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Title of the manuscript
A triple-probe fluorescent in situ hybridisation screening strategy for risk stratified therapy of acute lymphoblastic leukaemia in low resource settings.

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Abbreviation Table

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BCP-ALL</td>
<td>B cell Precursor Acute Lymphoblastic Leukaemia</td>
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<tr>
<td>FISH</td>
<td>Fluorescent in situ Hybridisation</td>
</tr>
<tr>
<td>HeH</td>
<td>High Hyperdiploidy</td>
</tr>
<tr>
<td>LH/NT</td>
<td>Low Hypodiploidy/ Near triploidy</td>
</tr>
<tr>
<td>iAMP21</td>
<td>Intrachromosomal Amplification of chromosome 21</td>
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</table>
Abstract

Karyotyping along with a 3-probe fluorescent *in situ* hybridisation (FISH) strategy was used to risk stratify therapy in 303 children with B-cell precursor acute lymphoblastic leukaemia (BCP-ALL). Of the 166 patients risk stratified, karyotype identified 91 (55%). FISH identified all karyotypes accurately, with the exception of hypodiploidy, and risk-stratified an additional 75 patients. The frequency of *ETV6-RUNX1* is lower and high hyperdiploidy, higher than reported in the west. An adapted 3-probe FISH strategy identified two patients with *ETV6-ABL1* fusion who received Imatinib. In limited-resource settings, a 3-probe FISH approach provides a practical approach for risk-stratified therapy in childhood ALL.
Introduction

B cell-precursor childhood acute lymphoblastic leukaemia (BCP-ALL) is characterised by prognostically significant cytogenetic subgroups. *ETV6-RUNX1* fusions and High Hyperdiploidy (HeH, 51-67 chromosomes) subgroups, observed in 40-50% of western patients, have cure rates exceeding 90% and are categorised as ‘low-risk’. [1, 2] *KMT2A* gene rearrangements, iAMP21 and *BCR/ABL1* fusions, seen in ~10%, identify a ‘high-risk’ cytogenetic subgroup. The rest occupy the ‘intermediate-risk’ category. Intensification of therapy, including addition of a tyrosine kinase inhibitor in *BCR/ABL1*, has improved outcomes in high-risk patients. [3] This pre-treatment cytogenetic risk stratification is a standard component of modern risk-adapted therapy in BCP-ALL. Cytogenetic analysis by karyotyping requires fresh samples, specialist laboratories and skilled scientists. In limited-resource countries, this is not readily available, resulting in suboptimal risk stratification and suboptimal therapy. Fluorescent in situ hybridisation (FISH) testing is rapid, does not require viable cells and is easily learnt. A triple-FISH strategy, with *ETV6/RUNX1*, *BCR/ABL1* fusion probes and a *KMT2A* break-apart probe reliably identifies major prognostic cytogenetic subtypes except low hypodiploidy/near triploidy (LH/NT). [4] The *ETV6/RUNX1* probe additionally identifies iAMP21 and HeH, as multiple *RUNX1* signals in this context signify copy number change in the absence of a fusion. [5, 6] The discrete extra *RUNX1* signals here indicate HeH which is confirmed by FISH analysis using additional centromeric probes in the absence of karyotyping.

This triple FISH strategy has been adopted by the Indian Childhood Collaborative Leukaemia (ICiCLe) group to aid centres without dedicated cytogenetic facilities, as part of the risk stratification strategy to de-intensify therapy in low-risk BCP-ALL.

*ABL1* rearrangements other than *BCR-ABL1* have been described in BCP-ALL and these cryptic fusions may respond to adjuvant Imatinib therapy [7]. Imatinib is widely
available in the developing world at affordable prices. This systematic cytogenetic testing strategy indicated the presence of \textit{ETV6-ABL1} fusion in two patients with newly diagnosed Pre-B ALL, enabling timely introduction of Imatinib therapy. Subsequent sequencing studies confirmed the presence of \textit{ETV6-ABL1} fusions in both the patients. Here, we report our experience using 3-probe FISH analysis to therapeutically risk stratify BCP-ALL patients, coupled with an extended cytogenetic strategy to identify other \textit{ABL1} fusions.
Patients and Methods

Consecutive paediatric patients (age <18 years) diagnosed with BCP-ALL, treated at the Tata Medical Center Kolkata between September 2011 and October 2016 were included in these analyses (n=303). Patients were treated after consent, on a standardised, risk-stratified, ethics-approved clinical protocol. Conventional karyotype and FISH analyses were performed on bone marrow aspirates using standard protocols as described. [8]. To reduce costs, FISH testing was performed as a two-step reflex study. Testing was performed first using the \textit{ETV6/RUNX1} dual-colour dual-fusion FISH probe (Abbott, Illinois, USA). Samples with a negative result for \textit{ETV6/RUNX1} fusion were sequentially probed with a \textit{BCR/ABL1} dual-colour dual-fusion probe (Abbott) and a \textit{KMT2A} dual-colour break-apart probe (Abbott), (Figure 1). Based on karyotype and triple-FISH results, patients were classified as standard and high risk or as B-others. In patients without successful karyotype results, the presence of discrete extra \textit{RUNX1} signals served as a screening tool indicating HeH ALL. HeH was confirmed through FISH analysis using centromeric probes to identify additional trisomies of chromosomes 4,10 and 17. The cytogenetic characterisation was complete within 5 days of receiving the sample. Patients with 9q34 rearrangement on karyotype and/or an extra \textit{ABL1} FISH signal were further investigated with metaphase FISH and an \textit{ABL1} dual-colour break-apart probe (Zytovision Bremerhaven, Germany) to identify \textit{ABL1}-rearranged non-\textit{BCR/ABL1} ALL. Metaphase FISH analysis was used to detect fusion partners of \textit{ABL1}. FISH analysis using a \textit{PDGFRβ} dual-colour break-apart probe (Zytovision) was performed in patients with poor day 7 prednisolone response and/or those with high end-induction minimal residual disease (MRD $\geq 10^{-4}$).
Results

Three-probe FISH strategy complements karyotyping

Karyotyping was performed successfully in 252 of 303 BCP-ALL patients (83%), 196 of which were abnormal (Supplemental Figure 1). Karyotyping alone identified prognostically significant subgroups in 91 patients (30%), including HeH in 73, KMT2A rearrangements in 7, BCR/ABL1 in 7 and LH/NT in 4. Three-probe FISH analysis identified all karyotype abnormalities except LH/NT. FISH analysis identified a further 75 patients (25%) with cytogenetic abnormalities, including 34 with ETV6/RUNX1 fusions; 3 with iAMP21; 35 with HeH (including 8 with normal karyotype and 27 with failed karyotype); 2 with BCR/ABL1 (1 with normal and the other with failed karyotype) and 1 with KMT2A rearrangement (normal karyotype). In all 35 patients with HeH, FISH analysis using centromeric probes for chromosomes 4, 10 and 17 confirmed HeH. Thus, the combination of karyotyping and FISH identified standard risk cytogenetics in 142 patients (47%) and high-risk cytogenetics in 24 patients (8%), with the rest (137) classified as intermediate risk (Supplementary Table 1).

Three-probe FISH strategy serves as a screening tool to detect Ph negative ABL1-rearranged BCP-ALL

Extended cytogenetic analysis identified 2 patients with ETV6/ABL1 fusions. In both these patients the BCR/ABL1 fusion probe revealed an additional ABL1 signal, which on metaphase FISH was observed to be on the short arm of chromosome 12 (12p). Metaphase FISH with cohybridisation of ETV6/RUNX1 and BCR-ABL1 probes on the same metaphase suggested an ETV6/ABL1 fusion. Sequencing of the genomic
fusion breakpoints in both the cases confirmed the *ETV6/ABL1* fusion (type B, genomic breakpoints in intron 5 of *ETV6* and intron 1 of *ABL1*) albeit with different break points.
Discussion

This study represents a large cohort of systematically cytogenetically characterised paediatric BCP ALL patients from India. [9, 10] Three-probe FISH analysis corroborated and complemented karyotyping, enabling cytogenetic categorisation in 166 of 303 patients (55%). FISH analysis alone additionally identified 25% patients with prognostically relevant cytogenetic subtypes. Hypodiploid ALL is however not detected using the 3-probe FISH strategy. In our setting, this limitation has been addressed by incorporating an inexpensive flow cytometry-based DNA ploidy analysis as part of routine diagnostic immunophenotyping. [11]

The study findings are concordant with the reported lower frequency of ETV6/RUNX1 fusion in Indian patients. [9] There was a higher incidence of HeH and thus the overall proportion of standard risk cytogenetics remains similar to those reported in the west. The frequency of high risk cytogenetic subtypes (BCR/ABL1, iAMP21, Hypodiploidy and KMT2A rearranged ALL) is similar to reported literature.

B-other ALL is a cytogenetically indeterminate group and constitutes nearly 50% of paediatric BCP-ALLs seen at our centre. In this cohort, 3-probe FISH analysis identified two patients with ETV6/ABL1 fusion. In both patients, the detection of an extra ABL1 signal on the short arm of chromosome 12 led us to suspect ETV6/ABL1 fusion. Since commercial ETV6/ABL1 fusion probes are not available, we further investigated by cohybridising BCR/ABL1 and ETV6/RUNX1 fusion probes on the same metaphase. In one of these patients an ABL1 break-apart probe was used to confirm ABL1 gene rearrangement. Where the ABL1 break-apart probe is not available, metaphase FISH analysis using the BCR/ABL1 probe can also detect
ABL1 gene rearrangements where, the extra ABL1 signal will be observed on a different locus or on a different chromosome.

Contemporary risk-stratified approaches have improved outcomes in BCP-ALL to over 90%. Absence of suitable cost-effective modern diagnostic studies limits risk stratification in resource-constrained settings and contributes to global inequality in outcomes in childhood ALL. [12] Diagnostic approaches using readily available, easily standardised and relatively inexpensive techniques such as the proposed three-probe FISH testing have the potential to successfully address these disparities.

Acknowledgments

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MP, MKS performed cytogenetics and analysed the data; RI and DS were involved in sample identification and performed, MLPA and molecular genetics experiments; MP, DKM, SK and VS were involved in designing the cytogenetic strategy and writing up the paper.

Conflict of Interest

All authors report no conflict of interest,
References


7209 Indian Patients with de novo Acute Leukemia: A Single Centre Study from India. *Journal of Cancer Therapy* 2016, 7:530-544.


Legends

Figure 1. FISH screening strategy to risk stratify precursor B cell ALL

Table 1. Cytogenetic risk stratification in 303 BCP-ALL patients based on karyotyping and FISH analysis

Supplemental Figure 1. Distribution of cytogenetic findings by karyotyping and FISH analysis in BCP-ALL (n=303)
<table>
<thead>
<tr>
<th>Cytogenetic Risk Group</th>
<th>Number (%)</th>
</tr>
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<tbody>
<tr>
<td><strong>Low</strong></td>
<td></td>
</tr>
<tr>
<td>High Hyperdiploidy</td>
<td>108 (36)</td>
</tr>
<tr>
<td>ETV6-RUNX1</td>
<td>34 (11)</td>
</tr>
<tr>
<td><strong>High</strong></td>
<td></td>
</tr>
<tr>
<td>KMT2A rearrangement</td>
<td>8 (3)</td>
</tr>
<tr>
<td>BCR-ABL1</td>
<td>9 (3)</td>
</tr>
<tr>
<td>Low hypodiploid/Near triploid</td>
<td>4 (1)</td>
</tr>
<tr>
<td>iAMP 21</td>
<td>3 (1)</td>
</tr>
<tr>
<td><strong>Intermediate</strong></td>
<td></td>
</tr>
<tr>
<td>Normal Karyotype</td>
<td>38 (12)</td>
</tr>
<tr>
<td>Chromosome9p abnormality*</td>
<td>21 (7)</td>
</tr>
<tr>
<td>TCF3-PBX1 fusion</td>
<td>15 (5)</td>
</tr>
<tr>
<td>Complex karyotype</td>
<td>16 (5)</td>
</tr>
<tr>
<td>Failed</td>
<td>13 (4)</td>
</tr>
<tr>
<td>6q deletion*</td>
<td>10 (3)</td>
</tr>
<tr>
<td>Abnormal 17p</td>
<td>5 (2)</td>
</tr>
<tr>
<td>1q duplication</td>
<td>8 (3)</td>
</tr>
<tr>
<td>-7/ 7p deletion*</td>
<td>7 (2)</td>
</tr>
<tr>
<td>14q32 rearrangement</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>t(8;14)(q24;q32)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>13q</td>
<td>2 (1)</td>
</tr>
</tbody>
</table>
ETV6-RUNX1 dual colour probe

- **Fusions**
  - Positive for ETV6/RUNX1

- **Multiple discrete RUNX1 signals**
  - Indicates high hyperdiploidy

- **Multiple RUNX1 signals in cluster or tandem step ladder pattern**
  - IAMP21

**In the absence of karyotyping or flow ploidy**

FISH using Centromeric probes for CEP4, 10 and 17 to confirm high hyperdiploidy

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**KMT2A Break apart probe**
Positive if split signals

**BCR/ABL1 dual fusion probe**
- Fusions: positive for BCR/ABL1
- Additional ABL1 signals: further investigate using metaphase FISH or ABL1 break apart probe

* Proceed with KMT2A and BCR/ABL1 FISH if discrete RUNX1 signals seen in absence of fusions
Supplemental Figure 1. Distribution of cytogenetic findings by karyotyping and FISH analysis in BCP-ALL (n=303)

Karyotype 303
   Successful 252
     | Abnormal 196
     |     | Normal 56
     |     |   | FISH
     |     |   | Abnormal 38
     |     |   |   | HeH: 27
     |     |   |   | BCR/ABL1: 1
     |     |   |   | ETV6/RUNX1: 9
     |     |   |   | KMT2A: 1
     |     |   | Normal 13
     |     | | Abnormal 24
     |     |   | HeH: 8
     |     |   | BCR/ABL1: 1
     |     |   | ETV6/RUNX1: 15
     | | Normal 32

* These patients showed abnormal karyotype with other abnormalities, ETV6/RUNX1 fusions were identified on FISH analysis.