3 months
MRD sensitivity in T-ALL

Clonotype 1_Vg10_Jg1/2 (47%)

<table>
<thead>
<tr>
<th>FU period</th>
<th>Total Reads</th>
<th>MRD read</th>
<th>Sensitivity</th>
<th>Triplicate Total Read</th>
<th>Triplicate MRD Read</th>
<th>Triplicate Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>895,367</td>
<td>52</td>
<td>5.80767E-05</td>
<td>2,622,164</td>
<td>151</td>
<td>5.7586E-05</td>
</tr>
<tr>
<td></td>
<td>722,119</td>
<td>23</td>
<td>3.18507E-05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,004,678</td>
<td>76</td>
<td>7.56461E-05</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>957,788</td>
<td>90</td>
<td>9.39665E-05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 year</td>
<td>856,086</td>
<td>21</td>
<td>2.45302E-05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>919,636</td>
<td>81</td>
<td>8.80783E-05</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Clonotype 2_Vg2_JgNone (36%)

<table>
<thead>
<tr>
<th>FU period</th>
<th>Total Reads</th>
<th>MRD read</th>
<th>Sensitivity</th>
<th>Triplicate Total Read</th>
<th>Triplicate MRD Read</th>
<th>Triplicate Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>895,367</td>
<td>64</td>
<td>7.14791E-05</td>
<td>2,622,164</td>
<td>135</td>
<td>5.14842E-05</td>
</tr>
<tr>
<td></td>
<td>722,119</td>
<td>25</td>
<td>3.46203E-05</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>1,004,678</td>
<td>46</td>
<td>4.57858E-05</td>
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</tr>
<tr>
<td></td>
<td>957,788</td>
<td>68</td>
<td>7.09969E-05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 year</td>
<td>856,086</td>
<td>19</td>
<td>2.2194E-05</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>919,636</td>
<td>65</td>
<td>7.06801E-05</td>
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</tr>
</tbody>
</table>
Clonal progression
Advantages of NGS-MRD Methodologies

• Standardize the workflow and testing in a regulated environment.

• Detect clones and newly emerging clones or subclones in follow-up samples. Offer concordant and harmonized testing worldwide through sequence-specific results.

• Test at a level of sensitivity only limited by the amount of input DNA interrogated.
NGS-based TCR/IgH rearrangements are considered feasible methods for detecting clonality and MRD targets in ALL.

However, many hurdles…
Correct quantification of MRD to avoid overestimate the MRD level

• 1) The first will include **housekeeping gene primers** included in the LymphoTrack master mixes, so the included software will report both prevalence of the malignant clones (several, if desired), as well as total cells in the specimen;

• 2) Another is a $10^{-4}$ **“low positive” control** that can be spiked in for testing specimens for the locus being tested (one for both $IGH$, $IGK$; the other positivd for both $TRG$, $TRB$).

• 3) The third is **an external spike in control** at a concentration of 50 copies/$\mu l$ that will allow investigators to add a known internal standard.
Summary

• MRD, the most important prognostic marker in ALL, is used for risk stratification in most current treatment protocol.

• IG/TCR amplicon NGS is a promising method to quantify MRD.

• Technical pitfalls and limitations of IG/TCR amplicon NGS must be addressed prior to implementation into clinical routine diagnosis.