



Role of Cytogenetics and FISH in Laboratory Workup of B Cell Precursor Acute Lymphoblastic Leukemia

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Abstract

Modern therapeutic protocols in acute leukemias risk stratify disease based on genetic characterization of the neoplastic cells and their response to treatment. Genetic characterization is routinely performed by cytogenetic testing of leukemic cells and is a standard component of modern risk-adapted therapy in acute lymphoblastic leukemia (ALL). High-throughput technologies like RNA sequencing have identified multiple novel subtypes in recent years. The cytogenetic strategy using GTG and fluorescent in-situ hybridization (FISH) has to be adapted to identify not only the primary principal chromosomal abnormalities but also the novel subtypes. In the review, we describe a systematic comprehensive cytogenetic strategy that integrates information from immunophenotyping, flow-based DNA ploidy, and karyotyping complemented by targeted FISH studies to identify more than 70% of genetic abnormalities described in B cell precursor ALL. The simplified strategy includes a four-probe FISH and flow ploidy strategy, \pm karyotyping that identifies high risk (*KMT2A*, *BCR::ABL1*, hypodiploidy, *iAMP21*) and standard risk (*ETV6::RUNX1* and high hyperdiploid) cytogenetic groups. The extended FISH panel includes probes targeting *MEF2D*, *ZNF384*, and *CRLF2* rearrangements that are used intuitively on integrating the immunophenotyping features that characterize these entities. The strategy also includes a systematic approach to identify masked hypodiploidy integrating targeted FISH analysis directed toward identifying monosomies of chromosomes 7, 15, and 17 and flow cytometry-based DNA ploidy analysis. The recently described PH-like ALL is characterized by *ABL* class fusions and rearrangements of *CRLF2* and *JAK2* genes. FISH analysis using break-apart probes can be used to identify these aberrations. The cytogenetic approach also includes FISH analysis to identify intragenic and whole gene deletions of the *IKZF1* genes that identify a subset of patients associated with high risk of treatment failure.

Keywords

- ▶ acute lymphoblastic leukemia
- ▶ *ETV6::RUNX1* fusion
- ▶ hyperdiploidy
- ▶ *BCR::ABL1* fusion
- ▶ *KMT2A* gene rearrangement
- ▶ *iAMP21*
- ▶ FISH in ALL
- ▶ karyotyping

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Introduction

Modern treatment protocols in acute lymphoblastic leukemia (ALL) risk stratify patients based on genetic characteristics of leukemic cells and response to treatment.¹ Genetic characterization is routinely performed by cytogenetic testing of leukemic cells and is a standard component of modern risk-adapted therapy in ALLs.^{2,3} Arising from the precursors of the lymphoid lineage, ALLs are either B cell precursor (BCP-ALL) or T cell type (T-ALL). BCP-ALL accounts for 75% of ALLs in children and 80% of ALLs in adults and comprises multiple genetic subtypes defined by their sentinel finding chromosomal abnormalities that are essential in risk stratification. T-ALLs, on other hand, are treated as a high-risk disease upfront and hence this review will focus on cytogenetic characterization of BCP-ALL.

Recurring Chromosomal Alterations in BCP-ALL

BCP-ALLs are characterized by a spectrum of finding chromosomal abnormalities that occur early in the course of the disease and are prognostic and predictive of outcomes.⁴ Copy number abnormalities and sequence mutations are cooperating secondary lesions in leukemogenesis and may either be acquired or enriched at disease progression (► **Table 1**).^{5,6} The clinically significant chromosomal abnormalities include aneuploidies and chromosomal translocations/gene rearrangements. High hyperdiploidy (HH) and *ETV6::RUNX1* fusions have more than 90% cure rates and are categorized as standard risk^{7,8} and *TCF3::PBX1* fusions are categorized as intermediate risk. Hypodiploidy, intrachromosomal amplification of chromosome 21 (iAMP21), *BCR::ABL1*, *TCF3::HLF1* fusions, complex karyotypes, and *KMT2A* rearrangements are categorized as high risk.⁹

The distribution of the cytogenetic subtypes is variable in different age groups with good risk cytogenetic subtypes, that is, HH and *ETV6::RUNX1* fusions being more frequent in pediatric patients and *BCR::ABL1* being more frequent in adults. *KMT2A* rearrangements are characteristic of infant ALL with prevalence rising in adults (10–15%).^{9,10}

Cytogenetic and Molecular Methods to Characterize BCP-ALL

Karyotyping

Chromosome banding technique is a morphological assessment of whole genome of a single cell and requires fresh samples and skilled manpower. The turnaround time varies from 5 to 10 days. The chromosomes are best studied at the metaphase stage of the cell cycle when the chromatin is condensed and chromosome morphology is well defined. The reliability of karyotyping analysis depends on obtaining good quality analyzable metaphases from neoplastic cells. Blasts with low proliferative potential and a proportion of HH cases may not yield analyzable metaphases, the results being interpreted erroneously as normal karyotype in such cases. Correlating FISH and flow ploidy results in these cases aids in accurately identifying the cytogenetic subtype.

Fluorescent in Situ Hybridization

Fluorescent in situ hybridization (FISH) is based on the principle of hybridization of single-stranded DNA probes labeled with fluorophores to their complementary genomic sequences. FISH does not require live cells, is relatively inexpensive, and allows transport of samples for testing at referral laboratories. FISH microscopy images can also be reviewed centrally to ensure standardization and diagnostic accuracy across treatment centers. Sensitivity varies from 1 to 5%.

Preanalytical Variables and Quality Control in Karyotyping and FISH

The first pull bone marrow aspirate sample is the preferred sample for cytogenetic studies that should be transported as soon as possible to the laboratory and processed with minimum delay. However, peripheral blood samples with high blast counts can also be utilized for FISH studies. Samples should be collected in heparin vials.

A minimum of 20 metaphases obtained from two independent cultures should be analyzed. In the presence of a clonal cytogenetic abnormality, evaluation of fewer than 20 metaphases is acceptable.

In FISH analysis, each probe should be validated for various thresholds, aberrant signal patterns to establish false positive/negative ranges. A minimum of 100 interphase nuclei by two analysts for diagnostic samples and 200 interphase nuclei for follow-up samples are recommended. The cutoff value for fusion probes is 1% and a higher cutoff for break apart probes is recommended. Cutoff values for each probe can be calculated by either using coefficient of variation with standard deviation and β inverse function or by using the Excel (Microsoft, Redmond, Washington, United States) statistical function CRITBINOM (n, p, α) with a confidence level of 95%. When results are just above the cutoff value, the report should mention that the clinical significance is unclear.¹¹

In patients with lymphoblastic lymphoma without bone marrow involvement the cytogenetic characterization can be performed on either touch preparations, or on formalin fixed paraffin embedded sections.

Single-Nucleotide Polymorphism Array

Single-nucleotide polymorphism (SNP) arrays are a useful tool to study copy number abnormalities, polymorphisms, and copy neutral loss of heterozygosity at a whole genome level. The technology is based on the principle of hybridizing fragmented nucleic acid sequences derived from patient's DNA that are labeled with fluorescent dyes on the allele-specific oligonucleotide probes immobilized on array chip. The hybridization signal is recorded using a detection system and results are interpreted. SNP arrays can identify aneuploidies, chromosomal duplications, deletions, and copy neutral loss of heterozygosity. High-density SNP array in ALLs identifies hyperdiploidy, hypodiploidy, hidden hypodiploidy, *IKZF1* deletions, and *IKZF1* Plus patients (deletions in *PAX5*, *CDKN2A*, *CDKN2B*, *PAR1* deletions with absence of *ERG* deletions).^{12,13} The utility of SNP array in routine

Table 1 Primary cytogenetic and secondary genetic abnormalities in BCP-ALL

Primary Cytogenetic Abnormalities				Secondary Genetic Abnormalities				
Cytogenetic Subtype	Characteristics	Frequency In Children	Frequency in Adults	Prognosis	Genes involved	Change	Frequency in children	Frequency in adults
High Hyperdiploidy	>50 chromosomes	25-40%	2%	Good	<u>DEVELOPMENT</u>			
<u>Hypodiploidy</u>	<46 chromosomes	2-3%	9-11%	Poor	PAX 5	Deletion/ Translocation/ Mutations	32%	30-35%
Near Haploidy	24-29 chromosomes				IKZF1	Deletions/ Mutations	15%	40-50%
Low hypodiploidy	30-39 chromosomes							
High Hypodiploidy	40-45 chromosomes				<u>CELL CYCLE REGULATION</u>			
Near Triploidy	66-79 chromosomes	1%	1%	Intermediate				
Near Tetraploidy	84-100 chromosomes	1%	1%	Intermediate	CDKN2A	Deletions	20-25%	
iAMP 21	Amplification		1%	Poor	TP53	Deletions/ Mutations	<2% (Increased at relapse)	<2% (Increased at relapse)
<u>FUSIONS</u>					<u>SIGNALLING</u>			
t(12;21)	ETV6::RUNX1	15-25%	<1%	Good	JAK1/2			
t(9;22) t(1;19)	BCR::ABL1 TCF3::PBX1	2-5% 2-6%	35% 3%	Poor Intermediate	CRLF2	Rearrangements	5-16%	4-6%
KMT2A (MLL)	Multiple partners	1-2%	10-15%	Poor	RAS	Mutations	Increased at relapse	Increased at relapse
(Ph like)	ABL1/CRLF2	10%	20-25%	Poor	<u>OTHERS</u>			
ZNF384	Multiple partners	1-5%	2-7%		CREBBP	Deletions/ Mutations	Increased at relapse	Increased at relapse
MEF2D	Multiple partners	3%	7%	Poor	NT5C2	Mutations	Increased at relapse	Increased at relapse
DUX4/ERG		7%	4-5%	Good	NR3C1	Deletions	Increased at relapse	Increased at relapse

IKZF1 Plus profile (deletions in IKZF1 deletions plus PAX5, CDKN2A, CDKN2B, PAR1 deletions with absence of ERG deletions) seen in 6% of paediatric ALL⁷⁴ and 21% of adult ALL⁹

diagnostics laboratories is limited by high cost per test and inability to identify fusions resulting from balanced translocations.

Transcriptome Analysis

Transcriptome sequencing and analysis identifies fusions and unique gene expression signatures identifying novel genetic subtypes like *BCR::ABL1* like, *ETV6::RUNX1* like, *KMT2A* like, and *PAX5* altered ALLs.^{14–16} High costs, lack of expertise in analysis of data, and limited access to the technology have restricted its wider use in routine diagnostic laboratories.

Cytogenetic Strategy in BCP-ALL

The cytogenetic strategy is based on karyotyping, FISH analysis using a panel of four probes and DNA index using flow cytometry. The cytogenetic strategy is designed to identify aneuploidies (hyperdiploidy and hypodiploidy), rearrangements (*ETV6::RUNX1*, *BCR::ABL1*, *KMT2A* rearrangements, *TCF3::PBX1*, *TCF3::HLF1*), and amplifications (iAMP21).

While karyotyping identifies aneuploidies (hyperdiploidy, hypodiploidy and near haploidy), balanced chromosomal rearrangements (*BCR::ABL1*, *TCF3::PBX1* and *KMT2A* rearrangements), and structural abnormalities (duplications and deletions larger than 5MB), the resolution of karyotyping is low (<5MB) and it fails to identify cryptic translocations like t(12;21)/*ETV6::RUNX1* and some of the *KMT2A* rearrangements. Karyotyping also serves as a discovery tool identifying multiple structural and numerical abnormalities and identifies complex karyotype (5 abnormalities) that is associated with very high risk in adult ALL.⁹

FISH-Based Strategy

We developed a cytogenetic strategy based on three probe FISH testing and flow ploidy to identify principal genetic subtypes of BCP-ALL (► Fig. 1).¹⁷ The three-probe FISH strategy

includes dual-color fusion probe targeting *ETV6::RUNX1* fusion, dual color fusion probe targeting *BCR::ABL1* fusion, and *KMT2A* break-apart probe. The three-probe strategy has evolved into four-probe strategy by adding the *TCF3* triple color probe to identify *TCF3::PBX1* and *TCF3::HLF1* fusions.

FISH analysis is performed in a stepwise manner, the first step involves testing using *ETV6::RUNX1* probe in pediatric ALL patients and setting up sample for karyotyping and flow based detection of DNA index/ploidy. Samples that test positive for *ETV6::RUNX1* fusion are not tested using additional probes. If the sample tests negative, reflex testing is performed using *BCR::ABL1* dual-color fusion probe, *KMT2A* break-apart probe, and *TCF3* break-apart probe. Adult ALL patients are tested for *BCR::ABL1* fusion in the first step.

Additional Findings Using ETV6::RUNX1 Dual-Color Fusion Probe

FISH analysis using *ETV6::RUNX1* dual color probe not only identifies the fusion but also identifies iAMP21 and is a screening tool to identify HH.^{3,17}

HH: Presence of three to four discrete additional *RUNX1* signals is suggestive of HH as these patients universally gain chromosome 21. Almost 25 to 30% of HH patients show normal karyotypes. In these patients (hidden HH) presence of additional *RUNX1* signals is suggestive of HH and can be confirmed by using centromeric probes targeting chromosomes 4, 10, and 17 and correlating with flow ploidy.

iAMP21: iAMP21 is defined by the presence of five or more total copies of *RUNX1* in interphase cells or three or more extra *RUNX1* signals on a single abnormal chromosome 21 in a tandem step ladder arrangement.¹⁸

Additional Findings Using BCR::ABL1 Dual-Color Fusion Probe

FISH analysis using the dual-color fusion probe identifies *BCR::ABL1* fusions and is also a screening tool to identify other *ABL1* translocations. Presence of additional *ABL1* signal

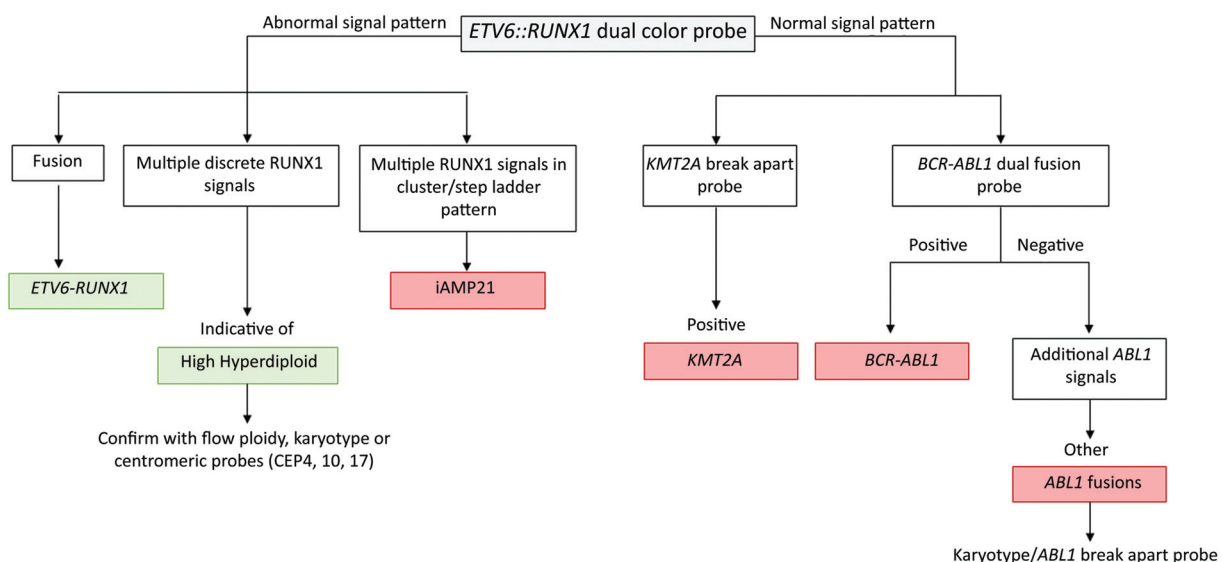


Fig. 1 Fluorescent in-situ hybridization (FISH) screening strategy to identify principal cytogenetic subtypes.

indicates the presence of *ABL1* rearrangements involving other partner genes. The *ABL1* rearrangement in these patients needs to be confirmed using *ABL1* break-apart probe.

Aneuploidies

The clinically relevant aneuploid entities in ALL comprises of HH, near triploidy, near-tetraploidy, near haploidy, and low hypodiploidy. HH and low hyperdiploidy are associated with intermediate prognosis.

High Hyperdiploidy

HH ALL forms one of the largest subgroups accounting for 30 to 40% of pediatric B cell ALL making it one of the most common malignancy in the pediatric population. HH ALL is characterized by non-random gains of chromosomes X,4,6,10,17,18, and 21 more frequently as trisomies followed by tetrasomies, with the modal chromosome number ranging from 51 to 67 chromosomes.¹⁹ HH ALL is generally associated with favorable clinical features like low white cell counts, age 2 to 7 years and a very low incidence of extramedullary disease.⁴ The outcome is superior with more than 90% overall survival in modern treatment protocols. Gains of specific chromosomes in HH ALL have been implicated as a significant factor affecting outcomes. Analyzing the cytogenetic data +6, +4/+10, +10/+17, and +4/+18 has been reported as good risk indicators in various studies.^{4,20,21} The Children's Oncology group uses the presence of triple trisomies (trisomies of chromosomes 4,10 and 17) as a good risk indicator.²² Lower modal numbers and the presence of trisomy 5 have been implicated as poor risk indicators in some studies.²⁰ However, these associations were not found to be consistent between the various clinical trials. Nearly 50% of HH patients show structural changes duplication of long arm of chromosome 1 being most common. Fusions are rare in HH patients. Patients with *BCR::ABL1*, *ETV6::RUNX1* or *KMT2A* rearrangements may be seen along with chromosomal gains and a hyperdiploid karyotype. These gains are usually secondary and the patients are assigned to the risk subtype based on the fusions present.

Diagnosis of HH ALL: Identified by karyotyping, flow cytometric-based DNA index, and SNP array. About 25 to 30% of HH patients show normal karyotype and diagnosis is based on flow cytometric DNA index and additional *RUNX1* signals on *ETV6::RUNX1* FISH analysis and CEP probes targeting chromosome 4,10, and 17.^{23,24} In patients with failed karyotype result, gain of *RUNX1* signals on *ETV6::RUNX1* FISH analysis suggests presence of HH, and the diagnosis is to be confirmed by FISH analysis using CEP probes targeting chromosomes 4,10, and 17.

Hypodiploidy and Near-Haploidy

Hypodiploidy is characterized by less than 46 chromosomes and is further subtyped into HH (40–43 chromosomes), low hypodiploidy (30–39 chromosomes), and near haploidy (25–29 chromosomes), the latter two being associated with extremely poor prognosis.²⁵ It is seen in 1% of childhood and 3 to 4% of adult BCP-ALL. Near haploid and low hypodiploid karyotypes

show monosomies of chromosomes 3,4, 7,13,15,16, and 17, while chromosomes X,14,18, and 21 show two copies.²⁶

Masked Hypodiploidy: In a proportion of patients with hypodiploidy, the hypodiploid clone can be masked through a process called endoreduplication. In these patients, the hypodiploid clone duplicates the number of chromosomes and the karyotype can reveal only metaphases derived from the endoreduplicated clone with 50 to 79 chromosomes.²⁷ The patients are erroneously categorized as HH ALL. Masked hypodiploidy can be identified by SNP arrays, where the chromosomes with two copies showing uniparental isodisomy and chromosomes with four copies showing 2:2 allelic ratios.²⁸ Flow-based ploidy analysis may show two peaks corresponding to the hypodiploid clone and the endoreduplicated hyperdiploid clone. Presence of characteristic pattern of gains of chromosomes also aids in identifying a masked hypodiploidy warranting further investigation. We devised a systematic cytogenetic strategy to identify masked hypodiploidy using a combination of flow ploidy, pattern of gains of chromosome on karyotype, and targeted FISH analysis directed to identify monosomies of chromosomes 3,7,15, and 17.²⁹

Diagnosis of Low Hypodiploidy/Near Haploidy

Diagnosis can be established by karyotyping, FISH, and SNP arrays. *TP53* mutations are seen in 90% of low hypodiploid patients.^{30,31}

Near Triploidy (66–79 Chromosomes)

There is evidence that near triploidy represents a hidden low hyperdiploid clone.²⁶ Once masked hypodiploidy has been ruled out, near triploidy is to be considered as an intermediate risk factor. Diagnosis rests on karyotyping, FISH, and SNP arrays.

Near Tetraploidy (84–100 Chromosomes)

Near tetraploidy is seen in 1% of childhood ALL and is more frequent in T ALL. Near tetraploidy does not carry any prognostic impact by itself.³² Once the presence of *ETV6::RUNX1*, *BCR::ABL1* fusions and *KMT2A* rearrangements have been ruled by FISH, near tetraploidy is to be considered as an intermediate risk factor.

Amplifications: Intrachromosomal Amplification of 21

iAMP21 is seen in 2% of pediatric ALL and is rare in adults.³³ It is characterized by gains and losses along the long arm of chromosome 21 with *RUNX1* gene being present in the common area of amplification.³⁴ The common regions of deletions involve subtelomeric region of chromosome 21.^{18,35} SNP array and whole genome studies have inferred that the formation of iAMP21 involves breakage-fusion-bridge cycle, resulting in amplification and formation of dicentric chromosomes and chromothripsis.³⁴ Associated with high relapse risk, intensive therapies have improved outcomes in recent years.³⁶

Diagnosis of iAMP21: On karyotype iAMP21 is seen as a grossly abnormal chromosome 21, the diagnosis being supported by FISH analysis showing more than or equal to three

copies of *RUNX1* gene on the arm of the chromosome 21.³⁵ On interphase FISH iAMP21 is defined as more than or equal three five copies of *RUNX1* gene per cell. On interphase FISH care should be taken to differentiate from HH as more than or equal to five copies of *RUNX1* may be seen in HH patients as well. In doubtful cases FISH targeting the subtelomeric region of chromosome 21 may help in diagnosis. SNP array analysis identifies a typical pattern of gains and losses associated with iAMP21 and is diagnostic in rare atypical cases where additional copies of *RUNX1* gene may be present on other chromosomes.³⁷

Rearrangements

The chromosomal translocations in BCP-ALL commonly involve transcription factors, epigenetic modifiers, cytokine receptors, and tyrosine kinases. The translocations deregulate gene expression either by forming a chimeric transcript or overexpression of a gene by juxtaposition of enhancers to the partner gene.

t(12;21)(p13;q22)/ETV6::RUNX1

ETV6::RUNX1 fusions are more frequent in childhood ALL and account for 25% of pediatric ALL in Western data and around 18% as per our data.^{2,17,38,39} It is associated with favorable outcomes. The *ETV6::RUNX1* fusion is known to occur prenatally where it gives rise to pre-leukemia state followed by secondary mutations that trigger overt leukemia.⁴⁰

Diagnosis: The t(12;21) is cryptic and is best identified by FISH analysis using dual-color fusion probes. The FISH approach has more advantages than the molecular RTPCR approach as the transcript expression can be low and also the FISH analysis using *ETV6::RUNX1* probe additionally identifies HH, iAMP21, and other *ETV6* fusions as well.

t(9;22)(q34;q11)/BCR::ABL1

BCR::ABL1 fusion accounts for 25% of adult ALL and is seen in 2 to 5% of childhood ALL. Associated with high risk,^{41,42} the outcomes have improved in recent years with the use of tyrosine kinase inhibitors in both children and adults.⁴³⁻⁴⁷ Ikaros deletions are common in *BCR::ABL1* positive patients and are associated with treatment failure and relapse.⁴⁸

Diagnosis: Identified by karyotyping, FISH and molecular methods.

TCF3 Rearrangements: t(1;19)(q23;p13)/TCF3::PBX1 and t(17;19)(q22;p13) /TCF3::HLF1

TCF3::PBX1 fusions occurs in approximately 6% of BCP-ALL and associated with intermediate risk.^{49,50} The fusion protein is formed by joining the homeobox (*HOX*) gene *PBX1* (for pre-B cell homeobox 1) on chromosome 1 with the two activation domains of the basic helix-loop-helix transcription factor *TCF3* on chromosome 19 leading to transcriptional activation of *PBX1*.

TCF3::HLF fusion is a rare genetic subtype seen in less than 1% of BCP ALL.⁵¹ Patients frequently present with disseminated intravascular coagulation, hypercalcemia,⁵² low

WBC counts, and absence of CD34 expression on immunophenotyping. It is associated with a dismal prognosis, despite treatment intensification and allogeneic stem cell transplantation.⁵³

Diagnosis: Identified by karyotyping, FISH, and molecular methods. The translocation t(1;19) may occur either as a balanced form or an unbalanced form (only derivative 19 present), the unbalanced form being more common.^{50,54} Not all patients with t(1;19) identified on karyotype carry the *TCF3::PBX1* transcript especially those associated with HH.⁵⁵ FISH analysis using a break-apart probe identifies these translocations accurately. The translocation t(17;19) can be identified by karyotyping and FISH. The *TCF3* break-apart probe is included in our FISH panel. Patients testing positive for *TCF3* rearrangement using break-apart probe are investigated using tricolor fusion probe that identifies the specific partners, that is, *PBX1* and *HLF1*.

KMT2A Rearrangements

KMT2A rearrangements are hallmark of infant ALL with an increased prevalence in adolescents and young adults (4%) and peaking in adults (15%). *KMT2A (MLL)* gene can rearrange with more than 80 partners and more than 100 different translocations have been described. They are strong drivers of leukemogenesis with very few secondary alterations and are associated with poor prognosis.⁵⁶

Diagnosis: Best identified by FISH analysis using break-apart probes. Karyotyping identifies partners and in patients with poor chromosome morphology match metaphase FISH analysis helps in identifying partner. *KMT2A* rearrangements involving genes located close to the *KMT2A* gene on chromosome 11 (*ATP5L* and *USP2*) show a normal FISH pattern and these rearrangements are best identified using molecular techniques or RNA sequencing.⁵⁷

B-Other ALL

B-Other ALL is a heterogeneous group defined by absence of all routinely assessed classifying cytogenetic abnormalities described above and are classified as intermediate risk cytogenetics. In the preceding decade, over 18 different genetic subtypes in the B-Other group have been identified, with differing treatment outcomes (► Fig. 2).¹⁴ Subtypes with good outcomes are *DUX4* fusions (associated with *ERG* deletions). Poor outcome subtypes include Ph-like ALL which may benefit from intensive therapy along with tyrosine kinase inhibitors.^{43,44} *MEF2D* and *ZNF384* fusions have been reported to be associated with intermediate prognosis.⁵⁸ Independent of these cytogenetic groups, somatic copy number alterations and gene mutations are also of prognostic significance. Among these, are deletions in *IKZF1* (*IKZF1*^{del}) especially when accompanied by deletions in *CDKN2A/B*, *PAX5* and the *PAR1* locus; deletions in *RB1*, *NR3C1*, *BTG1*; and mutations in *TP53*, and *PAX5*.^{8,59}

Role of Karyotyping and FISH in Identifying Different Subtypes of B-other BCP-ALL

Karyotype is a discovery tool and serves as a screening tool to identify structural abnormalities and rearrangements which

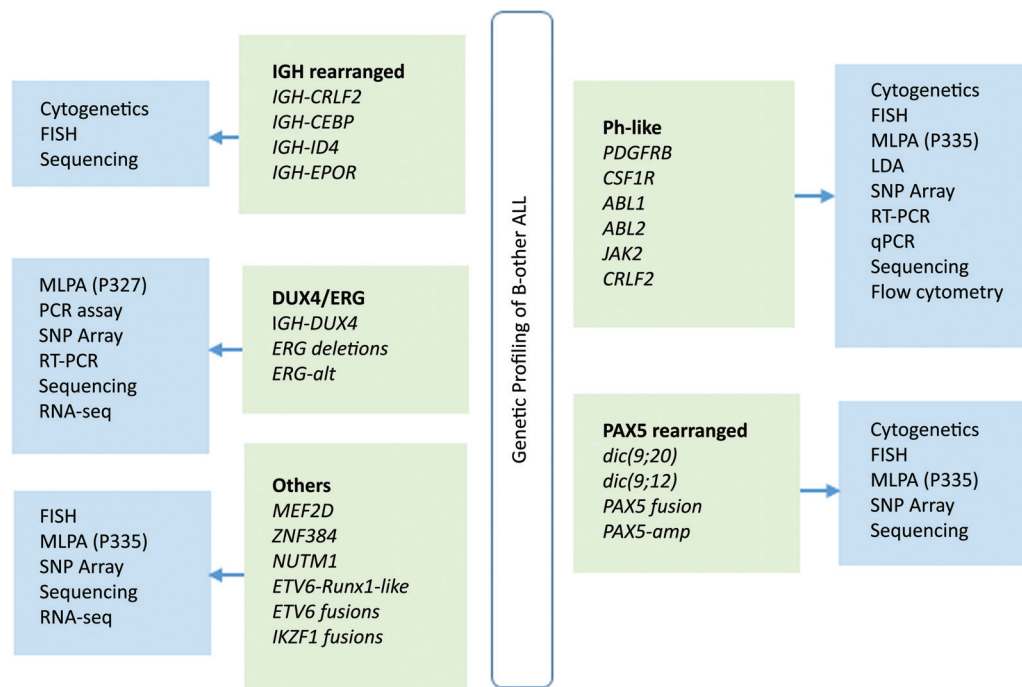


Fig. 2 Techniques available for genetic profiling of B-Other ALL.

can be confirmed through FISH analysis using specific probes.

MEF2D Rearranged BCP ALL

MEF2D (myocyte enhancer factor 2D)-rearranged ALL accounts for 4% of pediatric and 10% of adult ALL with a higher incidence in adolescents. It is characterized by a distinct immunophenotype (CD10⁻, CD38⁺) and is associated with a poor prognosis.^{58,60,61} The common fusion partner genes include *BCL9* (1q21), *HNRPULN1* (19q13.2), *DAZAP1* (19p13.3), *CSF1R* (5q32), *SS18* (18q11.2), *STAT6* (12q13.3), and *FOXJ2* (12p13.3). The fusions result in increased cell growth, resistance to dexamethasone, and increase of *HDAC9* expression. The most common fusion is the *MEF2D::BCL9* fusion which is the result of an interstitial deletion between 1q21 and 1q22. The fusion is cryptic and cannot be identified by karyotyping. *MEF2D* rearrangements lead to increased *HDAC9* expression, therefore amenable to histone deacetylase inhibitor treatment.⁵⁸

Diagnosis: The diagnosis is based on characteristic immunophenotype confirmed by FISH analysis using a *MEF2D* break-apart probe. The tricolor *MEF2D* break-apart probe differentiates between *MEF2D::BCL9* fusions and other partners

B- ALL with Zinc Finger Protein 384 (ZNF384) Rearrangements

ZNF384 rearrangements show a peak incidence in adolescents and young adults and account for 1 to 5% of pediatric and 2 to 7% of adult ALL.⁶²⁻⁶⁴ The *ZNF384* gene functions as a transcription factor and multiple partners have been described. The *ZNF384* rearranged ALL have a distinct transcriptomic signature and are associated with aberrant expression of myeloid markers (CD13 and CD33). With a

stem cell signature, the *ZNF384* rearrangements have been reported in biphenotypic, mixed phenotype, and acute undifferentiated leukemia.⁶⁵

Diagnosis: The aberrant expression of myeloid markers provides a clue. The rearrangements are undetectable by karyotyping and are identified by FISH analysis using a *ZNF384* break-apart probe.

BCR::ABL1 Like (Ph-Like ALL)

The *BCR::ABL1*-like (Ph-like) is characterized by gene expression profile similar to *BCR::ABL1* fusion positive patients but lacks the *BCR::ABL1* fusions.^{15,16} Ph-like ALL is seen in 12% of childhood, 21% of adolescent 27% of young adult, and 20 to 24% in adults more than 40 years of age. It is a heterogeneous group consisting of gene rearrangements, copy number alterations, and mutations that activate tyrosine kinase or cytokine receptor signaling. Similar to *BCR::ABL1* positive ALL, *IKZF1* deletions are found in 70 to 80% of Ph-like ALLs and are associated with poor outcomes.¹⁵

The definition of Ph-like ALL is based on gene expression profile, while the cytogenetic approach to identify this entity is based on identifying *CRLF2* rearrangements and identifying *ABL* class fusions using break-apart probes (→ Fig. 3).

CRLF2 Rearranged BCP-ALL

The *CRLF2* gene is located on the pseudoautosomal regions of the sex chromosomes X and Y. The two common genomic alterations resulting in *CRLF2* rearrangement include t(X;14)(p22.3;q32.33) or t(Y:14)(p11.32;q32.33) and *CRLF2::P2RY8* fusion resulting from 320KB interstitial deletion within the PAR1 region bringing the two genes together.^{5,66,67} The *P2RY8::CRLF2* fusions have also been identified with other primary cytogenetic abnormalities like HH and iAMP21. *CRLF2* rearrangements result in overexpression of the

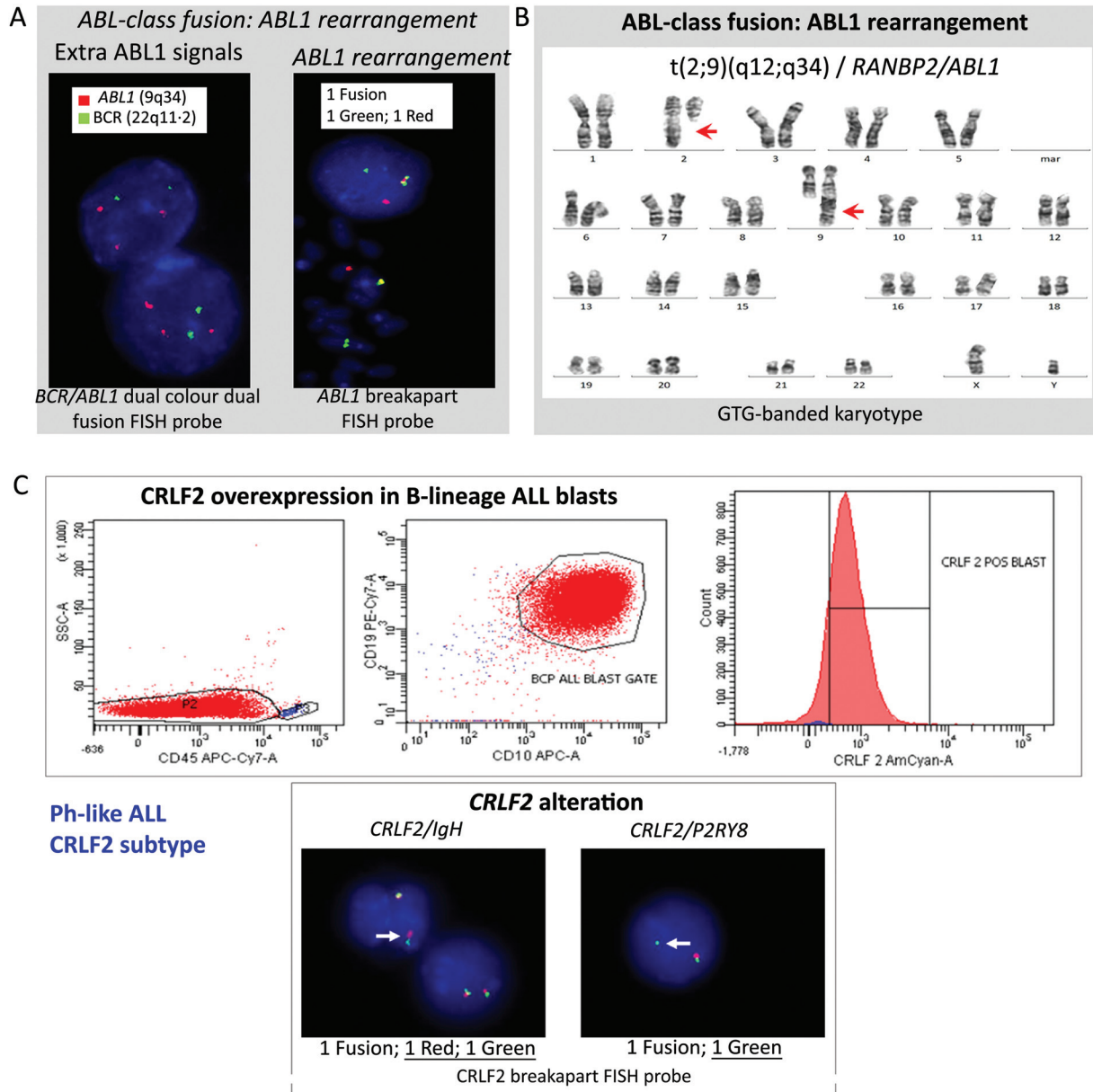


Fig. 3 Cytogenetic approach to identify Ph-like ALL with *ABL1* rearrangement (A and B) and *CRLF2* rearrangement (C).

CRLF2 protein that can be detected by multiparametric flow cytometry. The *IgH* translocation places the *CRLF2* gene under the impact of *IGH* enhancer and in *CRLF2::P2RY8* fusion under the influence of promoter sequences of the *P2RY8* gene.

Rearrangement of the *CRLF2* gene is seen in 50% of Ph-like ALL and in half of Down syndrome-associated BCP-ALL.⁶⁸ The *CRLF2* rearrangements are associated with poor prognosis in non-Down syndrome pediatric and adult patients.^{38,41,69}

Diagnosis: Both the *IgH::CRLF2* and *P2RY8::CRLF2* are cryptic and cannot be detected on karyotype. They can be detected by FISH using a *CRLF2* break-apart probe. *CRLF2::P2RY8* fusions can also be identified by detecting deletion in the *PAR1* region by using multiplex ligation-based probe amplification (MLPA) or chromosomal micro arrays or SNP arrays.

ABL Class Fusions

ABL class fusions although not so frequent in BCP-ALL (3–5% of pediatric ALL, 2–3% of adult ALL) are seen in 10% of Ph-like ALL.^{41,42,70} The *ABL* class rearrangements result in fusion of the 5' partner gene with 3' of the kinase gene resulting in transcripts that have intact tyrosine kinase domain resulting in activation of the kinase pathway.⁷⁰ The frequently involved kinase genes include *ABL1*, *ABL2*, *CSF1R*, *PDGFRB*, and *PDGFRFA*.⁶⁹

Diagnosis: The fusions can be identified by RNA sequencing and multiplex PCR assays. Cytogenetically, the presence of an additional *ABL1* signal on *BCR::ABL1* FISH is a screening tool and hints toward the presence of the *ABL1* rearrangements. Since the Ph-like ALL is associated with poor response to induction therapy and high MRD, our cytogenetic approach is based on FISH analysis using break-apart probes targeting

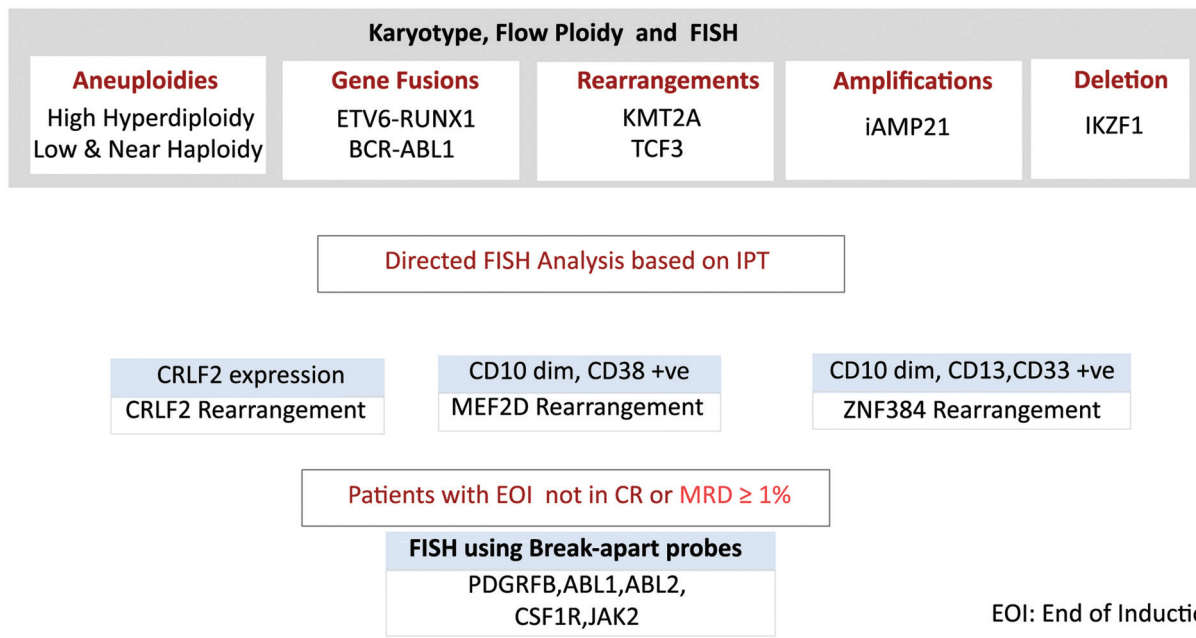


Fig. 4 Cytogenetic strategy to profile B-other ALL including Ph-like ALL.

ABL1, *ABL2*, *CSF1R*, *PDGRFB*, and *PDGRFA* in patients not in remission at end of induction or having a high MRD. Karyotype can also identify translocations involving the kinase genes that are confirmed using specific break-apart probes.

JAK Rearrangements and Mutations

Translocations resulting in rearrangement of *JAK2* gene are seen in 5% of pediatric Ph-like ALL and more frequently in young adults.⁶⁹ The *JAK2* rearrangements result in in frame fusion of the 5' of the partner gene with the 3' of the *JAK2* kinase gene keeping the tyrosine kinase domain intact. The *JAK2* fusions can be identified by molecular techniques, RNA sequencing, visible chromosomal rearrangements involving the 9p24 loci, and FISH analysis using the break apart probe. The *JAK2* mutations are frequent in *CRLF2* rearranged ALL and result in activation of the JAK-STAT pathway. The mutations can be identified using Sangers sequencing. The other JAK mutations are not so common and involve the *JAK1* gene.

IKZF1 Deletions and IKZF1 plus BCP ALL

IKZF1-deletions are known to have poor outcomes in ALL overall,⁷¹ and are associated with resistance to therapy. *IKZF1* deletions with co-occurring alterations in *CDKN2A/2B*, *PAX5*, and *PAR1* (pseudo-autosomal region) in the absence of *ERG* deletions are defined as *IKZF1* plus and are associated with poor outcomes and high rates of treatment failure.⁷²

The *IKZF1* deleted and *IKZF1* plus are commonly identified using MLPA or chromosomal microarray analysis.¹² In recent times, FISH probe targeting the exon 4–7 of the *IKZF1* gene has been used to identify the whole gene and intragenic deletions of the *IKZF1* gene.⁷³ Karyotyping identifies monosomy 7, deletions of short arm of chromosome 7, and dicentric translocations that result in deletion of the entire short arm of chromosome 7. Dicentric translocations between the long arm of chromosomes 7 and 9 result in

deletions of the entire short arms of chromosomes 7 and 9 and are consistent with the diagnosis of *IKZF1* plus.

Summary

Cytogenetic study including karyotype and FISH is an efficient tool in identifying the primary chromosomal abnormalities in BCP-ALL. The minimal diagnostic workup suggested is a FISH panel that includes probes targeting *ETV6::RUNX1*, *BCR::ABL1*, *KMT2A* rearrangements and *TCF3* rearrangements along with karyotyping and flow ploidy. In patients with failed karyotype, information from *ETV6::RUNX1* probe, flow ploidy complemented with additional centromeric probes targeting 4,10 and 17 can identify the aneuploidies including the hidden hyperdiploid and a fair proportion of masked hypodiploidy patients. The information from immunophenotyping can be integrated into the cytogenetic analysis and utilized for targeted FISH analysis (► Fig. 4). For example, information on dim to negative CD10 expression and CD38 positivity is an indicator to test using *MEF2D* break apart probe. Similarly, dim CD10 expression with aberrant CD13 and CD33 positivity is an indicator to test for *ZNF384* rearrangements. These patients commonly show *ETV6* deletions on *ETV6::RUNX1* FISH analysis. Reflex testing for *ABL* kinase and *JAK2* rearrangements in patients is suggested in patients with treatment failure at end of induction or with high MRD. The above strategy can identify genetic aberrations in more than 70% of BCP-ALL patients.

Authors' Contributions

R.I. and M.P. wrote the original draft, K.R., A.D. collated the data and figures; M.P. and R.I. have full access to all data and the final responsibility for publication. All authors reviewed the manuscript draft submitted for publication.

Conflict of Interest

None declared.

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