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Pediatric High Risk Leukemia — Molecular Insights

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Abstract

Acute leukemia comprises of 31% of all cancers in children making it the most common childhood malignancy. Significant strides have been made in treatment, partly through risk stratification and intensified therapy. A number of subtypes remain at high risk for relapse and poor outcome, despite current therapies. Here we describe risk stratification and molecular diagnosis used to identify high risk leukemias and guide treatment. Specific cytogenetic alterations that contribute to high risk B and T cell acute lymphoblastic leukemia (ALL), as well as infant leukemia are discussed. Particular attention is given to genetic alterations in IKZF1, CRLF2, and JAK, that have been identified by whole genome sequencing and recently associated with Ph-like ALL. Ongoing studies of disease mechanisms and challenges in developing pre-clinical patient-derived xenograft models to evaluate therapies are discussed.

Keywords: Acute lymphoblastic leukemia, Pediatric Leukemia, high risk leukemia, Ikaros, IKZF1, CRLF2

1. Introduction

Acute leukemia comprises 31% of all cancers in children, making it the most common childhood malignancy. Acute lymphoblastic leukemia (ALL) makes up 80% of these cases and the remaining are leukemias of the myeloid lineage. Among the lymphoblastic leukemias, there are two immunophenotypic groups: B cell precursor ALL (B-ALL, 80% of all ALL) and T cell ALL (T-ALL, 20%). In the following sections, the further classification of each of these subtypes of leukemia, based on their molecular characteristics, is discussed. Also discussed are the clinical importance, prognosis, and new therapies available for each subtype.

2. Overview of classification of pediatric leukemia

2.1. Definition of standard and high risk leukemias

The National Cancer Institute (NCI) has classified acute lymphoblastic leukemia in children based on age at diagnosis, initial white blood count, and the presence of extra medullary disease.

- Standard risk: initial WBC count less than 50,000/μL and age 1 to younger than 10 years

- High risk: initial WBC count 50,000/μL or greater and/or age 10 years or older

The Children's Oncology Group (COG) has classified acute leukemia into four risk groups using prognostic factors that are strongly predictive of outcome such as: 1) age; 2) initial white count; 3) gender; 4) presence of extra medullary disease at diagnosis (CNS or testicular disease); and 5) blast cytogenetic findings and ploidy, and 6) response to induction therapy [1, 2].

Based on these factors, the four risk groups for COG classification of newly diagnosed B-ALL for the AALL08B1 study are low risk, average risk, high risk, and very high risk. In T-ALL, high white count does not have a prognostic significance. The above classification applies to B cell phenotype cases. The presence of CNS or testicular disease, age <1 year, and trisomy 21 are considered high risk. The importance of tumor cell characteristics will be discussed in the following sections.

2.2. Molecular diagnosis and its importance

Immunophenotyping is used to classify leukemia into B-ALL and T-ALL [3, 4].

B-ALL: comprises 80% of ALL. B-ALL cells express cytoplasmic CD79a, CD19, HLA-DR. Surface CD10 (formerly known as common ALL antigen or CALLA) is seen in 90% of these cases. Subtypes are as follows:

- Common Precursor B Cell ALL (75% of cases): These cells are CD10 positive and express no surface or cytoplasmic Ig. This group has the best prognosis.

- Pro B ALL (5% of cases): Commonly seen in infants with MLL rearrangement. These cells are CD10 negative and express no surface or cytoplasmic Ig.

- Pre B ALL (20% of cases): These cells express cytoplasmic Ig.

T-ALL: These cells express cytoplasmic CD3, with CD7 plus CD2 or CD5 on leukemic blasts. T-ALL is associated with older age, male gender, high initial white count, and mediastinal mass.

Cytogenetic alterations: The presence of recurrent numerical and structural chromosomal abnormalities in both ALL and AML are very common and are associated with prognostic significance. Hence, cytogenetic characteristics are now used for risk stratification of patients with ALL [5, 6].
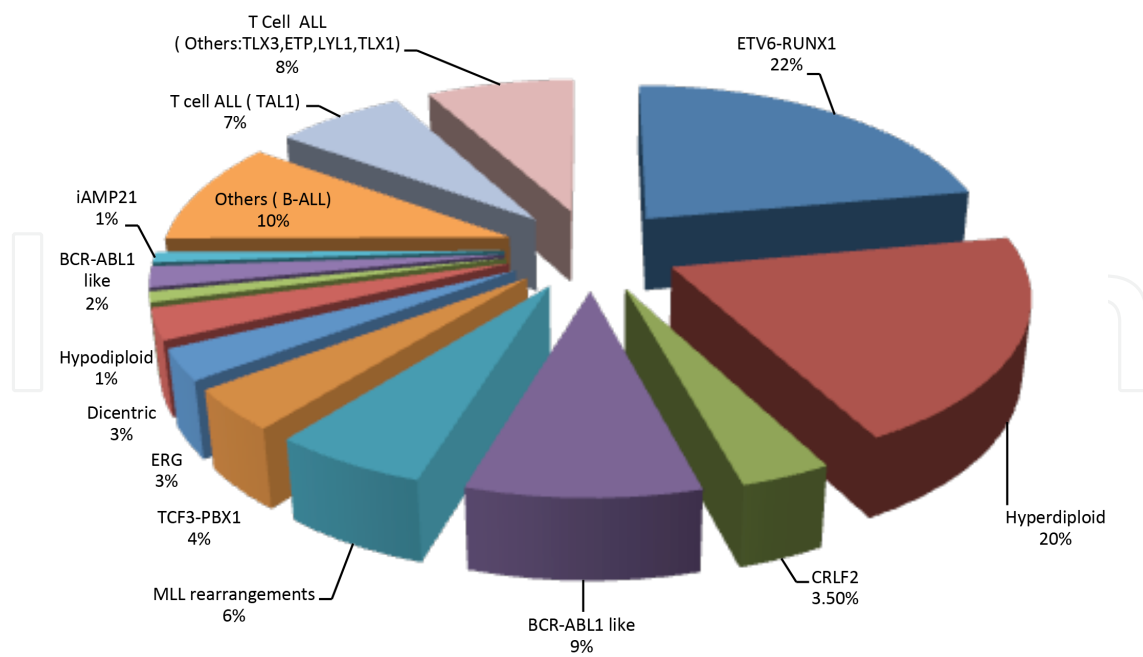
Figure 1. Frequency of Cytogenetic Abnormalities in ALL

Subtype	Frequency (%)	Clinical Significance
Hyperdiploid (>50 Chromosomes)	20-30	Good prognosis
t(12;21)(p13;q22)ETV6-RUNX1	15-25	Good prognosis; expression of myeloid antigens
t(1;19)(q23;p13)TCF3-PBX1	2-6	Good prognosis; associated with CNS relapse; more frequent in African American
CRLF2 rearrangements	5-7	55% OF Down Syndrome ALL; poor prognosis in non-DS ALL; IKZF1 and JAK1/2 alterations common
ERG deletion	7	Good outcome
t(9;22)(q34;q11.2)BCR-ABL1	2-4	Improved outcome with TKI and intensive chemotherapy
ABL1, PDGFRB, JAK2 rearrangements	2-5	'BCR-ABL1 like' ALL; high WBC count; associated with IKZF1 alteration
MYC rearrangement	2	Good outcome with short term intensive chemotherapy
PAX5	2	Unknown prognostic significance
t(4;11)(q21;q23) MLL- AF4	1-2	Infant ALL ; poor prognosis specially in <6 month age
Hypodiploid (<44 chromosomes)	1-2	Poor Prognosis; associated with IKAROS gene family and RAS pathway mutations

Table 1. Common Genetic Alterations and Clinical Significance B-ALL.

Risk stratification based on cytogenetic characteristics has revealed many subgroups of NCI standard and high risk groups that are now treated differently. Many treatment strategies using targeted therapies directed at specific genetic alterations in the tumor cells have opened up a new era of leukemia therapy. Figure 1 and Table 1 show cytogenetic abnormalities seen in ALL [6, 7].

3. Infant leukemia and leukemia with MLL rearrangement

3.1. Overview of infant leukemia

Infant leukemia comprises only 1–2 % of childhood ALL. The cells often show an immature pro-B phenotype. They present with very high WBC count and large extra medullary disease burden which confer poor prognosis in this subset of children [8].

3.2. Biology of infant leukemia

Rearrangements of the *KMT2A* (also known as mixed lineage leukemia (MLL)) gene located on chromosome 11q23 are seen in more than 80% of infant ALL, with increased frequency in patients under 6 months of age. They are also seen in ~50% of infant AML [8] and a majority of adults with therapy-related leukemia secondary to topoisomerase II inhibitors. Balanced translocations result in the fusion of *MLL* to a number of different partner genes, leading to the production of novel chimeric proteins with t(4;11)(q21;q23) *MLL-AFF1* (also called *MLL-AF4*) being the most common. Other rearrangements are t(11;19)(q23;p13.3) *MLL-/ELN*; t(6;11) (q27;q23) *MLL/MLLT4, also called MLL/AF6*; t(9;11)(p22;q23) *MLL/MFT3*, also called *MLL-AF9*; and t(10;11)(p12;q23) *MLL/MLLT10*, also called *MLL/AF10*. The *MLL gene product* plays a critical role in hematopoiesis by regulating the *HOX* group of genes, which sequentially influence hematopoietic stem cell renewal and leukemogenesis [9, 10].

3.3. Risk stratification, current therapeutic approaches and outcome in infant leukemia

Infant ALL is considered high risk for many reasons. The factors that confer poor prognosis are age younger than 6 months, extreme hyperleukocytosis (>300,000 white blood cells/μL), CD10 negativity, and the presence of *MLL* rearrangement. The type of the partner gene fused to *MLL* does not seem to influence outcome [9, 11, 12]. Studies by the BFM group and the international ALL protocol Interfant-99 have shown that patients who were early good responders to prednisone therapy had a better outcome [13, 14].

Infant ALL is currently treated with intensive chemotherapy including high dose cytarabine – "AML-like" therapy. The prednisone poor responders are classified as high risk and require intensification of therapy. Hematopoietic stem cell transplantation for infants with ALL in first remission remains controversial [15, 16].

4. FLT3 mutations in ALL

4.1. Incidence of *FLT3* alterations in ALL and its prognostic significance

Constitutive activation of FMS-like tyrosine kinase 3 (FLT3) plays an important role in the pathogenesis of hematopoietic malignancies. *FLT3* activating mutations, internal tandem duplications (ITD), and kinase domain (KD) mutations were initially discovered in acute myeloid leukemia (AML) and are associated with poor prognosis in both adult and pediatric AML. However, in lymphoblastic leukemia, FLT3 ITD did not cause significant change in overall survival and event free survival [17]. Recently, FLT3 overexpression has been seen in two types of ALL: 18% of infant ALL with *MLL* rearrangement and 21–25% of hyperdiploid ALL and in relapsed ALL samples [18, 19]. Studies have shown that high *FLT3* expression mutation identifies MLL-AF4+ (also called *MLL-AFF1+*) ALL patients at very high risk of treatment failure and poor survival, emphasizing the value of ongoing/future clinical trials for FLT3 inhibitors [20–23].

4.2. FLT3 inhibitors in the treatment of leukemia with FLT3 alterations

Recent gene expression studies have shown that in *MLL*-rearranged leukemia, FLT3, a receptor tyrosine kinase that plays a role in promoting cell proliferation and transformation is overexpressed. This has led to the pursuit of FLT3 inhibitors as targeted therapy for this disease [22]. Currently, Lestaurtinib (CEP-701™), an oral, highly selective small-molecule FLT3 inhibitor, is being used in COG - phase III trial wherein infants with *MLL*-rearranged ALL are being randomized to intensive chemotherapy with or without Lestaurtinib.

5. BCR-ABL positive leukemia

5.1. Incidence, current therapy, and outcome in BCR-ABL+ (Ph+) leukemia

Chimeric BCR–ABL1 protein is encoded by *BCR-ABL1* fusion gene created by reciprocal t(9;22) (q34;q11) translocation on chromosome 22, also known as the Philadelphia chromosome. The *BCR–ABL1* fusion gene is generated by joining most of the coding region of the *ABL1* tyrosine kinase gene (Abelson murine leukemia) on chromosome 9 to the breakpoint cluster region (*BCR*) gene on chromosome 22. The molecular consequence of all BCR–ABL1 fusion proteins is a hyperactive ABL1 kinase domain and aberrant phosphorylation of a variety of targets [23, 24]. There are two gene product variants: p190, which is seen in >90% of Philadelphia+ (Ph+) ALL in children, and p210, which is seen in CML.

Ph+ ALL comprises 3–4% of pediatric ALL, and about 25% of adult ALL cases. Prior to tyrosine kinase inhibitors, these patients had dismal outcomes despite the use of intensive chemotherapy and hematopoietic stem cell transplant in the first remission was the best available option [24].

5.2. Tyrosine kinase inhibitors in BCR-ABL+ (Ph+) leukemia and current challenges

Imatinib was the first generation of tyrosine kinase inhibitors that changed the face of treatment for Ph+ ALL and CML. The addition of Imatinib to intensive chemotherapy in childhood BCR-ABL1–positive ALL results in a 4-year event-free survival rate of 84%, more than double that of historical controls [25]. About 40% of the newly diagnosed Ph+ ALL patients carry point mutations within the kinase-binding domain of BCR-ABL that confers resistance to Imatinib.

Dasatinib and Nilotinib are second-generation tyrosine kinase inhibitors. Dasatinib is a multikinase inhibitor targeting several tyrosine kinases, including BCR-ABL and SRC kinases. It is 325 times more potent than Imatinib, binds to the active and inactive forms of BCR-ABL, and has excellent CNS penetration. These are effective in patients resistant to Imatinib, except those with the *T315I* mutation [26].

Currently, allogeneic hematopoietic stem cell transplant (HSCT) is the standard of care in second remission for patients with Ph+ ALL. It has been observed that most of the patients treated with tyrosine kinase inhibitor (TKI)-based therapy will eventually relapse without HSCT. However, benefits of allogeneic HSCT in first remission, after intensive chemotherapy and TKI therapy, has to be considered based on donor availability, minimal residual disease (MRD) status, and clinical status of the patient [27]. Recent studies in both adults and children have failed to prove clear benefit of allogeneic HSCT in the first remission for this group of patients mainly because of the small sample size [23, 28, 29].

6. *Ikaros*-altered ALL

6.1. Overview of *Ikaros*

Ikaros is a DNA-binding zinc finger protein encoded by the *IKZF1* gene. Ikaros is a transcription factor that functions as a regulator of gene expression and chromatin remodeling [30]. Ikaros regulates the development and function of the immune system and acts as a master regulator of hematopoietic differentiation. Genomic profiling studies identified *IKZF1* as an important tumor suppressor in ALL, particularly in ALL that is associated with poor prognosis [31, 32]. The mechanism by which Ikaros suppresses malignant transformation and the development of ALL is largely unknown. In the past few years, experiments by several groups have shown that Ikaros regulates expression of its target genes by recruiting them to pericentromeric heterochromatin, resulting in their activation or repression [33, 34].

6.2. Clinical importance of Ikaros deletion

IKZF1 has been established as one of the most clinically relevant tumor suppressors in high-risk ALL. Genome wide analysis studies have shown that 15% of all cases of pediatric B-cell ALL show deletion of a single *IKZF1* allele or mutation of a single copy of *IKZF1*, resulting in haploinsufficiency of *IKZF1*. Haploinsufficiency occurs with expression of a functionally inactive Ikaros splice form that acts as a dominant negative. Genetic inactivation of *IKZF1* is more rare in T-ALL where *IKZF1* alterations occur in ~5% of cases [35]. Over 80% of BCR-ABL1

ALL and 66% of chronic myeloid leukemia (CML) patients during lymphoid blast crisis show mutation or deletion of an *IKZF1* allele. One-third of BCR-ABL1 negative ALL patients also show *IKZF1* deletion or mutation. This group of patients is at increased risk of poor outcome with more than threefold increase in relapse rate [30]. Poor outcome associated with *IKZF1* alteration is frequently independent of age, sex, white cell count, and level of minimal residual disease – factors which are commonly used for risk stratification. Testing for *IKZF1* status at the time of diagnosis is now being explored in prospective trials. In recent genome profiling studies, *IKZF1*-altered high risk pediatric ALL cases have shown marked similarity to BCR-ABL positive ALL giving rise to a new subset of cases now called "Ph-like " or "BCR-ABL-like" ALL. This will be discussed further later in the chapter.

6.3. Regulation of Ikaros function in T cell leukemia

The role of Ikaros in normal T cell development is demonstrated by evidence that Ikaros regulates the expression of key genes in T cell differentiation. T cell differentiation is significantly impaired in Ikaros-deficient mice. Terminal deoxynucleotide transferase (*DNTT)* is a gene product critical for thymocyte differentiation that is regulated by Ikaros. Ikaros also regulates the expression of CD4, CD8, and IL-2 and plays a critical role in T cell differentiation [36, 37].

6.3.1. Regulation of Ikaros function

Casein kinase has been shown to phosphorylate Ikaros at multiple sites, and indeed, CK2 kinase is responsible for the majority of Ikaros phosphorylation. Studies by the Dovat group showed that a single phosphomimetic mutation at amino acid 13 or 294 caused the redistribution of Ikaros protein in the nucleus from pericentromeric localization to a diffuse nuclear staining pattern, while phosphoresistant mutations produced no changes in the subcellular localization of Ikaros. These data suggest that targeting of Ikaros to pericentromeric heterochromatin is regulated by its phosphorylation at specific amino acids [34, 38].

6.3.2. CK2 mediated phosphorylation of Ikaros impairs Ikaros function

Recent studies have demonstrated that CK2-mediated phosphorylation of Ikaros controls essential functions of Ikaros including DNA-binding, subcellular localization, and chromatin remodeling, as well as the level of Ikaros protein in cells (via ubiquitination and degradation). Phosphorylation of Ikaros by CK2 kinase also regulates cell cycle progression and Ikaros function in T cell differentiation [39, 40]. Since the overexpression of CK2 kinase and the loss of Ikaros function have been strongly associated with leukemogenesis, it is proposed that increased CK2 kinase activity leads to impaired function and/or degradation of Ikaros, which results in malignant transformation and the development of leukemia.

6.4. Inhibition of CK2 as a potential therapeutic approach to treat high risk leukemia

Casein Kinase II inhibition has been an attractive therapeutic strategy in several malignancies. A specific CK2 inhibitor, CX-4945, orally bioavailable small molecule is in Phase I clinical trial for solid tumors. The role of CK2 inhibitor as an antileukemic drug in ALL needs to be explored.

7. Hypodiploid ALL

7.1. Subclassification and treatment outcome of hypodiploid ALL

Hypodiploid ALL comprises 1–2% of all B-ALL cases and confers poor prognosis. Hypodiploid ALL is a chromosome number abnormality in the leukemic cells that results in 45 chromosomes or less. Hypodiploid ALL has been subdivided in various ways. In general, Hypodiploid ALL may be subclassified into the following four groups:

- near-haploid cases – 24–31 chromosomes

- low-hypodiploid cases – 32–39 chromosomes

- high-hypodiploid cases – 40–43 chromosomes

- near-diploid cases – 44–45 chromosomes

Patients with 44 or 45 chromosomes have a much better outcome than patients with fewer than 44 chromosomes [41].

7.2. Genomic profiles of hypodiploid ALL

Recently, a large group of 124 pediatric patients with hypodiploid ALL were analyzed using microarray profiling of gene-expression and copy-number alteration, and next-generation sequencing. These analyses indicate that near-haploid and low-hypodiploid ALL are distinctive from each other and from other types of ALL. In near-haploid ALL, genetic alterations target RTK (receptor tyrosine kinase) signaling, Ras signaling, and the lymphoid transcription factor gene *IKZF3*, while in low-hypodiploid ALL, genetic alterations involve *TP53*, *RB1*, and *IKZF2* [42].

RTK and Ras signaling alterations were present in more than two-thirds (70.6%) of near-haploid ALL cases, while they were much less common in low-hypodiploid (8.8%) and near-diploid ALL (31.8%). The RTK and Ras signaling alterations involve deletion, amplification, and/or sequence mutation of *NF1*, *NRAS*, *KRAS*, *MAPK1*, *FLT3*, or *PTPN11*.

The *TP53* alterations were present in 91.2% of low-hypodiploid ALL, but less than 5% of non-low-hypodiploid ALL. Meanwhile, *TP53* alterations are also present in non-tumor cells in 43.3% of the mutation-carrying cases, suggesting that the *TP53* mutations are inherited and the low-hypodiploid ALL might be a manifestation of Li-Fraumeni Syndrome (LFS).

The *Ikaros* gene family is very important for lymphoid development and differentiation. *IKZF2* is highly expressed in common lymphoid progenitors and pre-pro-B cells, while *IKZF3* is mainly expressed in more mature lymphoid precursors. *IKZF2* and *IKZF3* alterations occur in low-hypodiploid and near-haploid ALL, respectively, suggesting that low-hypodiploid ALL may arise from the transformation of a lymphoid progenitor that is a less mature lymphoid than those from which near-haploid ALL arises. More importantly, both low-hypodiploid and near-haploid leukemic cells show activation of Ras-signaling and PI3K-signaling pathways. In addition, PI3K and mTOR inhibitors can inhibit proliferation of both low-hypodiploid and

near-haploid leukemic cells ex vivo, which indicates that these drugs should be further investigated as a new therapeutic strategy for hypodiploid ALL [42].

8. Leukemias involving the *E2A-PBX1* translocation

8.1. Introduction: Wild type E2A and PBX1

The *TCF3* gene (also known as *E2A)* encodes two proteins, E12 and E47, which are generated by differential splicing events. Both proteins contain a C-terminal basic helix-loop-helix (bHLH) domain and two activation domains called AD1 and AD2. E2A proteins function as transcriptional activators by binding to E-box DNA sequence and recruiting histone acetyl-transferase complexes. E2A proteins contribute to various aspects of lymphocyte differentiation and development. In E2A deficient mice, B cell development is arrested at the early pro-B stage [43].

PBX1 was first described through its involvement in the translocation t(1; 19) [44]. *PBX1* itself is not expressed in lymphoid cells, though its related genes, *PBX2* and *PBX3*, are expressed in lymphocytes [45]. PBX1 contains a homeodomain involved in DNA binding and protein-protein interaction. It can cooperate with other homeodomian containing proteins of HOX and MEINOX classes to regulate the transcription of target genes [46].

8.2. Structure and function of the *E2A-PBX1* translocation

The chromosomal translocation between chromosomes 1 and 19 results in a fusion event between *TCF3* and *PBX1*, which is detected in 3–5% of all pediatric pre-B cell ALL cases [47, 48]. The E2A-PBX1 fusion proteins contain two-thirds of the N-terminal of E2A proteins (which retain the AD1 and AD2 domain, but lose the bHLH domain) and most of the PBX1 protein. Two forms of E2A-PBX1 proteins are detected, E2A-PBX1a and E2A-PBX1b, which differ at the C-terminus of PBX1 [49].

The E2A-PBX1 fusion protein is capable of transforming various cell types. Enforced expression of E2A-PBX1 induces lethal lympho-proliferative diseases in transgenic mice and aggressive myeloproliferative diseases in a murine bone marrow transplantation model [50].

8.3. Target genes of E2A-PBX1

Many efforts have been made to find the mechanisms by which E2A-PBX1 mediates transformation of pre-B cells. One important way is try to find potential target genes or pathways that are regulated by E2A-PBX1. Using ChIP-chip assay, Diakos et al. found 108 direct E2A-PBX1 targets [51]; however, few targets have been studied in detail. *Wnt16* and *EB-1* are two target genes of E2A-PBX1 that are well studied. Wnt16 belongs to the Wnt family, a group of signaling factors that plays important roles in many developmental processes including cell differentiation, proliferation, polarity, and migration [52]. Wnt16 is normally expressed in peripheral lymphoid organs but not in bone marrow. In contrast, Wnt16 is highly expressed in bone

marrow and cell lines that are derived from pre-B ALL patients carrying E2A-PBX1 [52]. *EB-1* expression was much lower in marrow from pre-B ALL patients without t(1; 19) than marrow from pre-B ALL patients and pre-B cell lines that contain the t(1; 19). EB-1 is a signaling protein containing a phosphotyrosine binding domain, and may play a role in the regulation of cell proliferation. [53].

8.4. Clinical treatment and outcome for E2A-PBX1 ALL

For pediatric B-ALL patients with the E2A-PBX1 fusion protein, the prognosis has been controversial. It was initially associated with poor outcome when treated with antimetabolite-based therapy [54, 55], but the recent development of intensified chemotherapy has much improved the outcome of this subgroup. However, in a trial conducted by St. Jude Children's Research Hospital, although patients with the t(1; 19) had an overall favorable outcome with intensified chemotherapy, this group had a significantly higher incidence of CNS relapse, suggesting intensive CNS-directed therapy is needed to further improve the outcome in this group of patients [56].
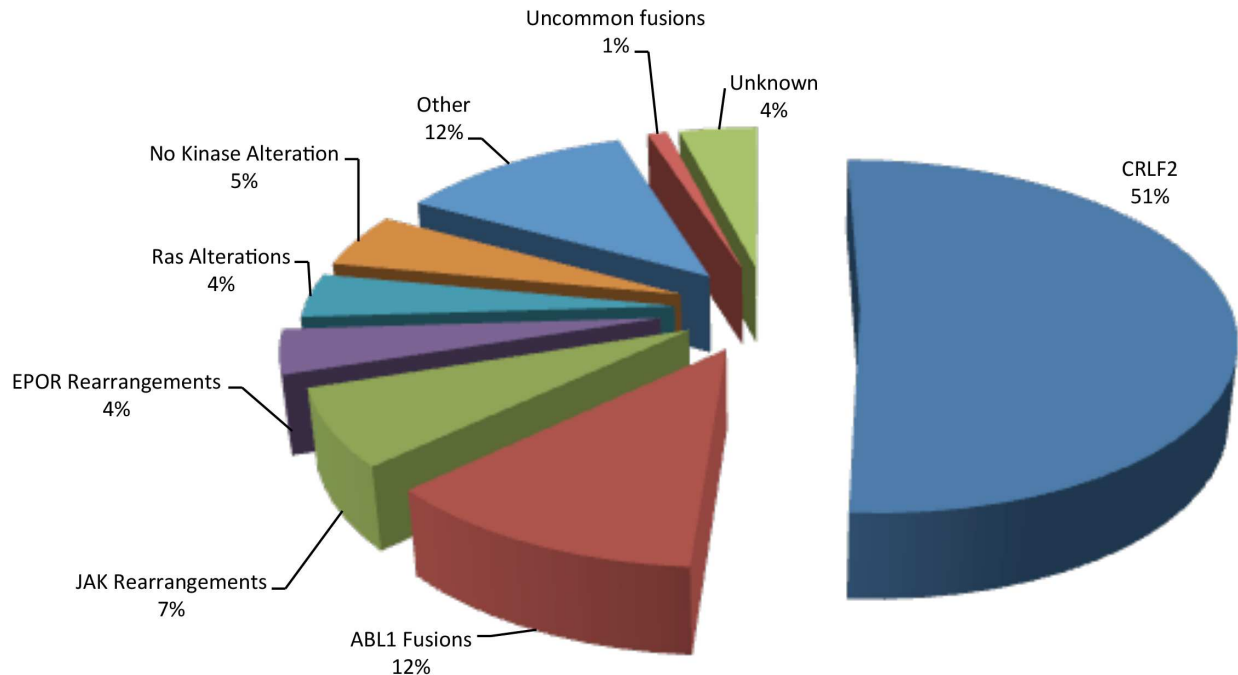
9. Ph-like ALL

9.1. Description and prevalence of Ph-like ALL

Ph-like (BCR-ABL1-like) ALL defines a distinct subtype of high risk ALL that is characterized by a gene expression profile similar to that of Ph+ ALL but lacking the characteristic t(9;22) translocation. This subtype represents about 15–20% of all cases of ALL [57, 58]. The prevalence of Ph-like ALL increases with age: 10–13% of ALL in children and 21–27% of ALL in adolescents and young adults. Patients with Ph-like ALL have higher leukocyte counts at presentation and are more likely to have minimal residual disease at the end of induction chemotherapy. The survival rates of patients with Ph-like ALL is significantly lower when compared to non-Ph-like ALL among all age groups, adults faring worse than children [58]. Genes that are involved in B cell development, including *IKZF1* (Ikaros), are deleted more frequently in Ph-like ALL as compared to non-Ph-like ALL (82% vs. 36%) [57, 58]. Genomic alterations involving kinase signaling have been observed in >90% of Ph-like ALL. These include alterations involving *ABL1, JAK, EPOR, CRLF2, IL7R, FLT3*, Ras, and others [58]. Subtypes of Ph-like ALL are shown in Figure 2.

9.2. Translocations involving ABL1 in Ph-like B-ALL

The *BCR-ABL1* translocation and the resulting Ph+ leukemia was described in Section 4. Other translocations involving the *ABL1* gene have been demonstrated in B-ALL as well as T-ALL. The *ETV6* gene encodes an Ets family transcription factor that is essential for hematopoiesis and vascular growth. The *ETV6-ABL1* fusion, t(9;12)(q34;p13) is a rare event that has been reported in 22 cases of hematological malignancies including 6 cases of B-ALL and one case of T-ALL [59]. The *RCSD1-ABL1* fusion, t(1;9)(q24;q34), has been identified in 3 cases of B-ALL

[60]. One case of B-ALL with *SFPQ-ABL1* fusion, t(1;9)(p34;q34), and another case of B-ALL with *ZMIZ1-ABL1* fusion, t(9;10)(q34;q22.3) have been reported [61, 62].

Figure 2. Ph-like ALL subtypes

The *NUP214* gene encodes a nuclear pore complex protein that is involved in nucleocytoplasmic transport. *NUP214-ABL1* is the second most prevalent fusion gene involving *ABL1*. It has been reported only in T cell ALL with a frequency of approximately 5%. So far, 60 cases of this fusion have been reported in the literature involving both childhood and adult cases of T-ALL [63, 64]. In one study by Graux et al., extrachromosomal (episomal) amplification of *ABL1* within the *NUP214-ABL1* transcript was observed in 5 out of 90 cases of T-ALL. Episomes are extrachromosomal DNA that are not visible by conventional cytogenetics [65]. The above mentioned study utilized FISH and microarray-based comparative genomic hybridization techniques to map the episomes and confirm the *ABL1* amplification. Roberts et al. have demonstrated that leukemias with ABL class fusions are sensitive in vitro to the ABL class inhibitors Imatinib and Dasatinib [58].

9.3. Genetic alterations in Ph-like ALL that include EPOR and JAKs

A number of *JAK* mutations have been identified in Ph-like B-ALL, specifically mutations in the *JAK1* pseudokinase domain, as well as the *JAK2* pseudokinase and kinase domain, and in *JAK3* [66]. However, the most common are those occurring in the *JAK2* pseudokinase domain – R683G [67]. Activating mutations in JAK kinases (*JAK1, JAK2*, and *JAK3*) have been reported in approximately 10% of Ph-like pediatric ALL. The JAK mutations were associated with

alterations in *IKZF1* and *CDKN2A/CDKN2B*, and with poor outcomes [58, 67]. Approximately 50% of CRLF2 B-ALL patients show *JAK* mutations [66, 68, 69] while rearrangement or translocations involving the *JAK2* gene are characteristic of ~7% of Ph-like ALL [58].

Erythropoietin (EPO) is a hematopoietic growth factor for the erythroid lineage and regulates the production of red blood cells. The binding of EPO to its receptor EPOR leads to downstream activation of JAK2-STAT5, PI3 kinase, and MAP kinase pathways [70]. Rearrangements that involve EPOR have been found in ~4% of Ph-like ALL [58].

9.4. Ras pathway genetic alterations in Ph-like ALL

Ras pathway mutations have been described in pediatric ALL cases. In a report from the Children's Oncology Group by Zhang et al., among 23 childhood B-ALL cases with a Ph-like gene expression profile, 9% had Ras pathway mutations. However, the proportion of Ras pathway mutated cases increased to 62% among ALL with focal *ERG* deletion [71]. Among Ph-like B-ALL cases, 4% are known to have Ras pathway genetic alterations. A majority of these involve missense mutations in *KRAS* and *NRAS* [58].

9.5. IKZF1 alterations in Ph-like ALL

Aberrations in the *IKZF1* gene have been observed in 80% of pediatric B-ALL cases that harbor the *BCR-ABL* rearrangement [72] and a similar pattern is seen in Ph-like ALL. Patients with *IKZF1* deletions have increased risk for relapse and poor treatment outcomes, making *IKZF1* an independent predictor for treatment outcomes [73–75]. More recently, alterations of the *IKZF1* gene have been associated with other genetic defects including *JAK* mutations and *CRLF2* rearrangements. Mullighan et al. demonstrated that 70% of *JAK* mutated cases harbored *IKZF1* alterations [67]. Dorge et al. also performed a study which showed a higher number of Ikaros deletions in patients that have *P2RY8-CRLF2* rearrangement compared to those that are negative for the rearrangement [73]. Further, in another study of 29 pediatric B-ALL cases that showed high CRLF2 expression accompanied by *CRLF2* rearrangement, ~70% of the patients had *JAK* mutations and 80% of cases had deletions or mutations in *IKZF1* [66]. These findings provide evidence that *CRLF2* rearrangements are significantly associated with alterations in *IKZF1*, as well as *JAK* mutations. Moreover, in patients harboring *CRLF2* rearrangements, *JAK* mutations and *IKZF1* deletions/mutations have a poor survival outcome, suggesting that these three lesions cooperate to worsen prognosis and treatment outcome of high-risk B-ALL. Therefore, they can serve as therapeutic targets, as well as prognostic tools that can be used effectively in risk-stratification and treatment strategies to improve treatment outcomes in CRLF2 and other high-risk B-ALL patients.

9.6. Rearrangement of CRLF2 in Ph-like ALL

Overexpression of CRLF2 is responsible for more cases of Ph-like ALL than any other genetic alteration [58]. The *CRLF2* gene is located in the pseudoautosomal region 1 (PAR1) of Xp/Yp. Three types of *CRLF2* gene alterations can result in overexpression of the CRLF2 protein on

the surface of the leukemic cells. One involves the juxtaposition of Xp/Yp into the immuno‐ globulin heavy chain locus at 14q32 resulting in *IgH-CRLF2* rearrangement. The other involves a focal deletion of PAR1 that juxtaposes the regulatory elements of the purinergic receptor gene *P2RY8* to *CRLF2* resulting in a chimeric fusion gene *P2RY8-CRLF2.* Thirdly, a less common missense mutation in exon 6 of *CRLF2*, F232C, results in constitutive CRLF2 dimeri‐ zation [66, 76]. CRLF2 overexpression is present only in B-ALL cases without rearrangements in *ETV6 (TEL)*, *KMT2A (MLL)*, *TCF3*, and *BCR-ABL.* It is also worth noting that more than half of Down syndrome associated ALL overexpress CRLF2 resulting in poor outcomes in these patients [69]. CRLF2 is overexpressed in approximately 8% of childhood and adult ALL and comprises ~50% of Ph-like ALL [58].

10. Biology of CRLF2 B-ALL – The most common form of Ph-like ALL

10.1. CRLF2–TSLP cytokine receptor component in CRLF2 B-ALL

CRLF2 is a type I cytokine receptor that, along with the IL7 receptor alpha (IL-7Rα), forms a heterodimeric receptor complex for the cytokine TSLP (thymic stromal lymphopoietin). In normal B-cell progenitors the expression of CRLF2 is undetectable by flow cytometry, although TSLP cytokine acts on these cells to induce proliferation [77]. In contrast, the high levels of CRLF2 on CRLF2 B-ALL cells can easily be detected by flow cytometry [68]. In CRLF2 B-ALL cells, TSLP has been shown to increase the phosphorylation of STAT5, AKT, and S6, indicating increased JAK-STAT and AKT/mTOR signaling [78]. Studies of TSLP effects in a range of cell types indicates that it can also indicate cell survival signals through the activation of several biological pathways including ERK, MAPK, PI3K/AKT/mTORC1, and NFκB, at least in some cell types [79]. The physiological significance of the signaling induced by TSLP in CRLF2 B-ALL cells remains to be determined [78].

10.2. Role of JAK mutations in CRLF2 B-ALL

Overexpression of *CRLF2* is believed to cooperate with other genetic lesions leading to leukemogenesis and chemoresistance. In most patients, overexpression of CRLF2 and *CRLF2* gene rearrangements has been associated with deletions and/or mutations of the *IKZF1* gene. Mutations in *JAK1* or *JAK2* are present in half of all cases of CRLF2-overexpressing B-ALL and can result in constitutive activation of this pathway [66, 68, 69].

To understand the contributions of CRLF2 and JAK2 to leukemogenesis, several groups have conducted studies using *CRLF2* and *JAK* mutants. Results from cellular models that utilized murine Ba/F3 cells transduced with combinations of patient-derived mutant *JAK1* or *JAK2*, and *CRLF2* achieved cytokine-independent growth in cells that contained both *CRLF2* and *JAK2* mutants. This cytokine independent growth did not occur in cells that were transduced with wildtype CRLF2 alone or JAK2 mutants alone. Co-immunoprecipitation studies demon‐ strated that human CRLF2 and phosphorylated mutant JAK2 interact physically [76, 80]. These research findings provide evidence that supports the hypothesis that CRLF2 and JAK2 can

cooperate to produce leukemogenesis. Indeed, Hertzberg et al. showed that almost all patients with *JAK* mutations harbor *CRLF2* gene rearrangements[80] This is in keeping with CRLF2 serving as a scaffold to facilitate signaling of mutant *JAK2* [81]. However, ~50% of CRLF2 B-ALL patients show no *JAK* mutations, suggesting that another factor might be inducing JAK activation. This is certainly consistent with the role of endogenous TSLP-induced CRLF2-mediated JAK phosphorylation in CRLF2 B-ALL. TSLP has been shown to increase both JAK-STAT and AKT/mTOR phosphorylation in human CRLF2 B-ALL cells – including those with activating *JAK* mutations [78].

10.3. Inherited background variability and susceptibility to CRLF2 B-ALL

Aside from these acquired genetic variations that contribute to leukemogenesis, other studies using Genome Wide Association techniques are employed to determine whether inherited (germline) genetic variations increase susceptibility to the development of high-risk Ph-like ALL. In a major study, 75 Ph-like ALL patients were compared to 6,661 non-ALL controls and two Single Nucleotide Polymorphisms (SNPs) within the *GATA3* gene achieved genome-wide significance (rs3824662 and rs3781093). The results showed that both SNPs were over-represented in Ph-like ALL and were associated with higher risk of relapse [82]. More importantly, the *GATA3* SNP (rs3824662) was associated with *CRLF2* rearrangement, *JAK* mutations, and *IKZF1* deletions [82]. *GATA3* is one of the six-membered *GATA* transcription factor family that is involved in the reprogramming of somatic cells to pluripotency[83]. More specifically, *GATA3* is critical for T cell development [84] and genetic alterations in *GATA3* have been observed in T-ALL patients [85] and in other hematopoietic disorders such as AML and Hodgkin Lymphoma [86, 87]. Taken together, these studies provide evidence that in addition to acquired genetic alterations such as *CRLF2*, inherited genetic variations such as *GATA3* SNPs may also contribute to increased susceptibility to developing CRLF2 B-ALL and increased risk of relapse. Thus, germline and somatic genetic alterations may play an important role in leukemogenesis.

10.4. CRLF2 B-ALL and health disparities

CRLF2 B-ALL is five times more prevalent in patients of Hispanic/Latino origin and after initial treatment they experience a higher rate of relapse. This high prevalence of CRLF2 B-ALL in Hispanics is further supported by studies that have been conducted in regions where there is a high proportion of Hispanics. For example, the highest incidence of childhood ALL is in Hispanics compared to other ethnic groups in California [88]. In a study of pediatric B-ALL patients, 18 of 51 (35.3%) Hispanic/Latino patients harbored *CRLF2* rearrangements compared to 11 of 154 (7.1%) patients of other ethnicities [66]. Harvey et al. further demonstrated that in addition to *JAK* mutations and *IKZF1* deletions, *CRLF2* rearrangement was also significantly associated with Hispanic/Latino ethnicity [66]. It has been established that populations originating from different ancestries can be distinguished by genetic polymorphisms that are unique to their ancestors [89]. Therefore, the current approach to studying the mechanisms that contribute to the racial disparities relating to incidence and outcome of ALL is to evaluate ancestry-related genetic alterations in conjunction with external factors such as the environ-

ment and socioeconomic status [90, 91]. Recent studies to identify ALL susceptibility genes in the Hispanic population demonstrated that *ARID5B* and *GATA3* SNPs are more frequent in Hispanics compared to other ethnicities [82, 92]. These SNPs have been associated with higher ALL incidence (ALL susceptibility), increased frequency of relapse, and poorer outcome [82, 92]. A possible explanation for the increased frequency of these SNPs in Hispanics is their shared genetic ancestry with Native Americans that also exhibit increased frequency of the SNPs [92]. While ancestry-related studies can provide a basis for disease, there is still a need for studies that will identify the biological factors that contribute to the health disparity. Thus, there is a need for the development of preclinical animal models that can be used to evaluate Hispanic patient samples, thereby allowing us to effectively study CRLF2 B-ALL in context of racial disparities.

10.5. Challenges of preclinical models of CRLF2 B-ALL

Current strategies to treat Ph-like ALL and more specifically CRLF2 B-ALL include: 1) stratification of patients using prognostic and clinical factors (age, gender, race, white blood cell count, CNS involvement, testicular involvement, steroid pretreatment and MRD, cell morphology, immunotyping and genetic alterations); and 2) administer treatment regimen based on risk group. Since 91% of the Ph-like ALL subset of patients (including CRLF2 B-ALL) is characterized by activated kinase signaling, tyrosine kinase inhibitors are currently recommended as part of the treatment regimen for these patients [58]. Recent studies were aimed at evaluating the effects of tyrosine kinase inhibitors, more specially JAK inhibitors and PI3K/mTOR inhibitors on CRLF2 B-ALL cells in vitro and in vivo [78, 93, 94]. Results from these studies have shown that *CRLF2* rearranged B-ALL cells are sensitive to these inhibitors in vitro and in vivo. While JAK inhibitors and all other tyrosine kinase inhibitors have shown some promise, they have not been effective in all Ph-like B-ALL and not all CRLF2 B-ALL harbor JAK mutations. Since TSLP has been shown to increase activation of the JAK-STAT, PI3K/AKT/mTOR signaling pathways, it is important to conduct studies of disease mechanisms and evaluate therapeutic candidates in context of TSLP and other cytokines that are often present in the cancer microenvironment. Though some in vitro studies were performed in the presence of TSLP [78], in vivo studies in context of TSLP stimulation are lacking. Therefore systematic evaluation of the contributions of TSLP to CRLF2 B-ALL leukemogenesis and the assessment of therapies to treat these patients are required to gain a better understanding of the etiology and treatment outcome of the disease.

Human-mouse xenograft models are ideal in vivo models for studying disease mechanisms and for evaluating therapies for leukemia, particularly in context of inherited genetic variability. NOD/SCID mice have been shown to be receptive to the engraftment of human leukemia cells [95]. The development of additional mouse strains, such as the NOD/Scid/IL- 2Rγ null (NSG) strain have significantly increased the ability of human ALL cells to engraft [96]. These animal models are effective because mouse cytokines act on the human leukemia cells by providing the necessary growth signals required to facilitate proliferation and maintenance of leukemia cells [97]. However, while most mouse cytokines can activate receptors on human cells, some mouse cytokines are species-specific, e.g., IL-3, GM-CSF, and TSLP [97]. This poses

a challenge in effectively recapitulating the systemic or BM microenvironment present in patients. It is important to conduct studies as far as possible with the full complement of cytokines present under physiological conditions in order to truly identify disease mechanisms and evaluate therapies.

10.6. Novel pre-clinical xenograft models that provide human cytokines

The lack of cross species cytokine activity has been partially addressed for studies of myeloid leukemia by the recent development of so-called "cytokine mice" [98]. These immune-deficient mice express human IL-3, SCF, and GMCSF, three cytokines that play important roles in the production of myeloid lineage cells and show no or poor cross species activity. Xenografts from cytokine mice showed enhanced AML engraftment [98]. Alternative strategies have been used to produce mice that express human IL-15 and FLT3 and other cytokines, which have resulted in enhanced production of functional human natural killer cells, dendritic cells, monocytes/macrophages, and erythrocytes [97]. However, human TSLP has not been included as part of the complement of cytokines used in these studies.

Current xenografts that evaluate therapeutic candidates for CRLF2 B-ALL do not include human TSLP [94], which is required to provide the human CRLF2-mediated signals that were demonstrated by previous groups in vitro [78]. There is a need to develop new preclinical animal models that include TSLP and other species-specific cytokines in order to accurately evaluate disease mechanisms. A model that includes TSLP and allows for modulating the levels of TSLP will be of great value to the study of CRLF2 B-ALL. The rationale for this is that CRLF2, the receptor for the endogenous ligand TSLP, is overexpressed on CRLF2 B-ALL cells and has contributed to increased pathway signaling in these cells. Additionally, the role of TSLP in the initiation and maintenance of CRLF2 B-ALL is unknown. Current studies in our laboratories are aimed at developing a human TSLP+ xenograft model that provides human TSLP to activate CRLF2-mediated signals in human CRLF2 B-ALL cells transplanted into xenograft mice. This model will be used to study disease mechanisms and identify therapies to effectively treat CRLF2 B-ALL.

11. Conclusion

In summary, great strides have been made in effectively treating pediatric leukemia. Risk stratification based on clinical features combined with intensified therapies has contributed to this outcome. Increasingly, more precise molecular phenotyping available from whole genome analyses make it possible to identify and study specific subtypes of high risk leukemias. Mechanistic studies that identify druggable targets in aberrant pathways are likely to fuel rapid advances in the future. This progress will depend on the development of relevant preclinical models for studying disease mechanisms and for evaluating candidate therapies.

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