Adult Low-Hypodiploid Acute Lymphoblastic Leukemia Emerges from Preleukemic TP53 -Mutant Clonal Hematopoiesis


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Low hypodiploidy defines a rare subtype of B-cell acute lymphoblastic leukemia (B-ALL) with a dismal outcome. To investigate the genomic basis of low-hypodiploid ALL (LH-ALL) in adults, we analyzed copy-number aberrations, loss of heterozygosity, mutations, and cytogenetics data in a prospective cohort of Philadelphia (Ph)-negative B-ALL patients (n = 591, ages 18–84 years), allowing us to identify 80 LH-ALL cases (14%). Genomic analysis was critical for evidencing low hypodiploidy in many cases missed by cytogenetics. The proportion of LH-ALL within Ph-negative B-ALL dramatically increased with age, from 3% in the youngest patients (under 40 years old) to 32% in the oldest (over 55 years old). Somatic TP53 biallelic inactivation was the hallmark of adult LH-ALL, present in virtually all cases (98%). Strikingly, we detected TP53 mutations in posttreatment remission samples in 34% of patients. Single-cell proteogenomics of diagnosis and remission bone marrow samples evidenced a preleukemic, multilineage, TP53-mutant clone, reminiscent of age-related clonal hematopoiesis.

SIGNIFICANCE: We show that low-hypodiploid ALL is a frequent entity within B-ALL in older adults, relying on somatic TP53 biallelic alteration. Our study unveils a link between aging and low-hypodiploid ALL, with TP53-mutant clonal hematopoiesis representing a preleukemic reservoir that can give rise to aneuploidy and B-ALL.
INTRODUCTION

B-cell precursor acute lymphoblastic leukemia (B-ALL) represents a rare malignancy in adults associated with a poor prognosis, especially in the older range of patients (1–3). Despite significant improvements provided by pediatric-inspired treatment regimens (4), there is still an important discrepancy between B-ALL outcomes in children and adults, likely owing to two major factors. First, treatment-related toxicity increases with age, which prevents the use of intensive chemotherapy (1, 5). Second, adult B-ALL have decreased sensitivity to treatments when compared with children due to different leukemia genomic backgrounds. Hence, large genomic studies have highlighted the age-related prevalence of distinct genetic aberrations, with a drop in low-risk abnormalities (i.e., high hypodiploidy and ETV6::RUNX1) beyond the age of 10, together with the progressive increase in high-risk subtypes, i.e., BCR::ABL1, BCR::ABL1-like, KMT2A-rearranged and low hypodiploidy (6–9). Therefore, a better understanding of the pathogenesis of high-risk adult B-ALL is needed to improve clinical management.

B-ALL with low hypodiploidy (LH-ALL) is a distinct entity primarily defined by karyotype harboring 32 to 39 chromosomes with a nonrandom pattern of chromosome losses (10). Afterward, this entity was renamed “low hypodiploidy/near triploidy” to include cases with duplicated low-hypodiploid genome sharing the same poor prognosis (11). Within childhood B-ALL, LH-ALL represents a very small subset associated with a dismal outcome despite being treated with the most intensive procedures (12). A previous study in pediatric ALL showed that most LH-ALL harbored TP53 mutations, with half of them being of germline origin, connecting LH-ALL to the spectrum of the Li–Fraumeni cancer-predisposing syndrome (13). In adults, LH-ALL is also associated with poor outcomes (8) and frequent TP53 mutations (14–16), but few data addressed the somatic origin of TP53 mutations.

Clonal hematopoiesis is a condition characterized by the clonal expansion of hematopoietic stem/progenitor cells (HSPC) carrying somatic mutations at detectable levels. Sequencing of large cohorts of healthy subjects has shown that the prevalence of clonal hematopoiesis increased continuously with age, defining age-related clonal hematopoiesis (ARCH; refs. 17–19). Importantly, TP53 is one of the most
frequent genes involved in ARCH. In addition, TP53-mutant clonal hematopoiesis is associated with an increased risk of developing myeloid malignancies, including secondary and treatment-related myeloid neoplasms (20–22).

Here, we identified and characterized a large cohort of adults with LH-ALL. Then, considering the dramatically increased proportion of LH-ALL with age, we sought to investigate the possible role of TP53-mutant clonal hematopoiesis in the pathogenesis of adult LH-ALL. Using paired diagnosis/remission samples and a single-cell multomics platform, we demonstrate that LH-ALL can arise from a preleukemic TP53-mutant HSPC clone.

## RESULTS

### DNA Sequencing-Based Assessment of Copy-Number Aberrations and Loss-of-Heterozygosity Is Critical for Identifying LH-ALL

We studied a prospective cohort of adults with newly diagnosed Philadelphia (Ph)-negative B-ALL with available diagnostic sample (n = 591; Fig. 1A). We used targeted-capture sequencing to carry out copy-number aberrations (CNA) analysis and classified cases by a modal chromosomal number based on both karyotype and DNA data (Fig. 1B; Supplementary Fig. S1A and Supplementary Table S1). Noticeably, hypodiploid leukemic cells can sometimes undergo endoreplication, leading to a high number of chromosomes. Therefore, we examined all the cases presenting with either ≤40 (n = 41) or ≥50 chromosomes (n = 87). All cases with ≤40 chromosomes corresponded to LH-ALL, except one having near haploidy, confirming that the latter corresponds to a subtype virtually not found in adult ALL. Within cases with ≥50 chromosomes, loss-of-heterozygosity (LOH) analysis enabled to identify cases with a duplicated low-hypodiploid genome, as harboring multiple uniparental disomies corresponding to the same pattern as that of chromosome losses in classic low hypodiploidy (Supplementary Fig. S1B). Importantly, LOH analysis unequivocally distinguished duplicated low hypodiploidy from high hypodiploidy, the latter corresponding to a distinct good-risk B-ALL subtype.

Nearly half (40/87, 46%) of all cases with ≥50 chromosomes turned out to have a duplicated low-hypodiploid genome (Fig. 1C). The ancestral low-hypodiploid clone was detectable by karyotype in only a few patients (5/40, 13%; Fig. 1D), suggesting that they could have been misclassified as high hypodiploidy based on cytogenetics only. Moreover, in 26 of 80 cases (33%) eventually classified as LH-ALL, conventional cytogenetics included to culture failure or normal karyotype, suggesting that leukemic cells failed to grow in vitro. We also identified two LH-ALL cases as having the canonical pattern of chromosome losses, albeit with a modal chromosome number >39 due to trisomy 21. Therefore, our data illustrate that CNA and LOH analyses are critical to allocate correctly B-ALL to distinct aneuploidies representing clinically relevant B-ALL subtypes.

**Figure 1.** Identification of a cohort of 80 LH-ALL cases by karyotyping and sequencing-based assessment of CNA and LOH. **A,** Flow diagram describing the study cohort of Ph-negative B-ALL adult patients. **B,** Distribution of B-ALL cases according to their modal chromosome number assessed by karyotyping and/or sequencing-based CNA analysis. **C,** Proportion of LH-ALL within cases with ≤40 chromosomes and cases with ≥50 chromosomes after integration of sequencing-based LOH analysis. **D,** Heat map of chromosome anomalies as determined by karyotyping or CNA/LOH analysis in the 80 LH-ALL cases.
Adult LH-ALL Evolves from TP53 Clonal Hematopoiesis

RESEARCH ARTICLE

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TP53 Mutations Are Detected at Remission in a Substantial Proportion of Adults with LH-ALL

Given the age-related distribution of LH-ALL and the role of TP53 mutations in ARCH, we hypothesized that LH-ALL may be secondary to TP53-mutant ARCH. To test this hypothesis, we looked for TP53 mutations in posttreatment remission bone marrow samples assessed for minimal residual disease (MRD). Among the 73 patients with available samples, we observed persistence of the TP53 mutation identified at diagnosis in 25 patients (34%), at VAF ranging from 2.6% to 51.2% (cutoff value for positivity set at 2%; Fig. 3A and B; Supplementary Table S3). DNMT3A and TET2 mutations were also detected in remission samples from 9 and 6 patients, respectively. MRD levels based on the quantification of clonal rearrangements of immunoglobulin or T-cell receptor genes (IG/TR) were either undetectable or measured at much lower levels (Supplementary Table S4), indicating that those mutations were not related to residual B-ALL leukemic cells.

It should be noticed that 5 patients had a TP53 mutation with a VAF >40%, raising the possibility of a germline origin, as previously observed in pediatric LH-ALL. This could be ruled out in two patients for whom TP53 mutation was not detected in nonleukemic cells (Supplementary Table S3). In the three remaining patients, including one with detectable TP53 mutation in sorted T cells and two without available nonleukemic material, a germline origin could not be ruled out.

Next, to evaluate the dynamics of the TP53-mutant cell fraction, we used digital droplet PCR (ddPCR) to quantify TP53 mutations in 9 patients with available longitudinal postremission bone marrow samples (Fig. 3C; Supplementary Fig. S3). In two patients with confirmed somatic TP53 mutation, the TP53-mutant clone remained in a steady state at high rates at all time points. Four patients displayed a reduction of the TP53-mutant fraction over time, yet, uncoupled from lower or undetectable IG/TR MRD levels. Noticeably, in two of these patients, the higher sensitivity of ddPCR allowed to detect a low fraction of persistent TP53-mutant cells not detected by sequencing. In three other patients, no TP53-mutant cells were detected. Overall, these results reveal that a substantial proportion of adults with LH-ALL carry somatic TP53 mutations in nonleukemic cells repopulating bone marrow after treatment.

Integrated Single-Cell Genotyping and Immunophenotyping Reveals Multilineage TP53-Mutant Clonal Population at Remission Stage

We sought to investigate the contribution of the TP53-mutant clone to the different hematopoietic lineages and infer the oncogenic route toward LH-ALL. Using antibody-derived tag (ADT) sequencing and a custom single-cell DNA-seq (scDNA-seq) panel (23–25), we performed simultaneous single-cell immunophenotyping and genotyping on three bone marrow specimens obtained after induction. The three

| Table 1. Demographic and clinical characteristics of patients at baseline. |
|-----------------|-----------------|-----------------|
|                  | LH-ALL          | Non LH-ALL      | P          |
| Patients, n     | 80              | 511             |            |
| Sex, n(%)       |                 |                 |            |
| Male            | 42 (53)         | 269 (53)        | 1          |
| Female          | 38 (47)         | 242 (47)        |            |
| Age, year       |                 |                 |            |
| Median          | 59              | 39              | -0.0001    |
| Range           | 18–84           | 18–84           |            |
| Age group, n(%) |                 |                 |            |
| 18–24 y         | 5 (6)           | 123 (24)        | -0.0001    |
| 25–39 y         | 3 (4)           | 136 (27)        |            |
| 40–54 y         | 17 (21)         | 136 (27)        |            |
| ≥ 55 y          | 55 (69)         | 116 (23)        |            |
| White blood cell count, Giga per liter |       |                 |            |
| Median          | 3.2             | 7.5             | -0.0001    |
| Range           | 0.4–87.1        | 0.4–712.0       |            |
| Marrow blast infiltration, % |       |                 |            |
| Median          | 79              | 92              | -0.0001    |
| Range           | 21–99           | 12–100          |            |
| CD10 expression, n(%) |       |                 |            |
| Positive        | 62 (79)         | 382 (76)        | 0.67       |
| Negative        | 16 (21)         | 118 (24)        |            |

*White blood cell counts were available for 582 patients.

*Marrow blast cell counts were assessed for 569 patients.

*CD10 expression was assessed by flow cytometry for 578 patients.

Two-tailed independent t tests and two-tailed Fisher’s tests were used for continuous and categorical variables, respectively, and statistical significance was defined as a P < 0.05.

Figure 2. Landscape of recurrent genomic alterations in adult LH-ALL. **A**, Heat map of recurrent CNA and mutations in the B0 LH-ALL cases. Focal alterations refer to CNA not involving whole chromosome arms and mutations. Cases with several mutations are referred to as multihit. **B**, Lollipop plot depicting TP53 mutations detected in both LH-ALL and other Ph-negative B-ALL. **C**, Correlation between TP53 mutation VAF and blast percentage, as assessed by flow cytometry after ficoll or morphology on marrow smear, using Pearson correlation coefficient. **D**, Proportion of TP53 mutations in LH-ALL and other Ph-negative B-ALL with available data from the study cohort (n = 499). Hit, heterozygous; Hom: homozygous; indel: insertion/deletion.
Adult LH-ALL Evolves from TP53 Clonal Hematopoiesis

**RESEARCH ARTICLE**

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Figure 3. Tracking of ARCH-related mutations in remission samples of LH-ALL patients. A, Heat map of mutations in paired diagnosis and remission samples for 71 patients. Data are based on sequencing data with a threshold for positivity at 2% VAF. B, Box plot for VAF of mutations detected by sequencing at remission. C, Longitudinal assessment of TP53-mutant cell fraction as determined by ddPCR (0.05–0.1% sensitivity), along with clonal IG/TR-based MRD quantification (0.01%–0.001% sensitivity), for six patients with TP53 mutation detected in remission samples, including two below the sequencing detection threshold.
Adult LH-ALL Evolves from TP53 Clonal Hematopoiesis

Patients were treated in the EWALL-INO trial and had a TP53 mutation detected at postinduction remission, whereas MRD measured on the same sample was either low or undetectable. Protein expression data allowed discrimination of 7 distinct clusters (Fig. 4A). Based on lineage-specific markers, we were able to identify confidently T cells, B cells, monocytes, dendritic cells (DC), and myeloid and erythroid cell clusters (Fig. 4B; Supplementary Fig. S4A and S4B). Cell identity could not be assigned to the remaining cluster owing to unspecific and weak labeling of frail cells (thereafter named...
Figure 4. (Continued) C, Same UMAP plots (top) for individual patient’s remission samples, with cells colored according to TP53 genotype. Histograms (bottom) show the proportion of each TP53 genotype within each cell cluster. D, UMAP plots for the EI_035 remission sample, with cells colored according to genotype for several heterozygous SNPs allowing LOH assessment. ref, reference allele; alt, alternative allele.

“unspecific labeling”). Of note, few B cells were detected, which may be related to therapy including B cell–directed antibodies. Cell type composition was comparable across patients, but analyses on each sample individually allowed better identification of discrete cell populations not detected in all patients, such as natural killer (NK) cells and plasmacytoid dendritic cells (pDC; Supplementary Fig. S5).

Projection of the TP53 genotype on the protein uniform manifold approximation and projection (UMAP) representation allowed us to describe the cell architecture of the TP53-mutant clone (Fig. 4C; Supplementary Fig. S6). Patients EI_031 and EI_047 with similar patterns with TP53-heterozygous mutant cells detected in substantial fractions of myeloid, erythroid, and monocyte/DC clusters (hereafter collectively named myeloid clusters). The “unspecific labeling” cluster was likely related to myeloid populations according to the similar proportion of mutant cells. T-cell populations also contained mutant cells, yet at lower rates. For patient EI_031, NK cells, pDC, and B-cell clusters could be identified and also harbored mutant cells. Of note, the observation of rare cells with a homozygous genotype was likely related to allelic dropout and biologically not relevant, in agreement with similar proportions observed for constitutional heterozygous single-nucleotide polymorphisms (SNP; Supplementary Fig. S7).

Patient EI_035 harbored a large fraction of cells in myeloid clusters carrying TP53-homozygous mutation, pinpointing a concomitant CNA or copy-neutral LOH in those cells. By contrast, T cells were mainly wild-type, with a minority being heterozygous. Including amplicons covering common SNPs in the scDNA-seq panel allowed us to investigate allelic imbalance throughout the genome at the single-cell level. Thus, we evidenced LOH at 17p (but not at 17q) in myeloid clusters, suggesting a cytogenetic aberration at 17p resulting in the loss of the wild-type allele (Fig. 4D; Supplementary Fig. S8). Those cells also had LOH at 5q and 16q, whereas no other LOH possibly related to the LH-ALL clone was observed. Thus, the presence of those aberrations restricted to the myeloid compartments may be related to a distinct, myelodysplastic clone, only sharing the TP53 alteration with the LH-ALL clone. Altogether, these results show that patients with LH-ALL carry...
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TP53-mutant HSPCs able to repopulate bone marrow after intensive treatment.

LH-ALL Arises from Preexisting TP53-Mutant Clonal Hematopoiesis

We next aimed to address whether the TP53-mutant multilineage cell population arose from clonal selection under the stress of cytoxic therapy for B-ALL or preexisted at diagnosis. We profiled 14,518 cells from three diagnostic samples, including two paired with remission samples already presented (EI_047 and EI_035). ADT-sequencing and IG/TR clono-specific sequences enabled to recognize B-ALL leukemic cells and to identify minor nonleukemic cell populations (Fig. 5A and B; Supplementary Fig. S9). In B-ALL cells from the 3 patients, TP53 and SNP single-cell genotyping showed homozygous (hemizygous) TP53 mutation and LOH at multiple loci (Fig. 5C; Supplementary Fig. S10), in agreement with bulk data (Fig. 1D; Supplementary Table S2). In EI_046 and EI_047, substantial fractions of TP53-heterozygous mutant cells were observed within myeloid and NK cell populations, as observed in the paired-remission sample of EI_047. Similarly, in EI_035, the myeloid population was predominantly TP53-homozygous mutant. By contrast, the small subset of TP53-heterozygous mutant T cells was likely related to the cell doublet artifact, in agreement with a similar rate of detection of clonal IG/TR rearrangements (Supplementary Table S5). Consistent results were obtained through analyses of the fluorescence-activated cell sorting (FACS) of cell fractions (Supplementary Table S6).

Overall, these results confirm that the TP53-mutant myeloid population preexisted at B-ALL diagnosis and indicate that TP53-mutant ARCH likely preceded the onset of B-ALL.

Other ARCH-Related Genes Can Be Involved in the Pathogenesis of LH-ALL

Because other ARCH-related mutations were identified in bulk diagnostic samples from several patients with LH-ALL, we aimed to investigate their relation with TP53-mutant clonal hematopoiesis and LH-ALL and decipher the sequence of mutation acquisition.

EI_047 had a TET2 mutation detected in bulk sequencing at diagnosis at 48% VAF. Unexpectedly, single-cell analysis of the diagnosis sample revealed that this mutation was restricted to B-ALL cells and was not carried by myeloid cells (Fig. 6A). Accordingly, it was not detected in the bulk remission sample.

EI_046 harbored a JAK2 V617F detected at 7% VAF in the bulk diagnosis sample. Single-cell analysis revealed that it was restricted to myeloid and erythroid cells, in a homozygous state, reminiscent of myeloproliferative neoplasms (MPN).

Moreover, the TP53 and JAK2 mutations appeared to be in independent clones, as no cell carried both mutations (Supplementary Fig. S11). Therefore, single-cell analysis uniquely evidenced a JAK2-mutant ARCH or MPN-like clone, concomitant but clonally unrelated to B-ALL.

EI_035 had a DNMT3A mutation at diagnosis (VAF 45%), still detected at remission (VAF 42%). Single-cell analysis of the diagnosis sample showed that almost all B-ALL and nonleukemic cells carried heterozygous DNMT3A mutation. The presence of TP53-wild-type DNMT3A-mutant cells in the remission sample (Supplementary Fig. S11) indicated that DNMT3A mutation occurred earlier than TP53 mutation. In addition, and as observed at remission, myeloid cells had LOH at 5q, 16q, and 17p, suggesting a premalignant, concurrent clone at B-ALL diagnosis (Fig. 6B). All B-ALL cells had LOH at these loci and at additional loci in relation with low hypodiploidy. However, the discordant homozygous genotype at 16q between B-ALL and myeloid cells indicated a branched rather than linear relation between the myeloid and B-ALL clones.

Overall, these data allow to reconstruct the sequence of mutation acquisition (Fig. 6C) and show that adult LH-ALL can develop on a background of clonal hematopoiesis resembling myelodysplastic syndrome (MDS) or MPN.

DISCUSSION

Our study establishes a comprehensive genomic characterization of the largest series of LH-ALL reported so far. By combining sequencing-based CNA and LOH analyses to cytogenetics data in a prospective cohort of 591 Ph-negative adult ALL, we identified a high proportion of LH-ALL, reaching 32% in the older range of patients. This increased prevalence in older adults is in agreement with, but higher than, that observed in previous studies, including the recent UKALL study of older adult ALL (9). Although the diagnostic pitfall of duplicated hypodiploidy/near triploidy is recognized, our data suggest that a number of LH-ALL cases are still being missed or misclassified owing to misleading normal or hypodiploid karyotypes. Hence, CNA/LOH analyses in our study allowed to rescue more than a quarter of LH-ALL cases with normal/failed karyotype, whereas duplicated low hypodiploidy represented up to half of all LH-ALL. Of note, we also showed that LH-ALL patients present with lower blast counts in bone marrow and peripheral blood at diagnosis, which may explain underrepresentation in retrospective molecular studies relying on frozen samples. These findings warrant the implementation of CNA/LOH analysis in diagnostic laboratory practices, because genetic classification is critical for risk-adapted treatment stratification in most modern pediatric and adult clinical trials. The improved genetic assignment will also allow the refinement of future clinical correlation studies.

Our single-cell sequencing data on diagnostic and remission samples indicate that TP53 mutation is a preleukemic event preceding aneuploidy with monosomy 17 leading to the loss of the TP53 wild-type allele. Although a dominant-negative effect of TP53 missense mutations has been proposed to mediate the nonmutational inactivation of wild-type TP53 (26), it is noteworthy that genomic alteration of the second allele seems to be mandatory in the pathogenesis of LH-ALL. The specific pattern of chromosomal losses in adult LH-ALL, including the core association of 3, 7, 16, and 17 monosomies, is consistent with that observed in childhood LH-ALL and may inform about key genes involved in LH-ALL oncogenesis. Loss of chromosome 7 results in haploinsufficiency for IKZF1, a critical gene for normal B-cell differentiation, the recurrent loss of which in B-ALL drives a pejorative impact (27). Chromosome 16 contains CREBBP, which is also targeted by focal alterations in B-ALL, especially in relapsed cases (28). Noteworthy, as the CD19 gene is also located on chromosome 16, LH-ALL having only one allele may be prone to CD19 expression loss in the context of selective pressure with CD19-directed therapeutic agents (29). Chromosome 3 contains several tumor suppressor genes previously shown to be involved in B-ALL development.
Figure 5. Single-cell analysis at LH-ALL diagnosis identifies a preexisting multilineage TP53-mutant clonal population. **A,** UMAP plots for individual diagnostic samples from three patients (EI_047, EI_046, and EI_035), having 5,228, 4,947, and 4,343 cells available for analysis, respectively. Cells are clustered by expression of cell-surface markers and colored according to assigned clusters. **B,** Same UMAP plots for patients EI_047 and EI_035, with cells colored according to the presence of specific clonal IG/TR sequences allowing to identify B-ALL cells. Histograms show the proportion of positive cells within each cell cluster. **C,** Same UMAP plots (top) with cells colored according to TP53 genotype. Histograms (bottom) show the proportion of each TP53 genotype within each cell cluster.
Figure 6. Integration of ARCH mutations into the clonal architecture of LH-ALL. A, UMAP plots for individual diagnostic samples from three patients with cells clustered by expression of cell-surface markers and colored according to genotypes of TET2, JAK2, and DNMT3A mutations in EI_047, EI_046, and EI_035, respectively. Histograms show the proportion of each genotype within each cell cluster. B, UMAP plots for the EI_035 diagnostic sample, with cells colored according to genotype for several heterozygous SNPs allowing LOH assessment. (continued on next page)
suggest their functional redundancy and an oncogenic cooperation with a TP53-deficient background. We identified NF1 as a frequent target gene in adult LH-ALL, whereas it was not observed in their pediatric counterpart (13).

$\text{DNMT3A}$ and $\text{TET2}$ were among the top mutated genes, found in 10% of patients. As these genes are the most frequently mutated in ARCH (17), these mutations may simply reflect the older age of LH-ALL patients. Single-cell analysis revealed different possible clonal involvement of those mutations not inferred by bulk sequencing. In one patient, $\text{DNMT3A}$ mutation was the earliest somatic event, found in an ancestral multilineage hematopoietic clone, as previously shown in acute myeloid leukemia (AML; ref. 32). By contrast, in another patient, $\text{TET2}$ mutation was found as a secondary genetic event restricted to leukemic cells, suggesting an unanticipated oncogenic role of $\text{TET2}$ in B-ALL. In addition, in one patient with $\text{JAK2 V617F}$ mutation detected in bulk diagnosis, the single-cell analysis demonstrated that it was clonally unrelated to $\text{TP53}$-mutant ALL cells, which is reminiscent of post-MPN $\text{TP53}$-mutant AML lacking the MPN driver mutation (33).

The major finding of our study is that $\text{TP53}$ mutations are not only frequently associated with LH-ALL, but they are indispensable genetic events preceding aneuploidy. Hence, we detected $\text{TP53}$ mutations in virtually all LH-ALL cases and demonstrated that $\text{TP53}$-mutant heterozygous cells constituted a preleukemic compartment. This oncogenic route relying on initiating $\text{TP53}$ mutation is thus distinct from that observed in other malignancies, including ALL (34), where $\text{TP53}$ mutations often occur during tumor progression as a late event. In LH-ALL, $\text{TP53}$ mutation likely acts as the triggering event for genome instability, allowing the emergence of the low-hypodiploid clone responsible for leukemia onset. The further step of whole-genome duplication, whereas otherwise rare in ALL, may be also related to $\text{TP53}$ deficiency, as reported in other tumors (35).

Moreover, we show that somatic $\text{TP53}$ mutations in LH-ALL affect preleukemic HSPCs that retain their ability to generate mature blood cells, defining clonal hematopoiesis. This

**Figure 6. (Continued)** C. Fish plots and phylogenetic trajectories depicting the clonal architecture and the sequential acquisition of genetic abnormalities.
connects LH-ALL to the spectrum of hematologic malignancies related to TP53-mutant clonal hematopoiesis, namely, secondary and therapy-related AML and MDS having TP53 biallelic alteration and complex karyotype (20–22). We also show that, at least in some cases, a myeloid premalignant clone may evolve concurrently to LH-ALL, which may support treatments aiming at eradicating not only B-ALL but TP53-mutant clonal hematopoiesis. Altogether, our study sheds light on an unsuspected link between LH-ALL, TP53-mutant clonal hematopoiesis, and related myeloid malignancies and paves the way for future investigations regarding its clinical and therapeutic relevance.

METHODS

Patients and Samples
The study cohort included patients with Ph-negative B-ALL ages 18 to 59 years enrolled in the GRAALL-2014 trial between 2016 and 2020 and French patients ages ≥55 years enrolled in the EWALL-INO trial between 2017 and 2022 (ClinicalTrials.gov number NCT02617004 and NCT03249870, respectively). The GRAALL-2014 protocol was an intensive pediatric-inspired treatment similar to the GRAALL-2005 (5), but with age adaptation of doses for patients 45 to 59 years old. The standard induction comprised predniisone, daunorubicin, vincristine, cyclophosphamide, L-asparaginase, and intrathecal prophylaxis. The EWALL-INO protocol was a treatment based on a low-intensity chemotherapy backbone and intrathecal prophylaxis with the addition of inotuzumab ozogamicin (INO) during the two-phase induction. The first induction phase comprised 3 doses of INO (D1: 0.8 mg/m², D8 and D15: 0.5 mg/m²) in combination with weekly vincristine 2 mg i.v. and dexamethasone 40 mg for 4 weeks, and the second phase comprised 2 doses of INO at 0.5 mg/m² in combination with dexamethasone 20 mg (D1 and D8) and cyclophosphamide 300 mg/m²/day i.v. D1–3. All the patients provided written informed consent for sample banking and analyses. The study was in accordance with the Declaration of Helsinki. Cytogenetic analyses at diagnosis were performed by local laboratories using standard procedures. Molecular analyses were performed centrally as previously described (36). Briefly, monoclonal cells from pretreatment bone marrow or peripheral blood samples were isolated by Ficoll centrifugation, and blast percentage was assessed by flow cytometry in most cases before nucleic acid extraction. MRD was assessed by the quantification of clonal IG/TR rearrangements, according to the EuroMRD guidelines.

Targeted DNA Sequencing
A custom scDNAseq panel was designed to cover relevant somatic mutations and common SNPs allowing the detection of LOH (Supplementary Table S8). Additional primers for amplicons of clone-specific IG/TR rearrangements were also included (Supplementary Table S9). Frozen bone marrow monoclonal cells were thawed, stained using the ADT panel TotalSeq-D Human Heme Oncology Cocktail, V1.0 (BioLegend; antibody list in Supplementary Table S10), and loaded into the Tapestrí single-cell DNA genotyping platform (Mission Bio) to perform microfluidic encapsulation, lysis, and barcoding according to the manufacturer’s protocol (Chemistry V2, PN_3360A). To improve erythroid cells identification, TotalSeq-D0574 anti-human CD235a (Glycophorin A) Antibody (BioLegend) was spiked at 1 μg during the resuspension procedure (available only for EI_046 diagnostic sample). DNA and ADT libraries were prepared and underwent 2 × 150 bp paired-end sequencing on a NextSeq 500 platform (Illumina).

Bioinformatics Analysis
Raw data were processed using Mission Bio’s Tapestrí Pipeline (DNA pipeline version 2.0.2 and Protein pipeline version 2.0.1) for preprocessing, alignment (reference genome hg19), cell barcode correction, cell identification, variant calling of DNA amplicons, and ADT-seq reads counting. Multimics h5 files were then analyzed on R version 4.1.2 (R Foundation for Statistical Computing). TapestríR package was used to annotate variants: a genotype was considered informative if the single-cell sequencing depth was ≥5 reads. Variants were categorized as wild-type (WT) if VAF ≤10%, heterozygous (het) if VAF comprised between 10% and 90%, and homozygous (hom) if VAF ≥90%. Variants covered by <5 reads were considered noninformative and labeled as “missing genotype.” No genotype filtering was performed before integration with ADT-seq data, in order to avoid data attrition, as all genotypes (i.e., WT, heterozygous and hemizygous/homozygous mutations) are expected. The rate of the allelic dropout was evaluated based on heterozygous SNPs. False homozygous genotypes related to allelic dropout were observed in 1% to 18% of cells (Supplementary Fig. S7). Patient-specific amplicons targeting clone-specific IG/TR rearrangements were considered informative if detected in the diagnostic sample. The IG/TR amplicons were considered positive if covered by ≥5 reads.

ADT read counts and cell barcodes were extracted from h5 files and were analyzed using Seurat package V4.1.0 (42). Remission samples were merged before proceeding to protein expression analysis as similar cell compositions were expected. Diagnostic samples were analyzed separately. Cells with total ADT-seq read counts ≥100k were excluded.
to minimize the presence of doublets in further analyses (Supplementary Table S1). Centered log-ratio transformation was performed to normalize reads across cells before scaling, and then principal component analysis was performed on UMAP embedding and K-nearest neighbors and Louvain clustering algorithms to identify cell populations based on ADT sequencing before undergoing manual cell population labeling based on cell-surface marker expression. Genotype data were then merged, and relative mutated proportions were calculated for each cluster having at least 10 cells for interpretation.

**FACS of Cell Populations**

As a cross-validation experiment, diagnostic samples from two patients were subjected to FACS in order to characterize genetic alterations in distinct cell populations. Briefly, frozen cells were thawed, stained with lineage-specific antibodies, and sorted on FACSAria III (BD Biosciences). Cell fractions underwent DNA extraction using QIAamp DNA Microkit (Qiagen) followed by targeted sequencing for somatic mutations and ddPCR quantitation for IG/TR clonal rearrangements.

**Data Availability**

Single-cell and bulk targeted sequencing data are accessible through the EGA database (https://www.ega-archive.org) under accession numbers EGAS00001006784 and EGAS00001006901, respectively. Other data are available upon reasonable request to the principal investigator.

**Authors’ Disclosures**

S. Gachet reports grants from Fondation de France during the conduct of the study. V. Asnafi reports personal fees from Servier outside the submitted work. L. Ades reports grants and personal fees from BMS, personal fees from Novartis, AbbVie, and JAZZ pharma outside the submitted work. Y. Chalandon reports other support from MSD, Novartis, Incyte, BMS, Pfizer, AbbVie, Roche, Jazz, Gilead, Amgen, Astra-Zeneca, Servier, MSD, Roche, Gilead, Amgen, Incyte, AbbVie, Janssen, Jazz, and Astra-Zeneca outside the submitted work. F. Huguet reports personal fees from Novartis, Incyte, Amgen, Pfizer, and Servier outside the submitted work. No disclosures were reported by the other authors.

**Authors’ Contributions**

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**Note**

Supplementary data for this article are available at Blood Cancer Discovery Online (https://bloodcancerdiscov.aacrjournals.org/).

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**REFERENCES**


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