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Review

Diagnosis and Molecular Pathology of Lymphoblastic Leukemias and Lymphomas in the Era of Genomics and Precision Medicine: Historical Evolution and Current Concepts—Part 2: B-/T-Cell Acute Lymphoblastic Leukemias

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Abstract: The diagnosis and treatment of lymphoid neoplasms have undergone a continuously progressive positive change in the last three decades, with accelerated progress in the previous decade due to the advent of genomics in cancer diagnosis. Significantly, there has been an increasing emphasis on integrating molecular genetics with clinical, morphological, immunophenotypic, and cytogenetic evaluation for diagnosis. As we think of moving forward with further advances in the genomics era, it will be first helpful to understand our current state of knowledge and how we achieved it in the challenging and complex field of lymphoid neoplasms, which comprise very heterogeneous neoplastic diseases in children and adults, including clinically acute lymphoblastic leukemias (ALLs) arising from precursor lymphoid cells and clinically indolent and aggressive lymphomas arising from mature lymphoid cells. This work aims to provide an overview of the historical evolution and the current state of knowledge to anyone interested in the field of lymphoid neoplasms, including students, physicians, and researchers. Therefore, I have discussed this complex topic in three review manuscripts, designated Parts 1–3. In Part 1, I explain the basis of the diagnostic classification of lymphoid neoplasms and its evolution up to the current fifth edition of the World Health Organization classification of hematolymphoid neoplasms and the crucial importance of diagnostic tumor classifications in achieving and advancing patient care and precision medicine. In the second and third manuscripts, I discuss current diagnostic considerations for B-ALL and T-ALL (Part 2) and common indolent and aggressive mature leukemias/lymphomas (Part 3), including significant updates in the WHO 2022 classification, newly described entities, and concepts, including genetic predisposition to ALLs and lymphomas, and emphasizing throughout the essential integration of molecular genetics with clinical, morphologic, immunophenotypic, and cytogenetic evaluation, as required for the precise diagnosis of the type of lymphoma/leukemia in any patient.

Keywords: genetics; World Health Organization classification; whole genome sequencing; germline predisposition; pediatric cancer; flow cytometry; chromosomal abnormalities; fluorescence in situ hybridization; chromosomal microarray; RNA sequencing

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1. Introduction

Lymphoid neoplasms comprise very heterogeneous neoplastic diseases in children, adolescents, and adults, including clinically acute lymphoblastic leukemias (ALLs) arising from precursor lymphoid cells and clinically indolent and aggressive lymphomas arising from mature lymphoid cells. In the last decade, intense research on understanding the biological bases of these neoplasms, primarily due to the introduction of genomics in cancer diagnosis, has led to significant advances in predicting prognosis and newer treatment options. This three-part work aims to provide an overview of the historical evolution and the current state of knowledge to anyone interested in the field of lymphoid

neoplasms, including students, physicians, and researchers. Part 1 provides a historical overview of lymphoma classifications and the principles of the diagnostic classification of lymphoid neoplasms and includes sections on B- and T-cell development in the bone marrow and the thymus, respectively, germinal center components and the origin of mature B-cell neoplasms, clonality analysis in lymphoid neoplasms, and the crucial role of the diagnostic World Health Organization (WHO) classification in achieving and advancing precision medicine [1]. Part 2 of this manuscript focuses on acute lymphoblastic leukemias/lymphomas (ALLs) as we understand them today in 2023. Part 3 is focused similarly on mature lymphoid neoplasms of B, T, or natural killer (NK) cell lineages [2].

2. Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia/lymphoma (ALL) is a neoplasm of precursor B or T lymphoid cells, representing the most common leukemia and cancer in the pediatric age group. ALL also occurs in adolescents, young adults (AYA), and older adults, although it is much less common in these age groups than in childhood. Most childhood ALLs are B-lineage (B-ALL), comprising about 85% of all ALLs. The clinical outcomes and our understanding of B-ALL have significantly improved in the last two decades. In most developed countries, cure rates approach or exceed 90% in childhood ALL, although they are still much lower, at 30–40%, in adult ALL. As discussed in Part 1 [1], the great strides in improving the outcomes of pediatric patients with ALL were achieved by collaboration [3].

Epidemiology of Acute Lymphoblastic Leukemia

In 2020, the incidence of ALL in children <15 years of age was 4.2 per 100,000 inhabitants in the USA, according to the Surveillance, Epidemiology, and End Results (SEER) program data [4]. As mentioned above, this age group shows the highest age-adjusted incidence of ALL. In contrast, the age-adjusted incidence rates of ALL in all other age groups are less than half the incidence rate of childhood ALL. In these different age groups, the age-adjusted incidence rates, each per 100,000 inhabitants in the USA in 2020, are as follows: 1.1 in ages 15–39 years, 1.0 in ages 40–64 years, 1.6 in ages 65–84 years, and 1.7 in ages 75 years and older [4].

When grouped by sex, the age-adjusted incidence rates of ALL are higher in males than females in all age groups except 40–64 years and >75 years. In those aged 40–64 years, the incidence was the same in both sexes at 1.0 per 100,000. Notably, in those aged 75 years and older, females have a higher incidence of 1.8 versus 1.6 in males, each per 100,000 individuals in the USA in 2020.

In contrast, in the <15 years age group, the incidence in females is 3.4 versus 4.7 in males per 100,000 individuals in the USA in 2020. And in the same year, the incidence of ALL in all ages was 2.0 per 100,000 individuals in males and 1.6 per 100,000 individuals in females in the USA.

Of note, in the USA, there has been a rising trend in the incidence of ALL in females, with an annual percentage increase of 0.8% per year from 2000 to 2019 among all ages. In contrast, while the incidence in males of all ages increased with a 2.5% annual percent change (APC) from 2000 to 2004, the APC in the incidence in males from 2015 to 2019 was not significant [4]. However, there is a rising trend in two age groups in both sexes, with the APC from 2000 to 2019 being as follows: (1) in ages 15–39 years, the APC was 1.1 in females versus 1.7 in males, and (2) in ages 40–64 years, the APC was 1.9 in females and 1.8 in males [4]. This latter age group of those aged 40–64 years appears to have had the highest rising APC for incidence [4]. In children aged <15 years, the incidence is increasing only in females, with an APC of 0.4 from 2000 to 2019 [4]. Finally, in 2023, 6540 cases of ALL are expected to occur in the USA in 3660 males and 2880 females, with 1390 total estimated deaths in 700 males and 690 females [5].

3. Diagnosis of Acute Lymphoblastic Leukemia/Lymphoblastic Lymphoma

The morphological diagnosis of acute leukemia requires the visual recognition of the presence of "blasts" or leukemic cells in peripheral blood or bone marrow, which should comprise at least 20% of all nucleated cells in the peripheral blood or bone marrow. The specific diagnosis of acute lymphoblastic leukemia requires the confirmation of the lineage of leukemic cells as lymphoid. Flow cytometric immunophenotyping (FCI), a technique requiring viable cells for analysis, is most often used to perform this lineage confirmation. As ALL involves the peripheral blood and bone marrow, a leukemic peripheral blood sample and bone marrow biopsy are obtained for diagnosis by visual morphologic evaluation and FCI. However, suppose a sample with viable cells is not available. In that case, immunohistochemical stains may be performed on formalin-fixed, paraffin-embedded tissue sections of a trephine core biopsy, bone marrow aspirate clot, or any biopsy from a tissue mass due to suspected leukemic infiltration.

In the case of presentation as a lymphoblastic lymphoma, leukemic cells primarily infiltrate tissues, and there is much less involvement (less than 25%) of the bone marrow than in lymphoblastic leukemia. In these cases, lineage determination may be performed by FCI if a fresh tissue sample is submitted at the time of biopsy or by immunohistochemical stains if only fixed tissue specimens are available. Of note, lymphoblastic lymphoma is most often T-lineage and has a characteristic clinical presentation involving young males with a mediastinal mass and a high white cell count. B-ALL may also occur as lymphoblastic lymphoma but much less frequently than T-lymphoblastic lymphoma.

B-ALL must not be confused with Burkitt leukemia/lymphoma, which is a neoplasm of mature B cells, discussed in Part 3 of this review [2]. In contrast, B-ALL comprises B lymphoblasts, which are immature B precursor cells in the bone marrow that normally differentiate into mature B cells, as shown in Figure 1 in Part 1 [1]. B-ALL arises from B lymphoblasts, T-ALL arises from T lymphoblasts, and B- and T-ALL must be differentiated from leukemia/lymphoma arising from mature B or T lymphoid cells. In this context, it should be noted that Figures 1 and 2 in Part 1 [1] of this work are based on FCI studies of normal bone marrow and fetal tissues, which were performed to elucidate the immunophenotypic profiles of the different stages of the precursor cells using specific panels of antibodies. Those panels included nuclear terminal deoxyribonucleotidyl transferase (TdT) for the B-cell precursor studies but not T-cell studies; therefore, Figure 1 showed the presence of TdT in the normal stages of B-cell precursors [1].

Like CD34, nuclear TdT is a marker of immaturity that is not lineage-specific. Nuclear TdT positivity can be very helpful in diagnosing B-ALL and T-ALL, especially T lymphoblastic lymphoma, in conjunction with other immunohistochemistry stains or as part of an FCI panel of antibodies in the appropriate clinical situation and with the required histopathologic features on a biopsy of a tissue mass. However, it is critical to remember that TdT positivity alone, even in a tissue mass, is insufficient by itself to diagnose a T lymphoblastic lymphoma. TdT positivity is also present in a benign extrathymic proliferation of T lymphoblasts, which have been shown to occur, even as recurrent masses, in the pediatric age group [6]. Such benign proliferations of extrathymic T lymphoblasts are included in the fifth edition of the WHO classification as indolent T-lymphoblastic proliferation [7].

Technically, analysis by FCI for the presence of nuclear TdT as a marker of immaturity and a panel of cytoplasmic CD3, CD22, and myeloperoxidase for lineage determination requires permeabilizing cells from the leukemic sample. This permeabilization allows the analysis of intracellular antigens. In B-ALL, cytoplasmic CD22 must be positive in addition to surface B-cell antigens, while cytoplasmic myeloperoxidase and CD3 must be negative. In T-ALL, cytoplasmic CD3 is positive in addition to other surface antigens, and cytoplasmic CD22 and myeloperoxidase are negative.

In contrast, immunohistochemistry stains for TdT can readily evaluate the presence or absence of TdT in neoplastic cells. However, the intensity of the expression of antigens in leukemic cells is best assessed by FCI, not immunohistochemistry. Therefore, FCI is

commonly used for measurable disease detection of ALL after treating ALL patients; see the review in [8].

It is also crucial to remember that B-ALL, a neoplasm of B lymphoblasts, may show the expression of surface light-chain immunoglobulin, kappa, or lambda, which are usually considered to be absent in B lymphoblasts [9]. Notably, surface light-chain restriction can be present in any early, intermediate-, and late-stage precursor B-cell ALL [9]. The presence of surface light-chain immunoglobulins indicates mature B-cells in the normal developmental pathway, as depicted in the normal developmental Figure 1 in Part 1 of this review [1]. However, leukemic cells are neoplastic cells, and a prominent feature of neoplastic cells is aberrant antigen expression that does not follow normal maturation patterns. Therefore, in any acute leukemia, the diagnosis of immature versus mature B-lineage neoplastic cells must not be based only on the presence or absence of specific antigens associated with mature or immature cells [9].

Further, based on FCI, the leukemic cells in ALL may appear to arise from and mimic a specific stage of precursor B cell development. However, in many cases, it is difficult to pinpoint the immunophenotype to a particular normal developmental stage due to the immense heterogeneity in the immunophenotypic profiles of leukemic cells [10] (p. 175). Nevertheless, some genetic subtypes of ALL are known to be associated with specific features by FCI. These associations are summarized subsequently in this review.

4. The Classification of Acute Lymphoblastic Leukemia/Lymphoma Requires Genetics

As described above, diagnosing ALL requires morphological evaluation to diagnose acute leukemia and immunophenotypic analysis by flow cytometry or immunohistochemistry to confirm the nature of the leukemic cells as lymphoblasts. However, the genetic features of the leukemic cells at diagnosis predict the prognosis of the type of B-ALL. Other factors predicting outcomes in ALL include patient age and the presenting white cell count. Therefore, the diagnostic WHO classification of B-ALL is based primarily on genetic features.

4.1. Historical Overview

Historically, the prognostic significance of chromosomal abnormalities in childhood ALL was first shown by Secker-Walker et al. in 1978 and 1982 [11,12]. They showed that ALL patients with a hyperdiploid karyotype, comprised of >46 chromosomes, had significantly more prolonged first remissions than patients with other chromosomal abnormalities. The latter abnormalities were grouped as hypodiploid (<46 chromosomes with rearrangements), diploid (normal 46 chromosomes), pseudodiploid (46 chromosomes with rearrangements), and mixed (when no dominant chromosomal abnormality was present). These authors defined a clone as two or more abnormal cells with identical additional chromosomes or rearrangements [11]. The pseudodiploid group included significantly more infants and older children, and these patients had the shortest survival [12] and the poorest responses to treatment [13]. Also, the modal chromosome number with the best prognosis in childhood B-ALL was established to be >50 chromosomes, and these patients had the best responses to treatment [13].

Subsequently, in 1984, the t(1;19) translocation was identified in cases of pre-B-cell ALL showing cytoplasmic IgM positivity [14,15], consistent with late-stage precursor B cells. Also, the t(11;14) translocation was identified in 4 of 16 cases of T-cell ALL [14]. Rearrangements of 11q23 in ALL were shown to be present in infants and young adults and associated with hyperleukocytosis and a high risk of intracranial bleeding due to disseminated intravascular coagulation. These leukemias with 11q23 rearrangements showed HLA-DR and CD19 positivity with absent common acute lymphocytic leukemia antigen (CALLA, or CD10), consistent with an early precursor B-cell stage [16,17].

In the 1990s, other associations of immunophenotype with genetic abnormalities were described in three types of B-ALL: (1) B-ALL with 11q23 abnormalities, comprising *KMT2A*,

previously called the mixed lineage leukemia (*MLL)* gene, rearrangements, (2) B-ALL with t(1;19)(q23;p13.3), and (3) B-ALL with t(12;21)(p13;q32) [18–20].

First, in 1991, B-ALL with 11q23 abnormalities was shown to include subsets of CD24-negative and CD15-positive blasts, in addition to CD19 and HLA-DR positivity and absent CD10, as described earlier [18]. The CD10−, CD24−, CD15+, and CD19+ immunophenotype in ALL was specific but present in only 62.5% of cases with the t(4;11) rearrangements [18].

Then, in 1993, the leukemic cells in B-ALL with t(1;19)(q23;p13.3) were shown to homogeneously express CD19, CD10, and CD9, with a partial expression of CD20 and complete absence of CD34 [19].

Finally, in 1998, the leukemic cells in B-ALL with t(12;21)(p13;q32), which can be cryptic in chromosome banding analysis, were shown to have low-intensity or absent CD9 expression. Similarly, these leukemic cells also showed low-intensity or absent CD20. The specificity and sensitivity of this immunophenotype for *ETV6* (previously called *TEL)* rearrangements are about 70% and 90%, respectively, and 92–93% for predicting the *ETV6::RUNX1* (previously called *TEL::AML1)* translocation [20].

The risk classification of ALL in 1998 was primarily based on the presence of hyperdiploidy (>50 chromosomes) or *ETV6::RUNX1* fusion in children, with both having a provisional low risk [21]. In contrast, the genetic types of ALL that determined risk in adults were *BCR::ABL1* fusion or *KMT2A (MLL)* rearrangements [21].

The WHO 2001 classification called ALL "precursor B lymphoblastic leukemia/lymphoma" and "precursor T lymphoblastic leukemia/lymphoma." The WHO 2008 classification changed these names to "B lymphoblastic leukemia/lymphoma" and "T lymphoblastic leukemia/lymphoma," respectively.

The WHO 2008 classification defined seven B-ALL types based on well-established recurring genetic abnormalities [22,23]. T-ALL was recognized as a single neoplastic disease [22,23], as shown in Table 2 in Part 1 [1]. The seven subtypes of B-ALL included two defined by aneuploidy, B-ALL with hyperdiploidy, and B-ALL with hypodiploidy. The remaining five genetic subtypes of B-ALL included (1) B-ALL with t(12;21)(p13;q22); *ETV6::RUNX1,* (2) B-ALL with t(v;11q23); *MLL* rearranged, (3) B-ALL with t(1;19)(q23;p13.3); *TCF3::PBX1,* (4) B-ALL with t(9;22)(q34;q11.2); *BCR::ABL1,* and (5) B-ALL with t(5;14)(q31;q32); *IL3::IGH*. If any of the above genetic abnormalities were not identified in a case of B-ALL, the diagnosis was "B-lymphoblastic leukemia/lymphoma, not otherwise specified," according to WHO 2008 criteria.

Of note, B-ALL with the t(5;14) abnormality may show peripheral blood eosinophilia with a relatively low percentage of blasts in the bone marrow; in this instance, identifying the genetic abnormality allows the diagnosis of the specific type of B-ALL. The presence of eosinophilia in any B- or T-ALL should prompt testing for *FGFR1;* if positive in a B-ALL, the correct diagnosis would be B-ALL with *FGFR1* abnormalities [23].

All genetic abnormalities defined in the WHO 2008 classification could typically be identified by cytogenetics and fluorescence in situ hybridization (FISH). Hyperdiploidy (>50 chromosomes), *ETV6::RUNX1* fusion, and trisomy 4, 10, and 17 predict a favorable outcome, while hypodiploidy (<44 chromosomes), t(9;22) or *BCR::ABL1*, or *MLL* rearrangements confer a poor outcome [24]. In 2016, the revised fourth edition of the WHO classification introduced two provisional B-ALL types and one T-ALL subtype [25,26], as shown in Table 2 in Part 1 [1].

4.2. The Genetic Abnormalities in "B-Other" B Lymphoblastic Leukemias/Lymphomas Were Incorporated in the Fifth-Edition WHO Classification in 2022

Childhood ALL is comprised of ~10% T-ALL and ~90% B-ALL, including 31% with high hyperdiploidy, defined as the presence of 51–65 chromosomes with the most frequent modal number being 55, 21% with *ETV6::RUNX1*, 7% with poor-risk cytogenetics {t(9;22)(q34;q11), 11q23 or lysine methyltransferase 2 (*KMT2A*) translocations, near-haploidy/low haploidy (<40 chromosomes), t(17;19)q22;p13)}, and other translocations

{t(1;19)(q23;p13), 14q32/*IGH* translocations} [27]. In contrast, Philadelphia chromosome (Ph)-positive B-ALL is most common in adults.

However, no specific genetic abnormalities were found earlier in about 30% of B-ALL cases by routine genetic methods. These cases were termed "B-other" ALLs. In the last decade, genomic analyses of large B-ALL cohorts identified new genetic subtypes in these B-other ALL cases. These studies were based on techniques beyond routine cytogenetic and molecular assays, comprising gene expression profiling and whole-genome sequencing, including improved copy number analysis [28–35]. The novel types of B-ALL discovered by these advanced genomic techniques were incorporated as definite types of B-ALL in the fifth edition of the WHO classification (WHO-HAEM5). The International Consensus Classification (ICC) also recognized the genetic types of ALL; in addition, the ICC recognized several provisional types of ALL.

The genomic methods that led to the discovery of novel types of ALL are not currently available in most clinical laboratories. Still, the field is constantly moving toward more comprehensive genomic analysis, and hopefully, including these new subtypes in the classification will help to define their clinical significance and improve clinical outcomes.

Table 1 shows the genetic subtypes of ALL included in WHO-HAEM5 [7,36] and the ICC [37]. The changes and additions to the genetic types of ALL in WHO-HAEM5 in comparison with the revised fourth edition of the WHO classification in 2017 are summarized below:

(1) Upgraded from provisional entities in WHO 2017:

B-ALL with *BCR::ABL1*-like features, B-ALL with intrachromosomal amplification of chromosome 21 (iAMP21), and early T precursor lymphoblastic leukemia/lymphoma were provisional entities in WHO 2017. All three types of ALL are definite subtypes in WHO-HAEM5 and the ICC.

(2) Terminology changes:
   a) WHO-HAEM5 includes only the name of the fusion in the names of the types of ALL, and the complete cytogenetic nomenclature is eliminated from the name change. However, as explained in Part 1, WHO-HAEM5 continues to emphasize the importance of cytogenetics throughout the classification [1]. This concern was explained by WHO-HAEM5 editors in a recent publication [38];
   b) B-ALL with hyperdiploidy in 2017 is termed B-ALL with high hyperdiploidy in WHO-HAEM5. The ICC uses the same term, B-ALL, hyperdiploid, as in WHO 2017;

(3) B-ALL with *ETV6::RUNX1*-like features and B-ALL with *TCF3::HLF* fusion are new types of B-ALL in WHO-HAEM5;

(4) WHO-HAEM5 created a new subgroup, B-ALL with other defined genetic features, which includes seven new types of B-ALL, as shown in Table 1;

(5) If comprehensive testing in a case of B-ALL does not identify any of the genetic abnormalities now defined by WHO-HAEM5, the diagnostic subtype would be B-ALL, not otherwise specified (NOS);

(6) However, if complete testing cannot be performed, the diagnostic term B-ALL/LBL, not further classified, should be used, not B-ALL, NOS [36]. This distinction in diagnostic terminology is a desirable change from WHO 2017, which should ideally be applied throughout the fifth-edition WHO classification for all tumors, as many countries will not have the resources to use advanced genomics methods to define tumor types. It is essential to differentiate between any cancer that is genuinely NOS and cannot be diagnosed as a specific cancer type after comprehensive testing versus a tumor that cannot be determined to be a particular genetic type because it was not possible to test for any reason, as previously discussed [39].

Table 1. Precursor lymphoid neoplasms in the fifth-edition WHO 2022 and International Consensus Classifications.

WHO-HAEM5 2022 Classification [7,36]	**International Consensus Classification [37]**
B-lymphoblastic leukemias/lymphomas	*B-acute lymphoblastic leukemia (B-ALL)*
B-lymphoblastic leukemia/lymphoma [a]	B-ALL with recurrent genetic abnormalities
B-lymphoblastic leukemia/lymphoma with high hyperdiploidy	B-ALL with t(9;22)(q34.1;q11.2)/*BCR::ABL1*
B-lymphoblastic leukemia/lymphoma with hypodiploidy	with lymphoid only involvement [b]
B-lymphoblastic leukemia/lymphoma with iAMP21 [b]	with multilineage involvement [b]
B-lymphoblastic leukemia/lymphoma with *BCR::ABL1* fusion	B-ALL with t(v;11q23.3)/*KMT2A* rearranged
B-lymphoblastic leukemia/lymphoma with *BCR::ABL1*-like features [b]	B-ALL with t(12;21)(p13.2;q22.1)/*ETV6::RUNX1*
B-lymphoblastic leukemia/lymphoma with *KMT2A* rearrangement	B-ALL, hyperdiploid
B-lymphoblastic leukemia/lymphoma with *ETV6::RUNX1* fusion	B-ALL, low hypodiploid
B-lymphoblastic leukemia/lymphoma with *ETV6::RUNX1*-like features [b]	B-ALL, near haploid
B-lymphoblastic leukemia/lymphoma with *TCF3::PBX1* fusion	B-ALL with t(5;14)(q31.1;q32.3)/*IL3::IGH*
B-lymphoblastic leukemia/lymphoma with *IGH::IL3* fusion	B-ALL with t(1;19)(q23.3;p13.3)/*TCF3::PBX1*
B-lymphoblastic leukemia/lymphoma with *TCF3::HLF* fusion [b]	B-ALL, *BCR*::*ABL1*–like, ABL-1 class rearranged [b]
	B-ALL, *BCR*::*ABL1*–like, JAK-STAT activated [b]
B-lymphoblastic leukemia/lymphoma with other defined genetic alterations [b]	B-ALL, *BCR*::*ABL1*–like, not otherwise specified [b]
B-lymphoblastic leukemia with *DUX4* rearrangement	B-ALL with iAMP21 [b]
B-lymphoblastic leukemia with *MEF2D* rearrangement	B-ALL with *MYC* rearrangement [b]
B-lymphoblastic leukemia with *ZNF384* rearrangement	B-ALL with *DUX4* rearrangement [b]
B-lymphoblastic leukemia with *PAX5*alt	B-ALL with *MEF2D* rearrangement [b]
B-lymphoblastic leukemia with *PAX5* p.P80R	B-ALL with *ZNF384(362)* rearrangement [b]
B-lymphoblastic leukemia with *NUTM1* rearrangement	B-ALL with *NUTM1* rearrangement [b]
B-lymphoblastic leukemia with *MYC* rearrangement	B-ALL with *HLF* rearrangement [b]
B-lymphoblastic leukemia/lymphoma, not otherwise specified	B-ALL with *UBTF::ATXN7L3/PAN3,CDX2* ("CDX2/UBTF") [b]
	B-ALL with mutated *IKZF1* N159 Y [b]
Precursor T-cell neoplasms	B-ALL with mutated *PAX5* P80 R [b]
T-lymphoblastic leukemia/lymphoma	(Provisional) B-ALL, *ETV6::RUNX1*-like [b]
T-lymphoblastic leukemia/lymphoma, not otherwise specified	(Provisional) B-ALL, with *PAX5* alteration [b]
Early T-precursor lymphoblastic leukemia/lymphoma [b]	(Provisional) B-ALL, with mutated *ZEB2* (p.H1038R)/*IGH::CEBPE* [b]
	(Provisional) B-ALL, *ZNF384* rearranged-like [b]
	(Provisional) B-ALL, *KMT2A* rearranged-like [b]
	B-ALL, not otherwise specified
	T-acute lymphoblastic leukemia/lymphoma (T-ALL)
	Early T-cell precursor ALL with *BCL11B* rearrangement [b]
	Early T-cell precursor ALL, not otherwise specified [b]
	T-ALL, not otherwise specified
	Other provisional entities [a] given in reference [37] (Provisional)
	Natural killer (NK) cell ALL [c]

[a] "B-lymphoblastic leukemia/lymphoma" is coded in the International Classification of Diseases (ICD)-11 as B lymphoblastic leukemia or lymphoma, not elsewhere classified [36]; [b] New entities in 2022, or upgraded to definite from provisional entities in the revised 4th WHO edition in 2017; [c] Provisional entity in the revised 4th WHO edition in 2017 not recognized by WHO-HAEM5, as described in [7].

The distribution of the types of ALL varies with age between children, AYA aged 15–39 years, and adults aged > 40 years. In children, the subtype distribution also varies with the white cell count and age. White cell counts less than 50×10^9/L at presentation and age between 1 and 9 years define standard risk. In contrast, white cell counts greater than 50×10^9/L at presentation and age less than one year or between 9 and 15 years define high-risk B-ALL in children [40].

4.3. Specific Genetic Types of B-ALL and T-ALL Described in the Fifth-Edition WHO Classification

The following sections describe salient features of the specific genetic types of B-ALL and T-ALL. Wherever there is a difference in terminology for the genetic subtypes of ALL between WHO-HAEM5 and the ICC, the explanation is given in the respective sections.

4.3.1. B-ALL with High Hyperdiploidy and B-ALL with *ETV6::RUNX1* Fusion

These two types of B-ALL have similar clinical features but different molecular genetic features.

B-ALL with high hyperdiploidy, defined to harbor 51–65 chromosomes, was first described in the early 1980s. It is the most common abnormality in childhood ALL and

the most common pediatric cancer [41]. This subtype of B-ALL and the subtype with *ETV6::RUNX1* fusion are much more common in children than adults, and both have excellent prognoses. These two subtypes comprise ~55% of B-ALL in childhood standard-risk B-ALL with age 1 to <10 years and white cell count < 50×10^9/L. They also comprise about 25% of childhood high-risk B-ALL, about 5% of ALL in AYA, and 2–3% of ALL in adults aged >40 years [40].

Both subtypes initiate *in utero*, occur early in life between the ages of 2 and 10 years, and are much less frequent in adults. There is evidence for a two-step pathogenesis with hyperdiploidy or *ETV6::RUNX1* fusion occurring as the first hit *in utero*, which requires secondary alterations to transform into leukemia. The secondary alterations occur only in a few of the cells with *ETV6:RUNX1*, which is not sufficient on its own for leukemogenesis. Infections early in life are protective, but if they do not occur, then infections later in life can trigger the development of ALL [42].

Despite the clinical similarities between B-ALL with hyperdiploidy and B-ALL with *ETV6::RUNX1* fusion described above, the mutational analysis differs between the two entities, indicating different pathogenetic pathways.

B-ALL with *ETV6::RUNX1* Fusion

As mentioned, the *ETV6::RUNX1* fusion occurs pre-natally, but this abnormality alone is not capable of causing leukemia, as confirmed by multiple lines of evidence. The presence of the *ETV6::RUNX1* fusion in normal healthy newborns has been investigated in several studies, as reviewed in [43]. These studies have shown varying percentages for positivity, considered to be variable due to the molecular method used for detecting gene fusion. At least 5% of 1000 healthy Danish newborns were found to be positive for the *ETV6::RUNX1* fusion abnormality by a highly sensitive and specific molecular method [43]. This incidence is much higher than the 1 in 10,000 incidences of childhood ALL harboring this fusion [43,44]. In addition, ALL with *ETV6::RUNX1* shows a long latency period of 2 to 14 years for developing leukemia, indicating that additional events are required for leukemic transformation.

The secondary events that lead to ALL with *ETV6::RUNX1* fusion occur post-natally. Many investigators have researched cytogenetic and copy number aberrations in ALL with *ETV6::RUNX1* fusion, as reviewed in [45,46]. This type of ALL harbors an average of 3.5 (range 0 to 13) copy number aberrations per case. Notably, these aberrations were recurrent in only 26% of all (n = 164) cases of ALL with *ETV6::RUNX1* fusion. The remaining 74% of cases showed heterogeneous and unique (non-recurrent) patterns [47]. The most common abnormalities were present in 46% of the recurrently altered regions and comprised small focal deletions encompassing only one or two genes. The most common genes with focal deletions were (1) *ETV6* (59%), (2) *CDKN2A/B* (22%), (3) B-cell-development genes, such as *PAX5* (20%), *TCF4* (7%), and *EBF1* (4%), (4) genes with an established function in the immune system, such as *CD200* and *BTLA* (13%), or (5) *TBL1XR1* (12%) [47]. The mechanism of the primary leukemogenic event in B-ALL with *ETV6::RUNX1* fusion is recombination activating gene (RAG)-mediated deletions. These deletions are enriched at promoters and enhancers of genes actively transcribed in B-cell development [48]. The deletion of 12p appears to be the most frequent deletion in B-ALL with *ETV6::RUNX1* fusion. Other secondary genetic changes in B-ALL with *ETV6::RUNX1* include deletions of chromosomes 6q and 9p, loss of entire chromosomes X, 8, and 13, duplications of chromosome 4q, and trisomy of chromosomes 21 and 16. However, the clinical significance of these secondary changes is unclear [46].

Diagnostically, the t(12;21)(p13;q22) translocation is cryptic by chromosome banding analysis. The chimeric *ETV6::RUNX1* fusion is detectable by FISH, reverse-transcriptase polymerase chain reaction (PCR), and RNA sequencing. By FCI, the leukemic cells show absent or partial positivity for CD9, CD20, and CD66c, with frequent expression of the myeloid antigens CD13 and CD33 [20,36,49]. Also, the leukemic cells in B-ALL with *ETV6::RUNX1* and B-ALL with *ETV6::RUNX1*-like features express CD27 and are negative

for, or show low-intensity expression of, CD44 [31,50]. A subset of *ETV6::RUNX1* fusion-positive B-ALL is reported to be positive for CD27 and CD44 [50].

B-ALL with High Hyperdiploidy

In contrast, B-ALL with high hyperdiploidy characteristically has a non-random gain of chromosomes X, 4, 6, 10, 14, 17, 18, and 21. Individual trisomies or tetrasomies are seen in over 75% of cases, and there are no recurrent gene fusions. Chromosome losses are rare. The chromosomal gains are an early feature, suggesting that the hyperdiploidy drives leukemogenesis. Mutational analysis shows frequent alterations in the receptor tyrosine kinase (RTK)-RAS pathway and histone modifier genes [51]. Single-cell sequencing studies recently revealed that the stable aneuploidy karyotypes in this type of B-ALL likely arise from a single tripolar mitosis, followed by low-level clonal evolution [52].

B-ALL with high hyperdiploidy, defined by 51–65 chromosomes, characteristically harbors non-random trisomies, most frequently of chromosomes X, 4, 6, 10, 14, 17, and 18, and trisomy/tetrasomy 21. FISH for trisomy 4, 10, 17, and 21 can help to identify high hyperdiploidy in normal or failed karyotype results. An additional three to five discrete *RUNX1* signals while using a FISH probe for the *ETV6::RUNX1* fusion also suggest high hyperdiploidy, which FISH studies for trisomies 4, 10, and 17 can confirm [36].

By FCI, the leukemic cells in B-ALL with high hyperdiploidy show a higher-intensity expression of CD9, CD20, CD22, CD58, CD66c, CD86, and CD123 and a lower-intensity expression of CD45 compared with the FCI expression of leukemic cells in B-ALL with another ploidy state, including diploid, low hyperdiploid, and near-tetraploid [53]. FCI was performed in this study using an 8-color or 10-color flow cytometry protocol based on a standardized staining protocol developed by the EuroFlow consortium [54].

4.3.2. B-ALL with Hypodiploidy

B-ALL with hypodiploidy shows 43 or fewer chromosomes. The hypodiploidy subtypes include near-haploid with 24–31 chromosomes, low-hypodiploid with 32–39 chromosomes, and high-hypodiploid with 40–43 chromosomes. All of these subtypes, near-haploid and low- or high-hypodiploid B-ALL, have a poor prognosis. Near-diploid B-ALL comprising 44–45 chromosomes is not included in this category, as it does not have a poor prognosis [36].

Low-hypodiploid B-ALL comprises about 15% of all B-ALLs in adults aged >40 years, about 5% of ALLs in AYA, and 2–3% of childhood ALLs [40]. These B-ALLs harbor *TP53* mutations in 90% of cases. Fifty percent of these cases carry germline *TP53* mutations found in Li-Fraumeni syndrome [55], an autosomal-dominant cancer predisposition syndrome [56–58].

IKZF2 deletions and *RB1* mutations are also characteristically present in low-hypodiploid B-ALL [36,55]. IKZF2 is a member of the IKAROS family of proteins, which are zinc-finger transcription factors involved in lymphoid development and differentiation. The founding member of the IKAROS family is IKZF1, encoded by *IKZF1*, which is essential for lymphopoiesis [59]. *IKZF1* is altered in high-risk ALLs, which is described subsequently. However, *IKZF1* is not altered in low-hypodiploid ALL [55].

In contrast, 70% of near-haploid ALLs show alterations leading to activated receptor tyrosine kinase and RAS signaling pathways. These alterations include deletion, amplification, and/or sequence mutations of *NF1*, *NRAS*, *KRAS*, *MAPK1*, *FLT3*, or *PTPN11*. Near-haploid ALLs have a high frequency of *IKZF3* alterations [55].

Cytogenetic analysis in hypodiploid B-ALL shows a non-random pattern of chromosome losses, including chromosomes 3, 7, 13, 15, 16, and 17. The chromosomes lost in the low-hypodiploid leukemic cells occur in two copies, and the initially retained chromosomes occur in four copies. The presence of a typical pattern of lost and gained chromosomes may suggest the diagnosis of B-ALL with low hypodiploidy [60,61].

Of note, a doubled-up hypodiploid state may present as “pseudo-hyperdiploid”, a near-triploid state with 60–78 chromosomes, or a “masked” hypodiploid state. Due to their markedly different prognoses, these pseudo-diploid states must be distinguished from

true high hyperdiploidy [60–62]. Chromosomal microarrays can definitively make this distinction, as the doubled-up hypodiploid state will show a copy-neutral loss of heterozygosity. The essential diagnostic criteria according to WHO-HAEM5 require (1) a diagnosis of B-ALL and (2) karyotyping, FISH, or both showing fewer than 44 chromosomes. In addition, single-nucleotide polymorphism analysis to better identify masked hypodiploidy is desirable according to WHO-HAEM5 criteria. The DNA index from flow cytometry may help to suggest the diagnosis if both hypodiploid and near-triploid clones are present. Still, flow cytometry cannot confirm the precise chromosomal losses [36]. In the absence of a microarray in low-resource settings, an algorithm based on careful examination of the gained and lost chromosomes has been proposed to diagnose hypodiploid B-ALL [63].

4.3.3. B-ALL with Intrachromosomal Amplification of Chromosome 21

B-ALL with iAMP21 is defined by $\geq$5 copies of *RUNX1* per cell, with 3 or more copies on a single abnormal chromosome 21 [36,64]. It comprises about 2% of ALLs in children and AYA and is rare in adults aged >40 years [36]. Females and males are equally affected, with a median age of 9 years and low white cell count (5×10^9/L) at presentation. In a large international study of 530 patients with iAMP21-positive B-ALL, the oldest patient's age was 30 years [65]. Patients with Down syndrome are rarely associated with this type of B-ALL, with one patient reported among 530 patients [65]. However, individuals with a constitutional ring chromosome 21 or the Robertsonian translocation, rob(15;21)(q10;q10)c, between chromosomes 15 and 21 have an increased risk of developing B-ALL with iAMP21 [65,66]. Robertsonian translocations occur between the short arms of acrocentric chromosomes 13–15, 21, and 22. Individuals carrying the Robertsonian translocation between chromosomes 15 and 21 have been estimated to have a 2700-fold greater risk of developing B-ALL with iAMP21 compared with the general population. The risk is not present for any other type of ALL or cancer and is very specific for B-ALL with iAMP21. As Robertsonian chromosomes are dicentric, comprising both centromeres of chromosomes 15 and 21, after cell division, there is a tendency for chromothripsis in these cells, which becomes the mechanism of cancer causation in this type of B-ALL [66]. The prognosis of B-ALL with iAMP21 is worse than that of standard-risk B-ALL, and intensive therapy as a high-risk B-ALL improves the outcome [65].

As there are significant implications for the prognosis and treatment of this type of B-ALL, accurate diagnosis is essential. Diagnosis requires genetic testing. Cytogenetic analysis in B-ALL with iAMP21 shows a grossly abnormal chromosome 21 in a karyotype. FISH can confirm the diagnosis on a metaphase preparation, which will show iAMP21 abnormalities on a single abnormal chromosome 21. Caution is required if using interphase FISH, as this technique can also show $\geq$5 copies of *RUNX1* per cell in B-ALL with high hyperdiploidy due to extra copies of the entire chromosome 21 in hyperdiploidy. That distinction between high hyperdiploidy and iAMP21 can be made by metaphase FISH.

Also, after FISH results, the diagnosis of iAMP21 can be confirmed by chromosomal microarrays, which show a distinctive pattern of telomeric loss and gain in chromosomal 21. Therefore, chromosomal microarray findings can validate FISH results or diagnose rare cases of iAMP21 wherein the cytogenetic pattern is not typical and involves rearrangements in other genes [36,67]. Of note, in rare instances, B-ALL with iAMP21 was found to occur with *ETV6::RUNX1* fusion, *BCR::ABL1* fusion, or hyperdiploidy [65]. B-ALL with iAMP21 also shows a unique spectrum of secondary genetic abnormalities, which can be used for improved diagnosis. These abnormalities include a gain of chromosomes X, 10, or 14 (in the absence of high hyperdiploidy), monosomy 7, deletion of 7q, deletions of 11q, including the *KMT2A* gene, *P2RY8::CRLF2*, and deletions of *ETV6* and *RB1* [65].

4.3.4. B-ALL with *BCR::ABL1* Fusion or Ph-Positive B-ALL

BCR::ABL1 fusion-positive (or Ph+) B-ALL is infrequent in children, comprising ~2–5% of childhood B-ALL cases. Its incidence increases gradually with age, with an overall incidence of 20–25% in adults [68]. *BCR::ABL1* fusion-positive B-ALL is critically important

to diagnose as distinct from *BCR::ABL1* fusion-negative (or Ph-) B-ALL, as tyrosine kinase inhibitors (TKIs) have significantly improved the outcomes of Ph+ B-ALL. Owing to TKI therapies, the previous adverse outcomes of Ph+ B-ALL are now better than those of Ph-negative B-ALL in adults [68,69].

Diagnosis requires demonstrating the *BCR::ABL1* fusion by any method, including karyotyping, FISH, PCR assays, and DNA or RNA sequencing. After diagnosis, these patients also need the specific fusion transcript to be identified for subsequent measurable residual disease (MRD) monitoring after therapy. MRD is a powerful predictor of outcomes in ALL. Long-term molecular remissions are associated with an increased chance of cure, which has become the goal of therapy.

The most prevalent *BCR::ABL1* transcript expressed in about 70% of Ph+ B-ALL is e1a2, representing the minor breakpoint with the p190 kDa protein. The e13a2 and e14a2 fusion transcripts represent major breakpoints with the p230 kDa protein. These major breakpoint fusion transcripts are expressed in most of the remaining cases. Additionally, rare cases express variant transcripts [70]. Secondary cytogenetic abnormalities are common in Ph+ B-ALL. They include, in order of descending frequency, +der(22)t(9;22), +21, abnormalities of 9p, high hyperdiploidy (>50 chromosomes), +8, −7, +X, and abnormalities, resulting in loss of material from 8p, gain of 8q, gain of 1q and loss of 7p [71]. The presence of additional der(22)t(9;22) at diagnosis has an increased risk of relapse [71,72]. *IKZF2* deletions or splicing abnormalities are frequent in Ph+ B-ALL.

Mutational analysis of the *ABL1* kinase domain is recommended to guide therapy in patients who relapse or do not respond to the initial TKI therapy [36]. It is important to note that conventional Sanger sequencing requires at least 10–20% of clonal cells to detect a mutation, while next-generation sequencing (NGS) can have much lower sensitivity. In a comparative study in Ph+ ALL, Sanger sequencing did not detect *ABL1* kinase domain mutations in 55% of samples, wherein mutations were detected by NGS [73]. The earlier detection of resistance mutations by NGS at a lower frequency than that detectable by Sanger sequencing has a clinical impact because it can alert the clinician to the possibility of relapse [74]. Further, *BCR::ABL1* kinase mutations may be present at low levels at diagnosis of Ph+ B-ALL [75–77]. Therefore, current guidelines recommend mutational analysis before starting therapy with TKIs and subsequently during treatment [36,77].

Ph+ B-ALL arising de novo can mimic the lymphoid blast crisis phase of chronic myeloid leukemia. The latter disease in the blast phase has an inferior outcome compared with de novo Ph+ B-ALL [78]. The diagnostic distinction can be challenging without a clinical history of chronic myeloid leukemia. The distinction may be made possible by the following clues: (1) A combination of all morphologic, immunophenotypic, and genetic features, including the presence of increased immature myeloid cells as a part of the chronic myeloid leukemia blast crisis phase, might help to suggest the diagnosis of the blast phase at presentation [79]. (2) If lymphoblastic leukemia is arising from chronic myeloid leukemia, then the *BCR::ABL1* fusion signal will also be detected in non-lymphoid cells at diagnosis, which could be determined by FISH analysis. In contrast, if *BCR::ABL1* fusion is present in only B-lymphoblasts, that would indicate a de novo B-ALL. A positive *BCR::ABL1* fusion signal in peripheral blood neutrophils is a readily available test to identify the blast crisis phase of chronic myeloid leukemia [80]. (3) The retrospective diagnosis of a lymphoid blast crisis in chronic myeloid leukemia might also be made during therapy if the *BCR::ABL1* transcript levels exceed and are not explained by the number of leukemic cells.

The WHO classification emphasizes that this distinction should be made, and the description of the lymphoid blast phase is present in the chronic myeloid leukemia section because that is the original disease from which lymphoblastic leukemia arises [36]. However, the ICC has subdivided Ph+ B-ALL into two subtypes, shown in Table 1. These ICC subtypes, "with lymphoid only involvement" and "with multilineage involvement," essentially represent de novo Ph+ B-ALL and the B-lymphoid blast crisis phase of chronic myeloid leukemia, respectively [81]. The names for the ICC subtypes are based on the developmental level of the original cell in which the *BCR::ABL1* transformation occurs,

i.e., committed lymphoid cell in de novo Ph+ B-ALL versus multipotent stem cell for the lymphoid blast crisis of chronic myeloid leukemia.

In addition, *BCR::ABL1* fusion may rarely occur in T acute lymphoblastic leukemia (T-ALL), T lymphoid crisis of chronic myeloid leukemia, and in the group of mixed-phenotype acute leukemias (MPALs); see the references in [36].

4.3.5. B-ALL with *BCR::ABL1*-like Features (or Ph-like B-ALL)

"B-ALL with *BCR::ABL1*-like features" lacks the Ph chromosome and *BCR::ABL1* fusion but shows a gene expression profile similar to that of Ph+ B-ALL and has an adverse prognosis. These B-ALL cases were first described by gene expression profiling in 2005 as B-ALL cases that clustered tightly with actual *BCR::ABL1*-positive cases but lacked *BCR::ABL1* fusion and alterations in *KMT2A* and transcription factor 3 (*TCF3*)::pre-B-cell-leukemia transcription factor 1 (*PBX1*) [82,83]. Subsequently, two groups, one Dutch and the other at St. Jude's Hospital in the U.S.A., described these cases with a poor prognosis in childhood ALL [84,85]. The Dutch group identified a new high-risk subtype of B-ALL with a gene expression profile most similar to that of *BCR::ABL1*-positive ALL and associated most often with treatment failures; this type of B-ALL comprised 15–20% of all precursor B-ALLs in the children in their cohort. The *BCR::ABL1*-like subtype of B-ALL was characterized by a high frequency of deletions in genes involved in B-cell development (82%), including *IKAROS*, *E2A*, *EBF1*, *PAX5*, and *VPREB1*, compared with other ALL cases (36%, $p = 0.0002$) [84]. In the U.S. cohort of 221 children with high-risk B-ALL, excluding Ph+ B-ALL, hypodiploid ALL, and infant ALL, St. Jude's investigators found *CDKN2A/B* alterations in 45.7%, *PAX5* alterations in 31.7%, and *IKZF1* alterations in 28.6% of patients. Like the Dutch study, the high-risk B-ALL cases with *IKZF1* alterations showed a gene expression profile similar to that of Ph+ B-ALL and had a poor prognosis [85].

The frequency of Ph-like ALL increases with age, so it is frequent in adults. It comprises >20% of adult ALLs, including 27.9% of ALLs in AYA aged 21 to 39 years, 20.4% of ALLs in adults aged 40 to 59 years, and 24.0% of ALLs in older adults aged 60 to 86 years with B-ALL [86]. Ph-like ALL is the most commonly observed type, comprising 53% to 62% of all ALL cases in individuals with Down syndrome [87–89]. Patients with Ph-like B-ALL present with significantly higher white cell counts (106×10^9/L) than patients with non-Ph-like B-ALL with leucocyte counts of 59×10^9/L [90]. However, the reported white cell counts in this type of B-ALL range from 4×10^9/L to 570×10^9/L [91] and 1×10^9/L to 603×10^9/L [92]. The disease is more common in males than females [91,92] and is more common in individuals of Hispanic ethnicity [92,93]. There is a high risk of induction failure and high MRD levels after induction [86,94].

Genetically, Ph-like ALL is heterogeneous. Similar to Ph+ B-ALL, Ph-like B-ALL cases also harbor *IKZF2* deletions. About half of Ph-like B-ALL cases overexpress cytokine receptor-like factor 2 (CRLF2), and about half of those harbor Janus kinase *JAK1* or *JAK2* mutations or rearrangements causing the constitutive activation of the JAK-STAT pathway [86,93]. CRLF2 overexpression occurs due to underlying *CRLF2* gene rearrangements, including those with *IGH* and *P2RY8* genes. The frequency of *JAK2* mutations and *IGH::CRLF2* translocations increases significantly with patient age [94]. Ph-like, *CRLF2*-rearranged, and JAK-activated B-ALLs have poor prognoses, and novel treatments are needed for these high-risk ALL patients [86,94]. Quantitative molecular methods may identify CRLF2 overexpression, and the expression of the *P2RY8::CRLF2* transcript has a poor prognosis [95]. *CRLF2* gene rearrangements may be identified by FISH or flow cytometry. FCI detects the overexpressed thymic stromal lymphopoietin receptor (TSLPR) caused by the *CRLF2* rearrangement [96].

Further, about 10% of Ph-like B-ALL pediatric patients harbor *ABL* class fusions in other tyrosine kinases. These alterations include fusions involving *ABL1*, *ABL2*, platelet-derived growth factor-receptor-beta (*PDGFRB)*, and colony-stimulating factor 1-receptor (*CSF1R)* genes [90,97]. The *ABL*-class fusions comprise about 4% of B-other-ALLs [98]. The *ABL*-class abnormalities are essential to detect because these patients may respond to

TKI therapy [91]. Other kinase-activating alterations may be present in Ph-like B-ALLs, including in *NTRK3*, *FLT3*, *PTK2B*, and *TYK2* genes. Some of these alterations, such as those in *NTRK3* and *FLT3*, are targetable by currently available agents [40,90].

Diagnosing B-ALL with *BCR::ABL1*-like features is currently a challenge for laboratories without access to RNA sequencing. The fusions are cryptic and not detectable by cytogenetics. As mentioned, *CRLF2* alterations may be detected by flow cytometry, FISH, and quantitative PCR assays. A clinical testing algorithm has been proposed primarily based on using flow cytometry and FISH assays [99].

4.3.6. B-ALL with *TCF3::PBX1* Fusion and B-ALL with *TCF3::HLF* Fusion

B-ALL with *TCF3::PBX1* Fusion

B-ALL with *TCF3::PBX1* fusion comprises about 5% of pediatric ALLs and is rare in adults. Precursor B-ALL with t(1;19)(q23.3;p13.3) was first described in the early 1980s to be associated with lymphoblasts having a pre-B-cell (cytoplasmic immunoglobulin-positive, surface immunoglobulin-negative) immunophenotype. The underlying fusion between *E2A* (the earlier name for *TCF3*) and *PBX1* genes was described in 1991 [100]. The clinical presentation is with high white blood cell counts. Historically associated with poor outcomes [101], this leukemia was subsequently shown to have favorable outcomes with an increased risk of central nervous system relapse [102].

The transcription factor 3 (*TCF3)* gene encodes two basic helix–loop–helix (bHLH) transcription factors, E12 and E47, through alternative splicing, which are crucial for B-cell development and in the process of immunoglobulin VDJ gene rearrangement [103]. *PBX1* is a transcription factor that critically regulates numerous embryonic processes, including morphological patterning, organogenesis, and hematopoiesis [104]. In most cases of B-ALL with t(1;19)(q23.3;p13.3) by karyotypic analysis, an underlying fusion occurs between exons 1–16 of *TCF3* at 19p13.3 and exons 3–9 of *PBX1* on 1q23, detectable by molecular methods [105]. However, in up to 10% of cases with the t(1;19)(q23.3;p13.3) translocation identified, the *TCF3::PBX1* fusion is not detected due to additional breakpoints within the genes that give rise to alternative transcripts [105]. Subsequently, FISH using *TCF3* break-apart probes allowed the detection of the usual and rare breakpoints in *TCF3::PBX1* fusions [106].

By flow cytometry, the leukemic cells express moderate-intensity CD10 and CD19, strong CD9, dim to negative CD34, and at least a partial absence of CD20. Of note, by WHO-HAEM5, the *TCF3::PBX1* fusion subtype does not include *TCF3* fusion gene partners other than *PBX1.* FISH using the *TCF3* break-apart probe is not sufficient because this probe will not distinguish *TCF3::PBX1* from *TCF3::HLF.* B-ALL with *TCF3::HLF* is classified separately [36].

Mate-pair sequencing has detected cryptic *TCF3::PBX1* fusions in B-ALL. This technique also identified additional genomic rearrangements in B-ALL without *TCF::PBX1* fusion but with atypical FISH patterns for *TCF3*, indicating the value of NGS in detecting *TCF3* gene fusions, including *TCF3::PBX1* and *TCF3::HLF*, in B-ALL [107]. Similarly, RNA sequencing in B-ALL identified cryptic *TCF3* fusions with several partner genes, including *FLI1* and *HLF*, in addition to *TCF3::PBX1*, indicating that standard cytogenetic methods are insufficient to detect all *TCF3* fusions. The prognostic significance of *TCF3::FLI1* fusion is not yet clear [108].

B-ALL with *TCF3::HLF* Fusion

B-ALL with *TCF3::HLF* fusion occurs due to a rearrangement between *TCF3* at 19p13.3 and the hepatic leukemia factor gene, *HLF*, at 17q22. *HLF* is not normally transcribed in lymphoid cells, and the protein belongs to a basic leucine zipper family of PAR proteins. This fusion was first described in 1992 in t(17;19)(q22;p13)-positive B-ALL, which was first described in 1991 [109]. The fusion leads to the leukemic cells becoming similar to stem cells. B-ALL with *TCF3::HLF* fusion is a rare type of leukemia, comprising <1% of B-ALL in pediatric patients, but it can also occur in adults [110]. Patients often have hypercalcemia

and coagulopathy (disseminated intravascular coagulation) at diagnosis and relapse early, with death within two years of diagnosis [110–113], in contrast with B-ALL with *TCF::PBX1*, with >85% five-year survival [114].

Of note, the diagnosis has recently been achieved, including retrospectively, by detecting cryptic *TCF3::HCF3* fusions by RNA sequencing and mate-pair sequencing [107,108]. Due to its dismal prognosis, this rare leukemia has been studied intensively genomically [115,116], with apparently no targeted genetic therapy yet. Nevertheless, immunotherapy treatment options may be promising. These patients may be treated with anti-CD19 immunotherapies, especially considering the reported high levels of CD19 expression on *TCF3::HLF* fusion-positive leukemic cells. Blinatumomab, a bispecific T-cell engager that binds CD3+ T-cells and CD19+ B-cells, led to a molecular remission in these patients in a preliminary study [40,117].

4.3.7. B-ALL with *KMT2A* Rearrangement

ALL with *KMKT2A* (previous name mixed lineage leukemia, *MLL*) rearrangement accounts for about 75% of infant ALLs, 5% of ALLs in older children, and ~10% of adult B-ALLs. Patients present with a high white cell count and central nervous system involvement. This leukemia has a poor prognosis [118–121]. There is strong evidence that the genetic abnormality is acquired *in utero*. However, the secondary events for transformation from a pre-leukemic state to leukemia occur post-natally in only a fraction of the pre-leukemic cases, similar to the other B-ALL-associated chromosomal abnormalities discussed earlier, including *ETV6::RUNX1* and *TCF3::PBX1*. In contrast with those abnormalities, *KMT2A* fusions have a short latency period for developing overt ALL. And *KMT2A* fusions may have the capability to initiate leukemia or the secondary mutations for leukemic transformation on their own (reviewed in [122]).

The lysine methyltransferase 2A (*KMT2A*) gene at 11q23 was first discovered in human acute leukemias in 1991 [123]. The *KMT2A* gene encodes a lysine methyltransferase that activates transcription by catalyzing the transfer of methyl groups from S-adenosylmethionine to the lysine 4 residue on the histone H3 tail. In this manner, *KMT2A* is crucial for the positive gene expression of many genes, including *HOX*, which are important in many developmental processes, including hematopoiesis. Consequently, alterations in *KMT2A* lead to many diseases, including developmental (non-neoplastic) and hematopoietic malignancies. The *KMT2A* gene is a hotspot target of chromosomal translocations in acute leukemias, which lead to a loss of the KMT2A methyltransferase function and an undifferentiated leukemic cell state [124,125].

In acute leukemias, the gene rearrangement occurs between *KMT2A* at 11q23 and one of many (>100) different fusion partner genes. The most common fusion partner for *KMT2A* is *AFF1* (previous name MLLT2) at 4q21.3-q22.1 in the t(4;11)(q21;q23) chromosomal abnormality [120,121,126]. The fusion partners, *AFF1*, *MLLT1*, and *MLLT3*, represented 87% of all (n = 2182) *KMT2A*-rearranged ALL patients between 2003 and 2022 in the largest study of *KMT2A*-rearranged acute leukemias, including ALL and acute myeloid leukemia, to date. In that large study, samples were analyzed for *KMT2A* rearrangement by PCR or targeted NGS [126]. According to this comprehensive analysis, the incidence of infant *KMT2A*-rearranged B-ALL peaks within the first year of life, with about 48% *AFF1*, 24% *MLLT1*, and 16% *MLLT3* as fusion partners among all cases. The incidence then declines during pediatric and young adult life, increases slightly until 55 years of age, and then presents a final decline due to decreasing numbers of patients [126].

Among all ALL patients (n = 2182), the following gene rearrangements were observed: *AFF1* (n = 1233; 56.5%), *MLLT1* (n = 404; 18.5%), *MLLT3* (n = 258; 11.8%), *MLLT10* gene (n = 80; 3.7%), *AFDN* (46; 2.1%), *EPS15* (n = 36; 1.6%), *USP2* (n = 21; 0.9%), *ELL* (n = 1; 0.05%), *KMT2A*-PTD (n = 1; 0.05%), and 43 other *KMT2A* rearrangements [126].

KMT2A rearrangement may be cryptic by karyotyping and FISH, and diagnosis may require NGS. Although usually identified by RNA sequencing, a recent study reported *KMT2A* rearrangements as the most commonly missed by this technique due to low

expression [127]. By flow cytometry, the leukemic cells have a CD19-positive, CD10-negative, and CD24-negative immunophenotype, often with one or more myeloid antigens expressed and negative TdT. Immunotherapy or chemotherapy may lead to a lineage switch to acute myeloid leukemia [36].

It is critically important to detect *KMT2A* rearrangements in acute leukemias, even from a targeted therapy perspective. KMT2A normally associates with menin in a macromolecular complex of highly conserved cofactors. Menin is a product of the *MEN1* tumor suppressor gene, which is mutated in heritable and sporadic endocrine tumors. The oncogenic *KMT2A* gene rearrangement continues to be associated with menin, and this association is required for *KMT2A*-mediated oncogenesis [128]. Revumenib, previously known as SNDX-5613, selectively inhibits the menin–*KMT2A* interaction and is being studied to treat *KMT2A*-rearranged acute leukemias [129].

4.3.8. New Subtypes of B-ALL Introduced in WHO-HAEM5

B-ALL with iAMP21 and B-ALL with Ph-like features were upgraded from provisional to definite subtypes of ALL. B-ALL with *TCF3::HLF* fusion was included as a new subtype of B-ALL; all three of these subtypes have been discussed above. This section briefly describes the other new genetic subtypes of B-ALL in WHO-HAEM5.

B-ALL with *DUX4* Rearrangement

B-ALL with *DUX4* rearrangement is one of the newer described subtypes of B-ALL. *DUX4*-rearranged B-ALL comprises 16% of the B-other cases or 4% of all pediatric B-ALL [28]. In B-ALL in AYA in Japan, *DUX4*, *ZNF384*, and *MEF2D* fusion genes account for about 40% of Ph-negative cases [29]. In B-ALL in Malaysia and Singapore, *DUX4*-rearranged B-ALL is the third-most-common subtype [130]. This leukemia has a favorable prognosis, similar to B-ALL, with high hyperdiploidy and *ETV6::RUNX1* fusion, despite the presence of high MRD levels [130,131].

The *DUX4* gene encodes a homeobox-containing protein and is located within a subtelomeric D4Z4 repeat region on 4q and 10q. The gene is present in 11–100 copies on each allele and is epigenetically silent in somatic tissues. The *DUX4* rearrangement occurs most frequently with *IGH* and less frequently with the *ERG* gene. In the *IGH*::*DUX4* fusion, a segment of the *DUX4* gene is relocated to *IGH*, leading to the overexpression of DUX4. This rearranged form of *DUX4* binds with a genetic region in the ETS-family transcription factor *ERG* (ETS-related gene), which leads to the expression of an *ERG* protein fragment that inhibits normal ERG function and causes leukemic transformation. *ERG* deletions are frequent secondary alterations in *DUX4*-rearranged B-ALL [28,29,132]. Also, *IKZF1* deletions co-occur with *ERG* deletions in *DUX4*-rearranged B-ALL. As the prognosis of *IKZF1* deletions depends on the co-occurring mutations in B-ALL, the usually adverse prognosis of *IKZF1* deletions can be overcome in these patients by chemotherapy based on MRD evaluation [133].

DUX4 rearrangements in B-ALL are complex and different from those in *CIC::DUX4* fusion-positive (non-Ewing) round-cell sarcoma (sarcoma described in [134]) [28]. The complexity of the genetic rearrangement is likely to be the reason why these abnormalities were not detected in the pre-genomics era. The gene expression profile for *DUX4*-rearranged B-ALL is distinctive [28]. Flow cytometry showed strong (aberrant) surface expression of CD371 on the leukemic cells in *DUX4*-rearranged B-ALL, which, when combined with the expression of CD2, diagnosed all cases of this type of B-ALL [135]. CD371 is predominantly expressed on myeloid cells [135] and is not expressed on mature lymphocytes (see image in [36]). *DUX4*-rearranged leukemic cells may also express CD66c, and the co-expression of CD66c and CD2 was almost exclusively found in *DUX4* fusion-positive B-ALL [136]. An immunohistochemical stain for detecting *DUX4* fusions showed immunohistochemical positivity in five of six molecularly-positive cases and negativity in three of three molecularly-negative cases [137]. *DUX4*-rearranged B-ALL leukemic cells may switch to

monocyte-like cells, which is a feature of CD371 expression [135,137], and this switch does not lead to a worse outcome [138].

The WHO-HAEM5 diagnostic criteria require RNA or DNA sequencing by NGS to diagnose this type of B-ALL. The desirable criteria include confirming the *DUX4* gene rearrangement, the presence of CD371 expression on leukemic cells by FCI, or both [36]. It is noteworthy that while RNA sequencing can diagnose *DUX4* fusions, the most extensive study of *DUX4*-rearranged B-ALL patients examined by whole-genome sequencing (WGS) in a single clinical trial in the U.K. showed that whole-transcriptome sequencing alone could not be relied upon to identify all *DUX4*-rearranged B-ALL cases in the absence of WGS. These investigators established an automated bioinformatics pipeline that improved the detection of *DUX4* fusions by WGS [139].

B-ALL with *ZNF384* Rearrangement

The zinc finger protein 384, *ZNF384*, gene is located on the chromosomal locus 12p13.31. The gene encodes for a zinc finger transcription factor that is ubiquitously expressed in the bone marrow and other tissues. The transcription factor appears to bind and regulate the promoters of the extracellular matrix genes [140]. *ZNF384* rearrangements may occur with at least ten different gene partners in about 5% of childhood B-ALLs, 10% of adult B-ALLs, and 48% of mixed-phenotype acute leukemia, B/myeloid-type [141,142].

In Japan, *ZNF384*-related fusion genes were identified in 4.1% of 291 B-ALL or about 9% of B-other ALL patients. All *ZNF384*-related gene fusions, including *TCF3::ZNF384* and *EP300::ZNF384*, showed weak or negative CD10 expression with aberrant CD13 and CD33 expression. But the clinical features differed depending on the specific fusion gene. Higher cell counts, younger age (median age five years), and more frequent relapses were present in *TCF3::ZNF384*-positive than in *EP300::ZNF384*-positive B-ALL patients. The latter group of B-ALL patients had a median age of 11 years [30,142]. FISH with break-apart probes or genomic sequencing (RNA or DNA) is required to diagnose the cryptic *ZNF384* rearrangement [36].

B-ALL with *MEF2D* Rearrangement

Myocyte-enhancer factor 2 (Mef2) transcription factors are necessary for early B-cell development [143]. *MEF2D*, located on 1q22, encodes one of these transcription factors. *MEF2D* was found to be rearranged in about 5% of pediatric B-ALL without recurring genetic abnormalities. *MEF2D* can rearrange with multiple genes (*BCL9*, *CSF1R*, *DAZAP1*, *HNRNPUL1*, and *SS18*), with *BCL9*, located on 1q21, being the most frequent.

MEF2D::BCL9-rearranged B-ALL presents at a median age of 14 years. Morphologically, the leukemic cells appear to be mature B-cell leukemia-like cells with high expression of HDAC [144]. They have a characteristic immunophenotype with weak or absent CD10, CD38 positivity, and cytoplasmic IgM positivity. The cytogenetic rearrangement is cryptic by karyotyping, and diagnosis requires FISH, gene expression profiling, or genomic sequencing. There is resistance to chemotherapy, with very early relapse in this high-risk leukemia [32,35,144].

B-ALL with *PAX5*alt and B-ALL with *PAX5* p.P80R

The *PAX5* gene encodes for a transcription factor that regulates numerous genes essential for normal B cell development. B-ALL with *PAX5*alt and B-ALL with *PAX5* p.P80R refer to two distinct types of B-ALL. Both of these types of B-ALL harbor molecular genetic abnormalities in *PAX5*, which lead to a loss of the normal PAX5 protein, initiating a precursor B lymphoblastic leukemia.

B-ALL with *PAX5* p.P80R is unique because this subtype of B-ALL is characterized by a single point mutation in PAX5 instead of the other types of abnormalities that are common in B-ALL, such as deletions and translocations. This point mutation, c.239C>G, p.P80R, causes a substitution of proline to arginine in the DNA-binding domain of *PAX5*. In a cohort of 170 adult B-ALL cases that were negative for the known genetic abnormalities

in B-ALL, gene expression data profiling showed four clusters corresponding to B-ALL with rearranged *ZNF384*, *DUX4*, *KMT2A*, and *BCR::ABL1*-like features [145]. A fifth cluster in this study comprised 14 patients with *PAX5* p.P80R and lacked any fusion gene. Sanger sequencing identified 16 additional cases with *PAX5* p.P80R in another cohort [145]. Cytogenetics showed structural rearrangements of 9p or 7p, including dic(9;20) and der(7;9). The second allele was deleted or inactivated, leading to biallelic loss of PAX5 [34,145]. Mutations of genes in the RAS pathway were also present [34,145].

B-ALL with *PAX5*alt includes leukemia-causing genetic abnormalities other than *PAX5* p.P80R. This type of B-ALL has a gene expression profile distinct from that of B-ALL with *PAX5* p.P80R [34]. It comprises about 3–5% of childhood ALLs and 9.6% of adult B-ALLs.

In contrast, B-ALL with *PAX5* p.P80R comprises about 1% of childhood B-ALLs and up to 5% of adult B-ALLs. By FCI, B-ALL with *PAX5* p.P80R shows a pro-B immunophenotype, with low CD20 and high CD45 expression on the leukemic cells. The leukemic cells are CD13-negative, CD33-positive, and CD2-positive and show stronger intensity CD10 expression than in *KMT2A*-rearranged B-ALL. The prognosis of B-ALL with *PAX5* p.P80R is better than that of B-ALL with *PAX5*alt abnormalities [33,34,131]. The diagnosis of these subtypes requires genomic sequencing.

B-ALL with *MYC* Rearrangement

This rare leukemia occurs in <1% of children, 1–2% of AYA, and 2–3% of adult B-ALLs [36]. These cases have a precursor B-ALL immunophenotype, including no expression of surface immunoglobulins, but they harbor *MYC* rearrangement. According to gene expression profiling, these leukemias cluster with precursor B cells and other B-ALLs, but not with Burkitt leukemia [146]. In adults, these leukemias are considered high-risk B-ALLs with poor prognoses [131]. Children with *MYC*-rearranged B-ALLs are usually treated with Burkitt lymphoma therapy, with a better outcome than adults with *MYC*-rearranged B-ALL [36].

B-ALL with *NUTM1* Rearrangement

NUTM1 rearrangement is more frequent in infants (about 3–5%) than in children (0.4–0.9%) with B-ALL, and this rearrangement has not yet been detected in adults with B-ALL [147].

The nuclear protein in the testes (NUT) is normally located in post-meiotic spermatogenic cells, wherein a global increase in hyperacetylation occurs for spermatogenesis. The NUT Midline carcinoma family member 1 (*NUTM1*) gene (also known as *NUT*), located on 15q14, was first discovered as a part of the fusion gene in a rare and aggressive carcinoma called NUT carcinoma [148,149]. NUT carcinoma harbors a reciprocal t(15;19)(q14;p13.1) translocation between *NUTM1* on chromosome 15q14 and the BET family gene *BRD4* on chromosome 19p13.1, leading to an in-frame *BRD4::NUT* fusion oncogene driven by the *BRD4* promoter [149]. Subsequently, with the increased evaluation of tumors by genomic sequencing approaches, the *NUTM1* gene was found to also be present with other fusion partner genes in different types of cancers, including sarcomas and B-ALL [150]. These fusions lead to aberrant NUTM1 overexpression, and the altered global chromatin acetylation might confer sensitivity to histone deacetylase inhibitors and possibly to bromodomain inhibitors for *NUTM1::BRD9* fusion cases [36].

Intriguingly, while NUT carcinoma is a highly aggressive cancer, *NUTM1*-rearranged B-ALL has a favorable prognosis. This type of B-ALL occurs in infants and comprises 21.7% to 30% of non-*KMT2A*-rearranged (or *KMT2A* germline) B-ALL in infants [147,151]. Among nine *NUTM1*-rearranged B-ALL patients with a median age of 8.8 months, *ACIN1* (n = 5), *CUX1* (n = 2), *BRD9* (n = 1), and *ZNF618* (n = 1) were identified as fusion partners [151]. Interestingly, this same study also identified other *KMT2A*-germline infant B-ALL patients with a median age of about 11 months who harbored *PAX5* fusion; those patients had a poor prognosis [151].

By immunophenotype, the leukemic cells in *NUTM1*-rearranged B-ALL may be CD10-positive or CD10-negative, in contrast with CD10-negative leukemic cells in *KMT2A*-rearranged B-ALL [147,151]. However, *KMT2A*-rearranged B-ALL may also be positive for CD10 [136], indicating that CD10 expression alone cannot be used to distinguish these two subtypes of ALL. The diagnosis can be made by FISH using a break-apart *NUTM1* probe or RNA or DNA sequencing [36,151].

B-ALL with *ETV::RUNX1*-like Features

This leukemia subtype comprises about 1–3% of childhood ALL [28] and about 2% of adult B-ALL [131]. Similar to Ph-like B-ALL, B-ALL with *ETV::RUNX1*-like features lacks the *ETV6::RUNX1* fusion, but the gene expression profile is similar to that of B-ALL with *ETV::RUNX1* fusion (see figure in [36]).

FCI shows the leukemia cells are CD24-positive and CD44-negative or low. However, note that this immunophenotype is not specific to this subtype of B-ALL and was also identified in B-ALLs with other genetic subtypes diagnosed by gene expression profiling [31]. Molecular analysis reveals combined *ETV6* and *IKZF1* alterations (rearrangements and deletions) in this type of leukemia [28]. Further, recent genomic studies showed biallelic *ETV6* inactivation [139] and the APOBEC mutational signature in *ETV6::RUNX1*-like childhood B-ALL patients [139,152]. Of note, B-ALL with *ETV6::RUNX1*-like features may also arise in patients with germline *ETV6* alterations [152].

4.3.9. Molecular Genetic Subtypes of B-ALL Defined by Standard Genetic Techniques and Whole-Genome Sequencing

Among the well-established genetic subtypes of B-ALL, t(9;22)(q34;q11)/*BCR::ABL1*, t(4;11)(q21;q23)/*KMT2A::AFF1*, and near-haploidy/low hypodiploidy are the high-risk abnormalities with the most impact on treatment and management. To a lesser extent, t(12;21)(p13;q22)/*ETV6::RUNX1* and high hyperdiploidy are abnormalities with an impact on good risk management [153]. These abnormalities can be detected by routine cytogenetic and molecular assays, including chromosomal banding analysis (karyotyping), FISH, and, for non-numerical abnormalities, reverse-transcriptase PCR, as was recommended in 2010 [153]. The latter assay provides a rapid, accurate, and sensitive method of detecting fusion transcripts in chromosomal translocations.

After the advent of genomics led to examining copy number abnormalities (CNAs) and sequence variants, additional prognostic markers based on genomic evaluation began to emerge, including *IKZF1* and *CDKN2A/B* deletions and rearrangements of *CRLF2*. These abnormalities are usually co-operating aberrations with the primary genetic abnormalities. Importantly, the pattern of CNAs was highly variable between primary genetic abnormalities in B-ALL [154]. Therefore, the genomic data available were then integrated into a cytogenetic and genomic risk stratification system that allowed appropriate risk-based patient management [155].

In the current era of tremendous progress due to genomic advances, there is now compelling evidence for WGS to become the first-tier test for all genetic abnormalities in ALL [139,156] to provide a diagnosis for the new genetic subtypes of B-ALL discovered only by applying genomics methods. Table 2 shows the well-established and more recently recognized genetic subtypes of B-ALL defined by standard genetic techniques and WGS with the prognostic significance of each subtype to help prioritize diagnostic workups for B-ALL.

Table 2. Genetic subtypes of B-ALL defined by standard genetic techniques and whole genome sequencing with their prognostic significance.

B-ALL Genetic Subtypes	Primary Genetic Aberrations	Prognostic Significance	FISH Probes	Fusion Genes	Detectable by Which Methods?		
					CBA and FISH	Molecular Assay	WGS
High hyperdiploidy	51–65 chromosomes	Favorable risk	Centromeric probes	Not applicable	Yes	CMA; not by RT-PCR	Yes
ETV6::RUNX1 fusion	t(12;21)(p13.2;q22.1)/*ETV6::RUNX1* [a]	Favorable risk	Dual-color fusion	*ETV6::RUNX1*	Yes [a,b]	Yes RT-PCR	Yes
Hypodiploidy	43 or fewer chromosomes: Near-haploid: 24–31 chromosomes; alterations in *NF1*, *NRAS*, *KRAS*, *MAPK1*, *FLT3*, or *PTPN11*; and *IKZF3*; Low-hypodiploid: 32–39 chromosomes; *TP53*, *IKZF2*, and *RB1* mutations; 50% of *TP53* mutations are germline; High-hypodiploid: 40–43 chromosomes	High risk	Screening probes may show a typical pattern of chromosomal gains and losses to suggest the diagnosis	Not applicable	Yes	Yes CMA; not by RT-PCR	Yes
Intrachromsomal amplification of chromosome 21	≥3 or more copies of *RUNX1* on a single abnormal chromosome 21 with frequent deletion of subtelomeric 21q sequences	Higher risk improved with intense treatment	*ETV6::RUNX1* probe [153]	Not applicable	Yes [c]	Yes CMA	Yes
BCR::ABL1 fusion	t(9;22)(q34.1;q11.2)	High risk improved with TKI therapies	Dual color or tricolor dual fusion	*BCR::ABL1*	Yes	Yes RT-PCR	Yes
BCR::ABL1-like features	*CRLF2* rearrangements, including *P2RY8::CRLF2*; *JAK* mutations; *ABL1*, *ABL2*, *PDGFRB*, and *CSF1R* fusions; and *NTRK3*, *FLT3*, *PTK2B*, and *TYK2* alterations	High risk	*CRLF2* BA	*P2RY8::CRLF2*	CBA: No FISH: Yes	Yes; MLPA	Yes
TCF3::PBX1 fusion	t(1;19)(q23.3;p13.3)	Favorable to intermediate	Dual color fusion	*TCF3::PBX1*	Yes	Yes RT-PCR	Yes
TCF3::HLF fusion	t(17;19)(q22;p13)	High risk	*TCF3* BA [d]	*TCF3::HLF*	Yes	Yes RT-PCR	Yes
KMT2A-rearranged	*KMT2A* (11q23) rearrangements	High risk	*KMT2A* BA	11q23 translocations	Yes [a]	Yes RT-PCR	Yes
DUX4-rearranged	*DUX4* fusions; DUX overexpression	Favorable, despite high MRD	Not applicable	*IGH::DUX4* or *ERG::DUX4*	No	Not by PCR [e]	Yes
ZNF384 rearrangement	*ZNF384* rearrangements; *EP300::ZNF384;* and *TCF3::ZNF384*	Favorable [157]; intermediate; depends on partner gene	*ZNF384* BA	*EP300::ZNF384; TCF3::ZNF384*	CBA: No	Not by PCR [e]	Yes
MEF2D rearrangement	*MEF2D* rearrangements; *MEF2D::BCL9* or *MEF2D::HNRNPUL1*	High risk	*MEF2D* BA	*MEF2D::BCL9* or *MEF2D::HNRNPUL1*	CBA: No	RT-PCR [17]	Yes

Table 2. *Cont.*

B-ALL Genetic Subtypes	Primary Genetic Aberrations	Prognostic Significance	FISH Probes	Fusion Genes	Detectable by Which Methods?		
					CBA and FISH	Molecular Assay	WGS
*PAX5*alt	*PAX5* abnormalities other than *PAX5* p.P80R: gene rearrangements, non-p.P80R sequence mutations, or focal intragenic amplifications, with the exception of *PAX5::JAK2* (Ph-like B-ALL) and *PAX5::ZCCH7*, which occurs in cases with other class-defining alterations [34,36]	Intermediate in children; high risk in adults	*PAX5* BA for rearrangements	Not applicable	CBA: No; FISH only for *PAX5* BA	Not by PCR [e]	Yes
PAX5 p. P80R	*PAX5* p. P80R	Intermediate in children; high risk in adults	Not applicable	Not applicable	No	Not by PCR [e]	Yes
MYC rearrangement	*MYC* rearrangement; *IGH::MYC*, *IGK::MYC*, or *IGL::MYC*	High risk in adults; better in children	*MYC* BA	*IGH::MYC*, *IGK::MYC*, or *IGL::MYC*	Yes	Not by PCR [e]	Yes
NUTM1 rearrangement	*NUTM1* (15q14) rearrangement	Favorable	*NUTM1* BA		CBA: yes [f] (subset)	Not by PCR [e]	Yes
ETV6::RUNX1-like features	*ETV6* fusions excluding *PAX5::ETV6*, *ETV6::ABL1*, and *ETV6::JAK2*; *IKZF1* fusion and/or deletion; *ETV6* biallelic inactivation if lacking other defining features [156]	Unfavorable [157] Favorable [g] [131,156]	Not applicable	Not applicable	No	Not by PCR [e]	Yes

[a] Cytogenetically cryptic; [b] FISH alone could not detect all cases of *ETV6::RUNX1* detected by WGS [156]; [c] Detectable by metaphase FISH; [d] *TCF3* BA FISH cannot distinguish between *TCF3::PBX1* and *TCF3::HLF* fusion; [e] These rearrangements can be detected by NGS (DNA or RNA sequencing) but not by PCR-based assays; [f] Karyotype can identify a subset of *NUTM1* rearrangements with aberrations at 15q14, but the exact band is often difficult to discern [36,147]; [g] No relapses or deaths at 10 years in the U.K. WGS study [156]. FISH; fluorescence in situ hybridization; BA, break-apart probe; CBA, chromosome banding analysis; CMA, chromosomal microarray; RT-PCR, reverse-transcriptase polymerase chain reaction; MLPA, multiplex ligation-dependent probe amplification; WGS, whole-genome sequencing; NGS, next-generation sequencing.

4.3.10. T-ALL, Not Otherwise Specified

T-ALL accounts for about 10–15% of all newly diagnosed ALLs, depending on the age range and ethnicity of the population [158]. Compared with B-ALL, T-ALL occurs more commonly in males and at an older age, often in AYA. T-ALL presents more often as a T-lymphoblastic lymphoma with a high white cell count and a mediastinal mass that may become a medical emergency.

The genetic basis for T-ALL is poorly understood; there is a higher incidence in Black individuals, but the cause is not yet known. The higher incidence in males has been linked to inactivating mutations and deletions in the X-linked *PHF6* tumor-suppressor gene; these mutations were not associated with *NOTCH1*, *FBXW7*, or *PTEN* mutations or with overall survival. Further, the known risk alleles for ALL have different effects on susceptibility to B-ALL and T-ALL [158].

T-ALL is also considered to arise from developing (precursor) T cell stages that normally occur in the thymus. The diagnosis requires confirming the T-precursor cell stage of the leukemic cells. However, these stages do not have independent prognostic significance.

In contrast with B-ALL, although many genetic abnormalities and dysregulated oncogenic signaling pathways, including dysregulated NOTCH1 signaling, have been identified in T-ALL, the genetic abnormalities in T-ALL have not been found to stratify risk [152,159]. Prognosis in T-ALL is primarily guided by MRD evaluation during therapy, and risk has been defined by combining molecular alterations with MRD [160]. The expression of a five-gene set (*ZPBP*, *GOT1L1*, *ACTRT2*, *SPATA45*, and *TOPAZ1*, all restricted to male germ cells) has been identified as an optimal classifier for prognostic stratification in T-ALL patients [161].

In 2022, ten different subtypes of T-ALL were identified based on RNA sequencing, with differences noted between adults and childhood T-ALL [162]. Also, the preliminary results of comprehensive genomic analyses of childhood T-ALL presented at the European Hematology Association annual meeting in 2022 revealed that >60% of driver lesions in T-ALL are non-coding [163]. This preliminary study showed 16 subtypes of T-ALL based on the clustering of RNA sequencing data [164].

Significantly, in late-2022, standard genetic techniques (chromosomal banding analysis, FISH, and molecular genetic analysis by reverse-transcriptase PCR, combined with whole-genome sequencing) classified T-ALL into nine distinct subgroups based on genetic alterations in *TLX1*, *TLX3*, *TAL1*, *HOXA9/10*, *MLLT10*, *NUP98*, *MYB*, *BCL11B*, and the presence of *SET::NUP214* fusion gene [165]. These genetic subgroups of T-ALL defined by standard cytogenetic techniques and whole genome sequencing are shown in Table 3, modified from Müller et al. 2023 [165].

The abnormalities shown in Table 3 could not be consistently detected by standard cytogenetics karyotyping due to a lack of dividing tumor cells or cryptic abnormalities. Therefore, FISH break-apart probes are needed for those abnormalities. Reverse-transcriptase PCR assays identified the fusion genes. The abnormalities that still could not be identified by these standard techniques were identified by WGS, which also confirmed all abnormalities detected by the traditional methods. Rearrangements of *BCL11B::TLX3*, *SET::NUP214*, and *STIL::TAL1* were not detectable by chromosome banding analysis due to the low resolution of the technique [165].

Table 3. Genetic subtypes of T-ALL defined by standard genetic techniques and whole-genome sequencing. (Table modified from Müller et al. 2023 [165]).

T-ALL Genetic Subgroups	Primary Genetic Aberrations	FISH Probes	Fusion Genes	Detectable by Which Methods?		
				Both CBA and FISH	Molecular Assay	WGS
TLX1	t(10;14)(q24;q11); *TRAD::TLX1*	*TLX1* BA		Yes	No	Yes
	t(7;10)(q34;q24); *TRB::TLX1*	*TLX1* BA		Yes	No	Yes
TLX3	t(5:14)(q35;q32);*BCL11B::TLX3*	*BCL11B::TLX3*, *TLX3* BA		FISH: yes; CBA: no	No	Yes
TAL1	t(1:14)(p32:q11); *TRAD::TAL1*	*TRAD* BA		Yes [b]	No	Yes
	del(1)(p32p32) [a]		*STIL::TAL1*	CBA: no [c]	Yes	Yes
HOXA9/10	inv(7)(p15q34); *HOXA::TRB*	*HOXA* BA		yes	No	Yes
SET::NUP214	del(9)(q34q34) [a]		*SET::NUP214*	No	Yes	Yes
MLLT10	t(10;11)(p12;q14)		*PICALM::MLLT10*	CBA: yes [c]	Yes	Yes
	t(X;10)(p11;p12)		*DDX3X::MLLT10*	CBA: yes [c]	Yes [c]	Yes
NUP98	t(4;11)(q23;p15)	*NUP98* BA	*NUP98::RAP1GDS1*	Yes	Yes [c]	Yes
MYB	t(6;7)q23;q34); *TRB::MYB*	*TRB* BA		Yes [b]	No	Yes
BCL11B	t(8;14)(q24;q32); *BCL11B::CCDC26* [a]			CBA: no [c]	No	Yes
	t(6;14)(q25;q32); *BCL11B::ARID1B* [a]			CBA: no [c]	No	Yes
	t(3;14)(p24;q32); *BCL11B::SATB1*			CBA: yes [c]	No	Yes
	BCL11B enhancer amplification			No	No	Yes
Rare	t(4;14)(q25;q11); *TRAD::LEF1*	*TRAD* BA		Yes [b]	No	Yes
	t(11;14)(p13;q11); *TRAD::LMO2*	*TRAD* BA		Yes [b]	No	Yes
	t(7;10)(q34;q24); *TRB::NKX2*	*TRB* BA		Yes [b]	No	Yes
	t(7;9)(q34;q34); *TRB::NOTCH1*	*TRB* BA		Yes [b]	No	Yes
	t(11;14)(p13;q32); *LMO2*			CBA: yes [c]	No	Yes
	Mutation in *MYB* enhancer			No	Yes [c]	Yes

[a] Cytogenetically cryptic; [b] Detectable by metaphase FISH only in conjunction with chromosome banding analysis because metaphase FISH identifies the partner chromosome of 14q11 (TRAD) or 7q34 (TRB); [c] Probes not available but detectable by FISH; for rare fusions, PCR has to be established. FISH; fluorescence in situ hybridization; BA, break-apart probe; CBA, chromosome banding analysis; WGS, whole-genome sequencing.

The BCL11B group showed more frequent granulocyte/macrophage progenitor and hematopoietic stem cells than the TLX1, TLX3, and TAL1 groups, which showed more frequent dendritic cells, Th1, and Th2 cells. In the BCL11B group, *NOTCH1* mutations, *PHF6* mutations, and *CDKN2A* deletions were absent, and *FLT3* mutations were frequent (7/10 cases, 70%), including both internal tandem duplication and tyrosine kinase domain mutations. There was a high expression of KIT and LMO2, low RAG1 and RAG2 expression, and *TCR* rearrangements were absent [165]. Their findings supported the hypothesis that the BCL11B type of T-ALL arises from a hematopoietic progenitor stem cell expressing ectopic BCL11B, which induces the T-lineage commitment in neoplastic cells [165,166]. *BCL11B* rearrangements are found in T-ALL, mixed-phenotype acute leukemia, and immature acute myeloid leukemia [166,167].

Early T Precursor Lymphoblastic Leukemia/Lymphoma

Early T-cell precursor lymphoblastic leukemia/lymphoma (ETP-ALL) is a subtype of T-ALL that arises from early T-cell precursors with stem-cell-like features [168]. About one-third of ETP-ALLs comprise the BCL11B group with structural alterations (described in the previous section and shown in Table 3) [166].

ETP-ALL has a distinctive immunophenotype; the leukemic cells are CD1a-negative, CD8-negative, CD5-negative, or CD5-weakly positive with <75% positive blasts and express at least one (or more) stem cell (CD34 and HLADR) or myeloid-associated surface antigen(s)

(CD11b, CD13, CD33, CD65, and CD117) on >25% blasts. The leukemic cells express cytoplasmic CD3, but surface CD3 is absent. CD7 is consistently expressed, and this feature, in conjunction with stem cell or myeloid marker positivity, serves to identify MRD by flow cytometry. Myeloperoxidase is absent or present in less than 3% of blasts [36,168]. CD123 expression has also been reported in ETP-ALL [169].

ETP-ALL has a poor prognosis, although, with current therapies, the outcomes are similar for non-ETP-ALL and ETP-ALL. Still, non-ETP T-ALL patients are more likely to relapse, and ETP-ALL patients are more likely to have refractory disease [163].

4.3.11. Summary of Specific Flow Cytometric Immunophenotypic Features in the Genetic Types of B-ALL and T-ALL

Many investigators have used flow cytometry to try to define the immunophenotypic profiles characteristic of specific genetic subtypes of acute leukemias. CRLF2 overexpression represents a rare example of a single antigen that can suggest a diagnosis of a particular genetic subtype of B-ALL with *BCR::ABL1*-like features. In conjunction with clinical features, FCI with an extensive antibody panel can help to suggest a differential diagnosis. Still, a definitive diagnosis of the genetic type requires genetic testing in most cases. In 2020, Ohki et al. [136] reported the FCI findings of >1000 childhood ALL cases, including 926 B-ALL and 118 T-ALL. They described the FCI findings of most genetic types of B-ALL. They classified the remaining cases as B-other [136]. Their study provides an excellent understanding of the heterogeneity of FCI findings in ALL.

It is worth noting that, in selecting their cohort cases, Ohki et al. excluded B-ALL cases with surface light-chain expression [136]. Therefore, their study could not determine surface light-chain expression in the genetic types of B-ALL. However, as previously mentioned, surface light-chain expression may be present in all three stages of B-ALL, i.e., pro-B, common ALL, and pre-B ALL, including in pediatric and adult B-ALL [9]. In that earlier study, B-ALL patients with the following cytogenetic abnormalities in the leukemic cells: hyperdiploidy, t(1;19)(q23;p13), t(12;21)(p13;q22), t(9;22)(q34;q11), and t(2;11)(p21;q23) showed unequivocal surface light-chain restriction [9].

Of note, in their study, Ohki et al. described the distribution of all genetic types of ALL in their cohort according to the early pro-B (CD10-negative cytoplasmic IgM-negative), intermediate CD10 (so-called common ALL), and late pre-B (cytoplasmic IgM-positive) stages of precursor B-cell differentiation. They also provided the percentages of cases showing >20% positivity for 23 antigens in each type of B-ALL.

Table 4 is presented based on the FCI findings by Ohki et al. [136] and other publications describing FCI findings in specific genetic types of ALL. Table 4 shows the distribution of the pro-B, common, and pre-B types of B-ALL among various types of B-ALL and the percentages of cases showing >20% positivity for a few selected antigens from their study.

Table 4. Summary of flow cytometric immunophenotypic profile of leukemic cells in specific genetic types of B-ALL based on 1044 consecutive childhood ALL cases in Ohki et al. 2020 [136] and other referenced publications.

B-ALL Genetic Subtypes	Total N in [136]	Distribution of Pro-B, Common, and Pre-B Cases for Each Genetic Type of B-ALL [136]			Percentages of B-ALL Cases Showing >20% Positivity for a Few Specific Antigens in [136]				Specific Features, if Any, of the Leukemic Cells by FCI Based on Referenced Publications
		CD10− cyt IgM− Pro-B	CD10+	cyt IgM+ Pre-B	CD10 % CD34 %	CD33 % CD13 %	CD66 %	CD27 % CD44 %	
High hyperdiploidy	179	0	85.5%	14.5%	CD10: 100% CD34: 87.7%	CD33: 9.5% CD13: 3.4%	CD66c: 73.7%	CD27: 10.1% CD44: 100%	Higher-intensity CD9, CD20, CD22, CD58, CD66c, CD86, and CD123, and lower-intensity CD45 compared with B-ALL with other ploidy status [53]; Strong CD123+ [170,171]
ETV6::RUNX1 fusion	164	0	86.1%	13.9%	CD10: 99.4% CD34: 72.6%	CD33: 42.4% CD13: 24.4%	CD66c: 0%	CD27: 70.6% CD44: 45.3%	Absent or partial positivity for CD9, CD20, and CD66c; frequent CD13+ and CD33+ [20,36,49,136]; CD27 + CD44(−)/low+ [31,50,136]; Uniformly low CD123+ [170,172] [a]
Hypodiploidy	6	0	66.7%	33.3%	CD10: 100% CD34: 85.7%	CD33: 42.9% CD13: 14.3%	CD66c: 85.7%	CD27: 20.0% CD44: 100%	DNA index by flow cytometry may suggest the diagnosis if both hypodiploid and near-triploid clones are present [36]
iAMP21	NA	NA	NA	NA	NA	NA	NA	NA	None
BCR::ABL1 fusion	46	0	84.4%	15.6%	CD10: 97.8% CD34: 97.8%	CD33: 34.5% CD13: 15.2%	CD66c: 91.3%	CD27: 41.9% CD44: 97.7%	Coexpressed CD66c+ and CD25+ [173]; higher intensity of CD13, CD33, CD66c, CD10, CD34, and CD25 than *BCR::ABL1*-negative [174,175]; CD123 higher-intensity expression [171]
BCR::ABL1-like features, kinase fusion-positive	11	0	63.6%	36.4%	CD10: 100% CD34: 100%	CD33: 36.4% CD13: 9.1%	CD66c: 36.4%	CD27: 44.4% CD44: 100%	Immunophenotype similar to *BCR::ABL1;* high CD20 and CD45RA expression; CD99+ (91%) TdT+ (100%), and cyt IgM+ (36.4%) [136]
BCR::ABL1-like features, *CRLF2*-rearranged	15	0	86.7%	13.3%	CD10: 100% CD34: 93.3%	CD33: 46.7% CD13: 0%	CD66c: 80.4%	CD27: 57.1% CD44: 100%	CRLF2 overexpressed [96,136]; all other features described here were similar to *BCR::ABL1* and *BCR::ABL1*-like kinase+ [136]

Table 4. *Cont.*

B-ALL Genetic Subtypes	Total N in [136]	Distribution of Pro-B, Common, and Pre-B Cases for Each Genetic Type of B-ALL [136]			Percentages of B-ALL Cases Showing >20% Positivity for a Few Specific Antigens in [136]				Specific Features, if Any, of the Leukemic Cells by FCI Based on Referenced Publications
		CD10− cyt IgM− Pro-B	CD10+	cyt IgM+ Pre-B	CD10 % CD34 %	CD33 % CD13 %	CD66 %	CD27 % CD44 %	
TCF3::PBX1 fusion	68	0	26.5%	73.5%	CD10: 98.5% CD34: 4.4%	CD33: 0% CD13: 0%	CD66c: 0%	CD27: 4.2% CD44: 100%	Homogeneous CD19+, CD10+, and CD9+, with partial expression of CD20; absent CD34 [19]
TCF3::HLF fusion	NA	NA	NA	NA	NA	NA	NA	NA	High expresssion of CD19 [36]
KMT2A::AFFI-rearranged	13	69.2	7.7%	3.1%	CD10: 7.7% CD34: 76.9%	CD33: 7.7% CD13: 0%	CD66c: 0%	CD27: 0% CD44: 100%	CD10–, CD24–, CD15+, and CD19+ blasts in B-ALL with t(4;11)(q21;q23) [18]; NG2+ [136,176]
KMT2A::MLLT3-rearranged	10 *MLLT3*	33%	11%	55.5%	CD34: 0%	CD33 [b] CD13: 0%	CD66c: 0%	CD27: 0% CD44: 100%	Aberrant CD7+, CD2+, and CD5+, more frequent in CD10+ cases; NG2+, CD15+, CD65+, CD117+, CD56+, CD99+, CD45RA+, and CD20− in CD10– cases [136]
DUX4-rearranged	20	5.9%	52.9%	41.2%	CD10: 95.0% CD34: 90.0%	CD33: 10.0% CD13: 15.0%	CD66c: 30.0%	CD27: 0% CD44: 94.1%	CD66c and CD2 coexpression-specific [136]; CD2+ [131]; CD20− TdT+ CD99−/rare +; CD56+ in 15% cases [136]; CD371+ [135]; and monocytic CD14, gain of CD45 and CD33, may be present at diagnosis and post-induction [36]
ZNF384 rearrangement	29	44.4%	51.9%	3.7%	CD10: 51.7% CD34: 100%	CD33: 82.8% CD13: 27.6%	CD66c: 10.3%	CD27: 10.5% CD44: 95.7%	Negative or dim CD10 with aberrant CD13 and/or CD33 expression [30,177]; monocytic differentiation may be present at diagnosis and early after induction [36], note [c]
MEF2D rearrangement	13	15.4%	23.1%	61.5%	CD10: 76.9% CD34: 38.5%	CD33: 15.4% CD13: 0%	CD66c: 0%	CD27: 0% CD44: 84.6%	Negative or dim CD10 and high expression of CD38 [32]
*PAX5*alt: *PAX5* fusion in [136]	11	0	72.7%	27.3%	CD10: 100% CD34: 81.8%	CD33: 9.1% CD13: 0%	CD66c: 63.6%	CD27: 0% CD44: 100%	CD20+ (64%), TdT+ (100%), CD34+ (82%), CD99+ (73%), and CD21+ (20%) [136]
B-other in Ohki et al. [136]	335	3.8	76.5	19.7	CD10: 95.5% CD34: 79.8%	CD33: 17.8% CD13: 4.7%	CD66c: 49.0%	CD27: 17.3% CD44: 91.9%	CD20+ (42%), TdT (16.7%), CD99 (65.7%), and CD45RA (51.3%) [136]
PAX5 p. P80R	NA	NA	NA	NA	NA	NA	NA	NA	CD2+ CD10+ CD33+ CD15- CD65- blasts [131]

Table 4. *Cont.*

B-ALL Genetic Subtypes	Total N in [136]	Distribution of Pro-B, Common, and Pre-B Cases for Each Genetic Type of B-ALL [136]			Percentages of B-ALL Cases Showing >20% Positivity for a Few Specific Antigens in [136]			Specific Features, if Any, of the Leukemic Cells by FCI Based on Referenced Publications
		CD10− cyt IgM− Pro-B	CD10+	cyt IgM+ Pre-B	CD10 % CD34 %	CD33 % CD13 %	CD66 %	CD27 % CD44 %
MYC rearrangement	NA	NA	NA	NA	NA	NA	NA	NA	Precursor B-cell immunophenotype; no specific features reported by FCI
NUTM1 rearrangement	NA	NA	NA	NA	NA	NA	NA	NA	CD10+ or CD10– blasts [151]
ETV6::RUNX1-like features	NA	NA	NA	NA	NA	NA	NA	NA	CD24+ and CD44– or low+ blasts [31]

[a] Normal hematogones (immature B-precursor cells) show discordant expression of CD123 and CD34; in contrast, lymphoblasts in B-ALL show a concordant expression pattern of CD123 [172]; [b] CD33 was positive in 16.7% of CD10+ and 50% of CD10(-) *KMT2A::MLLT3* B-ALL, as published [136]; [c] CD25+ CD10−/dim pro-B blasts reported in *ZNF384*-rearranged-like features [131]. cyt, Cytoplasmic; FCI, flow cytometric immunophenotyping, NA, not available.

4.3.12. Clinical Significance of the Newer Subtypes of ALL

ALL subtypes were stratified into risk groups based on the MRD-directed treatment of B-ALL and T-ALL. MRD evaluation was performed by peripheral blood and bone marrow biopsies at the following time points after induction: day 8 for peripheral blood, day 15 for bone marrow, and day 42 for bone marrow [157]. *ETV6::RUNX1*, high-hyperdiploid, and *DUX4*-rearranged B-ALL had the best prognosis. However, on day eight, peripheral blood MRD < 0.01% was found in 51.2% of *ETV6::RUNX1* B-ALLs, 21.1% of high-hyperdiploid B-ALLs, but not in any of the *DUX4*-rearranged B-ALLs. *TCF3::PBX1*, *PAX5*alt B-ALL, T-ALL, ETP-ALL, iAMP21 B-ALL, and hypodiploid ALL have an intermediate prognosis. *BCR::ABL1*, *BCR::ABL1*-like, *ETV6::RUNX1*-like, and *KMT2A*-rearranged ALL have the worst prognosis. Intensifying therapy based on the day-15 MRD $\geq$ 1% improved the outcomes of *DUX4*-rearranged, *BCR::ABL1*-like, and *ZNF384*-rearranged ALLs. Still, achieving day-42 MRD < 0.01% did not preclude a relapse of *PAX5*alt, *MEF2D*-rearranged, and *ETV6::RUNX1*-like B-ALLs [157].

5. Inherited Genetic Predisposition to ALL

Genetic predisposition to lymphoid neoplasms, including ALL and lymphomas, occurs in several constitutional inherited cancer predisposition syndromes and as non-syndromic germline predisposition, which may be inherited or de novo. These constitutional syndromes include Li-Fraumeni syndrome, constitutional mismatch repair deficiency syndrome, Bloom syndrome, Werner syndrome, ataxia telangiectasia syndrome, Nijmegen breakage syndrome, the RASopathies, including juvenile myelomonocytic leukemia, and Down syndrome. The interested reader is referred to a recent review of these inherited syndromes in the context of hematologic and lymphoid neoplasms, including ALL [178].

Of note, the overall low (<5%) incidence of germline-predisposing variants in ALL contrasts with the high incidences of specific (and high-risk) types of ALL in specific genetic diseases. These include *BCR::ABL1*-like ALL comprising about 60% of all Down syndrome (DS)-associated ALLs [86–88] and 50% of hypodiploid B-ALLs in Li-Fraumeni syndrome, as mentioned above. Treatment-related mortality in DS-ALL is high (50%) compared with that in non-DS-ALL, and the overall survival rate is significantly worse in DS-ALL (35.71%) than in non-DS-ALL (75.80%) [179–181].

Patients with B-ALL may harbor germline abnormalities in *PAX5*, *ETV6*, and *IKZF1* genes, all of which may also be somatically mutated in ALL [178]. In 2013, a heterozygous germline *PAX5* c.547G>A variant encoding p.Gly183Ser was identified in patients with B-ALL in two families, one Puerto Rican and one African American [182], followed by the identification of the same germline *PAX5* variant in a third family of Ashkenazi Jewish ancestry with B-ALL [183]. The leukemic samples showed a loss of chromosome 9p via the formation of an isochromosome of 9q, i(9)(q10), or the presence of dicentric chromosomes involving 9q, leading to an absent wild-type *PAX5* allele in all familial ALL cases. The germline mutation showed incomplete penetrance because it was present in all individuals with leukemia and in obligate carriers without disease, indicating that a complete loss of wild-type *PAX5* led to B-ALL [182,183]. B-ALL onset was at a young age (16 months, 21 months, and 55 months) [183].

In contrast, B-ALL manifested at older ages (11 years, 17 years, and 25 years) in another family with a germline *PAX5* heterozygous c.113G>A mutation leading to a p.R38H substitution [184]. Somatic loss of chromosome 9p was also observed in the B-ALL leukemic samples of this family [184]. A splice-site germline variant c.1013-2A>G in another B-ALL patient also showed i(9)(q10) as a secondary abnormality [185], suggesting that these somatic abnormalities of chromosome 9 could serve as a potential clue to the presence of a germline *PAX5* mutation in B-ALL.

Inherited mutations in *ETV6* and *RUNX1* comprise two inherited thrombocytopenia conditions predisposing to hematologic neoplasms, including ALL and other hematologic malignancies. Germline mutations in *ETV6* predispose to ALL and myeloid neoplasms, even including both lymphoid and myeloid neoplasms in the same family or patient; see

either cited reference for the clinical and genetic features of individuals in 27 families with inherited *ETV6* mutations [178,186]).

In 2021, B-ALL was reported in two families with familial platelet disorder (FPD) due to inherited *RUNX1* mutations [187]. Interestingly, germline *RUNXI* abnormalities were recently investigated in 6190 children with B- or T-ALL [188]. Pathogenetic germline *RUNX1* mutations were found exclusively in T-ALL, while the germline *RUNX1* variants in B-ALL were functionally minimally damaging. In that study, a history of FPD was unavailable. Germline *RUNX1* variants were present in 1.26% (n = 61) of 4836 B-ALL and 2% (n = 28) of 1354 patients with T-ALL. These germline alterations included 31 unique variants in 61 B-ALL patients and 18 unique variants in 26 T-ALL patients.

Of note, seven of those germline *RUNX1* variants were found in both B- and T-ALL [188], reminiscent of the situation with germline *ETV6* variants causing acute leukemias of lymphoid and myeloid lineages and further supporting the notion that, with germline predisposing variants, it is the secondary events that determine the leukemic cell lineage, as discussed earlier [186]. *JAK3* mutations were observed as secondary events in germline *RUNX1* variants predisposing to T-ALL with an early T precursor phenotype [188].

In another study, 28 unique germline *IKZF1* variants in coding regions were identified in 0.9% (45/4963) of children with presumed sporadic ALL [189]. Deleterious germline mutations in *IKZF1* are known to cause immunodeficiency, and ALL has been reported with an underlying *IKZF1*-associated immunodeficiency [190].

Further, in a consanguineous family of Eastern European Ashkenazi Jewish ethnicity, B-ALL harboring a germline SH2B adaptor protein 3 (*SH2B3*) gene mutation with homozygous loss of SH2B3 was reported. The phenotype showed growth retardation, mild developmental delay, chronic hepatitis, and Hashimoto autoimmune thyroiditis with B-ALL [191].

The spectrum of germline predisposition in familial and sporadic ALL is yet to be elucidated. Nevertheless, identifying a germline predisposition affects patient management and the families of the patients. Especially if a familial donor is being considered for an allograft, evaluating for germline predisposition is essential.

6. Conclusions

Immense progress has been achieved in understanding the biology of ALL relevant for risk-stratified patient management and treatment in the last decade. Precise diagnostic classification established by including molecular genetic tests in the diagnostic workup is now critical for lymphoblastic leukemias. The role of morphologic evaluation in ALL is mainly limited to visually identifying leukemic cells or blasts. Flow cytometric immunophenotyping is required to confirm the lineage of the leukemic cells as lymphoblastic. Further, in some types of B-ALL, the flow cytometric immunophenotypic profile may suggest a possible genetic subtype, which molecular genetic methods could then confirm. The genetic characterization of ALL allows the best possible treatment for patients with opportunities for clinical trials. With continued reductions in cost and improving technologies, WGS, when implemented in clinical laboratories, will likely enhance diagnostic capabilities and identify all genetic subtypes of ALL with prognostic significance. Also, the rapid shift to precisely dissect the biology of lymphoid neoplasms needs to be translated to clinical patient care in all countries, including resource-poor regions and institutions in developed countries and low- and middle-income countries, for better patient outcomes worldwide.

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