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ONCOLOGY: RESEARCH ARTICLE

Clonal dynamics in pediatric B-cell precursor acute lymphoblastic leukemia with very early relapse

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Published meeting abstract: Genomic architecture and clonal dynamics of early relapsed BCP-ALL. American Soci-

Abstract

Introduction: One-quarter of the relapses in children with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) occur very early (within 18 months, before completion of treatment), and prognosis in these patients is worse compared to cases that relapse after treatment has ended.

Methods: In this study, we performed a genomic analysis of diagnosis–relapse pairs of 12 children who relapsed very early, followed by a deep-sequencing validation of all

Abbreviations: AID, activation-induced deaminase; ALL, acute lymphoblastic leukemia; APOBEC, apolipoprotein B mRNA editing enzyme; BCP, B-cell precursor; BGI, Beijing Genome Institute; CNA, copy number alteration; DCOG, Dutch Childhood Oncology Group; DNA, deoxyribonucleic acid; DS, Down syndrome; MAF, mutant allele frequency; MLPA, multiplex ligation-dependent probe amplification assay; MRD, minimal residual disease; SBS, single-base substitution; WES, whole-exome sequencing; WGA, whole-genome amplification; WGS, whole-genome sequencing.

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identified mutations. In addition, we included one case with a good initial treatment response and on-treatment relapse at the end of upfront therapy.

Results: We observed a dynamic clonal evolution in all cases, with relapse almost exclusively originating from a subclone at diagnosis. We identified several driver mutations that may have influenced the outgrowth of a minor clone at diagnosis to become the major clone at relapse. For example, a minimal residual disease (MRD)-based standard-risk patient with *ETV6-RUNX1*-positive leukemia developed a relapse from a *TP53*-mutated subclone after loss of the wildtype allele. Furthermore, two patients with *TCF3-PBX1*-positive leukemia that developed a very early relapse carried E1099K *WHSC1* mutations at diagnosis, a hotspot mutation that was recurrently encountered in other very early *TCF3-PBX1*-positive leukemia relapses as well. In addition to alterations in known relapse drivers, we found two cases with truncating mutations in the cohesin gene *RAD21*.

Conclusion: Comprehensive genomic characterization of diagnosis–relapse pairs shows that very early relapses in BCP-ALL frequently arise from minor subclones at diagnosis. A detailed understanding of the therapeutic pressure driving these events may aid the development of improved therapies.

KEYWORDS
clonal dynamics, pediatric acute lymphoblastic leukemia, RAD21, TP53, very early relapse, WHSC1

1 | INTRODUCTION

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common pediatric malignancy.[1–4] Despite treatment improvements over the past decades, relapse still represents the most common cause of therapy failure.[5,6] Studies describing the genomic landscape of relapsed ALL have identified alterations in several genes associated with treatment resistance, including *IKZF1*, *CREBBP*, and *NT5C2*.[7–12] Selective pressure during upfront treatment can give competitive advantage to leukemic cells harboring these alterations, eventually leading to the rise of relapse. For example, activating mutations in the *NT5C2* gene, encoding cytosolic nucleosidase, accelerate depletion of intracellular purine nucleotides and lower proliferative potential of the tumor cells harboring these mutations.[10,13,14] During treatment with thiopurines, *NT5C2* mutations decrease cell vulnerability, thus giving the leukemic blasts a competitive and proliferative advantage compared to cells without these mutations. Nevertheless, exact mechanisms in which genomic alterations enable leukemic cells to develop resistance against chemotherapeutics are not fully elucidated. Furthermore, recent studies have shown that treatment failure may not always be the consequence of genomic alterations conferring treatment resistance and proliferative advantage, but rather persistence of leukemic cells in a protective niche.[15–17]

Over 40% of BCP-ALL relapses present late, 6 months after the end of treatment (>30 months from initial diagnosis).[18,19] These relapses may originate from leukemic (sub)clones that remained in a quiescent state during treatment or that could not be reached by the chemotherapeutics, for example, due to presence of physiological blood–organ barriers. On the other side of the spectrum are patients with very early relapses (<18 months from initial diagnosis), which represents approximately 25% of relapses that occur during treatment, and which are associated with dismal outcome.[18–20] Very early relapses may be different in that they display clonal outgrowth in the presence of chemotherapeutics. Alternatively, relapses may be the result of a suboptimal treatment, for example, due to toxicity-related discontinuations or omissions. Therefore, mechanisms driving relapses during treatment are not yet fully understood.

In this study, we explored the genomic abnormalities in 12 children with BCP-ALL who experienced very early relapse, and included one additional case with good initial treatment response and on-treatment relapse (within 24 months from diagnosis). We subsequently characterized the genomic alterations at diagnosis and relapse using whole-exome sequencing (WES) and digital multiplex ligation-dependent probe amplification (MLPA) assay, and investigated the clonal dynamics of these leukemias. These different cases revealed diverse but relevant lessons of on-treatment relapses in BCP-ALL.

2 | METHODS

2.1 | Patients and samples

In this study, we selected and analyzed a cohort of 12 children diagnosed with BCP-ALL, who experienced very early bone marrow relapse (<18 months after diagnosis), according to the definition of Berlin–Frankfurt–Münster (BFM) study group, and for which samples from

diagnosis, complete remission, and relapse were available. These children were treated according to a Dutch Childhood Oncology Group (DCOG) protocol ALL9 ($n = 6$), ALL10 ($n = 2$), ALL11 ($n = 3$), or Interfant-06 ($n = 1$). In addition, we included a child with favorable cytogenetics (*ETV6-RUNX1*-positive leukemia) and good initial treatment response, who developed an unexpected bone marrow relapse at the end of DCOG ALL10-based standard-risk treatment (23 months). Median age at diagnosis for the total cohort was 8 years (range 1–14 years), and median remission time was 13 months (range 6–23 months) (Table S1). Deoxyribonucleic acid (DNA) was isolated from mononuclear cells obtained from bone marrow or peripheral blood at the time of diagnosis, complete remission, and relapse using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Remission status of remission samples was determined according to the criteria of the respective study protocols, and all 13 patients reached complete remission. Informed written consent was obtained from all patients and/or their legal guardians before enrollment in the study. In addition, we collected 16 relapse samples from patients diagnosed with *TCF3-PBX1*-positive BCP-ALL. Primer sequences used to perform screening for mutations in *WHSC1* hotspots are given in Table S2.

2.2 | Whole-genome amplifications (WGA)

Due to limited amounts of genomic DNA in 17 samples from six patients, we performed WGA using the REPLI-g mini kit (Qiagen, Hilden, Germany). Master mix was prepared using 50 ng of genomic DNA. In order to limit unspecific amplification and random errors, incubation was done in four independent reactions of 12.5 μl. After an 8-hour incubation, the four reactions for each patient were pooled and purified. WGA material was used only for the discovery of somatic mutations using WES and all detected mutations were subsequently validated using targeted deep sequencing on non-amplified genomic DNA.

2.3 | WES and whole-genome sequencing (WGS)

WES was performed by the Beijing Genome Institute (BGI) in Copenhagen and Hong Kong using 3 μg of genomic DNA or whole-genome amplified material. DNA was randomly fragmented using Covaris technology and exon capture was done using Agilent SureSelectXT Human All Exon v4 or v5. Sequencing was performed using the Illumina HiSeq4000 platform. We achieved an average on-target sequencing depth of 93.3 (range 52–152.8; Table S3).

WGS was performed by BGI in Hong Kong using genomic DNA obtained from primary patient material. Genomic DNA was randomly fragmented using Covaris technology and sequenced on the BGISEQ-500 platform. We achieved an average sequencing depth of 45.7 (range 43.2–47.3; Table S4).

After excluding low-quality reads, clean reads were mapped to GRCh38 using Burrows–Wheeler aligner (BWA). Variant calling was done using HaplotypeCaller and Mutect2 of GATK. We excluded the following variants from further analysis: (i) likely germline variants (allele frequency >5% in the germline sample and variants detected in multiple independent germline samples); (ii) variants supported by only a single read; (iii) variants with multiple alternative alleles; and (iv) variants called within homopolymer regions. All remaining candidate somatic variants were manually curated using Integrated Genome Viewer (IGV) before subjecting them to deep-sequencing validation.

2.4 | Targeted deep-sequencing validation of somatic variants

In order to exclude possible false-positive somatic mutations and to obtain accurate estimates of the mutant allele frequency (MAF), we performed deep targeted sequencing using the SeqCap EZ HyperCap workflow (Roche, Rotkreuz, Switzerland). Library preparation was done according to the manufacturer's instructions using 100 ng of genomic DNA from diagnosis, relapse, and complete remission. One patient (P0135) underwent a stem cell transplantation and for this patient remission samples before and after stem cell transplantation were used for deep-sequencing validation. Following enzymatic fragmentation, adapter ligation, and pre-capture amplification of ~300 bp fragments, DNA capture was done at 47°C for 48 hours. After post-capture amplification, paired-end 2 × 150 sequencing was performed on Illumina NextSeq 500 sequencer.

2.5 | Digital MLPA assay

Copy number status of the most frequently affected genes in BCP-ALL was assessed using SALSA D007-X3 ALL digital MLPA (MRC-Holland, The Netherlands).[21] In brief, 100 ng of input DNA was denatured for 10 minutes at 98°C, followed by probe hybridization for 16 hours at 60°C. Ligation master mix was added at 48°C, and after incubation for 30 minutes at the same temperature, PCR reactions were performed (1 minute at 65°C, 45 cycles of 30 seconds at 95°C, 40 seconds at 65°C, and 90 seconds at 72°C). In total, 5 μl of each reaction was pooled and diluted to 10 nM amplicon library using 5 μl of mixture and 95 μl of water. Single-end 1 × 150 sequencing was performed on Illumina MiSeq sequencer.

2.6 | Bioinformatics analysis

Fish plots were made using the R package fishplot (version 0.5).[22] Mutational profiles were analyzed using R package MutationalPatterns (version 1.8.0).[23] Data visualization was done using R package ggplot2 (version 3.2.1) and cBioPortal MutationMapper.[24,25]

3 | RESULTS

A total of 13 cases (nine males and four females) with bone marrow relapse during upfront treatment (<2 years of initial diagnosis)

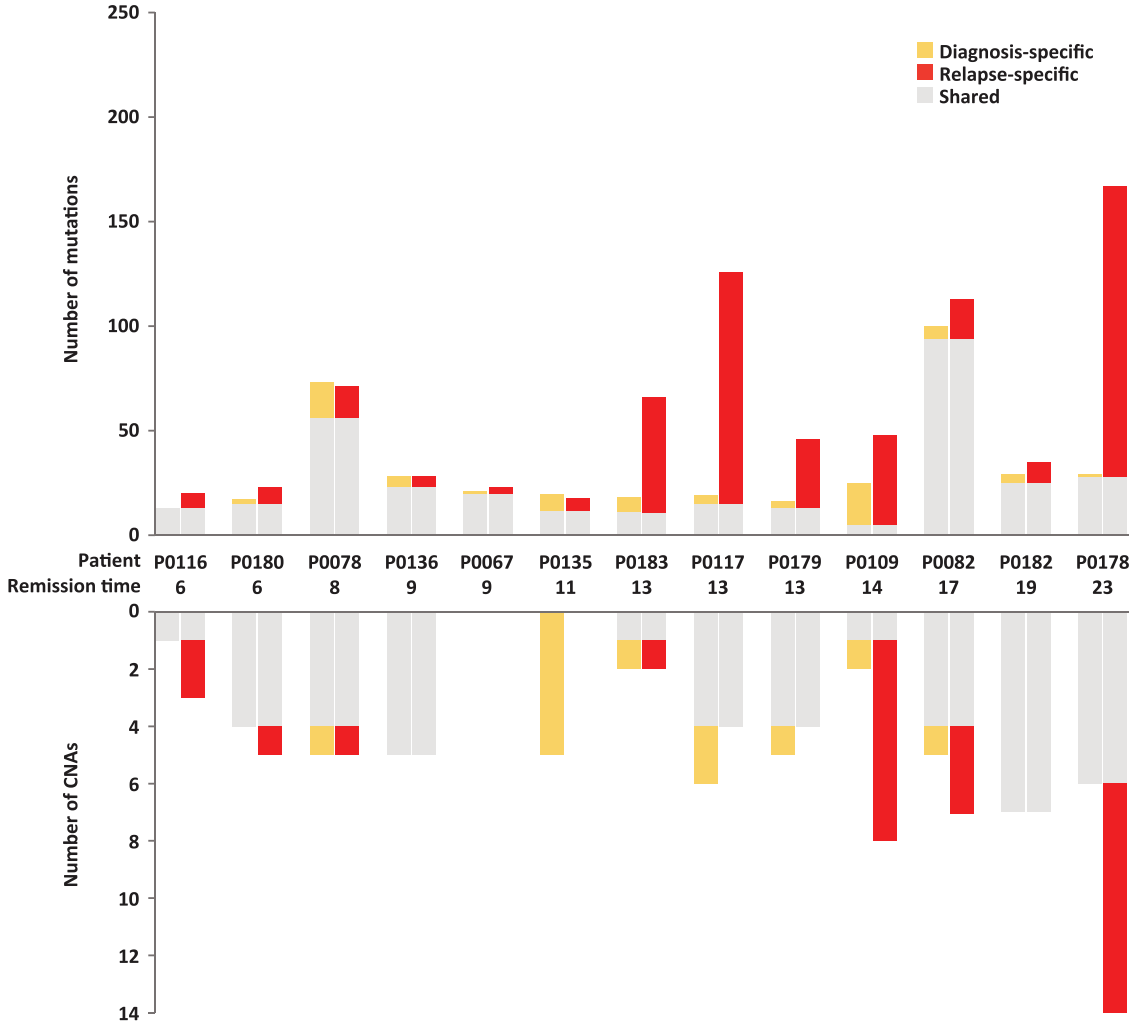

FIGURE 1 Bar plot showing number of mutations (point mutations and indels; upper panel) and copy number alteration (CNAs) (bottom panel) per patient in diagnosis (left bars) and relapse samples (right bars). Gray bars are shared mutations, whereas colored bars represent alterations that are unique at diagnosis (yellow) or relapse (red). Remission time is shown in months. Case P0135 had between 5% and 10% blast cells in the relapse sample, which is below detection limit of the digital multiplex ligation-dependent probe amplification assay

were included in this sequencing study (Table S1). Of these 13 cases, 12 relapsed very early (<18 months from diagnosis), while one *ETV6-RUNX1*-positive case with good initial treatment response experienced a relapse after 23 months while under standard-risk maintenance therapy. The total cohort consisted of patients with *ETV6-RUNX1* ($n = 3$), *TCF3-PBX1* ($n = 2$), hyperdiploid ($n = 3$), hypodiploid ($n = 1$), and B-other ($n = 4$) ALL subtypes, the latter including one patient with Down syndrome (DS) (Table S1). Median time between initial and relapse diagnosis of the total cohort was 13 months. Treatment initiated according to various upfront protocols was discontinued due to infections in three patients and stopped due to severe toxicity in one patient (Table S1).

Following WES of samples taken at diagnosis, complete remission, and relapse, all identified somatic variant calls (single-nucleotide variants and small insertions and deletions) were subjected to targeted capture and deep-sequencing (average depth 1658) in order to validate the mutations and obtain accurate estimates of their MAFs at each time point. These approaches resulted in a total of 861 confirmed somatic mutations with mutant allele frequencies varying from 0.1% to 100% (Tables S5 and S6). Validation rate was 88% for mutations with MAF ≥25%, and for subclonal mutations (MAF <25%) 56% were confirmed. In addition, we identified 99 somatic aneuploidies and copy number alterations (CNAs) using digital MLPA (Tables S5 and S7). Median number of mutations was 21 (range 13–100) for diagnosis samples and 46 (range 20–227) in those from relapse, while median number of aneuploidies and CNAs was five both at diagnosis (range 0–7) and at relapse (range 0–14) (Figure 1 and Table S5). We did not find a correlation between the number of mutations and CNAs in relapse and the duration of first complete remission. After filtering for mutations that were predicted to be pathogenic (non-synonymous, phyloP ≥2.5, CADD PHRED score ≥15), we detected in total 186 mutations in the leukemias of 13 patients (Table S6). The most commonly affected genes, both at diagnosis and relapse, were *KRAS* and *CDKN2A/B* (Figure S1).

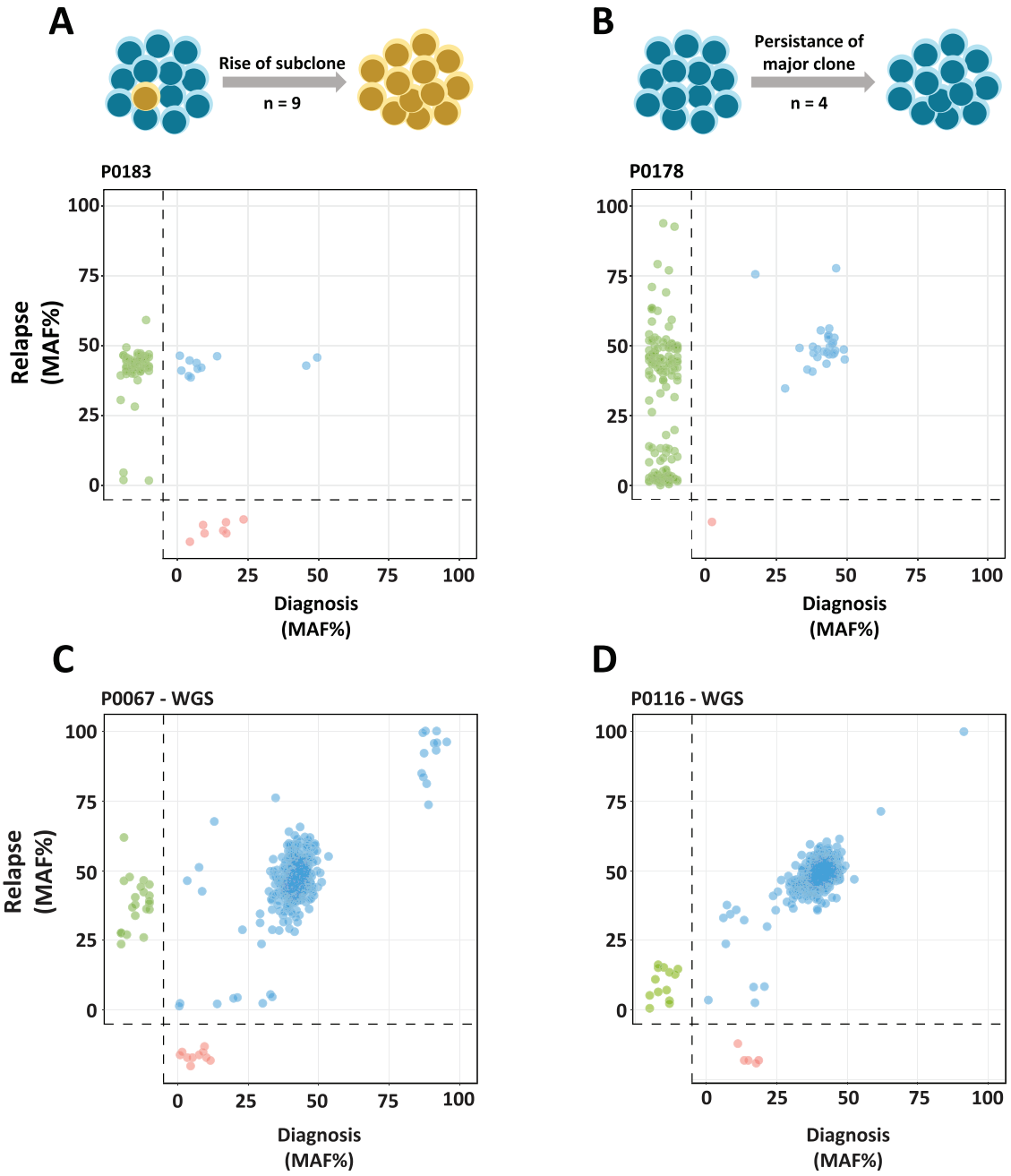

FIGURE 2 Schematic representation of clonal evolution models and scatter plots of representative cases showing clonal dynamics in the two groups of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) that relapsed during treatment. In both models, we observed new mutations at time of relapse. In the first model (A) the dominant clone at relapse originated from a subclone at diagnosis ($n = 9$), while in the second model (B) the dominant clone at relapse originated from the major clone at diagnosis ($n = 4$). Scatter plots from all cases are presented in Figure S2. (C and D) Whole-genome sequencing (WGS) analysis of two patients from the second group revealed that in both patients, there are few mutations that support that the relapse did originate from a minor subclone at diagnosis. Diagnosis and relapse-specific mutations are in red and green, respectively, while alterations shared between two time points are depicted in blue

3.1 | Clonal dynamics of BCP-ALL relapsing during treatment

Based on the clonal dynamics between diagnosis and relapse, we observed two patterns, which have been recognized in unselected diagnosis–relapse cohorts as well.[7,8,26–29] The first pattern was represented by a group of nine cases (69%), in which the dominant clone detected at relapse emerged from one of the existing subclones at diagnosis while acquiring new relapse-specific alterations (Figure 2A and Figure S2). A second pattern was observed in a group of four cases (31%), in which the dominant clone at relapse emerged from leukemic cells that were part of the major clone in diagnosis (Figure 2B and

Figure S2). In both of these groups, we observed a cluster of full-clonal mutations shared between diagnosis and relapse, indicating their origin from the common ancestral clone (Figure 2A,B and Figure S2). In order to further investigate the second pattern and to identify subgroup-specific mutations, we performed WGS of all samples of the two *TCF3-PBX1*-positive cases from this group. Both cases relapsed very early (6 and 9 months after diagnosis). Upon targeted deep-sequencing validation, somatic mutations were compared between all samples of the same patient, which confirmed that in both patients the far majority of mutations were preserved (Figure 2C,D). However, in both patients we also identified a small number of mutations in the major clone at relapse that were subclonal at diagnosis, suggesting that they represent the first pattern of clonal dynamics as well. Together, these findings indicate that the majority of the very early and on-treatment relapses in our cohort originated from a minor clone at diagnosis that branched off from the major clone.

3.2 | Recurrently mutated genes in relapse samples

We identified 118 predicted pathogenic mutations in major clones at relapse, including established drivers of BCP-ALL relapse like *NT5C2*, *CREBBP*, *WHSC1*, and *KRAS* (Figure S3). In addition, in two relapsed cases, we detected nonsense mutations in the cohesin complex component gene *RAD21*, a gene previously not associated with relapse or treatment resistance in BCP-ALL (Figure S3E). Both mutations resulted in truncation of the C-terminal domain of *RAD21*. *RAD21* is essential for sister chromatid cohesion, chromatin organization, and DNA looping through interaction with other members of the cohesin complex, while the C-terminal domain is involved in promotion of apoptosis in a positive-feedback manner.[30–33] Truncating mutations in *RAD21*, as well as other members of the cohesin complex, were previously reported as disease drivers in solid tumors and myeloid malignancies,[34–36] but not in BCP-ALL. Two cases, both with *TCF3-PBX1*-positive tumors, were found to carry a hotspot *WHSC1* E1099K mutation at diagnosis that was preserved at relapse. At diagnosis *TCF3-PBX1*-positive leukemia is associated with favorable outcomes[37–39] and relapses are rare. However, if relapses occur, these are often fatal, particularly if they present very early.[40] Therefore, we investigated whether *WHSC1* mutations were more common in *TCF3-PBX1*-positive relapses. In collaboration with eight other centers, we collected 16 additional *TCF3-PBX1*-positive relapses, of which six occurred very early (Table S2). Upon sequencing of the hotspot region of *WHSC1* in these tumors, one additional patient with very early relapse (1.3 years remission time) was found to carry a E1099K mutation in the relapse. In a previous study, which included 376 patients from two representative Dutch ALL cohorts, we showed that full-clonal *WHSC1* mutations occurred in 2% of pediatric ALL cases at diagnosis.[41] In *TCF3-PBX1*-positive ALL, these mutations were shown to be enriched at diagnosis (15%),[7,42] similar to the percentage we found in *TCF3-PBX1*-positive relapses (3/18; 16%). However, when considering only very early relapses, three of eight (38%) carried *WHSC1* mutations among the very early *TCF3-PBX1* relapses.

3.3 | Mechanisms causing hypermutation in BCP-ALL relapses during treatment

Three cases carried a high mutation burden (≥100), above the threshold of 85 mutations in the coding regions (>1.3 mutation/megabase), which we previously used to define hypermutation in pediatric ALL.[8] This hypermutation phenotype was restricted to relapse in two cases and was already present at the time of diagnosis in a third case. We and others have previously identified several mutational mechanisms being responsible for a high mutation load, including activation-induced deaminase (AID)/apolipoprotein B mRNA editing enzyme (APOBEC) activity.[8,43–45] These processes cause mutational patterns that can be recognized by analyzing single-base substitutions (SBSs) in specific three-nucleotide contexts.[46] Whereas in one case, the mutational profile was highly similar to the previously reported SBS1 mutational signature; we identified AID/APOBEC mutagenesis (SBS2/13) in the remaining two cases. In one of these cases, this mutational process was active at time of diagnosis, with 88% of the mutations being preserved at relapse (Figure 3), whereas the number of newly acquired relapse-specific mutations was low and unlikely to be driven by AID/APOBEC activity. In contrast, in the relapse sample of patient P0178, the far majority of relapse-specific mutations appeared to be driven by AID/APOBEC activity (Figure 3).

3.4 | JAK2-mutated clones in leukemia of a DS patient

In a patient with DS, we identified two dominant clones at diagnosis, each harboring different well-known *JAK2* hotspot mutations, R683S and R683G, with MAF of 24% and 27%, respectively (Figure 4). This observation indicates that most, if not all, leukemic cells in this patient carry either one of these mutations, illustrating the driving capacity of *JAK2* mutations in DS-ALL as reported by others.[47–49] The clone with *JAK2* R683S mutation expanded during treatment and gave rise to the sole dominant clone in relapse, while the clone with *JAK2* R683G was preserved as a minor subclone in relapse (MAF = 7%) (Figure 4).

3.5 | A standard-risk stratified patient with on-treatment relapse

One patient in our cohort was diagnosed with a low-risk *ETV6-RUNX1* subtype and, based on a good response to induction therapy, stratified as standard risk. Nevertheless, he relapsed just before the end of treatment. Genomic characterization revealed that this patient had a subclonal *TP53* R282W hotspot mutation at diagnosis (MAF = 18%). *TP53* mutations are rare in ALL, particularly at diagnosis, but in relapse these aberrations are associated with poor outcome.[40,50–52] Relapse occurred during 6-mercaptopurine and methotrexate courses in which the *TP53*-mutated clone emerged with a whole gene deletion of the second allele. Furthermore, this relapse appeared to be driven by an AID/APOBEC mutational mechanism (Figure 3).

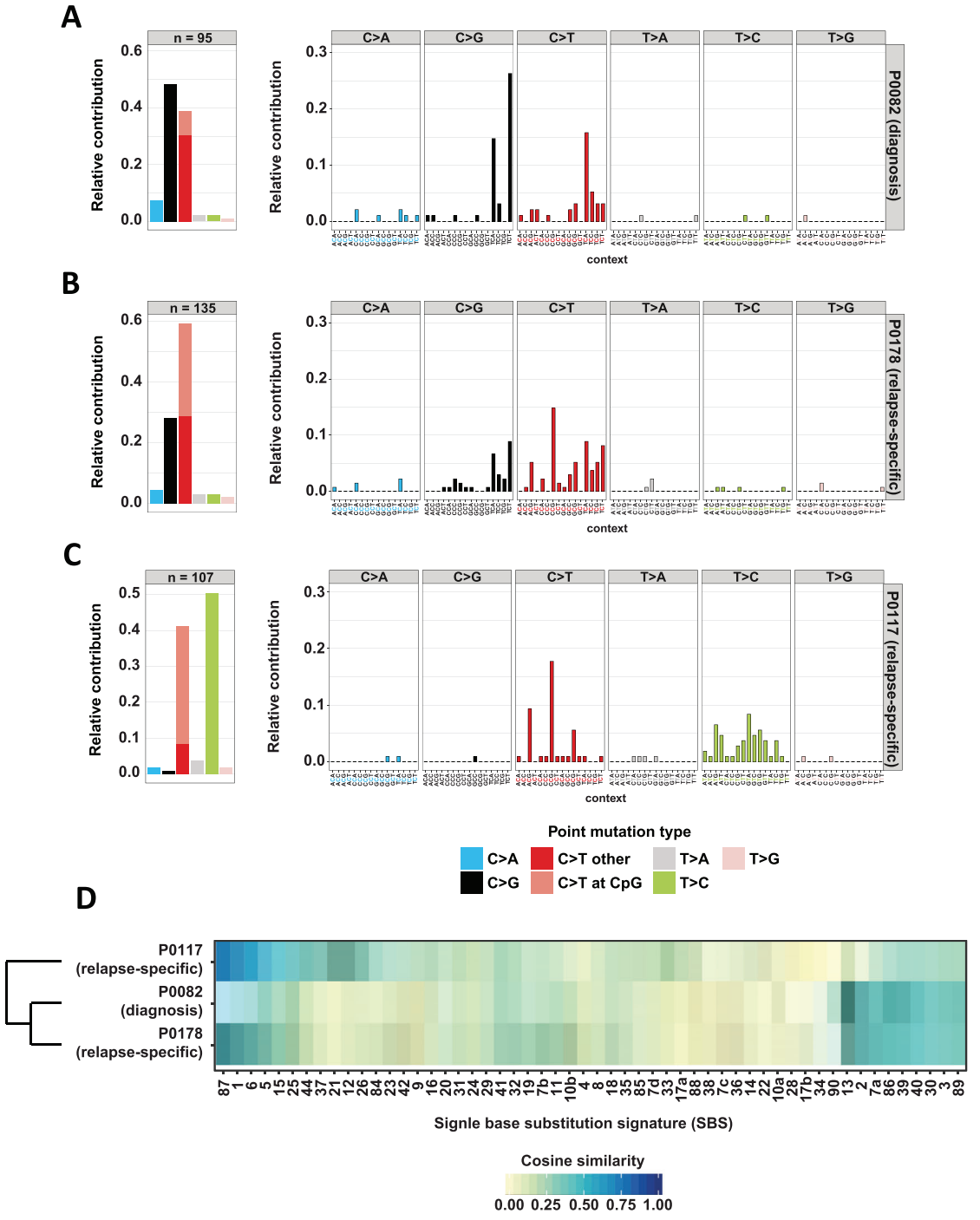

FIGURE 3 Mutational signatures at relapse. (A–C) Frequency of the substitutions (left panel) and mutational profile (right panel) of the three cases with high mutational load (>1.3 mutation/megabase). (D) Comparison of mutational profiles between four cases and known COSMIC signatures revealed high cosine similarity between SBS2 and SBS13 attributed to aberrant AID/APOBEC activity and SBS1 clock-like signature attributed to spontaneous deamination of methylated cytosine in CpGs

4 | DISCUSSION

During disease progression, multiple clones emerge, some of which may harbor alterations associated with treatment resistance. Chemotherapy can drive selection of these resistant clones, which may lead to their expansion and to relapse during treatment. Furthermore, intrinsic factors, such as drug toxicity and infections, can cause treatment interruption and subsequently potential relapse. The relapses in our study occurred during treatment and have poor prognosis compared to late relapses, after treatment has ended. In this study, we performed WES and digital MLPA of diagnosis–relapse pairs and complete remission samples of 12 cases who relapsed very early (<18 months from initial diagnosis) and one case with favorable prognosis and relapse at the end of treatment. Most cases (9/13)

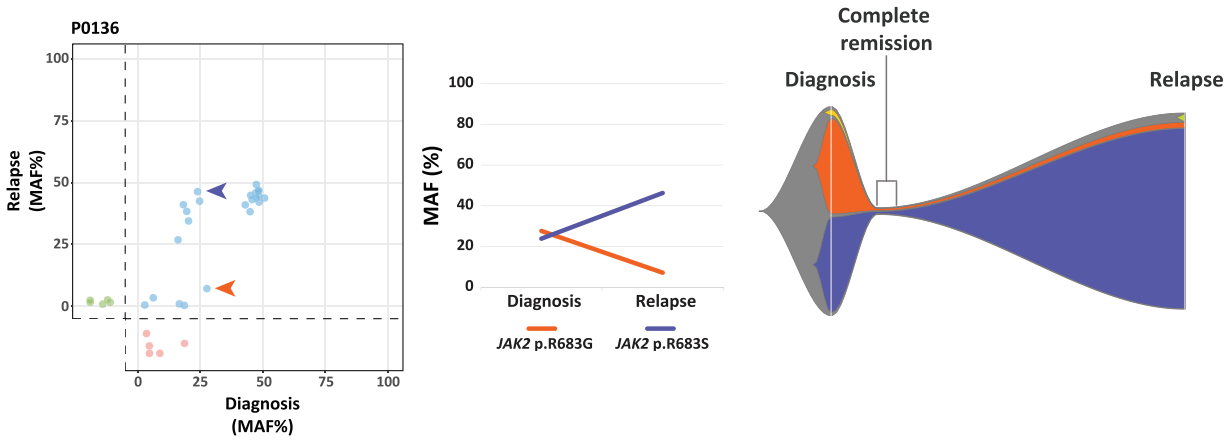

FIGURE 4 Clonal dynamics in a Down syndrome patient with two codominant clones in leukemia at diagnosis, each harboring *JAK2* mutations. One of the clones expanded and became a dominant clone in relapse, while the other clone decreased to a subclone at relapse

did not experience any treatment interruptions before the relapse occurred, indicating that treatment-driven clonal selection may be the dominant mechanism.

Our data indicate that clonal evolution in cases that relapse during treatment is a dynamic process, with the majority of relapses evolving from a subclone present at the time of diagnosis, which acquired additional mutations. In the majority of cases, we were able to identify preserved or newly acquired relapse-associated genetic alterations in the rising clone at relapse. This finding supports the model in which early relapses are associated with more dynamic changes in clonal evolution compared to late relapses.[8,53]

In this study, we identified several genes recurrently altered in the major clone of relapsed ALL, including *CREBBP*, *WHSC1*, *NT5C2*, *IKZF1*, *CDKN2A*, and *CDKN2B*, which were previously associated with early relapse, in different studies.[7–11,14,54] Alterations in epigenetic modifiers (for example, *ARID2*, *EP300*, *TOX*, and *ERG*) were rare or absent, which is in line with previous studies suggesting their association with late relapse.[8,44,53] In addition, we found two cases with mutations in cohesin complex member *RAD21*, a gene that was previously not associated with relapsed BCP-ALL. Studies have shown that mutations in cohesin complex genes are often truncating and have the potential to disrupt the entire complex.[35,36] However, myeloid leukemias with truncating mutations in cohesin complex genes usually do not have aneuploidies, suggesting that the mechanism in which these genes drive malignant transformation might include one of their non-canonical functions.[30,31,33,35,36] Although previous studies indicate importance of the conserved C-terminal domain for induction of apoptosis,[30,31] the relevance of *RAD21*, as well as other cohesin complex mutations, for BCP-ALL development and treatment failure still needs to be fully elucidated.

We have identified a hotspot *WHSC1* E1099K mutation in two patients with very early relapse and *TCF3-PBX1*-positive leukemia, a subtype with favorable outcome at initial diagnosis. In both cases, the mutation was preserved from a major clone already present at diagnosis, indicating that this alteration was an early event during disease development. Further screening of 16 relapsed cases with *TCF3-PBX1* leukemia revealed one additional relapse with the E1099K *WHSC1* mutation, which interestingly also relapsed very early. Furthermore, Ma et al. reported five very early *TCF3-PBX1*-positive relapses, three of which carried *WHSC1* mutations as well.[7,8] Although patients with *TCF3-PBX1*-positive BCP-ALL have favorable prognosis in modern treatment protocols and rarely relapse,[5,55,56] the outcome is poor when relapses occur.[57,58] Clinical diversity and discrepancies in outcome between relapsed and nonrelapsed patients suggest the presence of additional prognostic biomarkers.[58] *WHSC1* mutations may drive one of the mechanisms leading to (very early) relapse in *TCF3-PBX1*-positive leukemia. Mutations in the SET domain of methyltransferase *WHSC1* were shown to cause perturbations in epigenetic makeup through chromatin remodeling.[42,59] Among pediatric tumors, the frequency of *WHSC1* mutations is highest in BCP-ALL, especially in *ETV6-RUNX1* (20%) and *TCF3-PBX1* (15%) ALL subtypes.[42] Therefore, a *WHSC1* mutation is unlikely to be a strong risk factor for relapse in these subtypes by itself. However, considering the apparent higher frequency of these hotspot mutations in very early *TCF3-PBX1*-positive relapses, an epigenetic event may be involved, which is worth studying further, for example, by comparing the epigenetic landscape of *WHSC1*-mutated nonrelapsed and very early relapsed *TCF3-PBX1*-positive cases.

We did find interesting candidate mutations that could explain the observed clonal dynamics that preceded the relapse. For example, the proliferation of one of the two *JAK2* mutated clones in the patient with DS-ALL is in line with previous functional studies, which showed that *JAK2* R683S has higher proliferative advantage compared to R683G mutations in cell lines.[47,49] DS-ALL are more prone to treatment-related morbidity and mortality, which is the reason for reduction of treatment intensity in this group.[5,20,60] Our findings emphasize the fact that *JAK2* mutations may enhance proliferative capacities of DS-ALL cells, and support current ongoing trials using JAK inhibitors in DS-ALL to prevent relapses while reducing treatment intensity.

A second example was the patient with minimal residual disease (MRD)-based standard-risk stratification, who nevertheless relapsed while on treatment. This study identified a likely explanation for this particular patient as he carried a hotspot *TP53* mutation in a subclone at diagnosis, which grew out to relapse upon deletion on the

wildtype allele. *TP53* alterations are enriched in relapsed BCP-ALL,[7,8] and associated with unfavorable outcome even in patients with good initial response to induction therapy.[61] Previously, we have shown that subclonal (likely) pathogenic mutations in *TP53* are relatively common, but do not necessarily indicate a high risk for relapse, even in standard risk-stratified patients ($n = 9$).[41] Furthermore, mutations on both alleles are associated with poor outcome, as demonstrated in adult ALL,[62] but the risk of acquiring a second hit has not been studied in larger cohorts. Nevertheless, as *TP53* mutations are enriched in relapse,[8,44,50,51] the detection at time of diagnosis, even at subclonal level, may warrant careful monitoring by MRD analysis, particularly when stratified in low-risk treatment arms.

Taken together, our data reveal highly dynamic clonal evolution in cases that relapse very early, with subclones present at diagnosis emerging as new dominant clones in the majority of cases. While the small cohort size limits our capacity to generalize, specific mutations detected at diagnosis, like the *TP53* mutation in a standard-risk patient or the *WHSC1* E1099K mutations in three *TCF3-PBX1*-positive leukemias, are likely to have contributed to these relapses. Furthermore, we identified recurrent mutations in *RAD21* at relapse, a gene previously not associated with relapsed BCP-ALL.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The sequence data that support the findings of this study have been deposited in the European Genome-phenome Archive (EGA) under accession number EGAS00001005615.

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SUPPORTING INFORMATION

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