Keeping PACE with Ph Positive to Ph-Like Detection in B-Lineage Acute Lymphoblastic Leukemia: A Practical and Cost Effective (PACE) Approach in a Resource Constrained Setting

Sidharth Totadri¹ · Minu Singh¹ · Amita Trehan¹ · Neelam Varma² · Prateek Bhatia¹

Received: 10 January 2018 / Accepted: 31 July 2018 / Published online: 1 August 2018
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Abstract Philadelphia (Ph)-like or BCR-ABL like acute lymphoblastic leukemia (ALL) is defined on the basis of a gene expression profile that is similar to Ph-positive ALL. It comprises a wide spectrum of genetic lesions affecting primarily the cytokine receptor and/or kinase signalling genes. It accounts for approximately 10–15% of pediatric ALL, and is more common in patients who are high-risk according to the National Cancer Institute criteria. Presence of Ph-like mutations is an independent predictor of poor outcome. However, there is vast potential to utilize targeted therapy to improve survival in this group. The sizeable range of genetic lesions varying from translocations, fusions, point mutations and deletions make the diagnosis challenging. Hence, a practical and cost effective approach is required to enable identification in resource constrained settings. Patients with recurrent cytogenetic abnormalities such as ETV6-RUNX1, high hyperdiploidy, TCF3-PBX1, BCR-ABL1 and KMT2A (MLL) rearrangement need not be tested, as these are mutually exclusive with BCR-ABL like mutations. Detection of CRLF2 overexpression, which is the commonest abnormality, is employed as the first step. In patients lacking overexpression, testing for tyrosine kinase fusions can be performed. However, the goal should be to employ a combination of molecular diagnostic techniques such as reverse

transcriptase polymerase chain reaction (PCR), real time quantitative PCR, fluorescence in situ hybridization and Sanger sequencing to detect genetic lesions that are amenable to targeted therapy.

Keywords Copy number anomalies · High risk ALL · IKAROS · MLPA · Precision medicine

Introduction

The survival in pediatric acute lymphoblastic leukemia (ALL) currently approaches 90% in the developed world [1]. Comprehending the biological heterogeneity of this disease has played a vital role in this success story. Identification of underlying genetic aberrations in ALL enables risk stratification and precision medicine tailored to individual needs. B-lineage ALL accounts for 80–85% of pediatric ALL and is the most common subtype of pediatric leukemia [2]. Conventionally, numerical chromosomal aberrations such as hypodiploidy and hyperdiploidy; and structural aberrations such as t(12;21) (ETV6-RUNX1), t(1;19) (TCF3-PBX1), t(9;22) (BCR-ABL1) and 11q23 (KMT2A or MLL) rearrangements are included as part of the diagnostic panel [2, 3]. However, these are detected in merely two-thirds of children with ALL [2]. Relapse continues to occur in 15–20% of children treated for ALL, and studies have been focussing on unravelling the complete genetic signature of B-ALL to better understand disease biology [4].

In the last decade, rampant advances in next generation sequencing (NGS) and array technologies have witnessed the discovery of further genetic groups in pediatric ALL. The Philadelphia (Ph) chromosome [t(9;22), BCR-ABL1] is an established entity identified in 3–5% of pediatric ALL

✉ Prateek Bhatia
prateekbhatia16@gmail.com

¹ Pediatric Hematology-Oncology Unit, Department of Pediatrics, Advanced Pediatrics Center, Postgraduate Institute of Medical Education and Research, Chandigarh, India

² Department of Hematology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

[5]. Administration of tyrosine kinase inhibitors (TKI) in addition to conventional chemotherapy demonstrated significantly improved survival in Ph-positive ALL, thus establishing the paradigm of targeted therapy in ALL [5]. Ph-like or BCR-ABL like ALL comprises a myriad group of genetic mutations which share a gene expression profile that is similar to Ph-positive ALL [6]. Approximately 10–15% of childhood ALL; and 25–30% of adolescents and young adults (AYA) harbour Ph-like gene signature [7]. Diagnosis of these mutations is important as they portend an inferior survival outcome, as well as offer an opportunity for targeted therapy akin to Ph positive ALL. Additionally, they are standalone mutations which do not overlap with the conventionally detected genetic lesions in ALL [6].

Phenotype and Treatment Response of Ph-Like Like ALL

Patients with Ph-like ALL are likely to be treated with an intermediate or high-risk regimen for ALL, attributable to older age and/or higher total leukocyte count (TLC) at diagnosis [8]. In a large multicentric study which performed genomic profiling of 1725 patients with ALL; prevalence of Ph-like ALL was 10% in children with standard risk ALL (age 1–9 years and TLC < 50,000/ μ L), 13% in children with high-risk ALL (age 10–15 years and/or TLC $\geq$ 50,000/ μ L), 21% in adolescents (age 16–20 years) and 27% in young adults (age 21–39 years) [8]. Male preponderance increases with age, from 1.5:1 in children to 4:1 in young adults [7, 8].

European and American studies concur on the significantly higher post-induction minimal residual disease in children with Ph-like ALL [8–10]. Presence of Ph-like mutations is recognized as a strong, independent prognostic factor for poor survival outcomes in pediatric ALL [8, 9]. The 5-year event-free survival (EFS) was approximately 60, 40 and 25% in children, adolescents and young adults with Ph-like ALL and significantly lesser across all age categories when compared to other patients with non Ph-like ALL [8]. A study from the Dutch Childhood Oncology Group (DCOG) and German Cooperative ALL (COALL) trials demonstrated higher relapse rates in BCR-ABL like ALL [11].

Genomic Spectrum of Ph-Like Like ALL: “The Ph-Like Kinome”

Ph-like ALL is not a single genetic lesion but a heterogeneous group of genetic alterations which predominantly affect either the JAK-STAT or the ABL kinase signalling

pathway (Fig. 1). The difference between “Ph-like ALL” as described by the St. Jude Children’s Research Hospital/Children’s Oncology Group (SJCRH/COG) and “BCR-ABL1 like” by the Dutch/German groups is largely semantic [6]. However, some discordance can be ascribed to differences in the methodology of detection, selection of patients for genomic screening and ethnicity [6, 12]. The Ph-like group can be classified into 5 categories as follows [3].

1. CRLF2 rearrangements: These account for 50% of Ph-like ALL, and are often associated with mutations involving the IL7 receptor and janus kinase enzymes (JAK2 and JAK1) [13]. P2RY8-CRLF2 and IGH-CRLF2 are the predominant translocations in this class resulting in overexpression of CRLF2 [14]. It is noteworthy that ALL in individuals with Down syndrome is characterised by high incidence of CRLF2 rearrangement (30–50%) and JAK2 mutations [6, 14]. In patients with CRLF2 rearrangement lacking JAK mutations, there is invariable presence of other mutations activating JAK-STAT signalling [15]. For example, in a subset of patients with IL7R overexpression, concomitant reduced expression of the SH2B3 (also known as LNK gene) is seen, which is a negative regulator of JAK-STAT pathway. This combination is associated with Ikaros dysfunction and poor outcome [16, 17].
2. ABL1 class rearrangements involving the kinases ABL1, ABL2, CSF1R and PDGFRB. This class comprises various fusion genes that result in constitutive ABL kinase activation [13]. Tyrosine kinase fusion positive Ph-like ALL has been shown to have increased risk of relapse as compared to non Ph-like ALL [9]. This group is the most amenable to targeted therapy with conventional tyrosine kinase inhibitors (TKIs) such as imatinib and dasatinib [7, 19].
3. JAK2/EPOR rearrangements
4. Uncommon kinase mutations such as NTRK3, PTK2B, TYK2, FGFR1, BLNK, etc. Increased expression of FLT3 wild sequence and internal tandem duplications in the juxtamembrane domain of FLT3 can infrequently be seen in high-risk ALL [17].
5. RAS pathway mutations involving NRAS, KRAS, PTPN11 and NF1 (This last category is not constrained to this category of Ph-like like mutations and can occur

Fig. 1 Acute lymphoblastic leukemia (ALL) development, genetic risk stratification and Ph-like gene kinome. BCP: B-cell precursor, CNA's: copy number anomalies

in concomitance with other aberrations such as MLL rearrangement) [3].

The first two categories are the commonest while the latter three each account for $\leq 5\%$ of Ph-like mutations [8].

Copy number alterations (CNA's) in the genes responsible for B cell maturation and proliferation are implicated as the second hit in leukemogenesis (Fig. 1). Advanced genomic assays have enabled identification of such CNA's, that are detected in abundance in association with Ph-like ALL. A large study from the United Kingdom, employed multiplex ligase-dependent probe amplification (MLPA) to detect CNA's in the following genes: IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, RB1, and PAR1 [20]. Based on CNA's, the study delineated a poor risk group which included approximately 20% of all patients with B lineage ALL, distinct from the poor risk group with known high-risk recurrent cytogenetic aberrations such as t(9;22) and hypodiploidy [20]. This group was characterized by deletions in IKZF1, PAR1, EBF1 or RB1 [20]. Therefore, CNA's facilitate identification of the molecular signature of Ph-like ALL, and are useful to reaffirm the diagnosis of this entity [20, 21]. A genome-wide study conducted by the COALL and DCOG groups similarly demonstrated

significantly greater number of mutations in B cell development regulatory genes such as IKAROS (IKZF1), E2A (TCF3), EBF1 and PAX5 and the pre-B-cell receptor (pre-BCR) surrogate light chain VPBEB1 [19].

Diagnosis of Ph-Like Genetic Signature

The example of successful use of TKIs in Ph positive ALL, reinforces the importance of diagnosing this group of mutations which may be targeted in a similar fashion [22]. Further, they are thrice as common as Ph positive ALL [5, 6]. The SJCRH/COG study utilized a prediction analysis of microarrays classifier comprised of 257 gene probe sets, dedicated to the identification of Ph-like mutations [8, 12]. The Dutch study employed hierarchical clustering of 110 gene probe sets which were designed to detect all major genetic anomalies in ALL [12, 19]. A low-density array (LDA) card that has 8–15 probes has been designed by the collaborative efforts of COG and the National Institute of Health, that targets the most common mutations which are potentially responsive to tyrosine kinase inhibitors [14, 23]. However, the numerous genetic lesions constituting this group makes the task of diagnosis

daunting, especially in a resource limited setting, commonly encountered in lower/middle income countries (LMIC).

Patients with B lineage ALL who lack the characteristic genetic aberrations, namely, hyperdiploidy, ETV6-RUNX1, TCF3 rearrangement, MLL rearrangement, and BCR-ABL1 translocation, are referred to as “B-other” group [6, 11]. Greater than 50% of this group of patients harbour Ph-like mutations [11]. The Dutch/German genome-wide classification study demonstrated that none of the patients with these sentinel aberrations demonstrated a BCR-ABL1 like gene expression signature [19]. The other two sentinel aberrations in ALL defined by the revised World Health Organization classification, namely, hypodiploidy and t(5;14)(q31.1;q32.3) IL3-IGH are not employed to define the B-other group [11, 24]. Mutations in RAS and TP53 have been demonstrated to be additional driver mutations in ALL with near haploidy and low hypodiploidy [25]. IL3-IGH is a rare entity, seen in < 1% of patients with ALL. Gene deletions targeting lymphoid factors that are enriched in BCR-ABL1 like ALL have been demonstrated in association with this genetic abnormality [26].

A practical and cost effective (PACE) approach to diagnosing these lesions is to adopt an algorithm to perform diagnostic tests in a step wise manner (Fig. 2). A COG study described an algorithmic approach of using the LDA card for screening, followed by assessment of CRLF2 expression, and subsequent testing based on high or low CRLF2 expression [27]. We propose a similar simplified step wise PACE approach for diagnosis of Ph-like ALL in a resource constrained setting (Fig. 2).

PACE Algorithm for Diagnosis of Ph-Like ALL

1. *Selection of patients* The overall prevalence of Ph-like mutations in pediatric precursor B-ALL is 10–15% [6]. Therefore, it would be reasonable to restrict testing to a sub-group of patients who are most likely to test positive for these mutations. Since sentinel genetic abnormalities almost always do not overlap with this group, patients testing positive for ETV6-RUNX1, high hyperdiploidy, TCF3-PBX1, BCR-ABL1 and KMT2A (MLL) rearrangements can be excluded from analysis [6, 11, 19]. Further restriction of testing to high-risk patients with positive MRD at the end of

Fig. 2 Practical and cost effective (PACE) approach: Algorithm for Ph-Like kinome detection. The approach aims at detecting the most common targetable kinase lesions of Ph-like B-ALL which can be amenable to addition of targeted therapy such as tyrosine kinase inhibitors to chemotherapy. RT-PCR: reverse transcriptase

polymerase chain reaction, FISH: fluorescence in situ hybridization, BCP: B-cell precursor, RQ-PCR: real time quantitative polymerase chain reaction, MLPA: Multiplex ligation-dependent probe amplification, CNA's: copy number anomalies

induction therapy can be considered, if there is significant resource constraint. However, the SJRCH/COG study demonstrated negative end of induction MRD in 58% of Ph-like ALL, and Ph-like ALL was an independent predictor of poor survival irrespective of MRD [8].

2. *Screening for CRLF2 overexpression* CRLF2 gene rearrangement leading to overexpression of surface CRLF2 is the commonest abnormality detected in up to 50% Ph-like ALL [6, 13]. Interestingly, the Dutch study noted a lesser prevalence of 15–20% [9]. Quantitative real-time polymerase chain reaction (qRT-PCR) can be employed to measure CRLF2 overexpression. An expression level of ≥ 20 times of the median expression value in B lineage ALL patients was used by Cario et al. to define CRLF2 overexpression [28]. Alternatively, flow cytometric immunophenotyping can also be used to detect the CRLF2 surface antigen, as CRLF2 is not normally expressed by B cells [13].
3. *Further characterization of CRLF2 overexpression* Of the 2 sentinel aberrations associated with CRLF2 overexpression, P2RY8-CRLF2 is more common in children, as well as easier to detect with RT-PCR [6]. If negative, for this fusion transcript, a fluorescence in situ hybridization (FISH) may be performed for the less common IGH-CRLF2 translocation.

Since JAK2 mutations are associated with 50% of CRLF2 rearranged ALL, the next step would involve a PCR and sanger sequencing performed to detect JAK2R683G, which is the most common associated JAK2 mutation affecting the pseudokinase domain [6]. If the JAK2 mutation is not identified, mutations in the IL7 receptor gene (IL7R) can be screened for. PCR and targeted sequencing can be utilized to assess for mutation S185C in exon 5 and microdeletion in exon 6 of the IL7R gene as described by Shochat et al. [29].

4. *Screening for tyrosine kinase mutations in the absence of CRLF2 overexpression* Once CRLF2 overexpression is ruled out, then the second most common group among the BCR-ABL1 like mutations comprises the multiple fusion transcripts involving the tyrosine kinases. A multiplex PCR can be used as described by Reshmi et al. for common fusion mutations, namely, ETV6-ABL1, NUP214-ABL1, RCSD1-ABL1, RCSD1-ABL2, PAG1-ABL2, SSBP2-CSF1R and SSBP2-PDGFRB [27]. Roberts et al. and Imamura et al. have also described multiplex PCR for diagnosis of fusion mutations [8, 30]. Alternatively, in house primers for these fusions as well as for JAK2/EPOR rearrangement can be designed, and multiplex PCRs standardized. The overall costs involved would be significantly lesser than what would be required for using currently available commercial FISH probes for

detecting ABL1, ABL2, CSF1R, PDGFR α , JAK-2 or EPOR rearrangements. Laboratories where FISH is routinely used for BCR-ABL1, ETV6-RUNX1 and iAMP21 detection, and where well experienced technical and reporting staff are available, can consider utilizing a combination of 4–6 probes to detect ABL class and JAK/EPO rearrangements. RT-PCR, however, cannot detect all fusions, since there are ≥ 13 partners for ABL1, 3 for ABL2, 3 for CSF1R and 2 for PDGFRB. Developing multiplex PCRs would be technically demanding and a balance between detection of common fusions and use of FISH probes for others may offer a reasonably better approach. In settings where there is no resource constraint, RNA-sequencing would yield best results and detect novel transcripts too, albeit involving greater costs and requirement of specialized NGS equipment.

5. *Role of Multiplex ligation-dependent probe amplification (MLPA) in assisting in detection of Ph-like signature* MLPA analysis of genomic DNA, coupled with comparison to healthy controls, enables the detection of CNAs. This further contributes to the establishment of diagnosis of Ph-like ALL, since this technique is simple, cost-effective and has been well validated in large clinical trials (Fig. 3).

- A. Preservation of the PAR1 gene (absence of deletions) reiterates the absence of CRLF2 overexpression.
- B. P2RY8/CRLF2 fusion can be confirmed through assessment of CSFRA and IL3RA deletions.
- C. IKZF1 deletions that are significantly associated with BCR-ABL1 like ALL can be identified.
- D. JAK2 mutations can be predicted
- E. CNAs in the genes EBF1, CDKN2A/B, PAX5, ETV6, BTG1, and RB1 as described by Moorman et al. can be diagnosed (20).
- F. PDGFRB-EBF1, can be identified by presence of EBF1 deletion (only of exon 16). In Ph-like ALL, this represents the commonest fusion involving PDGFRB. It results in loss of EBF1 function, which is involved in B-lineage development. The presence of this fusion transcript translates into high rates of induction failure and relapse [31, 32].

Therapeutic Significance of Identifying Ph-Like Like ALL

The improved survival in Ph + ALL, with addition of TKIs such as imatinib and dasatinib to the chemotherapy backbone, ushered in the era of targeted therapy in ALL [5]. The

Fig. 3 Application of multiplex ligation-dependent probe amplification (MLPA) and capillary sequencing in detection of Ph-like signature. CNA's: copy number anomalies

successful employment of TKIs in Ph + ALL prompted a similar approach in Ph-like ALL with tyrosine kinase fusion transcripts, which was demonstrated to have positive results in individual patients [6]. The poor response to induction therapy, increased relapse rate and inferior EFS associated with Ph-like ALL necessitate an imminent need for application of targeted therapy to improve outcome in this entity. In vitro studies have established the efficacy of conventional tyrosine kinase inhibitors in cells expressing fusion transcripts involving the ABL class of genes such as ABL1, ABL2 and CSF1-R, as well as PDGFRB [8, 17]. Additionally, an inhibitory effect was identified with ruxolitinib in human leukemic cells expressing JAK2 and IGH-EPOR fusions, and with crizotinib in ETV6-NTRK3 [8]. In contrast BCR-ABL1 like leukemic cells have been identified to be highly resistant to conventional chemotherapeutic agents such as L-asparaginase and daunorubicin [19]. Clinical trials which involve addition of TKIs to the conventional chemotherapy backbone are being conducted under the aegis of major cancer centres and pediatric oncology groups including COG, SJRCH and MD Anderson Cancer Center, the results of which can be anticipated in the future [6, 7]. The sheer number of Ph-like mutations and paucity of patients identified with these aberrations pose hurdles to conducting clinical trials in Ph-like ALL [6]. Other non-TKI strategies inspired by the understanding of the biology of Ph-like ALL include, addition of vincristine/steroid pulses in maintenance therapy (effect being variable among different groups) in patients demonstrating IKZF1 mutations [6, 11]. Several potential targets for therapeutic exploitation can be identified in the downstream signalling pathways involved in Ph-like ALL, such as inhibition of mTOR, PI3K, HDAC and MEK [6, 14]. NTRK3 fusions can respond to crizotinib [7, 8].

Limitations

The PACE approach is intended to diagnose the commonest Ph-like mutations. However, it cannot detect all mutations which comprise this vast compendium. Certain

rare mutations such as fusion transcripts involving LYN and NTRK3 will not be detected. The strategy does not involve performing genome-wide analysis. Although CNAs in genes such as IKZF1, EBF1, CDKN2A/B, PAX5, ETV6, BTG1, and RB1 may be detected with MLPA; detecting mutations in TCF3 and VPBEB1 is beyond the scope of this approach. Though the proposed approach requires further validation, it is proposed as a practical and cost effective guide for laboratories in resource constraint settings.

Conclusion

Ph-like ALL or BCR-ABL1 like ALL represents an important sub-group of B lineage ALL. Although this entity portends inferior outcome, the genetic lesions offer vast opportunity for targeted therapy which can potentially improve survival. Diagnosis is challenging due to the genetic heterogeneity, however, a step wise practical and cost effective “PACE” approach can be rewarding.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest. Author identifying information is on the title page that is submitted separate from the manuscript.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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