

Research Article

Molecular Profiling Reveals Novel Gene Fusions and Genetic Markers for Refined Patient Stratification in Pediatric Acute Lymphoblastic Leukemia

Borbála Péterffy^{a,b}, Szilvia Krizsán^{a,b,c}, Bálint Egyed^{a,b,c}, Gábor Bedics^{a,b}, Anne Benard-Slagter^d, Sander Palit^d, Dániel János Erdélyi^c, Judit Müller^c, Tibor Nagy^{a,b,e}, Lajos László Hegyi^{a,b}, Anna Bekő^{a,b}, Lili Anna Kenéz^{a,b}, Zsuzsanna Jakab^f, György Péter^g, Marianna Zombori^g, Krisztina Csanádi^g, Gábor Ottóffy^h, Katalin Csernus^h, Agnes Vojcek^h, Lilla Györgyi Tiszlaviczⁱ, Krisztina Mita Gáborⁱ, Ágnes Kelemen^j, Péter Hauser^j, Krisztián Kállay^k, Gabriella Kertész^k, Zsuzsanna Gaál^l, István Szegedi^l, Gábor Barna^{a,b}, Ágnes Márk^{a,b}, Irén Haltrich^c, Zsuzsanna Hevessy^m, Anikó Ujfalusi^m, Béla Kajtárⁿ, Botond Timár^{a,b}, Csongor Kiss^l, Gergely Kriván^k, András Matolcsy^{a,b,o}, Suvi Savola^d, Gábor Kovács^c, Csaba Bódör^{a,b}, Donát Alpár^{a,b,*}

^a HCEMM-SE Molecular Oncohematology Research Group, Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary; ^b MTA-SE "Lendület" Molecular Oncohematology Research Group, Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary; ^c Pediatric Center, Semmelweis University, Budapest, Hungary; ^d MRC Holland, Department of Oncogenetics, Amsterdam, The Netherlands; ^e Department of Biochemistry and Molecular Biology, University of Debrecen, Debrecen, Hungary; ^f Hungarian Childhood Cancer Registry, Hungarian Pediatric Oncology Network, Budapest, Hungary; ^g Hemato-Oncology Unit, Heim Pál Children's Hospital, Budapest, Hungary; ^h Department of Pediatrics, Oncohaematology Division, University of Pécs Medical School, Pécs, Hungary; ⁱ Department of Pediatrics and Pediatric Health Care Center, Faculty of Medicine, University of Szeged, Szeged, Hungary; ^j Velkey László Child's Health Center, Borsod-Abaúj-Zemplén County Central Hospital and University Teaching Hospital, Miskolc, Hungary; ^k Pediatric Hematology and Stem Cell Transplantation Department, Central Hospital of Southern Pest, National Institute of Hematology and Infectious Diseases, Budapest, Hungary; ^l Division of Pediatric Hematology-Oncology, Institute of Pediatrics, Faculty of Medicine, University of Debrecen, Debrecen, Hungary; ^m Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Hungary; ⁿ Department of Pathology, University of Pécs Medical School, Pécs, Hungary; ^o Division of Pathology, Department of Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

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ABSTRACT

Risk-adapted treatment protocols conferred remarkable improvement in the survival rates of pediatric acute lymphoblastic leukemia/lymphoma (ALL/LBL). Nevertheless, clinical management is still challenging in certain molecular subgroups and in the presence of alterations associated with an increased rate of relapse. In this study, disease-relevant genomic and transcriptomic profiles were established in a prospective, multicenter, real-world cohort involving 192 children diagnosed with ALL/LBL. Gene fusions were detected in 34.9% of B-ALL and 46.4% of T-ALL patients, with novel chimeric genes involving *JAK2*, *KMT2A*, *PAX5*, *RUNX1*, and *NOTCH1*, and with *KMT2A*-rearranged patients displaying the worst 3-year event-free survival ($P = .019$). Nonsynonymous mutations were uncovered in 74.9% of the analyzed patients, and pairwise scrutiny of genetic lesions revealed recurrent clonal selection mechanisms commonly converging on the same pathway (eg, Ras, JAK/STAT, and Notch) in individual patients. Investigation of matched diagnostic and relapse samples unraveled complex subclonal variegation, and mutations affecting the *NT5C2*, *TP53*, *CDKN2A*, and *PIK3R1* genes, emerging at the time of relapse. *TP53* and *CREBBP* mutations, even as subclonal

These senior authors contributed equally: Csaba Bódör and Donát Alpár.

* Corresponding author.

E-mail address: alpar.donat@semmelweis.hu (D. Alpár).



aberrations, were associated with shorter 3-year event-free survival among all patients with B-ALL (*TP53* mutant vs wild-type: $P = .008$, *CREBBP* mutant vs wild-type: $P = .010$), and notably, B-ALL patients showing no measurable residual disease on day 33 could be further stratified based on *TP53* mutational status ($P < .001$). Our in-depth molecular characterization performed across all risk groups identified novel opportunities for molecularly targeted therapy in 55.9% of high-risk and 31.6% of standard/intermediate-risk patients.

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Introduction

Acute lymphoblastic leukemia/lymphoma (ALL/LBL) is the most common malignancy in childhood, accounting for over one-third of disability-adjusted life-years in children with cancer, thus representing the most prominent burden in pediatric oncology.¹ Although advances in therapeutic strategies have led to a significant improvement in the 5-year survival rates, reaching 85% to 90% in developed countries,² clinical management of ALL progression and relapse remains challenging. Clinical manifestation of pediatric ALL is typically preceded by a covert preleukemic phase, initiated by the first genomic driver aberration frequently occurring in utero as demonstrated in several genetic subtypes of the disease,^{3,4} followed by secondary alterations conferring a branching subclonal architecture in the leukemic cell population.^{5–8} During the past decade, substantial efforts have been made to understand the diverse and clinically relevant molecular landscape of ALL, which today is reflected by over 30 established or provisional subtypes of the disease.^{9–12} The advent of next-generation sequencing (NGS) enabled the identification of novel recurrent genetic alterations and transcriptional profiles, leading to the introduction of new disease subgroups with distinctive genomic and transcriptomic features.^{13–15} In a subset of ALL subtypes, survival rates still fall significantly below the average; therefore, advanced risk assessment supported by high-throughput sequencing is highly warranted to aid patient stratification and further improve therapeutic intervention.¹⁶ Moreover, in-depth genomic and transcriptomic profiling may uncover potential therapeutic targets in individual patients and contribute to the identification of novel biomarkers with prognostic relevance.

Relapse is the major cause of treatment failure among patients with pediatric ALL, with previous studies identifying distinct patterns of acquisition or enrichment of relapse-associated genetic aberrations.^{17,18} Improved understanding of evolutionary mechanisms, as well as identification of mutational targets conferring relapse, may have direct clinical implications. Early detection of aberrations driving disease progression can support the prediction of relapse and facilitate an informed therapy adjustment, potentially even guiding targeted treatment of relapse-fated clones prior to additional diversification and clonal selection, leading to clinically manifest secondary resistance.

In this study, we interrogated the genomic and transcriptomic landscape in a real-world cohort of unselected Hungarian patients consecutively diagnosed with pediatric ALL, using indication-optimized deep sequencing. Besides profiling and analyzing the cosegregation of leukemia-relevant alterations, we have uncovered disease progression-associated changes in the composition and abundance of somatic mutations and identified alterations with prognostic and/or therapeutic relevance. Our work facilitates a more refined risk assessment and patient stratification, which can aid the advanced diagnostics and improved clinical management of patients.

Materials and Methods

Patients and Samples

In the frame of the Hungarian Pediatric Leukemia Molecular Profiling Program, 192 patients (female:male ratio: 1.4:1) diagnosed with B-cell precursor acute lymphoblastic leukemia ($n = 150$) or lymphoblastic lymphoma ($n = 3$), as well as with T-cell precursor ALL ($n = 30$) or lymphoblastic lymphoma ($n = 9$), at a median age of 5 years (range: 1–17 years) were investigated. Diagnoses were established based on morphologic, immunophenotypic, and genotypical criteria in the Department of Pathology and Experimental Cancer Research—Semmelweis University, in the Department of Pathology—University of Pécs, or in the Department of Laboratory Medicine—University of Debrecen between 2018 and 2021. Patients were risk stratified and treated according to ALL IC-BFM 2002, ALL IC-BFM 2009, I-BFM NHL LL 2009, LBL 2018, and Interfant-06 protocols, with a median follow-up time of 29 months (range: 0–135 months).^{19,20} The diagnostic specimens contained, on average, 78% (range: 20% to 99%) of leukemic blasts as assessed by flow cytometry. Measurable residual disease (MRD) assessment was performed on bone marrow samples drawn on days 15 ($n = 177$), 33 ($n = 180$), and 78 ($n = 180$) in patients with ALL, using a 10-color BD FACSLytic or an 8-color BD FACSCanto II flow cytometer (BD Bioscience) and Kaluza 2.1.1 (Beckman Coulter), with at least 500,000 events analyzed at each time point.^{21,22} Baseline characteristics of the patients are summarized in [Supplementary Table S1](#). Ethical approval (45563-2/2019/EKU) from the Medical Research Council of Hungary, and written informed consent from the patients and/or from the parents or guardians was obtained for the study, which was conducted in accordance with the Declaration of Helsinki.

Diagnostic bone marrow ($n = 171$), peripheral blood ($n = 12$), lymph node ($n = 5$), pleural fluid ($n = 2$), or skin ($n = 2$) samples were collected and analyzed in this study. In addition, 26 bone marrow or peripheral blood samples drawn from 19 patients (15 B-ALL and 4 T-ALL patients) at the time of first, second, or third relapse were investigated. Mononuclear cells from bone marrow and peripheral blood were separated by density-gradient centrifugation in Lymphoprep density-gradient medium (Stemcell Technologies), following the manufacturer's protocol. Genomic DNA and RNA were extracted with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's recommendations.

DNA Copy Number Analysis

DNA copy number aberrations (CNA) were screened by multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA P335-C1, P383-A2, and P327-B2 probemixes and

by digitalMLPA using the SALSA digitalMLPA D007-X7 Acute Lymphoblastic Leukemia probemix (MRC Holland).

Copy number status of selected genes/regions (*EBF1*, *IKZF1*, *PAX5*, *CDKN2A/B*, *ETV6*, *BTG1*, *RB1*, and *PAR1*) recurrently altered in B-ALL/LBL were interrogated with the P335-C1 MLPA probemix. An additional analysis with the P202-C1 probemix was performed on B-ALL samples harboring *IKZF1* deletion and potentially fulfilling the criteria of *IKZF1*^{plus}²³ in order to test for the presence/absence of concurrent *ERG* deletion. In patients with T-ALL/LBL, *STIL::TAL1*, and *NUP214::ABL1* fusions along with CNAs in the *LEF1*, *CASP8AP2*, *MYB*, *EZH2*, *CDKN2A/B*, *MTAP*, *MLLT3*, *PTEN*, *LMO1*, *LMO2*, *NF1*, *SUZ12*, *PTPN2*, and *PHF6* genes were investigated using the P383-A2 probemix. MLPA reactions were performed according to the manufacturer's recommendations using 50 to 100 ng input genomic DNA. Amplified products were separated and quantified by capillary electrophoresis on an ABI 3500 Genetic Analyser (Life Technologies), with all data being processed and analyzed using *Coffalyser.Net* Software (MRC Holland). After intra- and inter-sample normalization, copy number status of each interrogated locus was determined, also considering the leukemic blast purity in the specimen as assessed by flow cytometry.²⁴

digitalMLPA analyses utilizing NGS readout were performed using a recently updated in-development version of the D007 ALL probemix (version D007-X7, MRC Holland), which includes (1) target probes for regions recurrently affected by copy number alterations in B-cell or T-cell ALL; (2) digital karyotyping probes covering all chromosome arms to identify gross chromosomal alterations and functioning as reference probes for data normalization; and (3) internal control probes for quality control and sample assessment. Genomic positions of probes are listed in [Supplementary Table S2](#). digitalMLPA reactions were carried out according to previously published protocols^{24,25} using 40 ng input DNA. Final PCR products were pooled and sequenced on a MiSeq platform (Illumina) using v3 chemistry with 115bp single-read configuration. Data processing and analysis were performed as described previously,^{24,25} using *Coffalyser* digitalMLPA software v221020.1234 (MRC Holland). After intra- and inter-sample normalization, copy number status of each interrogated locus was determined, also considering the leukemic blast purity in the specimen as assessed by flow cytometry.²⁴ Comprehensive descriptions of laboratory and bioinformatic protocols have been previously published.²⁵

Mutational Profiling by Deep DNA Sequencing

Targeted NGS was performed using a QIAseq Targeted DNA Custom Panel (Qiagen) covering 103 disease-relevant genes frequently (>2%) altered in ALL ([Supplementary Table S3](#)). Libraries were prepared using 40 ng genomic DNA (FFPE samples: 100 ng) according to the manufacturer's recommendations, including fragmentation, unique molecular index assignment, and target enrichment using region-specific primers. After equimolar pooling, libraries were sequenced on MiSeq platform (Illumina) using v2 chemistry with 150 bp paired-end configuration. Data processing and analysis were performed with the QIAseq Targeted DNA Panel Analysis pipeline (Qiagen) using the smCounter2 workflow utilizing unique molecular identifier-based variant calling which facilitates the accurate detection of low-frequency variants.²⁶ For reliable detection of high-confidence mutations, called variants were filtered and excluded if they did not pass the predefined quality criteria of smCounter2, occurred with a variant allele frequency (VAF) of <2% or the total coverage at the affected locus did not reach 100×. Furthermore, we excluded synonymous

variants, intronic variants, variants present with a minor allele frequency of >1% in The Genome Aggregation Database (gnomAD 2.1) or in the 1000Genomes database (Phase 3 v5a), as well as novel variants predicted as benign or likely benign by in silico pathogenicity predictors such as VarSome and Franklin²⁷ (<https://franklin.genoox.com>). Putative germline variants detected with a VAF of 45% to 55% and confirmed in available samples of remission were not reported. SnpSift version 4.3t was used for annotating variants with dbSNP (v151), ClinVar (2019-02-04), and COSMIC (v84) coding mutations, and variant consequence/impact was analyzed using Ensembl VEP (build 91).

Gene Fusion Screening by Targeted RNA Sequencing

Gene fusions were analyzed using the TruSight RNA Pan-Cancer Panel (Illumina) covering 1385 genes recurrently altered in various malignancies. Libraries were prepared following the manufacturer's instructions. After RNA fragmentation, reverse transcription, and adapter ligation, ligated cDNA products were amplified. Targeted regions of interest were hybridized with sequence-specific baits and captured using Streptavidin magnetic beads, followed by amplification of the enriched libraries. Normalized libraries were pooled equimolarly and sequenced on a MiSeq platform using v3 chemistry with 75bp paired-end configuration. Fusion transcripts were called with STAR-Fusion v1.9.0, FusionCatcher v1.20 and Pizzly v0.37.3 software tools.^{28–30} Identified gene fusions were validated by fluorescence in situ hybridization (FISH), real-time quantitative PCR, or Sanger sequencing.

Validation Cohort

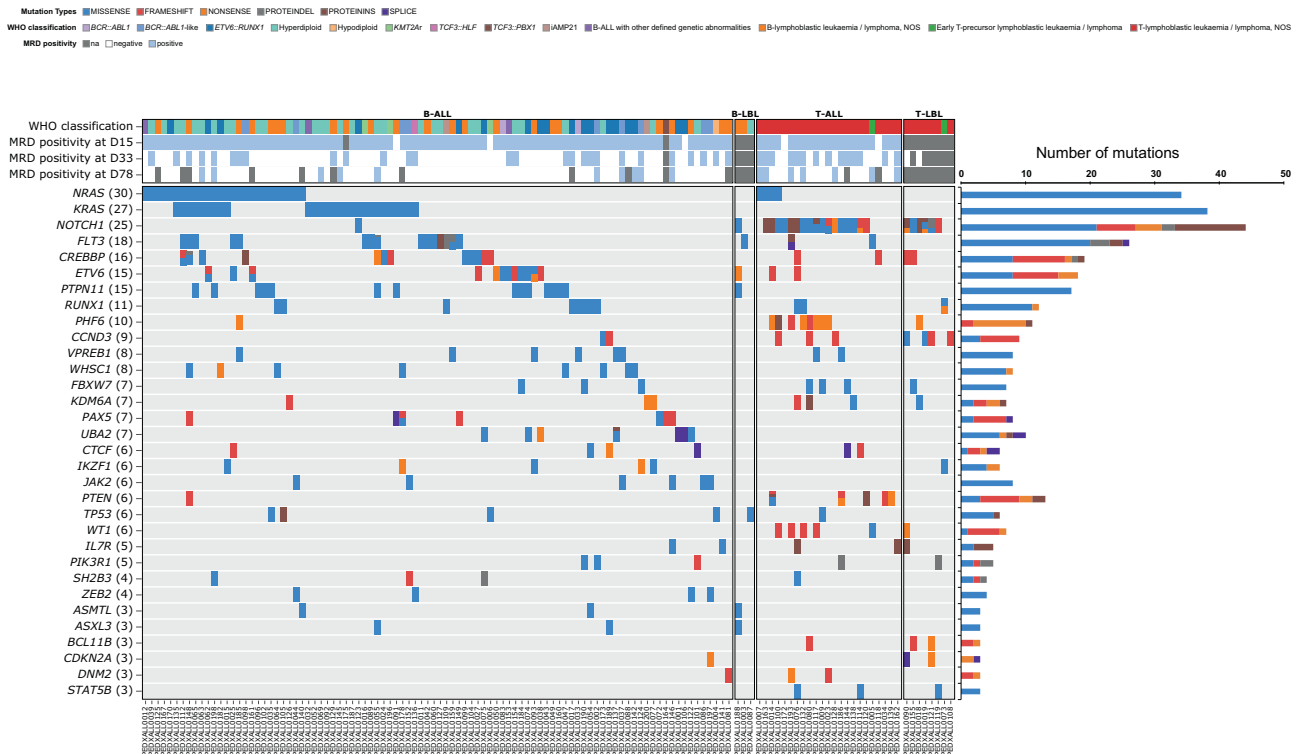
Two hundred sixty-five patients involved in the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative (<https://ocg.cancer.gov/programs/target>) ALL Phase 2 study (phs000464) were included in the validation cohort. Leukemic samples were genomically profiled at the Baylor College of Medicine using the SeqCap EZ Human Exome 2.0 protocol (Nimblegen). Data used for our analysis can be accessed at https://portal.gdc.cancer.gov/analysis_page?app=Projects. Patients diagnosed with B-ALL at age <18, and reported with event-free survival (EFS) time and mutational exome profile were included.

Statistical Analysis

The co-occurrence of genetic alterations and MRD positivity was evaluated using Fisher's exact tests. Overall survival (OS) and EFS, defined as the time to relapse or death, were estimated using the Kaplan-Meier method and statistically compared with log-rank test. Analyses were performed and figures were prepared using SPSS Statistics 28.0.1.0 (IBM Corporation) and GraphPad Prism version 8.0.2 (GraphPad Software Inc). Oncoplots and Disco plots were created using the PeCan ProteinPaint tool.³¹

Results

In total, diagnostic samples from 180 patients with B-ALL (n = 150) or T-ALL (n = 30) and 12 patients diagnosed with B-LBL (n = 3) or T-LBL (n = 9) were analyzed. Targeted DNA- and RNA-sequencing complemented with MLPA was performed in

**Figure 1.**

Short somatic variants detected by targeted DNA sequencing in diagnostic samples of 187 patients with pediatric ALL/LBL. Immunophenotype, World Health Organization classification, measurable residual disease (MRD) status on days 15, 33, and 78 of therapy, as well as mutation type and abundance of affected genes are also illustrated. Genes altered in at least 3 patients are displayed.

diagnostic samples of 165 patients (B-ALL: $n = 134$; T-ALL: $n = 27$; T-LBL: $n = 4$), whereas in 27 cases (B-ALL: $n = 16$; T-ALL: $n = 3$; B-LBL: $n = 3$; T-LBL: $n = 5$), either NGS (DNA-seq or RNA-seq) or MLPA results were unavailable. In addition, digitalMLPA was performed on 114 samples (B-ALL: $n = 90$; T-ALL: $n = 22$; T-LBL: $n = 2$; [Supplementary Fig. S1](#)). The distribution of World Health Organization subtypes in the whole patient cohort and across age groups is depicted in [Supplementary Figure S2](#).

Genomic Alterations Unraveled by Targeted Next-Generation Sequencing, Multiplex Ligation-Dependent Probe Amplification, and digitalMLPA

Targeted DNA sequencing identified 401 nonsynonymous single-nucleotide variants and short insertions/deletions in 187 diagnostic samples analyzed ([Fig. 1](#); [Supplementary Table S4](#)), with a mean of 1.78 (range: 0–10) mutations detected per patient. In total, 74.9% (140/187) of patients harbored at least 1 mutation, observed in 58/103 genes analyzed. Compared with B-ALL/LBL, the average number of detected mutations was significantly higher in T-ALL/LBL patients (1.5 ± 1.4 vs 2.7 ± 2.2 ; $P < .001$, [Supplementary Fig. S3A](#)). In B-ALL, mutations most frequently affected rat sarcoma pathway genes, such as *KRAS* (18.5%; 27/146), *NRAS* (17.8%; 26/146), and *FLT3* (10.3%; 15/146), whereas in T-ALL, *NOTCH1* (58.6%; 17/29), *PHF6* (27.6%; 8/29), *PTEN* (17.2%; 5/29), and *WT1* (17.2%; 5/29) were the most commonly altered genes. In terms of mutation classes, missense (64.1%; 257/401) mutations dominated in the patient cohort, with frameshift (15.2%; 61/401) mutations being the second most frequent alterations. The distribution of variant

allele frequencies in each altered gene is shown in [Supplementary Figure S3B](#).

Besides mutations reported in the public databases used for annotations, 175 variants previously not described as recurrent alterations were detected. Genes mutated in $\geq 5\%$ of the samples included *NRAS*, *KRAS*, *NOTCH1*, *FLT3*, *CREBBP*, *ETV6*, *PTPN11*, *RUNX1*, *PHF6*, and *CCND3*. In addition, targeted DNA sequencing uncovered rare but well-known mutations, such as *ZEB2* p.Q1072 (2.7%; 4/146) and *IKZF1* p.N159Y (1.4%; 2/146), which have putative clinical significance in B-ALL. Further scrutinizing the variants identified in *TP53*, we noticed that 3 of 6 mutations affected the p.R282 hotspot, with one of those—an in-frame insertion—not reported previously. All detected *TP53* variants affected regions coding the DNA binding domain. Mutations observed in the *IKZF1*, *ZEB2*, and *TP53* genes are shown in [Supplementary Figure S4](#).

DNA CNAs were identified by MLPA in diagnostic samples of 55.5% (81/146) of B-ALL and 66.7% (20/30) of T-ALL patients. Deletions most commonly affected the *CDKN2B* (20.5%; 30/146), *CDKN2A* (19.9%; 29/146), *PAX5* (16.4%; 24/146), and *ETV6* (16.4%; 24/146) genes in B-ALL patients. Twenty-three (15.8%; 23/146) patients with B-ALL harbored *IKZF1* deletion, with 7 of them displaying *IKZF1*^{plus} genotype (4.8%; 7/146). *CDKN2A* (56.7%; 17/30), *CDKN2B* (53.3%; 16/30), and *MTAP* (30.0%; 9/30) were most frequently altered in T-ALL patients, reflecting the common deletion of 9p21 in this subgroup of ALL ([Fig. 2](#); [Supplementary Table S5](#)).

digitalMLPA revealed additional CNAs in regions not covered by MLPA probemixes in 30 patients (24 B-ALL, 6 T-ALL). Using this more comprehensive technique, CNAs were detected in 95.6% (86/90) of B-ALL and 91.7% (22/24) of T-ALL patients, with an average of 12.6 CNAs per patient (mean subchromosomal alteration: 10.6, mean whole chromosome gain/loss: 2.0). The vast majority (79.8%)



Figure 2.

(A) Copy number aberrations detected by MLPA in diagnostic samples of 81/146 patients with B-ALL. (B) Copy number alterations detected in 20 samples of 30 T-ALL and 6 T-LBL patients, analyzed.

of whole chromosome copy number changes were observed in patients with hyperdiploid karyotype, with gains of chromosomes 14, 21, X, 6, 17, 18 being the most common alterations, and chromosomes 21 and X showing frequent acquisition of multiple copies. The 22 patients harboring high-hyperdiploid karyotype presented with modal chromosome numbers ranging from 51 to 58, whereas 1 patient harbored (PEDXALL113) 70 chromosomes, indicating a near-triploid karyotype (Supplementary Fig. S5). The most frequent subchromosomal alterations affected *IGLV4-60* (37.8%; 34/90), *VPREB1* (25.6%; 23/90), *CDKN2A* (23.3%; 21/90), and *CDKN2B* (22.2%; 20/90) in B-ALL patients, and *CDKN2A* (66.7%; 16/24), *CDKN2B* (62.5%; 15/24), *MTAP* (62.5%; 15/24), and *MLLT3* (29.2%; 7/24) in patients with T-ALL. Biallelic losses most commonly affected the *CDKN2A* (31/121; 16 B-ALL, 15 T-ALL), *CDKN2B* (25/121; 12 B-ALL, 13 T-ALL), and *MTAP* (19/121; 7 B-ALL, 12 T-ALL) genes. Additionally, we identified subclonal alterations in 42.1% (48/114) of the analyzed samples. All CNA detected by digitalMLPA are summarized in Supplementary Figure S5.

Gene Fusions Detected by Targeted RNA-Seq

Chimeric genes were uncovered in 34.9% (59/169) of B-ALL and 46.4% (13/28) of T-ALL patients, with *ETV6::RUNX1*, *P2RY8::CRLF2* and *TCF3::PBX1* being the most frequently detected alterations in B-ALL, and *STIL::TAL1* in T-ALL. In addition to gene fusions routinely

tested in clinical diagnostics and therefore validated by FISH, less common chimeric genes with known clinical relevance were also identified. Rearrangements driving *BCR::ABL1*-like phenotype were observed in 7.7% (13/169) of B-ALL patients and involved *ABL1*, *ABL2*, *CRLF2*, *EPOR*, and *JAK2* genes with various fusion partners. Additional uncommon fusions included *DUX4* (*DUX4::IGH*, *n* = 2), *MEF2D* (*MEF2D::BCL9*, *n* = 1), *NUTM1* (*ACIN1::NUTM1*, *n* = 1), and *ZNF384* (*EP300::ZNF384*, *n* = 1). Targeted RNA sequencing also allowed for the rapid and straightforward identification of *KMT2A* partner genes such as *AFF1*, *AFDN*, *USP2*, and *MLLT1*, typically not identified by FISH, requiring the use of multiple target-specific PCR tests or time-consuming and labor-intensive karyotyping in clinical diagnostics. Novel in-frame fusions involving *JAK2* (*KDM4C::JAK2*), *KMT2A* (*KMT2A::KNSTRN*), *PAX5* (*PAX5::MLLT10*), *RUNX1* (*RUNX1::DNAJC15*), and *NOTCH1* (*NOTCH1::IKZF2*) genes were also identified, with the latter one being detected in a T-LBL patient (Supplementary Table S6, Supplementary Fig. S6). Figure 3 summarizes all alterations including short variants, gene fusions, and CNAs in 165 samples analyzed by both targeted DNA sequencing and RNA sequencing as well as MLPA.

Cosegregation of Molecular Aberrations

The scrutiny of associations between individual genetic lesions revealed the simultaneous presence and mutual exclusivity of

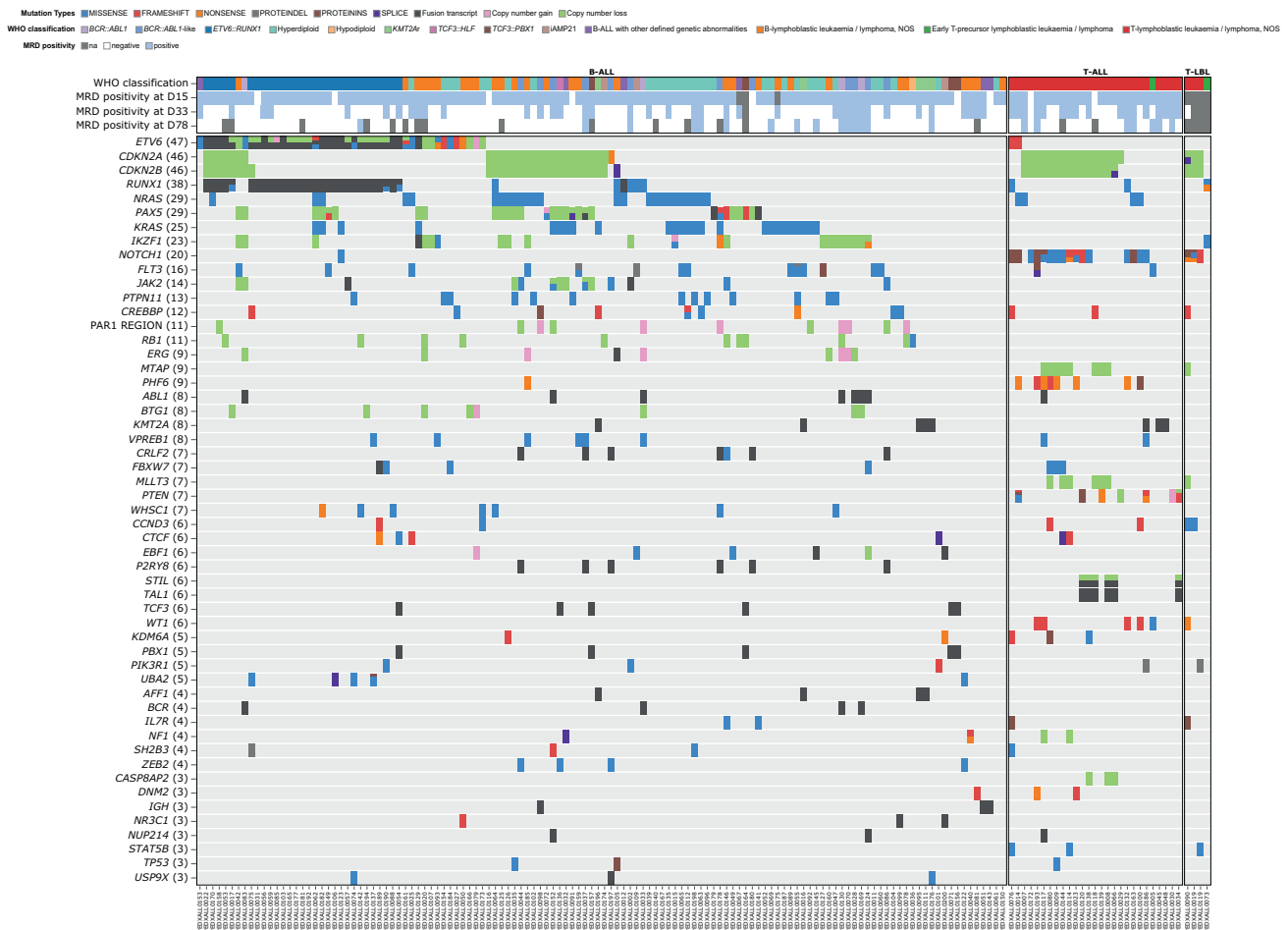


Figure 3.

Short somatic variants, copy number aberrations and gene fusions detected by targeted DNA sequencing, RNA sequencing and MLPA in diagnostic samples of 165 pediatric ALL/LBL patients analyzed by all 3 methods. Immunophenotype, World Health Organization classification, MRD status on days 15, 33, and 78 of therapy, and mutation type are also indicated. Genes altered in at least 3 patients are illustrated.

various aberrations. Pairwise analysis of small variants and CNAs revealed 51 positive associations in the whole patient cohort. For example, *KRAS* mutations frequently co-occurred with *NRAS*, *BRAF*, *FLT3*, and *CREBBP* mutations in B-ALL (Fig. 4A), whereas *STAT5B* mutations were associated with *JAK1* mutations, and *NOTCH1* mutations with *PHF6* mutations in patients with T-ALL (Fig. 4B). These findings suggest that recurrent clonal selection mechanisms commonly converge on the same or interconnected pathways in individual patients. In B-ALL, several small variants and CNAs showed nonrandom distribution across World Health Organization subtypes. For example, RAS pathway mutations (*NRAS*, *KRAS*, *PTPN11*, and *FLT3*) were highly prevalent (67.5%) among patients with hyperdiploid karyotype, whereas *CDKN2A/B*, *PAX5*, *ETV6*, *BTG1*, and *RB1* deletions were almost never detected in the hyperdiploid subgroup (Fig. 4A). The majority of *UBA2* mutations (85.7%; Figs. 3 and 4A) and *ETV6* deletions (66.7%; Figs. 2A and 4A) emerged in the *ETV6::RUNX1*-positive subgroup, and 81.8% of patients in this subgroup harbored clonal partial deletion of 22q11.22, as revealed by digitalMLPA (Supplementary Fig. S5). Among B-ALL patients, deletions affecting 6q were also predominantly observed in patients with *ETV6::RUNX1* fusion (85.7%; Supplementary Fig. S5). *IKZF1* deletions were detected in all but one *BCR::ABL1*-positive patients, and significant enrichment of *IKZF1*, *EBF1*, and *PAX5* deletions, *JAK2* mutation, *ZEB2* mutation,

and *PAR1* deletion was observed in the *BCR::ABL1*-like subtype. In T-ALL, *CASP8AP2* deletion was exclusively detected in patients harboring *STIL::TAL1* fusions, and besides frequent co-deletion of genes located in chromosome region 9p21, various positive associations were observed between *NOTCH1*, *PHF6*, *WT1*, *FBXW7*, *MLLT3*, and *MTAP* alterations (Fig. 4B).

Genetic Aberrations Detected in Relapse Samples

Twenty-six samples drawn from 19 patients with B-ALL ($n = 15$) or T-ALL ($n = 4$) at the time of relapse were analyzed. The average time from diagnosis to first relapse was 31 months (range: 5-109 months; Fig. 5A). Targeted DNA- and RNA-sequencing complemented with MLPA was performed on 17 samples, whereas in the rest of the cases, sample quality or quantity hampered RNA-seq or MLPA analysis. The relapse samples carried on average 2.4 mutations (range: 0-5), somewhat higher than observed in the diagnostic samples (average 2.0, range: 0-7). Thirty-three percent ($n = 21/64$) of the somatic mutations were present both at diagnosis and relapse, 27% ($n = 17/64$) were exclusively detected in diagnostic samples and 41% ($n = 26/64$) emerged during disease progression. Compared with diagnosis, *TP53* and *PIK3R1* mutations were enriched at relapse, whereas

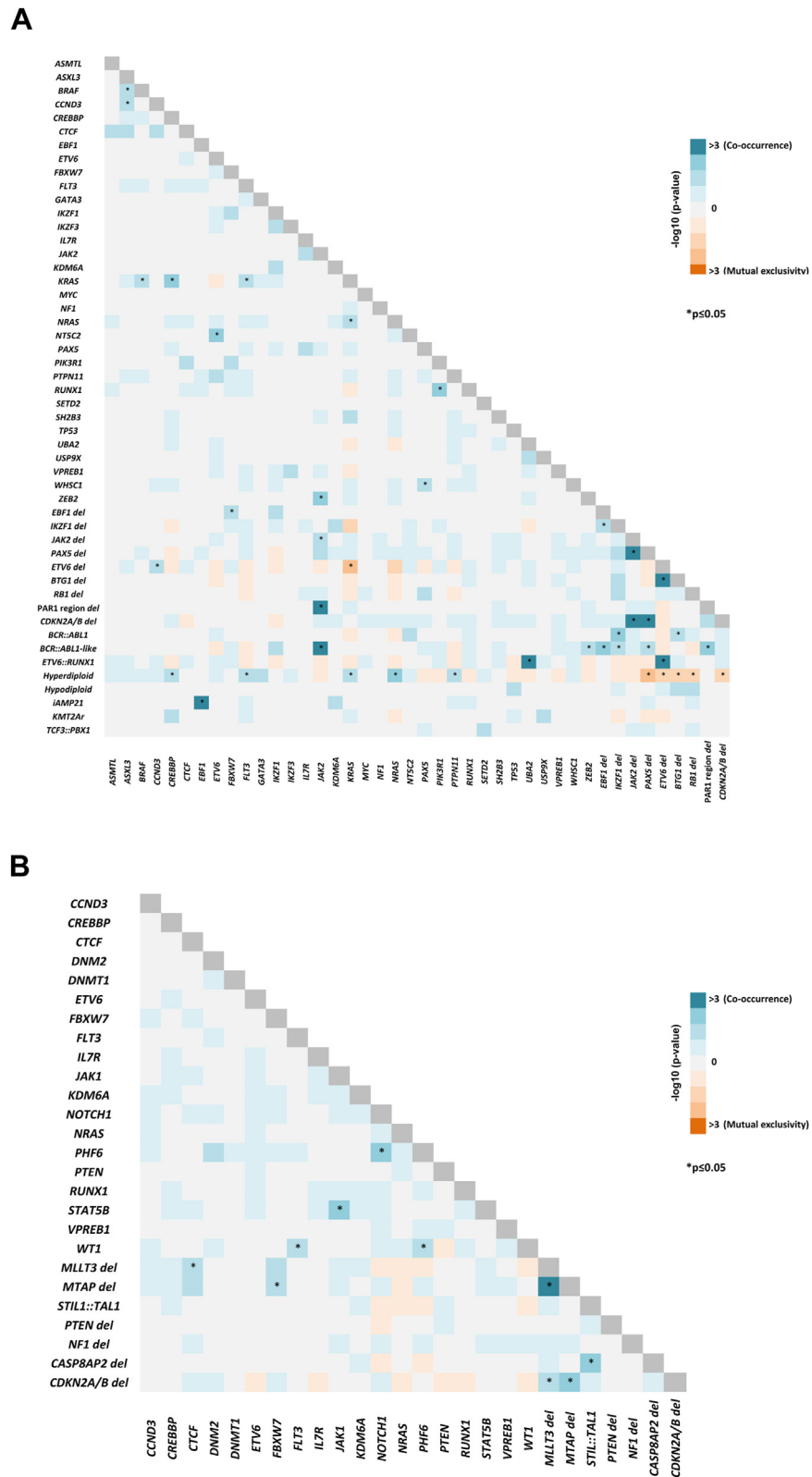
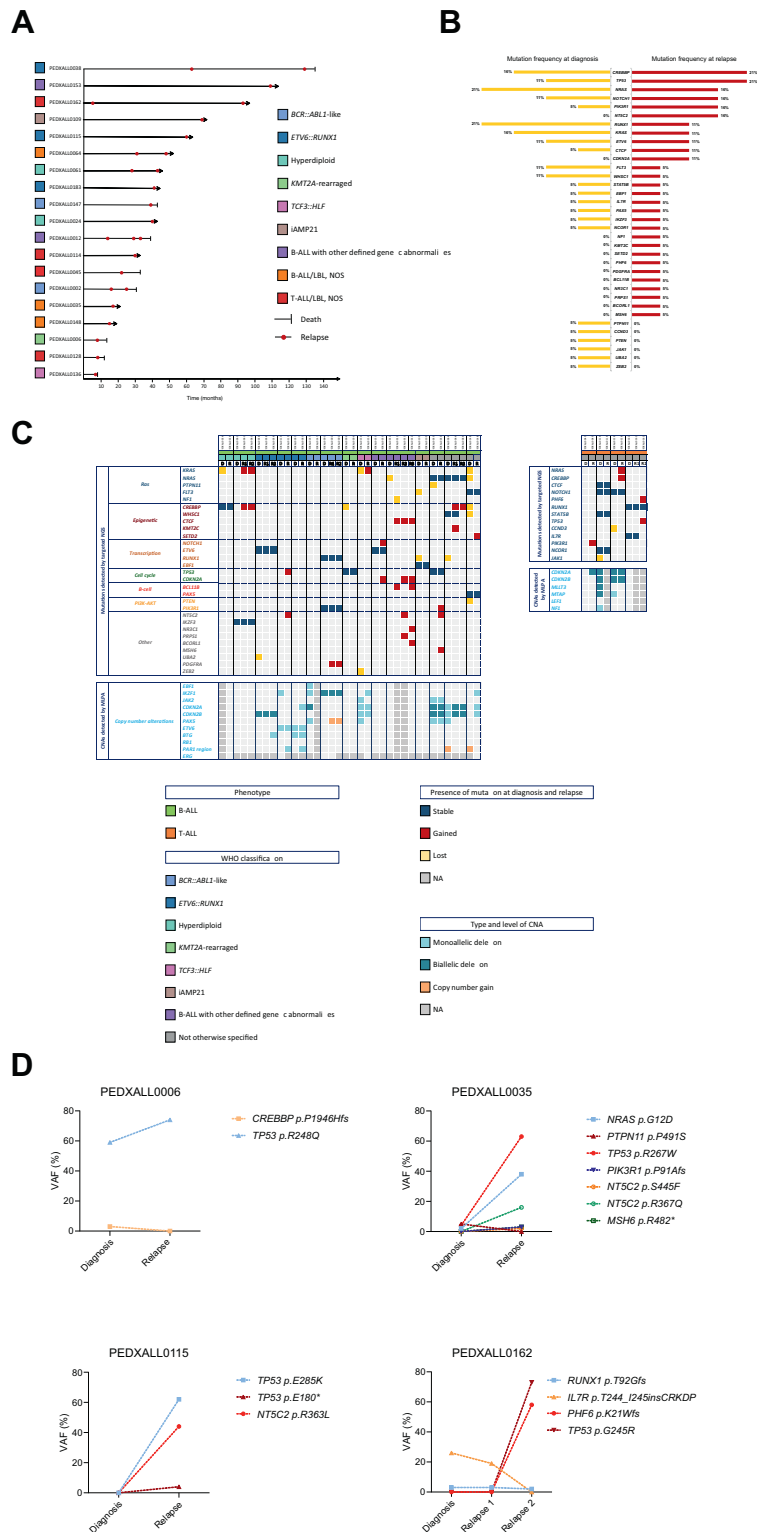
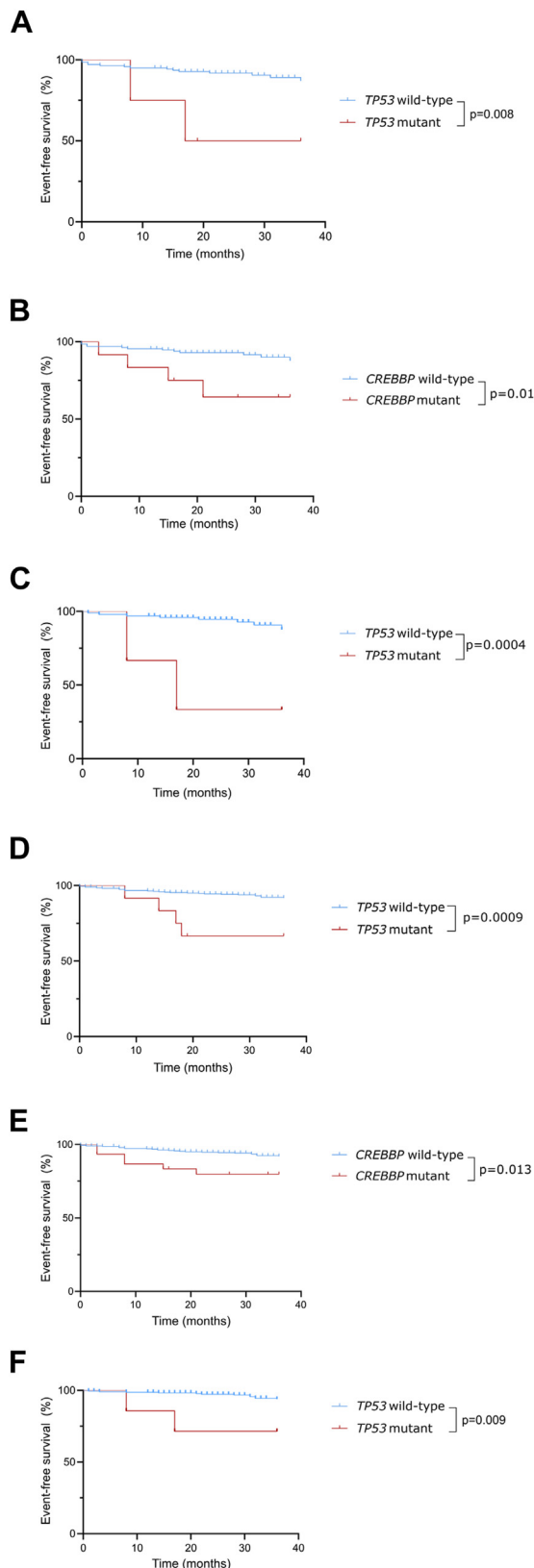


Figure 4.

(A) Cosegregation of short somatic mutations and subchromosomal copy number aberrations in B-ALL. (B) Co-occurrence and mutual exclusivity of short somatic variants and subchromosomal copy number aberrations in T-ALL. Associations statistically significant ($P < .05$) based on pairwise Fisher's exact test are indicated by asterisks.

**Figure 5.**

(A) Timeline and World Health Organization category of 19 relapsed patients. Red circles indicate the time from diagnosis to relapse. (B) Comparison of the mutation frequency in 35 altered genes between diagnostic and relapse samples. (C) OncoPrint displaying short somatic variants and copy number aberrations in 19 patients at the time of diagnosis and relapse. Distinctive colors are used for representing stable mutations (dark blue), which are present both at diagnosis and relapse, and unstable mutations, which are only present either at diagnosis (yellow) or at relapse (red). (D) Clonal dynamics and composition of mutations over the disease course in 4 selected patients harboring *TP53* mutation. World Health Organization subclassification of patients: PEDXALL0006 – B-ALL with *KMT2A*-rearrangement; PEDXALL0035 – B-ALL, NOS; PEDXALL0115 – B-ALL with *ETV6::RUNX1* fusion; PEDXALL0162 – T-ALL, NOS. (VAF: variant allele frequency).

**Figure 6.**

(A) Three-year event-free survival of B-ALL patients with or without *TP53* mutation in our in-house cohort. (B) Three-year event-free survival of B-ALL patients with or without *CREBBP* mutation in our in-house cohort. (C) Three-year event-free survival of B-ALL patients showing MRD negativity by flow cytometry on day 33 and

NT5C2 and *CDKN2A* mutations were exclusively observed at disease progression (Fig. 5B, C; Supplementary Table S7). The highest frequency of mutations at relapse was observed in *CREBBP* and *TP53*, both being altered in 21% (4/19) of the cases (Fig. 5B). *TP53* mutations were present at the time of diagnosis in 2 cases, and the alterations conferred temporally sustained survival advantage in both patients as demonstrated by increased variant allele frequencies at the time of relapse (VAF: 59% vs 74% and 4% vs 63%). The significant change in VAF from 4% to 63% at patient PEDXALL0035 suggests loss of heterozygosity at the time of relapse. In 2 additional patients, a detectable *TP53* mutant clone emerged at relapse; in 1 patient (PEDXALL0162), only at the second relapse, 7 years after diagnosis (Fig. 5D). In the majority of cases (13/19), at least 1 mutation persisted from diagnosis to relapse, with additional variants being eliminated by therapy in 9/19 patients, or emerging at relapse in 8/19 patients. In 4 cases, a completely different mutational profile was observed at the time of relapse (PEDXALL0012, PEDXALL045, PEDXALL115, and PEDXALL0136). Scrutiny of the evolution of detectable mutations in individual patients over time revealed complex subclonal dynamics with simultaneously rising and falling subclones in two-thirds of the cases (Supplementary Fig. S7).

Alterations Associated With Clinical Response, Outcome, and Potential Targeted Therapy

Investigating the presence of genetic alterations in light of MRD positivity assessed by flow cytometry, a strong association was found between *IKZF1* deletion and MRD positivity at days 33 and 78 after treatment initiation ($P = .021$ and $P < .001$, respectively). Strong positive association was also observed between *BCR::ABL1*-like subtype-associated gene fusions and MRD positivity at days 33 and 78 ($P = .014$ and $P = .005$, respectively). On the other hand, all but one *CCND3* mutant patient ($n = 5$) proved to be MRD negative on days 33 and 78.

The worst outcome across B-ALL subgroups was observed in patients harboring *KMT2A*-rearrangement (3-year EFS, presence vs absence of *KMT2A*-rearrangement; $P = .019$). Survival analysis of the B-ALL cohort also revealed significantly shorter 3-year EFS in patients with *TP53* (mutant vs wild-type EFS; $P = .008$, Fig. 6A) or *CREBBP* mutations (mutant vs wild-type EFS; $P = .010$, Fig. 6B). In our data set, the presence of variants in other frequently altered genes, such as RAS pathway mutations, whether clonal or subclonal, had no impact on EFS and OS. Notably, further subgroup analyses uncovered the adverse prognostic effect of *TP53* mutations even among patients showing favorable responses to therapy. Three-year EFS of patients with MRD negativity on day 33 remained inferior in *TP53* mutant cases (mutant vs wild-type EFS; $P = .0004$, Fig. 6C).

Because of the limited number of *TP53* and *CREBBP* mutant cases in our in-house cohort, we performed an additional focused analysis of 3-year EFS using a merged data set of 411 patients, also including 265 patients from the TARGET ALL Phase 2 study. In line

subclassified based on *TP53* mutation status. (D) Three-year event-free survival of B-ALL patients with or without *TP53* mutation in the expanded cohort, also comprising 265 patients from the TARGET ALL Phase 2 study. (E) Three-year event-free survival of B-ALL patients with or without *CREBBP* mutation in the expanded cohort. (F) Three-year event-free survival of B-ALL patients showing MRD negativity at the end of induction and subclassified based on *TP53* mutation status in the expanded cohort. Significantly shorter 3-year EFS was observed in patients with *TP53* mutations, even in case of MRD negativity on day 33.

with previous findings, survival analysis confirmed a dismal outcome of *TP53* ($n = 12$) or *CREBBP* ($n = 30$) mutant B-ALL patients in this larger cohort (3-year EFS; *TP53* mutant vs wild-type: $P = .0009$, Fig. 6D; *CREBBP* mutant vs wild-type $P = .013$, Fig. 6E). Furthermore, B-ALL patients showing MRD negativity at the end of induction could also be further stratified based on *TP53* mutational status (*TP53* mutant: $n = 7$) in the merged data set (3-year EFS, *TP53* mutant vs wild-type; $P = .009$; Fig. 6F).

In our in-house cohort, 7/13 B-ALL patients with very early or early event (EFS <24 months) were treated according to the intermediate-risk arm of the ALL IC-BFM 2009 protocol, and 4 of the 7 intermediate-risk patients harbored either *TP53* ($n = 2$) or *CREBBP* ($n = 3$) mutation. Of note, 3 of these 5 mutations were subclonal and fell in the VAF range of 3% to 5%, where conclusive interpretation of variants is typically challenging using standard NGS methods and sequencing coverage. Mutations emerging with a VAF $\geq 10\%$, and fusions affecting potentially targetable genes, such as *NRAS*, *KRAS*, *PTPN11*, *NF1*, *FLT3*, *JAK2*, *IL7R*, *SH2B3*, *CRLF2*, *EPOR*, *ABL1*, *ABL2*, *KMT2A*, and *NUP98* were observed in 55.9% (33/59) of high-risk and 31.6% (36/114) of standard/intermediate-risk patients treated according to ALL IC-BFM 2009 protocol (Supplementary Table S8, Supplementary Figs. S8 and S9). Additionally, deep sequencing enabled the confident identification of 39 mutations in targetable genes with a VAF <10%, affecting 11 patients in total.

Discussion

Survival rates of pediatric ALL have remarkably improved over the past decades, partially conferred by a gradually refined risk assessment and patient stratification, leading to the implementation of more personalized treatment approaches, and carefully guiding therapy selection during the disease course.^{32,33} The rapid development of advanced genomic and transcriptomic technologies has revolutionized the molecular characterization of leukemic samples, significantly deepening our understanding of disease biology and establishing the foundation for more precise clinical management of patients with ALL.^{18,34}

Our study provides a comprehensive genomic and transcriptomic characterization of disease-relevant, recurrent alterations in a nationwide consecutive cohort of pediatric ALL patients, with all samples being collected as part of the diagnostic workflow and integrated into the Hungarian Pediatric Leukemia Molecular Profiling Program. We have introduced a self-developed targeted DNA-seq panel, specifically designed for the deep genomic profiling of ALL, and demonstrated that combining rationalized DNA-seq and RNA-seq analyses with (digital)MLPA can be an effective strategy for the identification of subtype-defining alterations and potential therapeutic targets, eventually aiding the enhancement of clinical patient care.

A subset of our patients was also screened by digitalMLPA, which allows for the genome-wide screening of whole chromosome aberrations and large CNAs, besides the focused, exon-level interrogation of all ALL-relevant genomic regions recurrently affected by copy number changes.²⁴ Beyond modal chromosome number assessment, the technique can detect specific whole chromosome gains and losses, which is not achievable by DNA-index assessment, commonly unsuccessful by karyotyping and not cost effective using FISH. Although MLPA probes cover selected exons in a limited number of genes, digitalMLPA provides a more comprehensive screening for disease-relevant alterations in regions not covered by the routinely applied MLPA probemixes, facilitating a more precise risk assessment which can be translated

into enhanced patient stratification. The use of digitalMLPA for refining risk assessment was not specifically investigated in the present study, because we previously reported the added value of the method in a larger cohort of Hungarian patients with longer follow-up periods.³⁵ Moreover, digitalMLPA allows for a one-step identification of *IKZF1*^{plus}, a genotype reported to be associated with very poor prognosis in an MRD-dependent manner. In our cohort, all but one *BCR::ABL1*-positive patient harbored *IKZF1* deletion, and this aberration was also highly prevalent among patients displaying *BCR::ABL*-like features. Although, with a relatively short follow-up time, survival rates did not differ between patients with different *IKZF1* status, a high proportion of MRD positivity occurred among patients with *IKZF1* deletion on days 33 and 78 of the treatment.

Identification of gene fusions plays a pivotal role in the risk assessment and stratification of patients with pediatric ALL.^{10,36-39} Targeted RNA sequencing offers a valuable alternative to FISH and real-time quantitative PCR, which are still widely used in clinical diagnostics, by allowing for the simultaneous analysis of thousands of genes, thus enabling the detection of less common but therapeutically relevant fusions, beyond all subtype-defining rearrangements.⁴⁰⁻⁴⁴ In this study, we used a commercially available targeted RNA-sequencing approach, that had been proven to be suitable for the analysis of hematologic malignancies.^{41,43,45} The applied RNA-seq method unveiled clinically relevant fusions, such as chimeric genes involving the *DUX4*, *MEF2D*, *NUTM1*, and *ZNF384* genes, thus enabling the precise classification of affected patients into recently established subgroups of ALL.^{35,46-49} Furthermore, RNA-seq enabled the identification of partner genes in *KMT2A* rearrangements, frequently screened by break-apart FISH probes, allowing for treatment response monitoring using *KMT2A* fusions that are known to be reliable MRD biomarkers.⁵⁰⁻⁵² Although *KMT2A*-rearranged cases exhibited the most adverse outcome among B-ALL subtypes, menin inhibitors may provide promising targeted therapy options for this subgroup of patients.⁵³ Notably, we also observed several kinase fusions involving the *ABL1*, *ABL2*, *JAK2*, and *CRLF2* genes, thus having actionable potential, paving the way for targeted therapeutic interventions.^{37,39,54} Additionally, we identified novel, previously not described chimeric products involving the *JAK2*, *KMT2A*, *PAX5*, *RUNX1*, and *NOTCH1* genes. All these newly observed fusions were in-frame products, raising the possibility of their contribution to disease development, or in the case of *JAK2* fusion, even providing a potential therapeutic target.

Using deep DNA sequencing, we observed substantially different mutation patterns across ALL subtypes. Although mutations affecting RAS pathway genes commonly occurred in all molecular subgroups of B-ALL, the frequency of *NRAS*, *KRAS*, *PTPN11*, and *FLT3* alterations was most prominent among hyperdiploid patients. The association between hyperdiploidy and RAS pathway mutations was previously described,⁵⁵ with some studies reporting the correlation between RAS pathway mutations and prednisolone resistance, and sensitivity to MEK inhibition.^{56,57} Jerchel et al⁵⁸ found that only clonal mutations confer unfavorable prognosis, and Antić et al⁵⁹ also concluded that subclonal alterations are not reliable prognostic markers at the time of diagnosis. RAS pathway alterations did not affect EFS or OS in our data set, regardless of their clonal or subclonal presence. In B-ALL, *UBA2* mutations predominantly occurred in patients harboring *ETV6::RUNX1* fusion.^{60,61} Although prior studies have described the presence of *UBA2* alterations in this subtype of ALL,⁶² our data suggest that enrichment of *UBA2* aberrations among patients with *ETV6::RUNX1* positivity may be considerably higher than previously reported. Furthermore, digitalMLPA

revealed the partial deletion of 22q11.22, a region not covered by the applied MLPA probemixes, in the vast majority (81.8%) of *ETV6::RUNX1*-positive patients. Partial deletion of 22q11.22 has been described as a prevalent alteration in this subgroup; however, the frequency of the alteration was even more prominent in our cohort than observed in previous studies.⁶³ Although *VPREB1* is located in the region of *IGLV* segments, it is deleted focally in the majority of cases, independent of the *IGLVJ*-recombination, and its association with inferior clinical outcome has also been suggested.^{63,64} Our findings regarding the common co-occurrence of *VPREB1* deletion with *ETV6::RUNX1* fusion, which is typically associated with a favorable prognosis, may warrant further investigations.

Systematic deep mutational screening for potentially altered genomic regions also allows for the detection of uncommon point mutations with presumptive prognostic relevance, such as *PAX5* p.P80R, *IKZF1* p.N159Y or *ZEB2* p.H1038, and p.Q1072.^{9,10,65,66} We identified *ZEB2* p.Q1072 hotspot mutation in 4 cases and *IKZF1* p.N159Y in 2 patients, whereas *PAX5* p.P80R and *ZEB2* p.H1038 alterations were not observed in our cohort. Although the small number of mutant patients and the limited follow-up time did not allow us to draw firm conclusions regarding the prognostic significance of these mutations, previously published data suggests that *ZEB2* mutations are associated with shorter EFS and increased relapse rate, highlighting the importance of identifying these aberrations.⁶⁶ Accordingly, one of our patients with *ZEB2* mutation experienced a relapse already at month 7 and succumbed to their disease at month 8 after the start of treatment.

Notably, over half of the high-risk and approximately one-third of standard/intermediate-risk patients had potentially actionable mutations with a VAF >10%, or targetable fusions, which opens the door to the prospect of targeted therapy in a significant number of patients across all risk groups.^{37,54} Furthermore, deep sequencing unveiled putatively druggable variants with a low VAF in 11 additional patients, thus increasing the number of patients who may be eligible candidates for targeted therapy in the future.

Survival analysis performed on our B-ALL cohort revealed a significantly shorter EFS of *TP53* mutant patients compared with wild-type cases. *TP53* mutations occur in approximately 10% to 15% of ALL patients^{67,68} and are associated with therapy resistance, higher frequency of relapse, and dismal outcome.⁶⁷⁻⁶⁹ Additionally, previous studies suggested that *TP53* mutations are present in approximately 90% of low-hypodiploid patients.^{70,71} Although the proportion of low-hypodiploid cases was very low in our cohort ($n = 2$), both affected patients harbored *TP53* mutation, in line with previously published data. Upon examining B-ALL patients who tested negative for MRD on day 33 of therapy, we observed a notable decline in EFS in the presence of *TP53* mutation. This discovery is particularly intriguing, in light of the fact that assessment of MRD at the end of the induction period is widely considered a particularly powerful prognostic indicator.⁷²⁻⁷⁵ Our results suggest that *TP53* mutation status could enable further stratification of patients in this MRD-negative subgroup, which is generally associated with favorable prognosis. We believe this particular finding merits further investigation in larger patient cohorts; hence, as a first step, we performed a successful validation by complementing our results with data from the TARGET ALL Phase 2 study. *TP53* mutant cases in the validation cohort consisted of patients classified in 6 different subgroups, further highlighting the independent prognostic value of *TP53* alterations and the need for mutation screening across all subgroups of B-ALL.

Approximately half of the patients experiencing very early or early events in the first 2 years of therapy were classified in the intermediate-risk group, and *TP53* and/or *CREBBP* mutations were

detected in 4 of them, raising the question of whether these patients would have benefited from a more intensive therapeutic intervention. Importantly, 3 of these mutations were detected with a low VAF, underlining the added value of deep sequencing and its ubiquitous, unrestricted application across all risk groups. Of note, in chronic lymphocytic leukemia, our group and others found that low-burden *TP53* mutations also confer inferior prognosis; therefore, the detection of low-burden alterations at the time of diagnosis may also be of clinical relevance in acute leukemias.⁷⁶⁻⁸⁰

In a subset of patients, we also investigated disease progression-associated molecular changes by comparing matching genomic profiles at diagnosis and relapse. Notably, we observed the aforementioned 2 genes—*CREBBP* and *TP53*—showing the highest mutation frequency in relapse samples. *CREBBP* mutations are considered relapse-associated aberrations and have been linked to glucocorticoid resistance.⁸¹⁻⁸⁴ *TP53* mutations are detected more frequently in relapse samples,⁸⁵⁻⁸⁷ often emerging as a novel alteration, as it was also observed in 2 patients in our cohort. In patient PEDXALL0162, the *TP53* mutant clone was only detectable at the second relapse, several years after the treatment of the first relapse, suggesting a potential influence of previous cytotoxic therapy on disease evolution.⁸⁸ In 2 other *TP53* mutant cases, a pre-existing subclone expanded by the time of relapse, indicating an incomplete eradication of the respective cell population during initial treatment, and thus providing further evidence of the association between *TP53* mutations and chemotherapy resistance.^{68,87} All *NT5C2* mutations emerged only at the time of relapse, in line with previous studies describing these mutations as common relapse-specific alterations, conferring resistance to 6-mercaptopurine.⁸⁹⁻⁹² In the majority of cases, we identified branching clonal evolution with simultaneously expanding and diminishing subclones, suggesting complex evolutionary hierarchies of competing leukemic cells, which may pose significant challenges for effective therapeutic intervention.

In summary, we present a rationalized and affordable approach for deep and comprehensive molecular profiling of a nationwide cohort of children with ALL in Hungary, which can greatly facilitate the advanced molecular subtyping of patients and a fine-tuned risk assessment, translating into improved patient stratification and more precise therapy selection. Our study demonstrates that targeted RNA sequencing combined with digital MLPA is a robust diagnostic tool for identifying contemporary genetic subtypes of ALL, and it has the potential to serve as an alternative to current diagnostic workflows that are either time-consuming, labor-intensive, or technically challenging. Although identification of certain subgroup-defining gene expression profiles may be limited using targeted RNA-seq, some recent studies questioned the need for broad screening of expression signatures and rather articulated the importance of detecting druggable alterations to efficiently guide treatment-relevant clinical decisions.⁹³ Furthermore, our mutational screening method, specifically tailored to the scrutiny of ALL, demonstrated its clear added value by uncovering disease-relevant mutations with previously underappreciated prognostic importance and actionable alterations for targeted therapies. By revealing novel gene fusions and shedding light on associations between disease prognosis and *TP53* mutational status, we believe that our data set further illuminates the genomic landscape of patients with pediatric ALL.

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Author Contributions

D.A. conceived, D.A. and C.B. supervised the study. C.K. and G.K. spearheaded the design of the clinical protocol. B.E., D.J.E., J.M., Z.J., A.B., L.A.K., G.P., M.Z., K.Cs., G.O., K.Cs., Á.V., L.Gy.T., K.M.G., K.B., Á.K., P.H., K.K., G.K., Z.G., I.S., Z.H., A.U., G.B., Á.M., I.H., B.T., B.K., C.K., G.K., A.M., and G.K. provided patient samples and clinical/pathological annotations. B.P., S.K., and L.L.H. performed experiments. A.B.S. and S.P. conducted digitalMLPA experiments and data analysis, S.S. supervised and oversaw the data interpretation. B.P., S.K., B.E., G.B., T.N., C.B., and D.A. performed data analysis. B.P., S.K., B.E., C.B., and D.A. wrote the paper. All authors have read and critically reviewed the final version of the manuscript.

Data Availability

The materials used and analyzed during the current study are available from the corresponding author on reasonable request.

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Declaration of Competing Interest

A.B.S., S.P., and S.S. are employees of the MRC Holland (Amsterdam, Netherlands). The authors have no further conflicts of interest to disclose.

Ethics Approval and Consent to Participate

Ethical approval (45563-2/2019/EKU) from the Medical Research Council of Hungary and written informed consent from the patients and/or from the parents or guardians were obtained for the study, which was conducted in accordance with the Declaration of Helsinki.

Supplementary Material

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