Blood Spotlight

New oncogenic subtypes in pediatric B-cell precursor acute lymphoblastic leukemia

Henrik Lilljebjörn[1] and Thoas Fioretos[1,2]

[1]Division of Clinical Genetics, Department of Laboratory Medicine, Lund University, Lund, Sweden, and [2]Department of Clinical Genetics, University and Regional Laboratories Region Skåne, Lund, Sweden

Until recently, 20% to 30% of pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) could not be classified into any of the established molecular subtypes. Recent molecular studies of such cases have, however, further clarified their mutational spectrum and identified new oncogenic subtypes consisting of cases with *DUX4* rearrangements, *ETV6-RUNX1*–like gene expression, *MEF2D* rearrangements, and *ZNF384* rearrangements. In this review, we describe these new subtypes, which account for up to 50% of previously unclassified pediatric BCP-ALL cases. (*Blood*. 2017;130(12):1395-1401)

Introduction

When cytogenetic analysis of larger cohorts of malignancies became feasible in the late 1970s and early 1980s, it was demonstrated that cells from acute lymphoblastic leukemia (ALL) contain recurrent chromosomal aberrations that constitute independent prognostic factors.[1-4] This discovery occurred during the introduction of the risk-adapted multiagent treatment regimens that have now immensely improved childhood B-cell precursor ALL (BCP-ALL) survival; soon, cytogenetic information was incorporated in pediatric ALL risk stratification.[5] Further cytogenetic and molecular genetic studies of BCP-ALL cells have revealed a set of common, nonoverlapping aberrations that are regarded as separate subtypes. The consensus around the subtypes is highlighted by their inclusion in the classifications provided by the World Health Organization (WHO).[6] The established subtypes are based on the presence of high hyperdiploidy (50-67 chromosomes); hypodiploidy (fewer than 44 chromosomes); the *BCR-ABL1*, *ETV6-RUNX*, *IL3-IGH*, and *TCF3-PBX1* fusion genes; and *MLL* (*KMT2A*) rearrangements.[6] Together, these subtypes constitute 70% to 80% of both childhood and adult cases of BCP-ALL, although the frequency of each subtype differs substantially between children and adults.[7] In addition, 2 provisional entities were added to the 2016 update of the WHO classifications: "B-ALL with intrachromosomal amplification of chromosome 21" and "B-ALL with translocations involving tyrosine kinases or cytokine receptors" (*BCR-ABL1*–like).[6] The largest of these 2 subtypes (*BCR-ABL1*-like, also known as Philadelphia-like or Ph-like ALL) occurs in ~10% of pediatric cases, and was initially identified by 2 independent groups.[8,9] Mullighan et al and Den Boer et al both identified partially overlapping *BCR-ABL1*–like gene expression signatures that defined cases with poor outcome.[8,9] When applied to the same patient cohort, both of these signatures identify the cases with gene fusions involving tyrosine kinase genes, but they identify different proportions of cases with *JAK2* mutations and *CRLF2* fusions.[10]

The genetic events defining the established subtypes are assumed to be initiating events that require additional events for leukemia to develop. This is supported by various levels of evidence for the different aberrations. For example, studies of concordant childhood ALL in monozygotic monochorionic twins have revealed that such ALLs share primary genetic changes such as high hyperdiploidy,[11] *ETV6-RUNX1*,[12,13] *BCR-ABL1*,[14] or *MLL* fusions.[15] The primary genetic changes were concordant with shared genomic breakpoints or shared hyperdiploid chromosomal profile between the twin ALLs, whereas copy number changes and mutations presumed to be secondary events were discordant.[11-14] These findings are consistent with a model in which the leukemia originates from an early clone containing the primary (subtype-defining) genetic change that has been transferred from 1 twin to the other, in utero, through the shared placenta. Subsequently, this preleukemic clone has then developed into full-blown leukemia independently in the twins by acquisition of additional genetic changes after birth.

As initiating events, the genetic aberrations defining the established subtypes also appear to deregulate the transcriptional state to a higher degree than other genetic aberrations because the subtypes are associated with distinct gene expression patterns that are maintained by primary BCP-ALLs as well as BCP-ALL cell lines after passage.[16-18] Substantial efforts are under way to establish individualized therapies that target the biological processes altered in cancers.[19] The importance of the subtype-defining alterations in the leukemic process therefore makes these alterations and their downstream pathways particularly attractive targets, as exemplified by tyrosine kinase inhibitors such as imatinib and dasatinib that target BCR-ABL1 tyrosine kinase activity; the inclusion of such inhibitors in current treatment regimens has significantly improved the event-free survival for *BCR-ABL1*–positive ALL.[20,21] Targeted treatment is also being evaluated for other fusions. For example, EPZ-5676, a drug that inhibits DOT1L (a methyltransferase required for *MLL*-mediated leukemogenesis), is evaluated for treatment of *MLL*-rearranged leukemias, and ruxolitinib, which inhibits JAK2 (a tyrosine kinase required for CRLF2 signaling), is evaluated for treatment of *BCR-ABL1*–like ALL with *CRLF2* and/or *JAK2* rearrangements (www.clinicaltrials.gov #NCT02141828 and #NCT02723994).

Recently, we and others have been able to delineate the genetic makeup of a large fraction of the remaining 20% to 30% of BCP-ALL cases not belonging to the established genetic subtypes currently used for risk stratification. Analyzing these cases with modern high-resolution techniques such as RNA-sequencing (RNA-seq), whole exome sequencing, and whole genome sequencing has revealed additional groups with distinct gene expression profiles, in most cases associated with recurrent nonoverlapping rearrangements, analogous to the established subtypes. In this review, we will summarize these recent

discoveries of new oncogenic subtypes within BCP-ALL and their underlying somatic mutations.

DUX4-rearranged ALL

We and others recently discovered that *DUX4* rearrangements identify a distinct subtype of adult and pediatric BCP-ALL,[22-25] accounting for 4% to 5% of the childhood cases (Figure 1).[23,25] However, the first hint of this subtype's existence was noted in microarray-based gene expression studies of childhood BCP-ALL in which a group of cases outside of the established subtypes displayed a distinct uniform gene expression profile.[16] Further genomic studies revealed that ~50% to 70% of these cases displayed intragenic *ERG* deletions, a genomic aberration that was practically nonexistent in other BCP-ALL cases.[26,27] This subtype was found to be associated with high CD2 expression and a favorable prognosis, even together with *IKZF1* deletions, which are otherwise associated with a poor prognosis.[28,29] Although *ERG* deletions are common in this subtype, a number of features associated with the *ERG* deletions suggested that they were not the primary genetic abnormality in these cases: (1) they were not present in all cases with the distinct gene expression profile, (2) the *ERG* deletions were sometimes found to be subclonal at diagnosis, and (3) the *ERG* deletions were either lost at relapse or did not maintain the genomic breakpoints from diagnosis.[28-30] Instead, all cases with this distinct gene expression pattern were found to harbor rearrangements involving the transcription factor *DUX4*.[22-25] At least 1 truncated copy of the *DUX4* gene, normally located within subtelomeric D4Z4 repeat regions on 4q and 10q, was found to be inserted into the *IGH* locus in a large majority of the cases. A less common variant involved an insertion of *DUX4* into an intron of *ERG*.[23]

The small size of the rearrangements, the repeat nature of the *DUX4* gene (which is present in 11-100 copies on each allele in a normal genome), and its subtelomeric localization are the most likely explanations as to why the *DUX4* rearrangements evaded detection until the introduction of RNA-seq. Both *IGH-DUX4* and *ERG-DUX4* result in the expression of a 3′ truncated *DUX4* transcript, with additional nucleotides added from noncoding regions of *IGH* or *ERG*, leading to a DUX4 protein with the C-terminal end replaced with 0 to 50 (random) amino acids from noncoding parts of the partner region (Figure 2A). The relocation of *DUX4* results in: (1) *DUX4* expression, most likely from nearby enhancers and lack of repression from the native locus; (2) C-terminal truncation of the DUX4 protein; and (3) increased *DUX4* messenger RNA stability resulting from the presence of poly-A signals in the partner region. *DUX4* is a transcription factor normally expressed in germinal tissues.[31] Its expression is partly regulated by the repeat structure of the D4Z4 domains, where a sufficient number of repeats is required to prevent ectopic expression of *DUX4*.[31] Reduction of the number of D4Z4 repeats below 11 copies leads to ectopic *DUX4* expression in muscle cells and causes the disease facioscapulohumeral muscular dystrophy from toxicity of DUX4 to muscle cells.[31]

It is currently unclear how expression of *DUX4* fusions contributes to leukemia development. Yasuda et al studied the leukemogenic potential of the fusion in vivo by retroviral expression of *IGH-DUX4* and native *DUX4* in mouse pro-B cells transplanted into immunocompromised mice. They found that expression of *IGH-DUX4* but not native *DUX4* resulted in in vivo expansion of immature pro-B cells and gave rise to pro-B cell leukemia with a relatively long median latency period of 157 days.[22] Zhang et al studied *DUX4* expression in relation to the *ERG* deletions and reported that DUX4 binds to a cryptic promoter

within the *ERG* gene and directly induces the expression of an alternative *ERG* transcript, ERGalt. They suggested that the alternative *ERG* transcript is important for leukemia transformation and hypothesize that increased transcriptional activity in this region makes it more prone to RAG-mediated deletion, resulting in the characteristic *ERG* deletions.[25]

In children, 4% to 5% of all BCP-ALL harbors DUX4 rearrangements (Figure 1), making this the sixth largest subtype of childhood BCP-ALL (slightly larger than *BCR-ABL1*).[23,25] Besides the common *ERG* deletions, *DUX4*-rearranged cases have been found to harbor aberrations common in other forms of BCP-ALL such as deletions targeting cell-cycle regulator genes *CDKN2A* and *CDKN2B*, lymphoid transcription factor genes such as *IKZF1* and *PAX5*, and activating mutations in *NRAS* and *KRAS*.[24,25] In addition, they harbor mutations in genes that are not commonly mutated in other types of ALL, such as the transcription factor genes *MYC*, *MYCBP2*, *MGA*, and *ZEB2*.[25]

ETV6-RUNX1–like ALL

In a study of gene expression–based subtypes and gene fusions in 195 BCP-ALLs, we identified 6 B-other cases with a gene expression pattern identical to *ETV6-RUNX1*–positive cases despite lacking this fusion gene, as confirmed by reverse transcriptase polymerase chain reaction (6/6 cases) and fluorescence in situ hybridization (4/6 cases), in addition to RNA-seq.[23] This finding was unexpected given the strong correlation between primary genetic changes and global gene expression pattern in BCP-ALL.[16,17] Cooccurring *ETV6* and *IKZF1* aberrations (gene fusions or deletions) were detected in all cases in which comprehensive analysis could be performed at both the DNA and RNA level (5/6 cases; Figure 2B). We could identify a similar set of 4 *ETV6-RUNX1*–negative cases with an *ETV6-RUNX1*–like gene expression pattern in an independent data set. Only RNA-seq data were available for this data set, yet gene fusions involving *ETV6* could be detected in 3 of the 4 cases. No *IKZF1* aberrations were identified, possibly because of a lack of single nucleotide polymorphism array data. An additional 5 *ETV6-RUNX1*–negative cases with an *ETV6-RUNX1*–like gene expression profile, all harboring *ETV6* aberrations, were also recently described, further confirming the presence of this subtype.[32] In addition to sharing a gene expression profile with *ETV6-RUNX1*–positive cases, these cases exhibited a CD27pos/CD44low-neg immunophenotype, otherwise associated with *ETV6-RUNX1*–positive cases.[32,33] The cases were enriched for *IKZF1* rearrangements (3/5 cases) as well as intragenic *ARPP21* deletions (3/5 cases).[32] In addition, several previously published BCP-ALL data sets contain potential *ETV6-RUNX1*–like cases that exhibit gene expression or methylation patterns identical to *ETV6-RUNX1*–positive cases,[8,34,35] although it is unclear to what extent canonical and noncanonical *ETV6-RUNX1* fusions were excluded in those cases.

The 2 studies describing *ETV6-RUNX1*–like ALL so far indicate that 1% to 3% of all childhood BCP-ALL cases belong to this subtype.[23,32] There are no data available on *ETV6-RUNX1*–like cases in adult BCP-ALL. Although very few relapses have been reported among the *ETV6-RUNX1*–like cases, studies of larger uniformly treated cohorts are required to elucidate the prognostic significance of this subtype.

ZN384-rearranged ALL

The first *ZNF384* (also known as *CIZ* and *NMP4*) fusions were described in BCP-ALL in 2002,[36] but it was not recognized until
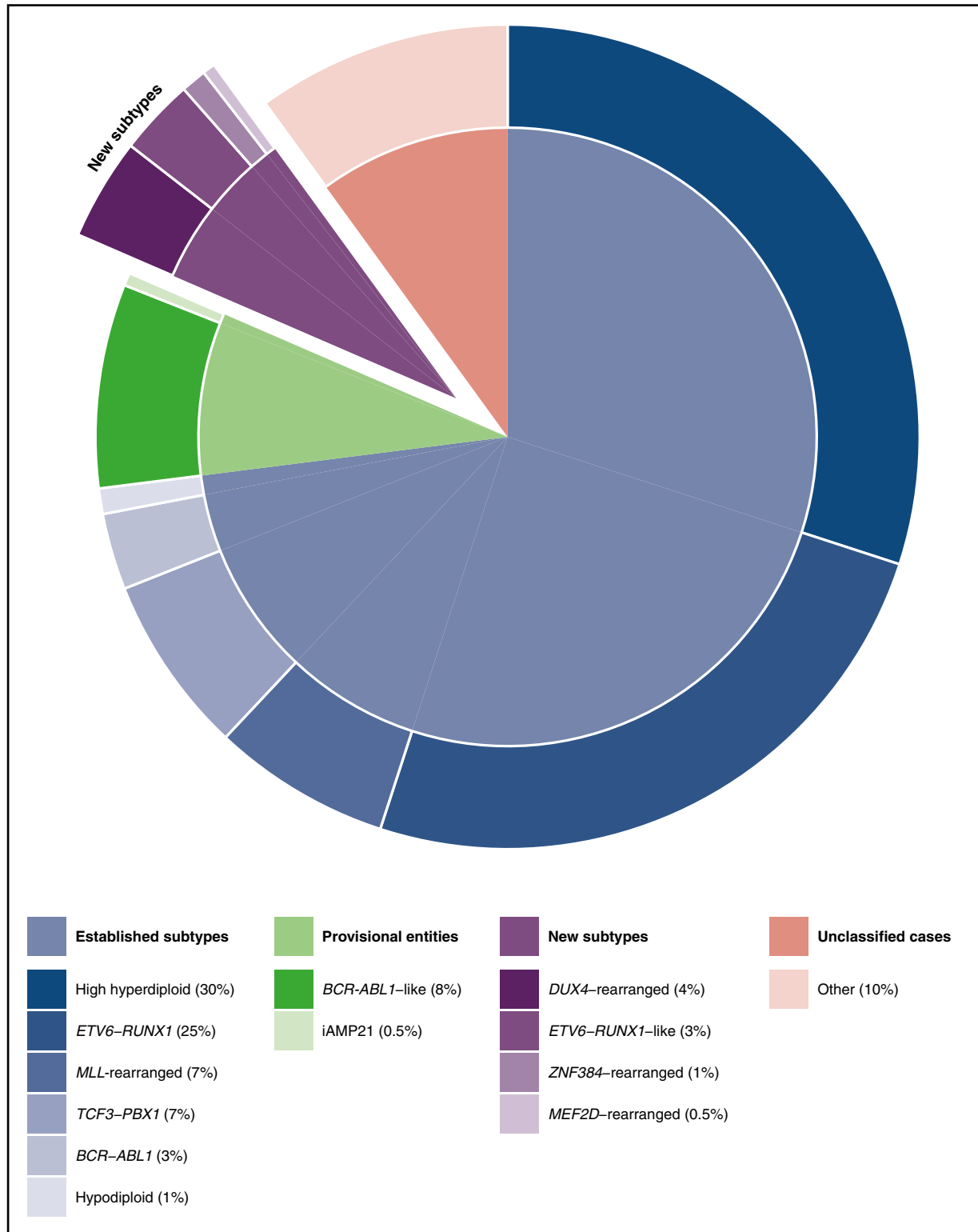
Figure 1. Estimated frequencies of BCP-ALL subtypes in children. Data were adapted from Lilljebjörn et al.[23] The outer donut chart describes the genetic subtypes of BCP-ALL. In the inner chart, established subtypes as defined by WHO[6] are indicated in blue, provisional entities in green, new subtypes in purple, and unclassified cases in red. The very rare established subtype *IL3-IGH* is not included in the chart.

recently that as much as 1% to 6% of childhood BCP-ALL cases and 5% to 15% of adult BCP-ALL cases harbor *ZNF384* fusions.[22,24,37-39] So far, 9 different 5′ fusion partners to *ZNF384* have been identified in BCP-ALL: *ARID1B, BMP2K, CREBBP, EP300, EWSR1, SMARCA2, SYNRG, TAF15,* and *TCF3* (Figure 2C-D).[24,36-42] Despite the high

number of fusion partners, cases with *ZNF384* rearrangements have a distinct gene expression pattern and a characteristic immunophenotype with low CD10 expression and expression of the myeloid markers CD13 and/or CD33.[38] Further studies are needed on the prognostic relevance of *ZNF384* rearrangements in children, although an
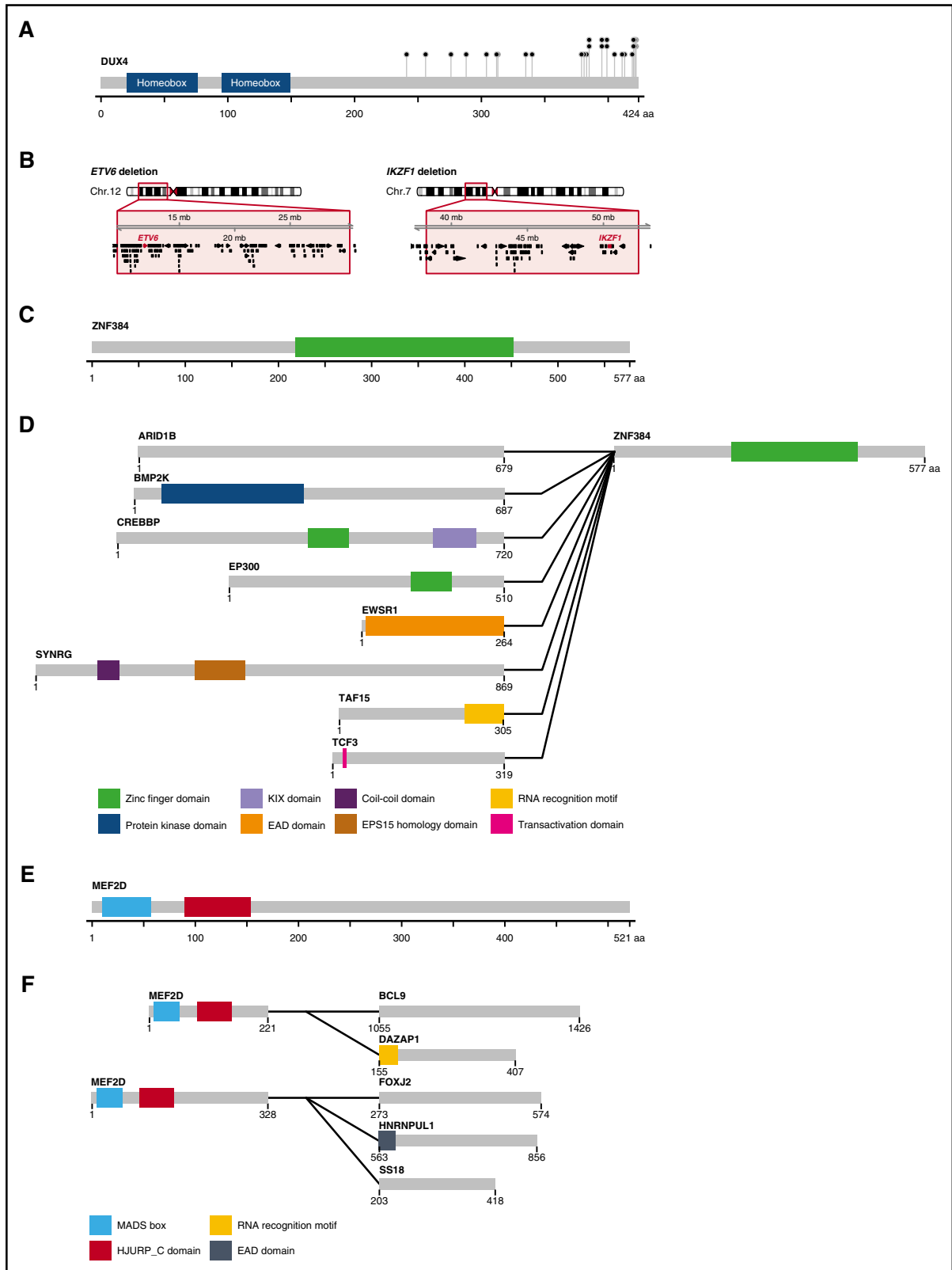
Figure 2. Genetic rearrangements associated with new BCP-ALL subtypes. (A) Schematic overview of the DUX4 protein with the position of the C-terminal truncations for 28 *DUX4*-rearranged BCP-ALL cases indicated by black circles. Data were adapted from Lilljebjörn et al.[23] (B) Two examples of aberrations associated with *ETV6-RUNX1*–like ALL are depicted: cooccurring intrachromosomal deletions of 12p and 7p resulting in deletion of *ETV6* and *IKZF1*. Besides deletions, the near ubiquitous *ETV6* and *IKZF1* alterations in this subtype can occur as small mutations or gene fusions with various partners. (C) Wild-type ZNF384 protein structure. (D) Schematic overview of reported ZNF384 fusion proteins.[36-39] Alternate breakpoints have been reported in addition to those illustrated here for several of the fusions. A *SMARCA2-ZNF384* fusion has also been reported,[42] but is not represented because no breakpoint information was provided. (E) Wild-type MEF2D protein structure. (F) Schematic overview of MEF2D fusion proteins detected in *MEF2D*-rearranged BCP-ALL cases.[23,42] MEF2D-CSF1R is not included because it reportedly does not share the *MEF2D*-rearranged gene expression profile.[42]

intermediate to favorable prognosis has been described in small cohorts.[24,38] Epigenetic regulators appear to have an important cooperative role in *ZNF384*-rearranged ALL because the genes *CREBBP*, *EP300*, *KDM6A*, *CHD4*, or *CHD8* are altered in 82% of the cases, either by sequence mutations or by being a fusion partner to *ZNF384*.[39] Mutations in RAS signaling pathway genes such as *NRAS*, *KRAS*, *PIK3CD*, and *PTPN11* are also common in this subtype.[39]

MEF2D-rearranged ALL

Fusion genes involving *MEF2D* (myocyte enhancer factor 2D) have recently been reported to be present in 1% to 4% of childhood BCP-ALL and 7% of adult BCP-ALL. *MEF2D* is the 5′ partner in all described fusions, and a total of 6 3′ partners have been described (*BCL9*, *CSF1R*, *DAZAP1*, *FOXJ2*, *HNRNPUL1*, and *SS18*; Figure 2E-F).[22-24,42-44]

MEF2D-rearranged cases have an inferior outcome.[42] Apart from *MEF2D-CSF1R*, which confers a *BCR-ABL1*–like gene expression profile, the rearranged cases also share a distinct gene expression profile that includes deregulation of MEF2D targets.[42]

Leukemic cells from *MEF2D*-rearranged cases have showed in vitro sensitivity to HDAC inhibitors, possibly from inhibition of *HDAC9*, which is a specifically overexpressed target of *MEF2D*. Hence, the addition of HDAC inhibitors could improve treatment regimens for this subtype.[42] Mutations in the RAS signaling pathway (*NRAS*, *KRAS*, *NF1*, *PTPN11*) are common in *MEF2D*-rearranged cases.[42]

Unclassified cases

With the addition of the previously mentioned novel subtypes, ~10% of pediatric BCP-ALL cases remain (Figure 1) that do not belong to either the established subtypes (*BCR-ABL1*, *ETV6-RUNX1*, high hyperdiploid, hypodiploid, *IL3-IGH*, *MLL*-rearranged, and *TCF3-PBX1*), the WHO provisional entities (*BCR-ABL1*–like and intrachromosomal amplification of chromosome 21), or the novel subtypes (*ETV6-RUNX1*–like, *DUX4*-rearranged, *MEF2D*-rearranged, and *ZNF384*-rearranged).[23,45] For adult patients, the frequency of unclassified cases is higher.[45] The molecular data on the remaining unclassified cases are somewhat conflicting. We reported that the majority of such cases (14/17) harbor in-frame fusions in a consecutive series of pediatric BCP-ALL,[23] whereas Gu et al detected a smaller proportion of in-frame fusions (65/187 cases) in BCP-ALL cases from more heterogeneous age groups.[42] Presumably, the cases lacking in-frame fusions harbor other aberrations responsible for leukemia development.

The in-frame fusions among these cases include recurrent fusions, possibly indicating very rare subtypes such as *TCF3-HLF*, associated with a very unfavorable prognosis,[46] and fusions involving *IGH* and *CEBP* transcription factor family genes.[47] Rare fusions involving *NUTM1* have been reported in several studies, but whether these cases have uniform characteristics and hence should be considered a separate subtype remains to be elucidated.[23,24,42,48]

Fusions between *PAX5* and a range of partners can be found in 40% of cases with dicentric chromosomes involving 9p such as dic(7;9), dic(9;12), and dic(9;20), whereas 50% of such cases instead show deletion of *PAX5*.[49] However, these dicentric chromosomes sometimes occur together with established primary aberrations such as *BCR-ABL1* and *ETV6-RUNX1*, and it is unclear whether ALL with dicentric chromosomes involving 9p should constitute a separate subtype.[49-51] Similarly, the fusions *IGH-CRLF2* and *P2RY8-CRLF2*, resulting in upregulation of *CRLF2*, can occur both together with primary aberrations such as *BCR-ABL1*, *ETV6-RUNX1*, and high hyperdiploidy and as a standalone fusion gene outside of established subtypes. Hence, it is possible that these 2 gene fusions in some cases constitute primary aberrations, whereas in other cases they constitute secondary aberrations.

Conclusions

The recent years have witnessed a dramatic progress in BCP-ALL classification. The new oncogenic subtypes described in this review, together with the *BCR-ABL1*–like subtype, explain around two-thirds of previously unclassified pediatric BCP-ALL cases. We expect that this increased molecular knowledge will be incorporated in clinical risk stratification, although, as of yet, standardized assays for identifying the *DUX4*-rearranged, *ETV6-RUNX1*–like, and *BCR-ABL1*–like subtypes are lacking. The latter 2 subtypes, being defined by gene expression profiles, will most likely require screening by expression-based assays such as RNA-seq or specific low-density expression arrays.[52] In the longer perspective, we expect the increased knowledge of BCP-ALL biology will permit the development and deployment of targeted therapies and thereby further reduce the mortality and side effects caused by the intense treatment regimens currently in use for this disease.

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ORCID profiles: H.L., 0000-0001-8703-1173; T.F., 0000-0002-3235-6154.

Correspondence: Henrik Lilljebjörn, Department of Clinical Genetics, Lund University, BMC C13, 221 84 Lund, Sweden; e-mail: henrik.lilljebjorn@med.lu.se; or Thoas Fioretos, Department of Clinical Genetics, Lund University, BMC C13, 221 84 Lund, Sweden; e-mail: thoas.fioretos@med.lu.se.

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