HHS Public Access

Author manuscript

Best Pract Res Clin Haematol. Author manuscript; available in PMC 2022 December 01.

Published in final edited form as:

Best Pract Res Clin Haematol. 2021 December ; 34(4): 101329. doi:10.1016/j.beha.2021.101329.

Redefining the biological basis of lineage-ambiguous leukemia through genomics: *BCL11B* deregulation in acute leukemias of ambiguous lineage

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Abstract

Acute leukemias of ambiguous lineage (ALAL), including mixed phenotype acute leukemia (MPAL) and related entities such as early T-cell precursor acute leukemia (ETP-ALL), remain diagnostic and clinical challenges dues to limited understanding of pathogenesis, reliance of immunophenotyping to classify disease, and the lack of a rational approach to guide selection of appropriate therapy. Recent studies utilizing genomic sequencing and complementary approaches have provided key insights that are changing the way in which such leukemias are classified, and potentially, treated. Several recurrent genomic alterations define leukemias that straddle immunophenotypic entities, such as *ZNF384*-rearranged childhood B-ALL and B/myeloid MPAL, and *BCL11B*-rearranged T/myeloid MPAL, ETP-ALL and AML. In contrast, some cases of MPAL represent canonical ALL/AML entities exhibiting lineage aberrancy. For many cases of ALAL, experimental approaches indicate lineage aberrancy arises from acquisition of a founding genetic alterations into a hematopoietic stem and progenitor cell. Determination of optimal therapeutic approach requires genomic characterization of uniformly treated ALAL patients in prospective studies, but several approaches, including kinase inhibitors and BH3 mimetics may be efficacious in subsets of ALAL.

Keywords

ALAL; lineage ambiguous; 14q32; *BCL11B* ; enhancer hijacking; mixed phenotype acute leukemia

Acute leukemias of ambiguous lineage (ALAL) encompass multiple poorly understood subtypes of acute leukemia that pose challenges in diagnosis and clinical management. Most ALAL cases are diagnosed as mixed phenotype acute leukemia (MPAL), with blasts exhibiting both a myeloid and T- or B-lymphoid immunophenotype or, more rarely, B/T or B/T/myeloid immunophenotypes. The remaining cases of ALAL lack lineage-defining markers and are diagnosed as acute undifferentiated leukemia (AUL) (Table 1). Together,

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MPAL and AUL account for 3–5% of newly diagnosed adult and pediatric acute leukemia cases [1–3]. Outcomes are generally poor [4,5], and due to their relative rarity, advances in our understanding of the genomic and cellular basis of ALAL have lagged behind more prevalent subtypes of acute leukemia. Moreover, diagnosis is based on few markers aimed to distinguish myeloid (e.g. MPO) and lymphoid (e.g. CD3, CD19) lineages [6]; however, recent data suggest that such immunophenotypic-based classifications often fail to capture biological similarities between different clinical entities of acute leukemia. For example, MPO is often used to distinguish T/myeloid MPAL from early T cell precursor acute lymphoblastic leukemia (ETP-ALL), despite the fact that both entities express myeloid and stem cell markers [7–9], frequently harbor similar mutations (e.g. in *WT1*, *RUNX1*, and *FLT3*) [8,10,11], and exhibit similar gene expression profiles [12]. Molecular similarities between leukemias with myeloid and T-lymphoid phenotypes have led to the proposal of entirely new diagnostic entities to capture these “interface” cases [13,14]. However, whether this is warranted requires specific investigation of the genomic and cellular basis of diverse ALAL and related (e.g. ETP-ALL, acute myeloid leukemia (AML)) cases.

Several recent studies have performed genomic analyses of ALAL/MPAL. In a study of 8 pediatric and 18 adult patients, Xiao and colleagues identified recurrent and largely mutually exclusive alterations in *PHF6*, *DNMT3A*, and *WT1* in MPAL with a T-lineage immunophenotype [15]. Notably, *NOTCH1* mutation was only identified in a single case. In contrast, a study focused exclusively on 31 cases of adult MPAL identified *NOTCH1* mutations in 29% of T/myeloid MPAL cases [16]. This study also found recurrent alterations of *DNMT3A*, as well as *IDH2, NRAS,* and *KRAS.* However, in contrast to Xiao et al, no *WT1* or *PHF6* mutations were identified. Both studies identified *BCR-ABL1* and *KMT2A* (MLL) rearrangements in B/myeloid cases. Discrepancies between these studies, for example regarding the prevalence of *NOTCH* or *WT1* mutations, could be due to small numbers of cases profiled or differences in the genetic origins of pediatric and adult ALAL.

We recently performed whole exome and genome sequencing in a cohort of 115 pediatric MPAL/ALAL cases [12] which confirmed that *WT1* mutations are among the most recurrent alteration in pediatric T/myeloid MPAL (20/49 cases, 40.8%). We also identified activating *FLT3* alterations in 42.9% of cases (including 70% of *WT1*-mutated cases) as well as mutations in *RUNX1* and *PHF6*. In B/myeloid MPAL, fusion genes involving the *ZNF384* transcription factor gene were identified in nearly 50% of cases. *ZNF384* fusions are also common in B-ALL [17,18] which had an overlapping gene expression profile with *ZNF384*-rearranged B/myeloid MPAL, supporting the notion that genomic alteration, rather than immunophenotype, more accurately classifies certain subtypes of leukemia. These fusions were not identified in adult B/myeloid cases [16] confirming that subsets of pediatric and adult MPAL have distinct genetic origins. *DNMT3A* mutations, for example, were only observed in adults and suggest that mutations associated with age-related clonal hematopoiesis might contribute to adult-onset MPAL. Importantly, all mutations described in these studies also occur in AML and/or ALL, leaving open the question of whether genomic alterations exist that specifically drive a lineage ambiguous phenotype.

Towards a transcriptional and genomic roadmap of acute leukemia

Although these studies identified several recurrent genomic alterations associated with ALAL, an important limitation was the selection of cases using current immunophenotypic criteria for ALAL/MPAL, precluding the identification of leukemia subtypes that might transcend diagnostic boundaries, and often, limited genomic characterization and a focus on protein coding alterations and fusion genes. Motivated by these challenges, we sought to perform an unbiased analysis of all acute leukemia subtypes in order to delineate biological heterogeneity, irrespective of diagnostic classification or immunophenotype, with an emphasis on comprehensively characterizing the genomic and molecular features that define lineage-ambiguous leukemia in the context of other leukemia subtypes. Here we summarize our pan-leukemia study that identified multiple unrecognized subtypes of acute leukemia, with a key finding being identification of distinct subgroup of lineage ambiguous leukemia driven by structural variants deregulating *BCL11B* that spans current diagnostic categories [19]. We discuss the implications of these findings on the cellular origins of lineage ambiguous leukemias, clinical diagnosis, and treatment.

To perform this study, we compiled transcriptome sequencing (RNA-seq) data from 2,573 cases spanning the major leukemia subtypes: major subgroups of B-ALL [20] and T-ALL [11], ETP-ALL, AML, MPAL, and AUL samples. We identified biologically-defined subgroups within each leukemia subtype through gene expression clustering analyses, using both unsupervised hierarchical clustering and t-distributed stochastic neighbor embedding (tSNE) approaches. As reported previously [20], B-ALL cases segregated into over 20 subgroups, each with defining genomic alterations (Figure 1A). Of note, the majority of immunophenotypically-defined B/myeloid MPAL cases clustered with B-ALL subgroups with matched genomic alterations, indicating that B/myeloid MPAL as a single distinct diagnostic entity may be misguided. The majority of T-ALL and AML cases also clustered according to genomic alteration, albeit less distinctly as compared to B-ALL cases, with many subtypes reported previously [11,21]. However, this analysis identified two novel gene expression clusters, highlighting the power of large, diverse cohorts to resolve biological heterogeneity (Figure 1B). The first cluster included 1.7% of T-ALL cases and was defined by *LMO2* rearrangement to non-T cell receptor loci, most frequently *STAG2*. The second cluster had an unknown genomic driver and encompassed 30–40% of T/myeloid MPAL and ETP-ALL cases, in addition to 3.8% of AML and 50% of AUL cases. Identification of this new subgroup, which spanned at least 4 different diagnostic entities, has broader implications for diagnosis and classification of leukemias with stem, myeloid and T-lymphoblastic features.

BCL11B deregulation defines a new subtype of ALAL

Initially, the only notable recurrent alterations identified in this subgroup were *WT1* mutations (16/61, 26.2%) and activating *FLT3* alterations (50/61, 82%) as previously reported in T/myeloid MPAL [12] and ETP-ALL [10,11]. As they were not exclusive to this subgroup, we suspected the presence of non-coding alterations that had eluded standard transcriptome and exome-based analysis. Using cis-X [22], a tool that uses matched transcriptome and whole-genome sequencing (WGS) data to identify genes with

allele-specific expression (ASE), we found that all 53 cases in this subgroup with matched RNA-seq and WGS data exhibited ASE of the T cell transcription factor gene *BCL11B*, and harbored non-coding structural variants (SVs) with breakpoints near the *BCL11B* gene on chromosome 14q32.2. These SVs included translocations to chromosomes 2,3,6,7,8,12 and 21, as well as cases with a focal amplification in *cis* with *BCL11B* (Figure 2A). Collectively, these genomic data nominated *BCL11B* as the likely driver of this gene expression subgroup.

BCL11B as regulator of T lineage differentiation and oncogene

BCL11B plays important roles in both normal and malignant hematopoiesis. *BCL11B* encodes a C_2H_2 zinc finger transcription factor first expressed in CD34+CD7-CD1a- thymic progenitor cells (corresponding to the double negative 2, or DN2, stage of murine thymocyte development) [23,24]. *BCL11B* expression continues to increase during subsequent maturation stages [23]. Acting as both a transcriptional activator and repressor, BCL11B controls the expression of hundreds of genes in a developmental context-specific manner [25], including upregulation of T-lineage genes (e.g. *Rag1/2*, *Tcf7*, *Gata3*, *Cd8a/b*, *Lck*, *Cd3d*) and downregulation of stem and progenitor cell genes (e.g. *CD34, Kit, Spi1*) during T cell commitment [26,27], and genes related to positive selection, apoptosis, and alternate lineage fates in mature T cell populations [28,29]. Progenitor T cells lacking *BCL11B* are not able to progress past the DN2 stage and fail to recombine T cell receptor genes; moreover, these cells retain myeloid and natural kill cell differentiation potential which is otherwise blocked in *BCL11B*-expressing T cells [29,30]. *BCL11B* is therefore a critical “gate-keeper” of the T cell lineage.

BCL11B was first shown to have a tumor suppressor role in 2003 when nearly 50% of gamma ray-induced T cell lymphomas in mice were found to harbor *Bcl11b* mutations [31]. Shortly thereafter, *BCL11B* mutations were identified in human T-ALL and are now known to occur with a frequency ranging from ~9–15% [11,32–34]. *BCL11B* mutations typically result in amino acid substitutions within the zinc finger domains which are predicted to interfere with, or abrogate, DNA binding and thus disrupt BCL11B function. Loss of *BCL11B* through deletion is also reported [33]. Notably, most *BCL11B*-mutated T-ALLs retain a wild-type *BCL11B* allele, indicating that complete loss is either not tolerated or is otherwise not tumorigenic; rather, functional haploinsufficiency—either through deletion or mutation of one allele—confers *BCL11B* with tumor suppressor properties. Interestingly, and consistent with this notion, low *BCL11B* expression was associated with induction failure [33] and poor overall survival [34]. Despite clear evidence as a tumor suppressor, *BCL11B* knock-out in several T-ALL cell lines resulted in apoptosis and cell death [35], suggesting that *BCL11B* may also possess oncogenic functions in some contexts.

The nature of *BCL11B* alterations in ALAL

Unlike the *BCL11B* alterations described for T-ALL (primarily amino acid substitutions and deletions), the 14q32 structural variants identified in lineage ambiguous leukemias leave the *BCL11B* coding region intact, suggesting that these alterations may influence *BCL11B* expression. The observation of allele-specific, generally high expression of

BCL11B suggested the SVs deregulated expression of one *BCL11B* allele, potentially in a hematopoietic stem and progenitor cell (HSPC) where *BCL11B* is normally repressed, resulting in lineage aberrancy. Enhancer hijacking is one such mechanism whereby SVs (e.g. translocations, deletions, or inversions) result in the "re-wiring" of enhancer-promoter interactions [36,37]. Because enhancers typically only regulate genes within one megabase, these rearrangements frequently lead to the aberrant activation or upregulation of newly acquired target genes. Using chromatin-based assays in patient samples, we confirmed that the *BCL11B* SVs did indeed result in enhancer hijacking, and we could use these data to glean further insights into the cellular origins of *BCL11B*-deregulated leukemia, as discussed below (Figure 2B).

Active enhancers are readily identified by high levels of the post-translational histone modification acetylation of histone H3 at lysine 27 (H3K27ac) [38–40]. Using publicly available H3K27ac chromatin immunoprecipitation followed by sequencing (ChIP-seq) data from human CD34+ HSPCs [41], we found that the SV breakpoints of all *BCL11B*-group patient samples occurred within several hundred kilobases (kb) of highly active CD34+ HSPC enhancers—also known as super-enhancers [42,43]. To interrogate the resulting enhancer-promoter re-wiring, we performed H3K27ac Hi-ChIP [44] in 5 patient samples to simultaneously survey H3K27ac and long-range chromatin interactions which identified chromatin interactions between the *BCL11B* gene and rearranged super-enhancers in all cases, even when the genomic distance was >300 kb. Additionally, all 7 CD34+ HSPC super-enhancers involved in *BCL11B* SVs showed activity in each patient sample, supporting the epigenetic similarities between *BCL11B*-deregulated ALAL and HSPCs.

Enhancer hijacking in acute leukemia

Enhancer hijacking as a mechanism of oncogenic gene expression has been described previously in acute leukemia and lymphoma, including aberrant activation or upregulation of *MN1* [45], *EVI1*/*MECOM* [46–48], and *FLT3* [49] in AML, and *TAL1*/*2*, *TLX3*/*5*, *LYL1*, and *LMO1*/*2* in T-ALL [50]. Additionally, most translocations in leukemia involving the immunoglobulin genes (i.e. T cell receptor (TCR) and immunoglobulin heavy/light chain (*IGH*/*IGK*/*IGL*) genes) result in overexpression of oncogenes through the activity of tissue-specific enhancers in these loci. For example, Burkitt lymphoma often results from t(8;14) translocations which juxtapose enhancer elements within the 3' *IGH* locus near the *MYC* gene to drive *MYC* overexpression [51,52], and subsets of Philadelphia chromosome-like ("Ph-like") B-ALL harbor *IGH* rearrangements to *CRLF2*, resulting in *CRLF2* overexpression and JAK/STAT pathway activation [53,54]. As most previously described enhancer hijacking events are driven by one or two recurrently targeted enhancers, the diverse array of at least 7 distinct enhancer hijacking events described for *BCL11B* was striking, and might reflect a particularly susceptible window in early hematopoietic development for the acquisition of translocations involving these highly transcriptionally active loci.

BETA: a novel mechanism of oncogenic enhancer formation in cancer

The translocations described above occur in ~80% of the *BCL11B*-deregulated group. The remaining 20% of cases lacked an identifiable translocation involving 14q32; instead, these cases harbored a high-copy amplification event of a relatively short (~2.5 kb) non-coding region located 730 kb downstream of *BCL11B*. We were not able to identify any cell type in which this region corresponded to a highly active enhancer, although weak H3K27ac of this amplified region was present in normal HSPCs and T cell progenitors. Using H3K27ac HiChIP, we discovered that this region contained extremely high levels of H3K27ac in patient samples with the amplification, which showed evidence of looping to the *BCL11B* gene. Using long-read PacBio sequencing, we confirmed that the 2.5 kb element was amplified in tandem, ruling out the possibility that multiple copies were scattered throughout the genome. We term this amplification "*BCL11B* Enhancer Tandem Amplification", or BETA. Other reported cases of enhancer amplifications in acute leukemia have been described and primarily occur within the ~2 megabase *MYC* locus, resulting in *MYC* overexpression. These include duplication of the NOTCH1-driven enhancer (NMe) in T-ALL [55] and amplifications of a more distal enhancer cluster, originally called "E1–E5" [56] and later as the B cell Enhancer Cluster, or BENC [57], in AML [58,59]. To our knowledge, BETA represents the first description of oncogenic enhancer formation by amplification of a genomic region not previously demarcated with high levels of H3K27ac.

14q32 rearrangements in mixed-lineage leukemia

Although we formally identified the *BCL11B*-deregulated leukemia subgroup through unbiased genomic approaches, several prior reports described chromosomal rearrangements consistent with *BCL11B*-deregulating SVs: three case reports published between 1990 and 2003 described a total of 13 pediatric T/myeloid mixed-lineage leukemia cases with 14q32 translocations identified from karyotyping, most notably t(6;14)(q25;q32) [60–62]. *BCL11B* was subsequently identified as the likely target of these rearrangements through fluorescent in situ hybridization (FISH) analysis of bacterial artificial chromosome (BAC) probes [63], followed by description of 5 additional cases, all with t(6;14) and a T/myeloid leukemia phenotype, including the first reports in adult patients [64–66]. Abbas and colleagues were the first to suggest that *BCL11B* might be the target of aberrant gene regulatory activity following 14q32 rearrangements, and the first to report the striking co-incidence with *FLT3* mutations [67]. The first translocation event *not* involving chromosome 6 was reported in 2018, with identification of two patients harboring rearrangement to 8q24 (i.e. distal to *MYC*) [68]. More recently, elevated BCL11B protein expression was identified in around 50% of T/myeloid MPAL cases examined in one study, although 14q32 rearrangements were only identified in a small subset [69]. The authors hinted at the possibility of other cryptic SVs that might upregulate *BCL11B* but which eluded karyotype and FISH analyses. Indeed, coincident with our study, Di Giacomo and colleagues reported on a further two 14q32 rearrangement events involving 2q22.2 (i.e. *ZEB2*), and 7q21.2 (i.e. *CDK6*), in addition to previously reported 8q24.2 and 6q25 [70]. The authors also identified that 14q32 rearrangements defined a unique gene expression group comprised of 20 T/myeloid MPAL, ETP-ALL and AML samples. Our study identified a further 3 previously undescribed translocation events, including 3p24.3 (near *SATB1*), 12p13.2 (within *ETV6*), and 21q22.12

(within *RUNX1*). Moreover, we identified the novel amplification event, BETA, which we now know accounts for 20% of *BCL11B*-deregulated cases. Because of the small size (35–50 kb after amplification), this event would likely escape detection by standard FISH analyses. Importantly, through mapping 3D chromatin contacts in patient samples we were able to uncover the mechanism by which 14q32 rearrangements deregulate *BCL11B*. Knowing that aberrant enhancer activity drives oncogenic *BCL11B* expression paves the way to investigate therapeutics that directly target this mechanism, for example by globally repressing enhancer function through bromodomain inhibitors [42], or by targeting the factors that mediate hijacked enhancer activity, which remain undefined.

Is a new entity of acute leukemia warranted?

Molecular similarities between acute leukemias with myeloid and T-lymphoid features have led to the proposal of new clinical entities termed “acute myeloid/T lymphoblastic leukemia” (AMTL) [13] or “interface acute leukemia” (IAL) [14] which would encompass all ETP-ALL and T/myeloid MPAL samples, as well as the subset of AML cases with T cell phenotypes (e.g. T cell receptor rearrangements), and the subset of T-ALL with myeloid phenotypes, irrespective of genomic driver. We argue that this broad classification is inappropriate, as it continues to give primacy to immunophenotype, rather than (genomic) leukemogenic drivers to guide classification, without providing useful insight into disease biology that may meaningfully impact diagnosis or therapy. As shown in multiple recent genomic studies of AML and ALL, designation of new subtypes should be determined by improved knowledge of the genomic drivers of disease, which are commonly distilled into distinct leukemic gene expression profiles. As we have shown, *BCL11B*-deregulating SVs effectively segregate 30–40% of T/myeloid MPAL and ETP-ALL into one biological group. Thus, classifying all ETP-ALL and T/myeloid MPAL into a single entity would mis-represent the impact of *BCL11B* SVs on driving distinct leukemia biology, particularly as other ETP-ALL and T/myeloid MPAL cases are driven by very different oncogenic events. Our analysis of outcome data suggested that ETP-ALL patients with *BCL11B*-deregulating SVs have better outcomes compared to those lacking these alterations, although these results require confirmation as the number of uniformly treated cases with outcome data was small. Future work will determine whether existing or novel therapeutic interventions will be effective against *BCL11B*-deregulated leukemia; however, based on the collective observations that *BCL11B* SVs are a recurrent, subgroup-defining genomic alteration that have not been identified in any cancer sample outside of this subgroup, we support the definition of 14q32 SVs targeting *BCL11B* as a new entity of acute leukemia. This recommendation is further supported by confirmation of the gene expression profile, characteristic immunophenotype and recurrent *BCL11B* alterations in an independent cohort of T, T/myeloid MPAL and ETP-ALL cases treated on Eastern Cooperative Oncology Group protocols.

Implications for the cell of origin of acute leukemias of ambiguous lineage

Multiple hypotheses have been advanced for the basis of lineage ambiguity/plasticity of lineage ambiguous leukemias. These include: 1) that oncogenic transformation occurs in a cell that retains myeloid and lymphoid differentiation potential (i.e. an HSPC) [71]; or 2)

the cellular transformation, whether in a stem or committed progenitor, leads to oncogenic deregulation of gene expression programs that manifest, in part, as aberrant multi-lineage marker expression [72]; or 3) that transformation occurs in a committed T-lineage cell followed by de-/cross differentiation to acquire stem and myeloid markers [13]; or 4) that leukemia samples with multiple immunophenotypic populations are comprised of at least two genetically distinct and independently derived leukemias in a single sample; that is, that immunophenotypic variegation is driven by mutational evolution.

Several observations suggest the first hypothesis is most likely in many cases. The most direct observation is that immunophenotype may shift during disease progression, e.g. from ETP-ALL to AML or T-ALL, or B/myeloid ALL to B-ALL with retention of the truncal oncogenic drivers, as exemplified by cases with *ZNF384* rearrangements [17,73]. This suggests that lineage plasticity is inherent to the leukemia, but does not exclude mutational evolution as a driver of lineage shift/switch. However, experimentally, we and others have shown that the same somatically-acquired mutations were present in immunophenotypically-defined subpopulations of MPAL samples harboring multiple subpopulations, including hematopoietic stem cells (HSCs) [12,74]; moreover, these subpopulations individually recapitulated the immunophenotypic diversity of the bulk tumor sample upon xenografting [12]. These data are consistent with a model where transformation occurs in an HSC or early progenitor that retains multi-lineage differentiation capacity. In our current work, epigenetic and gene expression analyses provide further support to an HSPC as the cell of origin (Figure 3). Namely, all 7 CD34+ HSPC super-enhancers implicated in 14q32 translocations showed enhancer activity (H3K27ac ChIP-seq signal) in patient tumor samples, including the *ARID1B* super-enhancer that was only active in HSPCs and not T cell progenitors. Additionally, the T cell enhancer elements ThymoD (which is required for normal *BCL11B* expression [75,76]) and NMe are only activated in recently immigrated thymic progenitor cells, yet these elements were inactive in all patient tumor samples. Assuming the activity status of these enhancers faithfully reflects the cell of origin, these data would support that transformation occurred in a cell that had yet to enter the thymus. Single cell analyses supported these inferences, with patient tumors showing the strongest similarity in both gene expression and open chromatin profile with activated HSPCs, not T lineage cells. The universal lack of T cell receptor rearrangements, which were present in 56.7% and 37.2% of non-*BCL11B* group ETP-ALL and T/myeloid MPAL samples, respectively, further ruled out the possibility that a T lineage cell underwent de-differentiation. These data support a model where 14q32 SVs occurring in an HSPC result in the aberrant activation of *BCL11B* to drive a lineage-ambiguous stem cell leukemia (Figure 3).

Implications for therapy

Does expression of both T and myeloid features in these leukemias reflect the differentiation capacity of HSPCs, or is this expression aberrant and rather reflects oncogenic transcription factor activity? Would distinguishing between these possibilities have implications for therapy, which relies primarily on the decision between AML- or ALL-directed regimens [5,77]? Using viral overexpression, we demonstrated that BCL11B is sufficient to upregulate T cell differentiation gene expression programs in CD34+ HSPCs which was accompanied

by cytoplasmic CD3 detection in vitro, raising the possibility that the T lineage phenotype could be driven exclusively by BCL11B activity in an otherwise stem/myeloid leukemia.

If *BCL11B*-group leukemias are immature myeloid leukemias with aberrant T cell marker expression, might one expect that AML-directed therapy would be more effective compared to ALL-directed therapy? Multiple retrospective analyses support that ALL-directed regimens result in superior outcomes compared to AML regimens in MPAL patients[4,77,78]. Thus, even if the T lineage phenotypes characteristic of *BCL11B* group leukemias are truly aberrant (i.e. do not reflect the cell of origin), these phenotypes are not necessarily clinically irrelevant and might confer the requisite lymphoid characteristics that make ALL-directed therapy more effective in treating T/myeloid MPAL.

Despite the improved outcomes of patients treated with ALL- versus AML-directed therapy, overall survival of MPAL patients in general is poor and targeted therapies are greatly needed. Activating mutations in the FMS-like tyrosine kinase 3 gene, *FLT3*, were identified in 80.1% of *BCL11B* group samples. This frequency greatly exceeds that reported for AML, where *FLT3* mutations are the most common alteration (15–30%) [79–83], as well as T/myeloid MPAL (11–37%)[12,16,84] and ETP-ALL (26–35%) [10,11,85]. This striking co-occurrence is notable, as it suggests these leukemias are dependent on FLT3 signaling and raises the possibility that FLT3 inhibitors might be therapeutically relevant in *BCL11B*-deregulated leukemia. The FLT3-specific tyrosine kinase inhibitor gilteritinib achieved Food and Drug Administration (FDA) approval for relapsed/refractory AML in 2018, and other non-selective FLT3/tyrosine kinase inhibitors have shown efficacy in multiple AML clinical trials [86–89]. No clinical trial has yet evaluated the efficacy of FLT3 inhibitors in T/myeloid MPAL or ETP-ALL, and case reports are scarce, although one report details the successful treatment of two adult T/myeloid MPAL patients with non-selective FLT3 inhibitors (midostaurin and sorafenib) [90].

Another promising therapeutic option includes the small molecule BH3 mimetics that block antiapoptotic activity in multiple leukemia cell types [91–93] (reviewed in ref. [94]). The finding that ETP-ALL is selectively dependent on the BCL-2 family of antiapoptotic proteins nominated the BCL-2-specific BH3 mimetic venetoclax for clinical use [95]. The first successful treatment of two patients with refractory ETP-ALL treated with venetoclax were reported in 2018 [96] and a recent study reported favorable outcomes in a phase I dose escalation study of venetoclax in combination with low-dose navitoclax, which targets both BCL-2 and BCL-XL, in relapsed/refractory T-ALL [97]. Notably, this study included 5 patients with ETP-ALL that had failed prior therapy, highlighting the potential of BCL-2 inhibitors in leukemias with an ETP immunophenotype. Similar to FLT3 inhibitors, venetoclax has yet to be tested in patients diagnosed with T/myeloid MPAL. Given the unknown genetic basis of most of these cases, genotype-response correlation of BH3 mimetics and FLT3 inhibitors is needed.

Conclusions

Recent transcriptomic and mutational profiling studies emphasize the limitations of using immunophenotype alone to classify individual leukemias with multi-lineage markers. The

increasingly widespread use of clinical RNA and genome sequencing suggests that the diagnostic approach to classifying leukemia should be revisited, and specifically, these new entities of lineage ambiguous/overlap leukemias be incorporated. Indeed, recognition of the clinical importance of identification of such entities provides increasing support for adoption of such genomic diagnostic approaches. While identification of this entity required analysis of a very large data set [19,98], one can now readily identify the entity on a case-by-case basis. Moreover, the *BCL11B* entity may be suspected from the characteristic immunophenotype (Table 1).

Moving forward, it will be important to define clinical cases with 14q32 SVs to enable future evaluation of therapeutic approaches and outcomes. However, even with therapeutic strategies in practice today, outcomes of patients with ALAL remain poor, especially for adults, underscoring the need to develop faithful preclinical models that recapitulate the mixed-lineage phenotypes associated with these diseases. In conclusion, by focusing on genomic and transcriptional alterations, we have identified a clinically relevant demarcation in lineage-ambiguous leukemias that provides clarity on cellular origins and possible novel therapeutic approaches.

Funding

The authors are supported by the St. Jude Children's Research Hospital Chromatin Collaborative, the Henry Schueler 41&9 Foundation (to C.G.M.), a St. Baldrick's Foundation Robert H. Arceci Innovation award (to C.G.M.) and NCI NRSA F32 CA254140 (to L.E.M)

Declaration of competing interests

Lindsey Montefiori declares no conflict of interest. Charles Mullighan has received research funding from Abbvie, Pfizer and Loxo Oncology, and consulting fees from Illumina.

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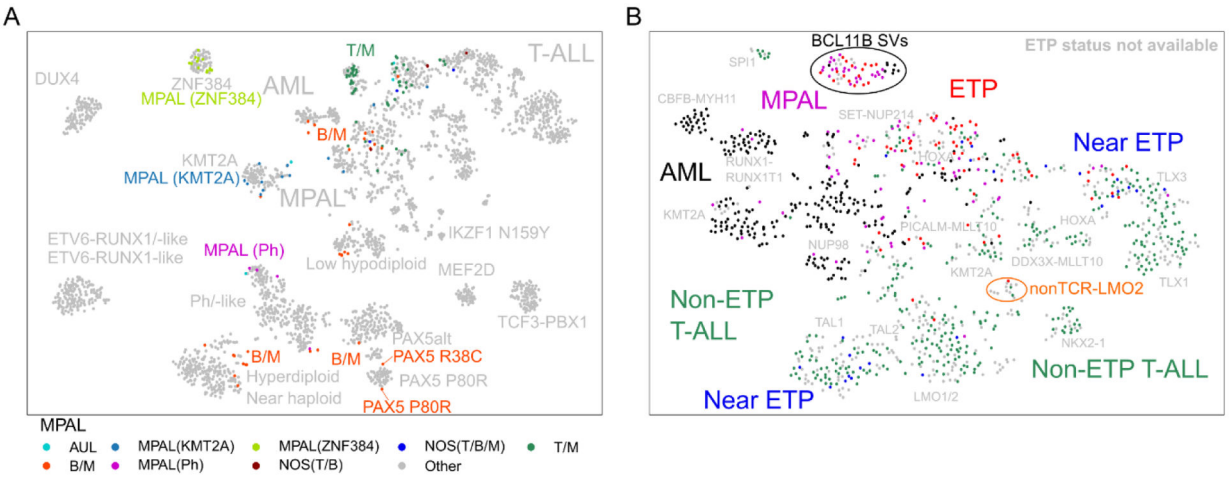

Figure 1. Gene expression profiling of acute leukemia reclassifies lineage ambiguous cases.

(A) tSNE analysis of 2,573 acute leukemia samples. All cases diagnosed as MPAL are shown according to MPAL subtype and/or known genomic alteration. The majority of B/myeloid MPAL cases cluster with B-ALL subtypes with shared genomic alteration. (B) tSNE analysis of all non-B-ALL cases. Two previously undescribed subgroups were identified, including cases with *BCL11B* SVs (circled in black) and cases with non-TCR *LMO2* rearrangements (circled in orange). Figure reproduced from ref. [19].

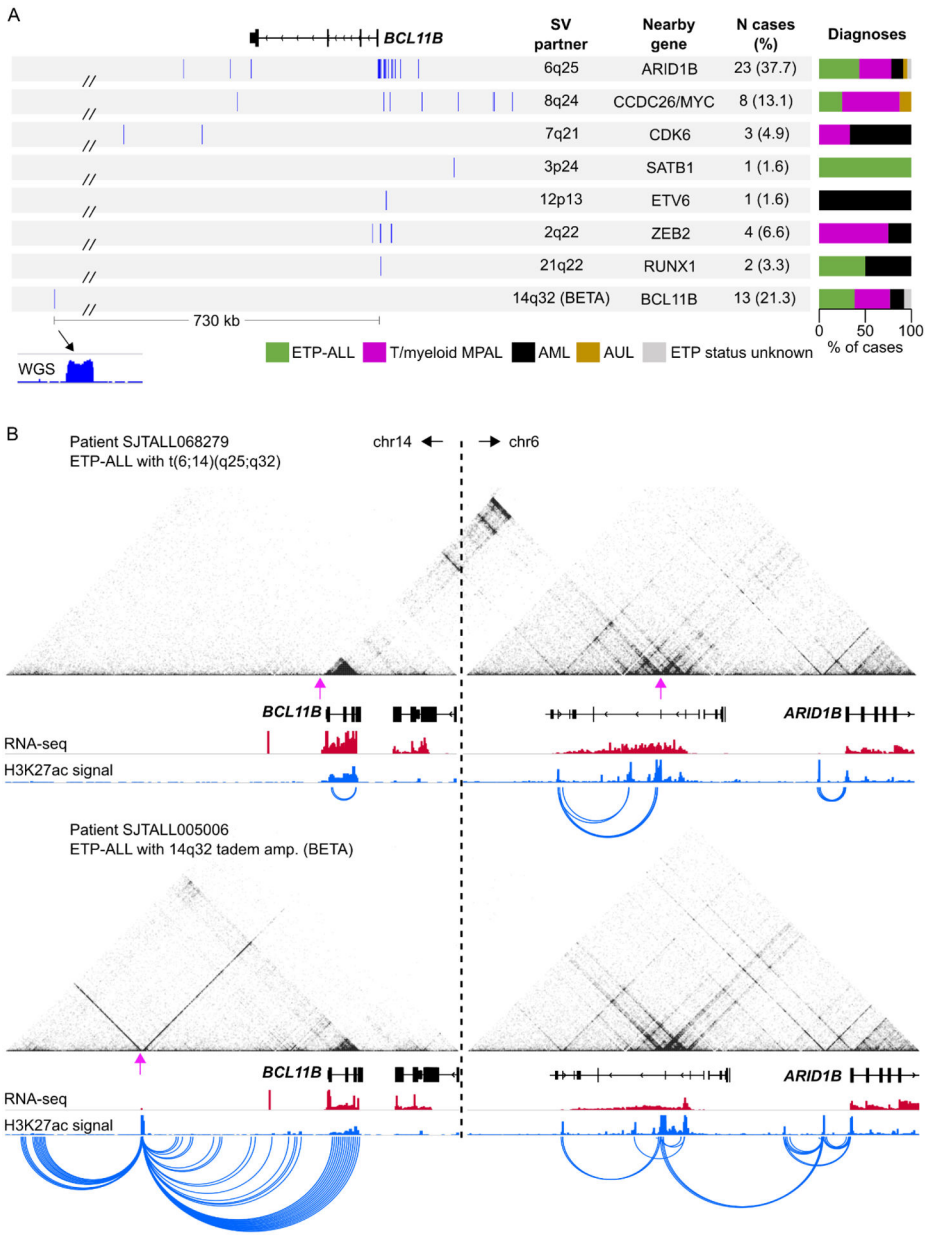

Figure 2. *BCL11B* SVs result in deregulated BCL11B expression through various enhancer-mediated mechanisms.

(A) Summary of patient-specific breakpoints organized by SV partner locus. Only breakpoints within the viewing region are shown and only for cases with an identifiable SV (6 cases excluded that lacked WGS). (B) H3K27ac HiChIP data for two patient samples showing enhancer hijacking by the *ARID1B* enhancer at the 6q25 locus (top) and de novo enhancer formation (BETA) resulting in *BCL11B* activation (bottom). The same genomic windows are shown for each sample to demonstrate that chromatin interactions between chromosome 14 and chromosome 6 are only observed in the case with the t(6;14) translocation (top). Magenta arrows show the breakpoint positions.

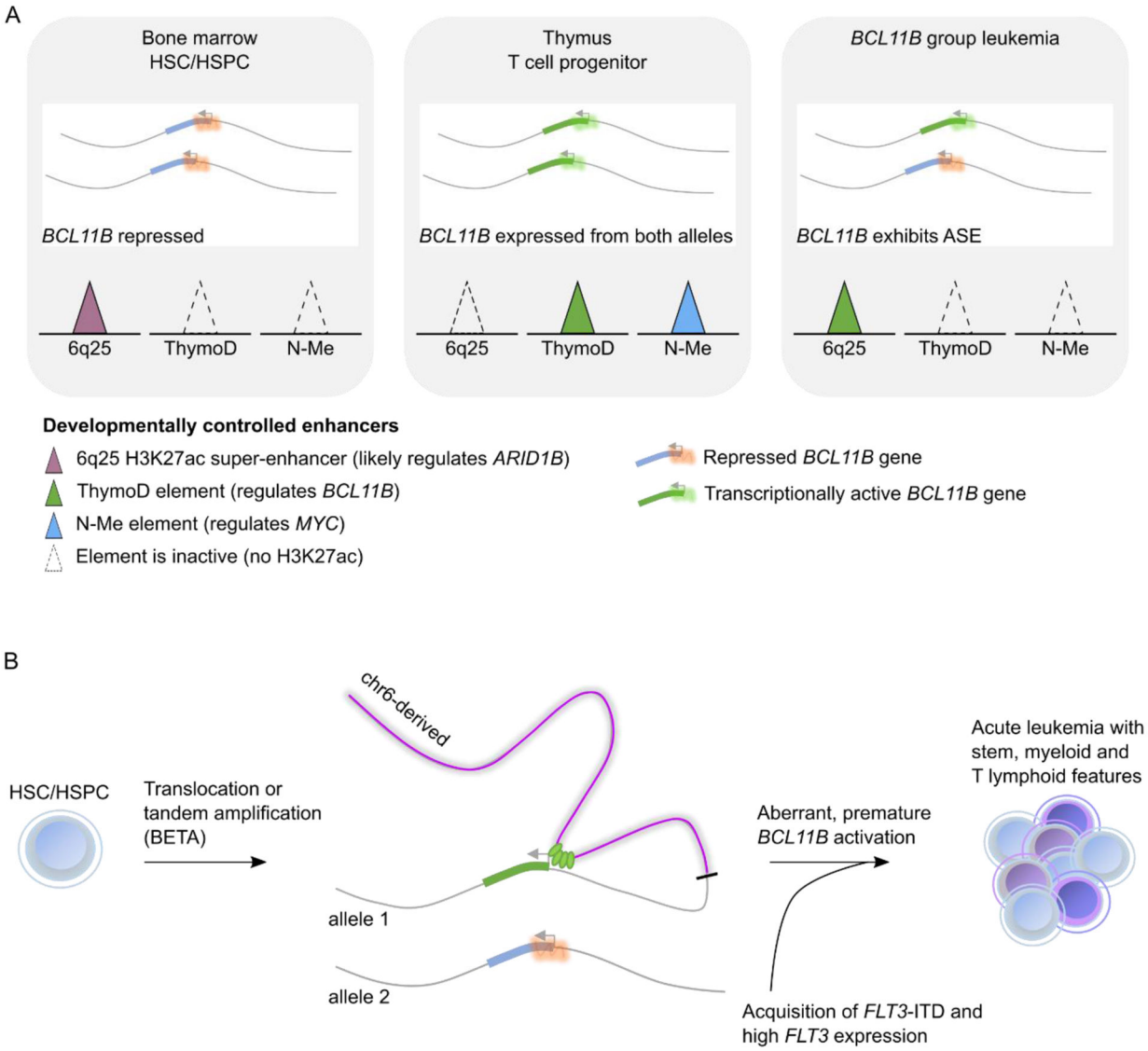

Figure 3. Model of *BCL11B*-mediated lineage ambiguous leukemia guided by epigenetic analysis.

(A) representation of the two *BCL11B* alleles in normal and leukemic cells. In bone marrow HSCs/HSPCs (left panel), both alleles are actively repressed and the two T-lineage enhancers, ThymoD (which regulates *BCL11B*) and N-Me (which regulates *MYC*) are not active (dotted triangles), whereas the *ARID1B* enhancer with an unknown function is active (purple filled triangle). In normal T cell progenitors that have immigrated to the thymus (middle panel), *BCL11B* is expressed from both alleles, and the T lineage enhancers are active whereas the *ARID1B* enhancer no longer shows H3K27ac signal. In *BCL11B*-deregulated leukemia (right panel), *BCL11B* is only expressed from one allele, and the enhancer landscape reflects that of normal HSCs/HSPCs, suggesting *BCL11B* expression reflects an abnormal gene regulatory state. (B) Working model that depicts the mechanism of aberrant *BCL11B* expression. SVs result in enhancer hijacking (e.g. of the *ARID1B* enhancer depicted) or de novo enhancer formation (BETA). Together with constitutive FLT3 activation, either through mutation or upregulation, these genomic events drive development

of a lineage ambiguous acute leukemia with stem, myeloid and T lineage features that result from a combination of the cell of origin (HSPC) and driving genomic lesion (*BCL11B*).

Table 1.

Flow cytometry-based diagnostic criteria for major acute leukemia subtypes.

Cell surface and intracellular markers used in the diagnosis of acute leukemia are shown, based on the WHO classification system[2]. The immunophenotype of cases with *BCL11B* SVs is shown at the bottom ("BCL11B deregulated AL"). Note that MPO, which is used to distinguish ETP-ALL from T/myeloid MPAL, is variable in this new subgroup.

Diagnostic entity	Immunophenotypic criteria
AUL	Lack of CD3, CD19, MPO, positive for CD117/CD34, positive for 1–2 of CD13/CD33, frequently CD7 and/or TdT positive
AML	MPO positive and/or positive for multiple markers of myeloid/monocytic differentiation (CD13/CD33/CD11b/CD11c/CD14/CD15/CD64/CD65), negative for cCD3, CD19, often CD117 and/or CD34 positive, variably CD7 positive
T/myeloid MPAL	Myeloid lineage: MPO or at least 2 markers associated with monocytic differentiation (CD11c, CD14, CD64).
	T lineage: strong cytoplasmic or surface CD3
ETP-ALL	cytoplasmic CD3 positive, but CD1a and CD8 negative, CD5 on <75% of blasts, variable for CD2, usually CD117 and/or CD34+, CD13/CD33/CD11b positive, negative for MPO
Typical T-ALL	Surface CD3 positive, CD8 positive, CD5 >75% of blasts, variable for CD34, infrequent for CD13/33/11b, negative for MPO
B/myeloid MPAL	Myeloid lineage: MPO or at least 2 markers associated with monocytic differentiation.
	B lineage: strong CD19 AND at least one of CD79a, cCD22, CD10
Typical precursor B-ALL	Positive for CD19/CD10/CD79a, negative for CD117, frequently CD34+, negative for CD3, myeloid markers
BCL11B deregulated AL	CD117 and/or CD34+, usually cCD3+, always CD2+, always CD5−, always CD8−, variable for MPO, positive for other myeloid markers